Oxidative Stress and Aging of the Male Reproductive Tract

By

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Abstract

The global demographic shift towards population aging will result in a dramatic increase in the numbers of elderly in the population. In order to cope with these changing demographics, it is imperative that we better understand aging and age-related pathologies. The reproductive tract provides an excellent system in which to study aging in that it is affected by aging, without compromising the overall health of the individual. In the Brown Norway rat model, the male reproductive tract (testis and epididymis) shows numerous signs of aging when other systems remain relatively unaffected by age, thus making this system ideal for studies on underlying causes of aging. One of the many theories proposed to account for the aging process is oxidative stress. Moreover, some of the changes that take place in the aging epididymis are suggestive of oxidative stress. In order to understand the contribution of oxidative stress to aging of the epididymis, we undertook global gene expression studies of the tissue in the young animal and then assessed how this gene expression was affected by age. We manipulated oxidative stress by caloric restriction and antioxidant (vitamin E) supplementation and deficiency. In characterizing the longitudinal gene expression in the young epididymis, we identified many genes never before reported in this tissue. We found that age profoundly affects gene expression in the epididymis and that the expression of oxidative stress related transcripts decreased, most dramatically in the distal (corpus and cauda epididymidis) regions of the tissue. Caloric restriction attenuated or reversed many of the gene expression changes that took place with age. The effect of caloric restriction was greatest for transcripts associated with protein synthesis and mitochondrial function. Finally, we found that long term antioxidant (vitamin E) deficiency resulted in increased expression of oxidative stress transcripts and exacerbated the effects of age on the accumulation of oxidative stress damage and sperm maturation. Together these studies demonstrated that the ability of the epididymis to respond to oxidative stress is altered with age and that if oxidative stress load is increased some parameters of aging of the tissue are exacerbated.

Resumé

Le changement démographique global vers une population vieillissante résultera en un nombre accru de personnes âgées dans la population. Afin de faire face à ces changements démographiques, il est impératif de mieux comprendre le vieillissement et les pathologies reliées à l'âge. L'appareil génital est un système idéal pour étudier le vieillissement puisqu'il est affecté par l'âge et ne compromet pas la santé générale de l'individu. L'appareil génital du rat Brown Norway (testicule et l'épididyme) démontre plusieurs signes de vieillissement alors que d'autres systèmes demeurent relativement non affectés par l'âge. Par conséquent, ce système est propice à l'étude sur l'origine des causes du vieillissement. Une des nombreuses théories proposées attribue le processus du vieillissement au stress oxydatif. De plus, certains des changements qui prennent place dans l'épididyme vieillissant suggèrent la présence du stress oxydatif. Afin de comprendre la contribution du stress oxydatif au vieillissement de l'épididyme, nous avons fait des recherches sur l'expressivité génétique des tissus chez le jeune animal et avons évalué comment ces derniers étaient affectés par l'âge. Nous avons manipulé le stress oxydatif en terme de restriction calorique et en carence et surplus d'antioxydant (vitamine E) En caractérisant l'expressivité génétique longitudinale dans le jeune épididyme, nous avons identifié plusieurs gènes qui n'avaient jamais été rapportés dans ces tissus auparavant. Nous avons trouvé que l'âge affecte profondément l'expression des gènes dans l'épididyme et que l'expression des gènes reliés au stress oxydatif diminue, notamment dans les régions éloignées du tissu (le corps et la queue de l'épididyme). La restriction calorique atténue ou renverse plusieurs des changements de l'expression du gène qui prennent place durant le processus de vieillissement. L'effet de la restriction calorique est majeur en ce qui concerne la transcription associée à la synthèse protéinique et la fonction mitochondriale. Finalement, nous avons trouvé que la carence à long terme d'antioxydant (vitamine E) causait une augmentation de l'expression de la transcription des gènes reliés au stress oxydatif et exacerbait les effets de l'âge sur l'accumulation des dommages du stress oxydatif et de la maturation des

spermatozoïdes. Ensemble, ces études ont démontrées que la capacité de l'épididyme à répondre au stress oxydatif est modifiée avec l'âge et que si la charge du stress oxydatif est augmentée, certains paramètres du vieillissement des tissus son exacerbés.

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List of Abbreviations

IGF-1 - insulin-like growth factor 1

GH - growth hormone

ROS - reactive oxygen species

mtDNA - mitochondrial DNA

ETC - electron transport chain

$O_2^{\bullet -}$ - superoxide

H_2O_2 - hydrogen peroxide

$OH^{\cdot}$ - hydroxyl radical

MDA - malondialdehyde

4-HNE - 4-hydroxy-2-nonenal

4-HHE - 4-hydroxyhexenal

ARE - antioxidant response element

SOD - superoxide dismutase

CuZnSOD - copper-zinc superoxide dismutase

MnSOD - manganese superoxide dismutase

SIR2 - silent information regulator 2

α-TTP – α-tocopherol transfer protein

HPA - hypothalamic–pituitary axis

GnRH - gonadotropin-releasing hormone

LH - luteinizing hormone

FSH - follicle-stimulating hormone

BN - brown Norway

List of Original Contributions

1. Segment specific gene expression in the BN rat epididymis was studied at the RNA level using cDNA arrays containing 1178 cDNAs. Prior to this study the highly complex and regionalized nature of gene expression in the epididymis had never been reported in such a comprehensive manner. This resulted in the characterization of the expression of many genes and gene families never before found to be expressed in the epididymis.

2. Insight was provided into the mechanisms underlying the highly regionalized nature of epididymal gene expression through the characterization of transcription factor gene expression along the epididymis.

3. The identification of transcripts expressed in a region specific manner along the epididymis provided new perspectives on the expression of genes that may be important in the establishment of the epididymal luminal microenvironment required for sperm maturation processes.

4. The effect of age on gene expression in the BN rat epididymis was determined at the RNA level using cDNA arrays containing 1178 cDNAs. This study demonstrated that age affects epididymal gene expression in a segment specific manner.

5. It was determined that the most pronounced effect of age on epididymal gene expression was to decrease the relative intensity of expression for the majority of the transcripts studied; the magnitude of gene expression changes was greatest in the distal (corpus and cauda) part of the epididymis.

6. The effect of age on the expression of oxidative stress related genes, heat shock proteins (HSP), cathepsins and proteasome components was established; the expression of HSPs and proteasome components was affected in a segment specific manner with the greatest effect occurring in the distal (corpus and cauda) epididymis.

7. The effects of caloric restriction on the aging of the epididymis were studied at the RNA level using cDNA arrays containing 1178 cDNAs. Caloric restriction was shown to attenuate or reverse age-related gene expression changes throughout the epididymis; this effect was most pronounced in the distal (corpus and cauda) part of the epididymis.

8. It was determined that caloric restriction had a dramatic effect on the age-related decreased expression of genes associated with protein synthesis and mitochondrial function in all regions of the epididymal epithelium.

9. The effects of long term vitamin E treatment on gene expression in the epididymis were determined; both vitamin E supplementation and deficiency affected gene expression in the aging epididymis.

10. It was demonstrated that long term vitamin E deficiency resulted in increased expression of oxidative stress related transcripts; this effect was most pronounced in the corpus epididymidis.

11. The presence and localization 4-HNE, a marker of oxidative stress damage to lipids, along the aged epididymis was found to be dependent on the level of exposure to vitamin E. Long term Vitamin E deficiency increases the amount, and alters the localization of oxidative stress damage along the epididymal epithelium; this effect was most notable in the corpus epididymidis.

12. The effects of long-term vitamin E deficiency and supplementation on the shedding of the cytoplasmic droplet were determined; vitamin E deficiency increases the proportion of sperm that retain their cytoplasmic droplet.

Format of the thesis

This thesis is a manuscript based thesis and conforms to section I.C. of the "Guidelines for Thesis Preparation" of the Faculty of Graduate Studies and Research of McGill University. The manuscripts are presented in the form in which they were published or submitted for publication. We retain the right to include these manuscripts in this thesis according to the copyright agreements of the respective publishers (Elsevier publishing and Biology of Reproduction by the Society for the Study of Reproduction) provided that this thesis is not published commercially or used for commercial purposes. Chapter one, is a general introduction that describes aging research, current hypotheses about the etiology of aging, research tools employed in this thesis to study aging, animal models used for the study of aging, an overview of aging in the male and an introduction to the epididymis. Chapter one ends with the rationale for the studies presented in this thesis and the objectives of the thesis. Chapters 2-4 are published manuscripts and Chapter 5 is a manuscript that has been submitted for publication. Connecting texts are included between the chapters in order to ensure that the thesis has continuity. Two appendices are included at the end of Chapter 5. Appendix one addresses the methodology used in these studies and Appendix two contains additional results not published or submitted for publication. Chapter 6 is a general discussion of the findings of this thesis. References are included at the end of each chapter. All of the experiments and analyses were completed by the candidate.

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Chapter 1

Introduction

1. Aging

1.1 Increased Life Expectancy

In the past century average life expectancy has dramatically increased. As a direct result of improvements in modern medicine, sanitation and living conditions, people born between 2000-2005 can expect to live an average of 65.4 years, 20 years longer than people born in the 1950's (*Source:* Population Division of the Department of Economic and Social Affairs of the United Nations Secretariat (2003). *World Population Prospects: The 2002 Revision. Highlights.* New York: United Nations). In Canada, average life expectancy has increased from 59 in the early 1920's to 79.3 in 2002 (Statistics Canada). Interestingly, while in average life expectancy (age that 50% of the population can expect to survive) has increased dramatically; the maximum life span potential (life span of the oldest living members of a population) of humans has remained approximately unchanged at 90-100yrs.

The dramatic increase in average life expectancy, in conjunction with declining birth rates, has resulted in a demographic shift towards increased numbers of elderly in the population around the world. In the more developed regions of the world, the percentage of elderly (60 years of age or older) in the population will increase from 19% in 2002 to 32% in 2050: in Canada the percentage of elderly will increase from 17% to 30%. In less developed regions, the percentage of elderly in the population will more than double (8-20%) in the same period of time. While the elderly comprise a smaller percentage of the population in less developed regions, the growth of this sector is occurring at a much faster rate than in the more developed world. Worldwide, the fastest growing segment of the population (average annual growth rate 2000-2050) is the oldest old (80 years of age or older); the proportion of oldest old will grow 2.24 percent and 4.2 percent in more developed and less developed regions, respectively (*Source:* Population Division of the Department of Economic and

Social Affairs of the United Nations Secretariat (2003). *World Population Prospects: The 2002 Revision. Highlights.* New York: United Nations). This demographic change towards an aging population raises complex health, social and economic issues in both developed and developing nations (reviewed in Anderson and Hussey, 2000; Jacobzone, 2000; Canadian perspective reviewed in Denton and Spencer, 1999; Scarth, 2003) and as a consequence there is great research initiative to better understand and address the problems presented by population aging.

1.2 Aging Research

Research on aging can be divided into two branches. The first branch of aging research concerns itself with population aging. To begin with, the economic and social impact of increasing numbers of elderly within a population must be understood and addressed. Additionally, there are many non-communicable disease conditions, such as Alzheimer's, Parkinson's, osteoporosis, heart disease and cancer, that are associated with advancing age. These disease conditions, along with other age related chronic diseases, are a significant and costly cause of disability and reduced quality of life in modern society (Active Ageing: A Policy Framework: A contribution of the World Health Organization to the Second United Nations World Assembly on Ageing, Madrid, Spain, April 2002). Therefore, the goal of this branch of aging research is to study the social and economic impact of an aging population and to find ways to delay or prevent the onset, and improve the interventions for age related diseases.

The second branch of aging research concerns itself with the process of aging itself. The objective of this type of research is to discover what determines the maximum life span potential of an organism, the intrinsic mechanism behind why we age. Many theories purporting to account for the aging process have evolved out of this branch of aging research. Any theory that serves to explain

the mechanistic underpinnings of the aging process however, must be understood in the context of the evolutionary hypotheses about why we age.

1.2.1 Evolutionary Considerations

The question of how aging has evolved is an interesting one. How has a trait that is clearly disadvantageous to the organism, as it affects both survival and fertility, evolved? Aging has broad phylogenic distribution, but it is not universal as not all species show age associated increased mortality and decreased fertility (Finch, 1990; Kirkwood and Austad, 2000). Aging is therefore not considered to be simply a 'biological wearing out' of an organism. In nature, senescence does not contribute significantly to mortality; even though all animals have the potential to age, in the wild very few of them ever do. Rather, it is extrinsic mortality (infection, starvation, predation, and cold) that is the cause for mortality in nature, and this occurs mainly in the young (Kirkwood, 2002). Since few animals in the wild live long enough to grow old, aging has little effect on survival, and the force of natural selection has little opportunity to act (Kirkwood, 2002; Kirkwood and Austad, 2000). This makes it unlikely for a specific aging gene to evolve. The weakening of the force of selection with advancing age is essential in considering the evolution of aging. Based on these basic assumptions, three major hypotheses have been proposed to explain the evolution of the aging process (Kirkwood and Austad, 2000).

The first evolutionary hypothesis is the mutation accumulation theory (Medawar, 1952) which states that aging results from mutations that exert their deleterious effects only late in life. These late acting deleterious mutations are able to accumulate in the germ line over generations because the force of selection is too weak by the time they exert their effects to select against them.

In the second evolutionary hypothesis, called pleiotropy or antagonistic pleiotropy (Williams, 1957), aging is thought to occur when the action of a gene is beneficial early on in life but becomes deleterious late in life. Such genes are maintained in the germ line because of the fitness advantage that they confer at

some point early on in life. Since the force of selection on a trait depends both on the size of the effect and the probability of surviving to be affected by it, a beneficial effect early on in life will outweigh an adverse effect in late life (Kirkwood and Austad, 2000).

The third prevalent evolutionary hypothesis is the disposable soma theory (Kirkwood, 1977). In this theory, aging occurs because there is a prioritization in the allocation of available resources, whereby an organism must choose between reproduction and maintenance of the soma. Somatic maintenance is necessary to keep an organism in good condition only as long as the organism has a reasonable chance of survival (Kirkwood and Austad, 2000). All processes, like DNA repair for example, required to maintain the soma are energetically demanding (Kirkwood and Austad, 2000). Thus in the wild, if cold will kill you before a damaged soma will, an organism will benefit from investing metabolic resources in thermogenesis or reproduction rather than mechanisms that will repair somatic damage, even if this means that damage will accumulate later in life and eventually cause aging.

1.2.2 Mechanistic Theories

Many mechanisms have been proposed to explain the aging process, here only the five prevailing theories will be addressed. For a more exhaustive review refer to Medvedev, 1990 and Warner et al., 1987.

1.2.2.1 Defective Genome Maintenance Hypothesis of Aging

The first clue that defects in genome maintenance are involved in the aging process was provided by human segmental progeroid syndromes. A segmental progeroid is a heritable syndrome where some, but not all, of the signs of aging are accelerated (Hasty et al., 2003). Some of the better-known progerias include Hutchinson-Gilford, Werners and Cockaynes syndromes. Werners syndrome is thought to be the most realistic premature human aging

syndrome because of the age of onset and striking resemblance to normal aging (Martin, 1978). Most progeroid syndromes are caused by mutations in genes that are involved in the sensing or repair of DNA damage (Hasty et al., 2003). Therefore, the hypothesis is that since the etiology of these premature aging syndromes is defective genome maintenance, normal human aging must be caused by the slow accumulation of cellular damage that results from imperfect genome maintenance. Many mouse models with defective genomic integrity and accelerated aging phenotypes have been created (Blasco, 2002; Hasty et al.; 2003). These models provide genotype-phenotype correlations that give valuable insight into some of the basic mechanisms of aging, especially at the level of specific tissues (aging of the epidermis for example). The problem with this hypothesis is that none of the progerias, or the animal models, completely mimics normal human aging. At this point, while it seems as though defective or imperfect genome maintenance contributes to the aging phenotype, it has not been convincingly demonstrated that it plays a causative role in the aging process.

Yet there is a growing body of evidence that the expression of a wide variety of specific genes can affect an animal's longevity. Much of the work on the genetics of aging has been done in lower organisms and many genes that effect lifespan have been identified. In many instances, genes that affect longevity affect the ability of an organism to respond to stressors, including oxidative stress. A list of these genes and their relationship to several of the theories outlined below is presented in table 1.

1.2.2.2 Telomere/telomerase Hypothesis of Aging

The telomere/telomerase hypothesis of aging, while a component of the genomic maintenance theory of aging, is deserving of particular attention, not least because of the interest is has garnered in the field and popular press. Telomeres are tandem arrays of a GT rich sequence (5'TTAGGG'3) found at the end of linear chromosomes that, along with binding proteins, serve to cap and

protect chromosome ends from degradation or fusion. In the normal replicative process of somatic cells, telomeres shorten with each cell division because of what is referred to as the end replication problem (Levy et al., 1992; Olovnikov, 1996). Briefly, due to the mechanics of the replication process, the ends of linear molecules in lagging strand DNA synthesis are not completely replicated (Levy et al., 1992; Olovnikov, 1996). Telomerase is a ribonucleoprotein enzyme that elongates telomeric repeats; when expressed, it will prevent or delay telomere shortening (Levy et al., 1992). In the 1960's it was observed that cells cultured under optimal conditions would only undergo a finite number of population doublings before entering a senescent state (Hayflick and Moorehead, 1965). This number was a uniform and fixed number of ~ 50 population doublings, even in different cultures initiated from different single cells (Hayflick, 1965). Based on this observation, it was assumed that all normal cells have an intrinsically fixed replicative limit, referred to as the Hayflick limit (Hayflick, 1965). This phenomenon, the 'clock' intrinsic to all cells that determines when a cell enters senescence, was thought to be responsible for the aging of tissues in vivo and thus be the cause of aging of a whole organism.

Since telomeres shorten with successive rounds of replication, the telomere hypothesis of aging postulates that telomere shortening is the 'clock' that determines when replicative senescence will occur. Several lines of evidence give credence to this hypothesis. To begin with, telomere length in human cells does decrease with increasing age (Lindsey et al., 1991). Moreover, normal human cells in culture do not express telomerase so telomeres do shorten with successive rounds of replication until they reach a critically short length that induces growth arrest (Counter et al., 1992). If telomerase is expressed, telomere length is maintained and the lifespan of human cells in vitro is greatly increased (Counter et al., 1992). The question is, does the replicative senescence that occurs in vitro occur in vivo, and if so what are the consequences for the organism?

This issue has yet to be resolved, and unfortunately the vast majority of the literature on replicative senescence is contradictory. Many studies have

been criticized for failing to differentiate growth arrest that is induced by the stress of culture conditions from growth arrest that occurs due to critically short telomeres (Wright and Shay, 2002). Another problem has been that mouse cells in culture, a frequently used model system, do not undergo replicative senescence due to critically short telomeres, but rather senesce in response to the stress of culture conditions (Rubin, 2002). In fact, the weight of the experimental evidence suggests that this is also true of human cells in culture (Rubin, 2002). Many of the initial observations / assumptions (Rubin, 1997) have thus proved to be incorrect and raised skepticism about the relevance of replicative senescence in vivo. Work in this field continues to try and identify the physiological effects of replicative aging in vivo (Rubin, 2002; Wright and Shay, 2002); however, until these effects are demonstrated, there is not enough evidence to indicate that telomeres or telomerase play a causal role in the aging process.

1.2.2.3 Aging and the Immune System

Like many other physiological systems, the immune system is affected by advancing age. This 'immunosenescence' is evidenced by changes in the T cell repertoire, decreased antibody production, shortened duration of humoral protective immunity after immunization, decreased specific killer cell activity, increased non-specific killer cell activity, altered production of cytokines and increased cellular and humoral auto-immunity (Grubeck-Loebenstein and Wick, 2002; Pletz et al., 2003). These changes are thought to be adaptive, as opposed to degenerative. For example, the involution of the thymus, a major feature of immunosenescence (Franceschi et al., 2000b), reflects a trade-off: the decreasing usefulness of the thymus after the T cell repertoire has been established balanced against the cost of maintaining an organ in which an enormous number of cells are wasted daily (more than 95% of cells in the thymus die by apoptosis) in order to get rid of potentially autoreactive clones (George and Ritter, 1996). The changes in the immune system that occur as a

result of immunosenescence affect the ability of an organism to respond to immunologic stressors and this is thought to cause a shift towards a chronic pro-inflammatory state with age. Inflammation is a localized protective response elicited by injury or destruction of tissues, which is designed to destroy, dilute or sequester both the injurious agent and the injured tissue. However, if triggered inappropriately, inflammation causes tissue damage and is detrimental to the organism. This is illustrated by a number of age related diseases such as Alzheimer's, sarcopenia, osteoporosis and atherosclerosis (Franceschi et al., 2000a) that have an inflammatory pathogenesis. Therefore, it has been proposed, that changes in immune function and the establishment of a inflammatory state, create the necessary conditions for age related diseases to occur in susceptible individuals. Interactions between genes and the environment can thus affect how an individual ages. Immunosenescence is a controversial theory and much of the data on age-related changes in the immune system is contradictory or inconclusive.

1.2.2.4 Endocrine Regulation of Aging

The concept that the endocrine system plays a role in regulating the aging process has come about more recently, largely due to work on the genetics of aging. The basis for this theory is experimental evidence that indicates that dozens of the genes known to influence longevity are involved in hormonal signalling (Tatar et al., 2003). These genes and the systems in which they operate are largely conserved among eukaryotes.

The most powerful and well-studied example of endocrine involvement in aging is the insulin/insulin-like growth factor (IGF-like) signaling pathway. The first evidence that insulin/IGF-like signaling regulates aging was provided by the discovery of C. elegans mutants that demonstrate remarkable longevity. These long-lived worms, referred to as age-1 (ageless-1) or daf-2 (dauer formation-2), have mutations in genes that affect dauer formation, a non-feeding, stress resistant larval state that c.elegans will enter during times of stress or adverse

environmental conditions in order to survive (Dorman et al., 1995). Age-1 and daf-2 mutant worms will bypass dauer to become incredibly long-lived adults. Genetic analysis revealed that the age-1 and daf-2 genes are involved in insulin/IGF-like signal transduction thus putting this pathway as a central regulator of aging in C.elegans (Lin et al., 2001). The longevity of both age-1 and daf-2 mutants is dependant on a gene called daf-16, a fork head transcription factor that is a key regulator of heat and oxidative stress resistance, fat storage, developmental arrest, fertility and metabolism (Tatar et al., 2003). Together, this provided evidence that in C.elegans, aging is regulated by a central pathway that converges on the ability of an organism to withstand stress. It has since been demonstrated that insulin/IGF-like signaling pathways also regulate aging in insects (drosophila) (Tatar et al., 2001). Although the situation is more complex in mammals, there is evidence to suggest that this pathway operates in rodents. Four of the six genetic mouse models for longevity involve deficiency in pituitary endocrine action (Tatar et al., 2003), and all of these mice have impaired production of pituitary hormones such as growth hormone (GH), thyroid stimulating hormone and prolactin. GH directly affects IGF-1 synthesis. Thus, in rodents the insulin/IGF-1 signaling pathways may act downstream of GH/pituitary endocrine action to regulate longevity.

The problem with this theory is that GH and IGF-1 levels decline with age in rodents and in humans, and studies suggest that GH supplementation is beneficial (Blackman et al., 2002; Rudman et al., 1990). GH is widely promoted as an anti-aging hormone (Longo and Finch, 2003). It has been suggested that the withdrawal of GH and IGF-1 causes senescence rather than delays it. GH deficiency in humans can lead to reduced life expectancy (Longo and Finch, 2003) and GH and IGF-I deficiencies in humans are associated with major defects and diseases (Longo and Finch, 2003). While evidence in other species and rodent models points to GH and IGF-I as promoters of aging and age-related diseases, the effects of reduced GH and IGF-1 on human health and longevity appear to be more complex and have yet to be resolved.

1.2.2.5 Oxidative Stress and Aging

Free Radical Theory of Aging

In the1950s Denman Harman observed that exposure to ionizing radiation, which induces the formation of free radicals, produces phenotypic parallels to aging. This led him to propose the free radical theory of aging (Harman, 1956; Harman,1992a; Harman,1992b) where he hypothesized that free radicals produced as a by-product of metabolism (oxidative phosphorylation; see below) cause cumulative damage to cells, ultimately resulting in aging.

Biologists had long noted that there was an inverse relationship between the metabolic rate of an organism and its longevity (maximum lifespan potential); this observation formed the basis for Pearls 'rate of living theory' of aging (Pearl, 1928). Harman's free radical theory of aging provided a mechanism for metabolism to affect longevity; free radicals are produced as a by product of metabolism, and they cause the cellular damage that ultimately results in aging. This theory was an appealing one. Free radicals are ubiquitous in biology and their production and damage can be modified by genetics and environment, thus accounting for the heterogeneity in the aging process between individuals and species.

The Role of Mitochondrial Aging

The mitochondrial hypothesis of aging is a refinement of the free radical theory of aging. It postulates that there is mitochondria specific oxidative damage, and that the accumulation of oxidatively damaged mitochondria results in the aging process (Harman, 1972; Miquel et al., 1992). Since mitochondria are the most significant intracellular source of reactive oxygen species (ROS) (see below), mitochondrial proteins, lipids and DNA are directly exposed to high levels of ROS. Mitochondrial membranes are quite sensitive to oxidative damage because of their high lipid content and proximity to sites of free radical

production. Furthermore, because mitochondrial DNA (mtDNA) lacks introns and is not associated with histones or other DNA-associated proteins, the probability of oxidative modification of a coding region of mtDNA is very high (Lenaz, 1998) Mitochondrial DNA repair capabilities are also limited and often not sufficient to overcome significant damage (Lenaz, 1998).

Oxidative damage to mitochondrial membranes and protein components compromises the ability of the mitochondria to function and can result in respiratory chain defects (Ozawa, 1995; Ozawa, 1997) and increased production of ROS (Lenaz, 1998). Similarly, mutations in mtDNA caused by unrepaired oxidative DNA lesions can lead to coding errors in components of mitochondrial enzyme complexes resulting in impaired electron transfer and oxidative phosphorylation (Lenaz, 1998). These errors are stochastic and will be randomly transmitted during mitochondria and cell division (Lenaz, 1998). The resulting cycle of oxidative damage to mitochondria that results in the production of more free radicals and more damaged mitochondria ultimately causes damage to the cell and causes aging.

Since its inception, Harman's free radical hypothesis of aging has become the most popular and widely tested aging theory. The free radical theory and any other theory that places oxygen free radicals and the damage they cause at the center of the cause of aging are now generally referred to as the oxidative stress hypothesis of aging. Oxidative stress results when the balance between the production and elimination of free radicals is such that there is an overproduction of free radicals that causes damage to the cell.

1.3 Oxygen and the Production of Free Radicals

The 'oxygen paradox', that oxygen is toxic to the organisms that rely on it for energy production, is a key concept underlying the concept of oxidative stress. In aerobic respiration, 90% of cellular oxygen uptake is used by the mitochondria in oxidative phosphorylation to generate energy in the form of ATP (Jackson et al., 2002). Briefly, oxygen serves as an electron sink, undergoing

four sequential electron reduction reactions to ultimately generate water and a proton-motive force that is coupled to ATP production (Cadenas and Davies, 2000). While the mitochondrial electron transport chain (ETC) is very efficient, it is not completely without leaks; an estimated 1-2% of oxygen consumption goes towards the production of superoxide anion radicals ($O_2^{\bullet -}$) by the single electron reduction of oxygen in side chain reactions of the mitochondrial ETC (Cadenas and Davies, 2000; Jackson et al., 2002). These side chain reactions are thought to occur most abundantly at complexes I (NADPH dehydrogenase) and III (cytochrome C reductase) of the mitochondrial ETC (Finkel and Holbrook, 2000), although under normal physiological conditions complex III is the main site of free radical production. Enzymes in the mitochondrial outer membrane, not linked to respiration, are another important source of superoxide radicals (Cadenas and Davies, 2000). The superoxide anion radical, once produced, spontaneously dismutates to form hydrogen peroxide (H_2O_2). In the presence of free transition metals such as copper and iron, both $O_2^{\bullet -}$ and H_2O_2 will form the highly reactive hydroxyl radical ($OH^{\bullet}$) via the Fenton (figure 1, A) or Haber-Weiss reaction (figure 1, B) (Yu, 1994). Metal catalysis is a biologically important generator of free radicals in vivo (Yu, 1994).

(**A**) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet}$

(**B**) $Fe^{2+} + O_2^{-\bullet} + H_2O_2 \longrightarrow Fe^{3+} + O_2 + OH^- + OH^{\bullet}$

Figure 1. Fenton and Haber Weiss reactions. Fe^{2+}, reduced or ferrous iron; Fe^{3+}, oxidized or ferric iron.

Collectively, these partially reduced metabolites of oxygen are called free radicals or ROS and the mitochondria is the primary intracellular source of their

production. ROS are ubiquitous in cells, and they play an essential role in cellular signaling pathways and regulating cell function (Finkel and Holbrook, 2000; Jackson et al., 2002). In spite of their physiological relevance, ROS are highly reactive molecules that can be toxic and injurious to cells by reacting indiscriminately with cellular proteins, lipids and DNA.

1.3.1 Oxidative Damage to Lipids

A major feature of free radical mediated injury in cells is lipid peroxidation (Yu, 1994). Polyunsaturated fatty acids, a key component of cell membranes, are most easily damaged by free radical attack. The easiest hydrogens for a free radical to abstract, i.e., the weakest C-H bonds, are those in the bis-allelic methylene position; polyunsaturated fatty acids have a greater proportion of bis-allylic positions and are therefore more susceptible to free radical attack (Schafer et al., 2002). The abstraction of hydrogen from a polyunsaturated fatty acid generates a fatty acid free radical (figure 2, A) which rapidly takes up oxygen to produce a fatty acid peroxyl radical (figure 2, B). This fatty acid peroxyl radical is highly reactive and will abstract hydrogen, generating a lipid hydroperoxide and another radical (figure 2, C). If the peroxyl radical attack was on another fatty acid, the radical produced is another fatty acid free radical and the chain reaction of damage to lipids is continued (Griffiths et al., 2002; Schafer et al., 2002).

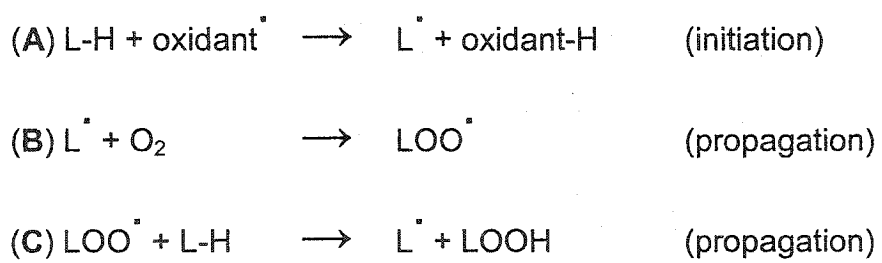

Figure 2. Schematic of chain reactions in oxidative attack on lipids. L-H, lipid; LOO•, peroxyl radical; LOOH, lipid hydroperoxide.

This kind of oxidative attack on lipids causes structural damage to membranes. It also results in the formation of cytotoxic secondary products via the fragmentation, rearrangement or cyclization of lipid hydroperoxides (Montine et al., 2002). The most abundant secondary products are produced via fragmentation. In general, these products are highly reactive electrophiles that, because they are more diffusible than free radicals, are able to travel to distant sites and cause damage. Biologically active aldehydes such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE) and 4-hydroxyhexenal (4-HHE) are the most commonly produced, and therefore measured, end products of lipid hydroperoxide fragmentation. These aldehydes are cytotoxic, mutagenic and are capable of inducing protein cross-links that inactivate/alter the function of many cellular components (Montine et al., 2002).

A minority of lipid hydroperoxides undergo internal cyclization to generate endoperoxide intermediates. These intermediates can be converted into multiple isomers with structures analogous to prostaglandins, i.e., contain a prostane ring. The reduction of endoperoxides derived from free radical attack of arachadonic acid results in the formation of isoprostanes, a relatively novel class of compounds that is frequently used as a marker of oxidative stress in vivo (Roberts and Morrow, 2002). Isoprostanes are also biologically active, they have been shown to affect gene expression, inhibit proliferation, and induce differentiation; they can also act as potent vasoconstrictors (Roberts and Morrow, 2002).

1.3.2 Oxidative Damage to Proteins

Proteins, more so than DNA and lipids, represent a diverse target for free radical attack. Therefore, many different kinds of damage can be done to cellular protein, including oxidative modification to both the peptide backbone and amino acid side chains (Stadtman, 2001). Different amino acids have different susceptibilities to oxidative attack, and thus the end product of oxidative attack

also depends on the specific protein target (Stadtman, 2001). The principal result of free radical attack on protein is the generation of a carbonyl group. This can occur through a variety of mechanisms including oxidation of some amino acid residues (lysine, arginine, and proline residues), cleavage of the peptide backbone via the α-amidation pathway or oxidation of glutamyl residues, and secondary reactions of amino acid side chains with lipid oxidation products, such as 4-HNE, or with reducing sugars or their oxidation products (Stadtman and Levine, 2000). These protein carbonyls are reactive and can result in intra- or inter-molecular protein cross links (Stadtman and Levine, 2000). The oxidatively modified proteins are targeted specifically to the 20S proteasome for degradation, but they are often resistant to proteolysis and can even inhibit the activity of the proteasome (Szweda et al., 2002). The result is that these modified proteins can loose function/activity and can accumulate and be cytotoxic to the cell. Because of their abundance and physiological relevance, carbonylated proteins are often used as molecular markers of oxidative damage to proteins (Stadtman and Levine, 2000).

1.3.3 Oxidative Damage to DNA

Over 100 different types of DNA lesions have been attributed to oxidative damage (Hasty et al., 2003). These include single and double strand breaks, interstrand cross links, abasic (apurinic/apyrimidinic) sites and base modification. The most common type of oxidative DNA damage is base modification. Of the 24 different types of base modifications identified so far, 8-hydroxyguanine is one of the most abundant and widely studied lesions (Wilson et al., 2003). Unrepaired 8-hydroxyguanine lesions cause GC-TA transversions and are therefore mutagenic. The abundance and physiological relevance make 8-hydroxyguanine lesions a commonly used marker of oxidative stress damage to DNA. Thymine glycol is another well studied oxidative base modification that occurs abundantly. Unlike 8-hydroxyguanine, thymine glycol is not mutagenic. Rather, it results in significant local distortion of the helix and causes a replication

block that is thought to be cytotoxic (Wilson et al., 2003). Of all the cellular macromolecules that are damaged by free radical attack, DNA has the most specific repair mechanisms designed to cope with oxidative damage. Base excision repair is the major pathway for removing and repairing oxidative DNA lesions although nucleotide excision repair, and transcription coupled repair play a role in the removal of helix distorting and replication blocking lesions respectively (Gros et al., 2002).

1.4 Cellular Responses to Oxidative Stress

Due to the essential yet potentially harmful nature of ROS, cells have evolved rather elaborate defense systems to protect against free radical induced damage. (Yu,1994). These defense systems include both enzymatic scavengers and a variety of low molecular mass molecules such as ascorbate, pyruvate, flavenoids and carotenoids. One of the most important and ubiquitous antioxidant defenses is glutathione, a tripeptide (glu-cys-gly) thiol-based antioxidant that is present in reduced form in millimolar concentrations in all living aerobic cells (Dickinson and Forman, 2002). The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) is a critical determinant of redox status of a cell (Pastore et al., 2003). The mechanisms by which glutathione can act as an antioxidant are many and include scavenging ROS directly and acting as a reducing agent in a number of enzymatic reactions (Dickinson and Forman, 2002) such as the glutathione S-transferase catalyzed conjugation of reactive electrophiles (figure 3, A) and the glutathione peroxidases catalyzed detoxification of peroxides (figure 3, B, C). In this way, glutathione protects the cell against both oxygen radicals and their toxic by-products.

Other important and ubiquitous enzymatic defenses against free radicals include superoxide dismutases (figure 3, D) which catalyze the dismutation of superoxide ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2) and catalase (figure 3, E) and which converts H_2O_2 to water (Yu, 1994).

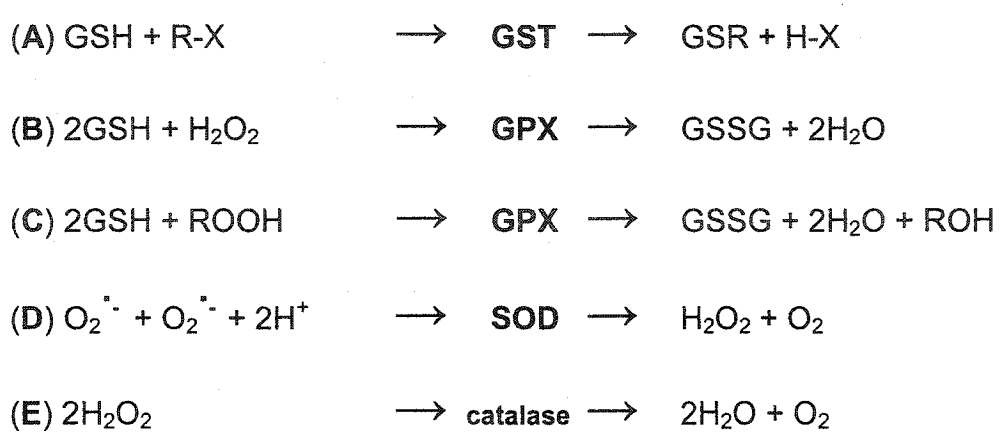

Figure 3. Schematic of reactions involving glutathione, superoxide dismutases and catalase. GSH, reduced glutathione; GSSG oxidized glutathione; GST, glutathione S-transferase; GPX, glutathione peroxidase; SOD, superoxide dismutase; R, electrophile; ROOH, peroxide.

The regulation of cellular antioxidant defense systems is complex and depends on the system in question. In general antioxidant defense systems are stress responsive and are regulated at the level of transcription. A number of proteins that help control the cellular redox status and defend against oxidative damage are ubiquitously expressed and coordinately induced under the regulation of the antioxidant response element (ARE), a *cis*-acting enhancer sequence. For example, enzymes associated with glutathione biosynthesis, redox proteins with active sulfhydryl moieties, and glutathione S-transferases (Nguyen et al., 2003) are transcriptionally regulated via signaling pathways that converge on the ARE (figure below). The glutathione content of a cell is determined by the balance between depletion from use and replacement by either reduction, or *de novo* synthesis. De novo synthesis of glutathione is constitutive to maintain basal levels but is also regulated in response to stress, for example in response to compounds that form glutathione-conjugates or generate ROS (Dickinson et al. 2003). The regulation of the different glutathione peroxidase isoforms that rely on glutathione is tissue specific and depends on

both post translational and transcriptional mechanisms including selenium availability and mRNA stability (Arthur, 2000; Brigelius-Flohe, 1999). All three isoforms of superoxide dismutases are controlled by extra- and intracellular conditions and are transcriptionally regulated in response to various mechanical, chemical, and biological messengers such as heat shock, UVB- and X-irradiation, heavy metals, hydrogen peroxide, nitric oxide, cytokines and arachadonic acid (Zelko et al., 2002).

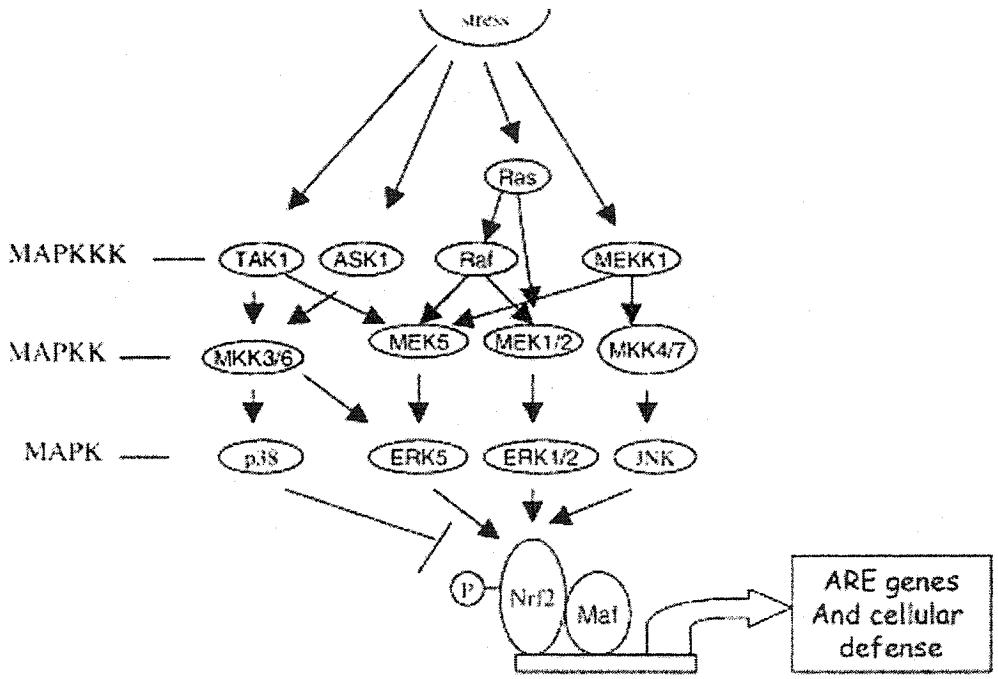

Figure 4. A model of the response to stress resulting in activation of the MAPK signaling pathway and Nrf2/Maf-dependent anti-oxidant responsive genes including phase II detoxifying enzymes and other stress enzymes. Modified from Owuor and Kong, 2002.

1.5 Accumulation of Oxidative Stress Damage with Age

Some of the earliest evidence that implicated oxidative stress in the aging process was the accumulation of oxidative stress damage with age. For

example, a classic (long recognized) 'hallmark' of an aging cell is the accumulation of lipofuscin, a brown-yellow, autofluorescent, electron-dense material, in lysosomes (Brunk and Terman, 2002). Although its composition has never been fully defined, lipofuscin is made up of oxidatively damaged cellular macromolecules that accumulate in lysosomes because the oxidative modifications render them resistant to degradation by lysosomal enzymes (same ref). More specifically, carbonyls (primarily aldehydes) from lipid peroxidation are thought to react with amino compounds to produce Schiff bases that display autofluorescent properties (Brunk and Terman, 2002). The relationship between oxidative stress and lipofuscin was confirmed when it was demonstrated that oxidative stress increases lipofuscin formation whereas antioxidant defenses prevent it (Terman and Brunk, 1998). Advances in the detection of oxidatively damaged cellular macromolecules have since provided ample evidence that the amount of oxidative damage to protein (Grune et al., 2001), lipids (Spiteller, 2001) and DNA (Bohr, 2002) increases during aging in a variety of tissues from a variety of species (Sohal and Weindruch, 1996).

The increased accumulation of oxidatively damaged molecules with age can reflect a number of possible changes in cell function with age. One possibility is that age-related increases in oxidative damage reflect increased production of oxygen free radicals in the cell. Indeed, it has been shown that rates of mitochondrial $O_2^{\bullet -}$ and H_2O_2 production increase as a function of age in a variety of organisms (Sohal and Weindruch, 1996). Furthermore, longevity has been shown to decrease under conditions where cells produce more ROS.

Another possibility is that the other side of the equation, the antioxidant defense systems that protect the cell against ROS are impaired with age. The effects of age on antioxidant levels are variable and no consistent trend has been reported (Sohal et al., 2002). Evidence suggests that supplementation of antioxidant defenses is effective in vitro (Saretzki and von Zglinicki, 2002) and in lower organisms (Melov et al., 2000). However, in mammals antioxidant supplementation, while effectively attenuating oxidative stress associated

disease processes, has had no effect on longevity (Meydani et al.,1998). Genetic manipulation of components of cellular antioxidant systems in lower organisms has been somewhat successful in increasing longevity. For example, in some studies, over expression of superoxide dismutase (SOD) alone or in combination with catalase increases mean life span of adult drosophila flies by 12% (CuZnSOD), 16% (MnSOD) or 34% respectively (Sun and Tower, 1999; Sun et al., 2002; and Orr and Sohal, 1994). In other studies, overexpression of SOD or catalase alone in adult flies had little or no effect on longevity (Orr and Sohal, 1992 (catalase); Orr and Sohal, 1993 (SOD); Seto et al., 1990). The reasons for these discrepancies are unclear, but it has been suggested that the background of the drosophila strain used in the study may account for the differences (Orr and Sohal, 2003). Overexpressing CuZn SOD in mice had no effect on lifespan (Huang et al., 2000). Together these studies suggest that increasing oxidative defenses is not the most promising way to impact lifespan.

The final possibility is that the ability of a cell to cope with oxidative damage is impaired with age. Since young healthy cells exhibit some degree of oxidative damage, it is clear that repair or elimination processes designed to deal with oxidative stress are imperfect to begin with. Age affects these processes in a number of ways. The ability of a cell to respond to oxidative stress is blunted with age (reviewed in Roy et al., 2002), as is the ability of a cell to repair oxidative lesions in both nuclear and mitochondrial DNA (Bohr, 2002). The proteasome is a multi-catalytic proteolytic complex, which recognizes and selectively degrades oxidatively damaged and ubiquitinated proteins (Carrard et al., 2002). Evidence for age-related impairment of proteasome structure and function has been reported and is suggested to be dependent on at least three different mechanisms: decreased proteasome expression; oxidative alterations and/or replacement of proteasome subunits, and the inhibitory actions of oxidatively damaged proteins on proteasome activity (Carrard et al., 2002). The other component of intracellular proteolysis, the lysosome, may also be affected by age; lysosomes accumulate lipofuscin with age (see above); this may affect lysosomal function (Szweda et al., 2002).

1.6 Tools for the Study of Aging

1.6.1 Caloric Restriction

Caloric restriction, the undernutrition without malnutrition of an organism, is the single most powerful manipulation available to researchers in the field of aging. Caloric restriction is the only regimen known to result in the consistent, robust and reproducible extension of the maximum life span (age at death of the 10^{th} percentile of survivors) of a number of organisms. Moreover, caloric restriction delays or attenuates a variety of age-associated impairments and pathologies without causing irreversible developmental or reproductive defects. Caloric restriction has been studied for a long time. In the early 1900's, the effects of caloric restriction on carcinogenesis (Rous, 1914) and longevity (Osborne et al., 1917; Osborne and Mendel, 1915) in rodents were beginning to be documented. These initial studies were followed up in the 1930's by those of McCay and et al. who concluded that caloric restriction in rodents results in an extension of life span, and that this extension is due to growth retardation (McCay et al., 1935; McCay et al., 1939). By the 1970's research into the anti-aging effects of caloric restriction intensified and several conclusions were reached:

- overall reduction of caloric intake, not restriction of a particular nutrient, is responsible for the extension of lifespan (Yu, et al., 1985)
- caloric restriction initiated in the young adult (6mo) rat is as effective as caloric restriction initiated at 6wks of age (Yu, et al., 1985)
- caloric restriction initiated in the middle aged (12mo) rat significantly increases life span although not as markedly as caloric restriction initiated at or just after weaning (4wks) (Weindruch and Walford, 1982)
- caloric restriction only during rapid growth from 6wks to 6mos of age is not nearly as effective at extending life span (Yu, et al., 1985)

These results refuted McCay's original hypothesis, that caloric restriction extends life span by growth retardation; they established that simply a reduction of caloric intake (25-60% less than control animals fed ad libitum) will extend life span in a remarkable range of organisms.

The question remains, how does reducing calories result in life extension? While there is as yet no definitive resolution to this question, several hypotheses have been proposed. Early on, it was thought that the life extending actions of caloric restriction were due to a reduction of body fat (Berg and Simms, 1960), or decreased metabolic rate (Sacher, 1977) however experimental evidence (Bertrand et al., 1980; McCarter and Palmer, 1992) has since refuted these hypotheses. The three theories that are currently garnering the most attention will be discussed below.

1.6.1.1 Oxidative Damage Attenuation Hypothesis

The idea that caloric restriction works by attenuating oxidative stress damage (Sohal and Weindruch, 1996) came out of the observation that caloric restriction animals accumulate less oxidative stress damage with age. Caloric restriction attenuates the age-associated increase in lipid peroxidation (Matsuo et al., 1993), the accumulation of oxidized proteins (Dubey et al., 1996), and the oxidative damage of DNA (Lopez-Torres et al., 2002; Sohal et al., 1994). Caloric restriction has no consistent effect on antioxidant enzymes (Sohal and Weindruch, 1996), however age-related increases in oxidant production are attenuated in mitochondria from caloric restriction animals as compared to AL control animals. (Lopez-Torres et al., 2002; Sohal and Dubey, 1994). There is also evidence to suggest that caloric restriction enhances the repair of oxidatively damaged molecules. DNA repair capabilities are increased (Haley-Zitlin and Richardson, 1993), and the age related decline in proteolytic removal of damaged proteins is attenuated in caloric restriction animals (Merker et al., 2001). Caloric restriction has a profound effect on the gene expression in a number of tissues and organisms including genes involved in the response to

oxidative stress and mitochondrial respiration (reviewed in Longo and Finch, 2003; Prolla, 2002; Weindruch et al., 2002). For example in mouse gastrocnemius muscle, caloric restriction completely prevented or attenuated age-related increases in expression of genes involved in the stress response and energy metabolism. (Weindruch et al., 2002). Similar effects were noted in a rat muscle (Sreekumar et al., 2002), rhesus monkey muscle (Kayo et al., 2001), liver (Cao et al., 2001) and mouse brain (Weindruch et al., 2002).

Caloric restriction also modulates the activity of redox sensitive transcription factors such as AP-1, NFκB and HIF-1 and so may affect the cellular response to oxidants by altering signal transduction pathways (Kim et al., 2002). Taken together, these results provide evidence that caloric restriction alters oxidative stress load and the ability of a cell to respond to oxidative stress. Until the role of oxidative stress in the aging process is established, the validity of the hypothesis remains unproven.

1.6.1.2 Stress Response

The ability to withstand stress is clearly associated with longevity (Table 1); genetic manipulations of yeast, nematodes, drosophila and mice that affect lifespan also affect the ability of the organism to withstand stress, such as UV radiation, heat and oxidative stress (Masoro, 2000a). The idea that caloric restriction is a chronic low-intensity stressor (caloric restriction animals have moderately elevated glucocorticoid levels, an indicator of stress) that enables an organism to better withstand stress has been demonstrated in a number of different organisms under a number of different stress conditions (Masoro, 1998; Masoro, 2000b). The mechanism by which caloric restriction affects the ability of an organism to withstand stress is controversial but recent discoveries in the mechanics of caloric restriction in yeast are beginning to shed some light In 2000, it was demonstrated that the caloric restriction pathway in yeast is dependant on a gene called SIR2 (silent information regulator 2) (Lin et al., 2000). SIR2 codes for a histone deacetylase that, by altering chromatin

conformation, can regulate the expression of other genes (Hekimi and Guarente, 2003). In yeast, the SIR2 protein is targeted to ribosomal DNA where it induces transcriptional silencing through histone deacetylation (Hekimi and Guarente, 2003; Imai et al., 2000). This promotes longevity as it prevents the accumulation of extrachromosomal ribosomal DNA circles that are toxic to the cell (Hekimi and Guarente, 2003). Mutations in the SIR2 gene prevent the longevity conferred by caloric restriction (Lin et al., 2000). The SIR2 protein is dependant on a co-factor, nicotinamide adenine dinucleotide (NAD), for its activity (Imai et al., 2000) and so the hypothesis is that SIR2 senses caloric restriction through changes in the NAD/NADH ratio and induces transcriptional silencing that results in longevity (Lin and Guarente, 2003). In 2003, it was demonstrated that yeast lifespan extension by caloric restriction is also dependant on another gene, PNC1, which encodes an enzyme that metabolizes nicotinamide (Anderson et al., 2003). Nicotinamide inhibits the SIR2 protein and thus PNC1 is necessary for SIR2 protein activity. Interestingly, the PNC1 gene appears to be responsive to other life-extending treatments in yeast in addition to caloric restriction (Anderson et al., 2003). These results suggest that in yeast, diverse stimuli can converge on and activate SIR2 to extend lifespan and therefore SIR2 functions as a general regulator of longevity. These results provide insight into the mechanism of action of caloric restriction; however, whether or not the SIR2 gene plays a role in caloric restriction in higher animals has yet to be established.

1.6.1.3 GH-IGF Axis

Many physiological changes occur in animals that have been calorically restricted. These include lower core body temperature; lower levels of plasma glucose, insulin, growth hormone (GH), thyroid hormones, and insulin-like growth factor-1 (IGF-1); increased insulin sensitivity; increased levels of glucocorticoids.; decreased body size and body fat (Longo and Finch, 2003). In worms and flies, mutations that downregulate components of the GH/IGF-1 pathway confer remarkable longevity and stress resistance (Table 1). The role of GH and IGF-1

in lifespan determination is also conserved in mice; mutations that affect pituitary production of GH or the IGF receptor increase lifespan and confer resistance to stress (see table 1). These long lived mutant mice, like calorically restricted mice, have lower plasma levels of GH, IGF-1, glucose and insulin (Longo and Finch, 2003). These observation have led to the hypothesis that caloric restriction extends lifespan by decreasing the activity of the GH/IGF-1 pathway.

GH and IGF-1 can promote cellular damage in vivo; both have been shown to decrease the activities of antioxidant enzymes and IGF-1 attenuates the stress response (Longo and Fabrizio, 2002). High levels of IGF-1 are also associated with several human diseases, including some cancers (Monzavi and Cohen, 2002). However, in rodents and in humans, GH and IGF-1 levels decline with age, and short-term supplementation in aged humans restores some aspects of function (Savine and Sonksen, 2000). In fact, GH deficiency in humans can lead to reduced life expectancy; therefore, decreased levels of GH and IGF-1 are often thought to cause aging rather than prevent it (Conceicao, et al., 2001). Moreover, when calorically restricted, long-lived GH/IGF-1 deficient mice (ames dwarf or prop-1df mice) live longer than their ad libitum fed counterparts (Bartke et al., 2001b) or wild type mice on caloric restriction alone. These observations suggest either that the mechanisms by which caloric restriction and GH/IGF-1 deficiency confer longevity may be different or that, under the test conditions, neither was able to produce their fullest potential gain in lifespan.

1.6.2 Vitamin E

The term vitamin E generally refers to a group of lipid-soluble molecules consisting of a chromanol ring and an isoprenoid side chain that have potent antioxidant activity. Eight such compounds are produced in nature; four tocopherols and four tocotrienols, the most abundant of which is α-tocopherol (figure 5).

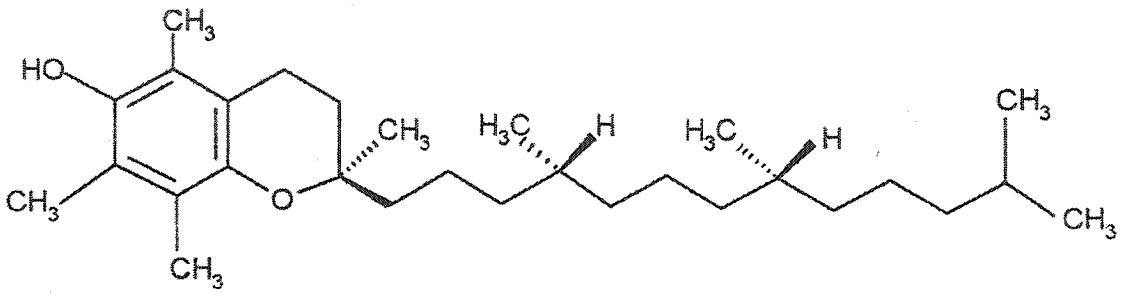

Figure 5. RRR-α-Tocopherol. Gao et al., 2002)

Vitamin E was first discovered as a micronutrient that was obligatory for reproduction in female rats in 1922 (Evans and Bishop, 1922). RRR-α-tocopherol (formerly d-α–tocopherol) is the only naturally occurring stereoisomer of α-tocopherol and the most potent in biologic assays (fetal resorption assays) (Brigelius-Flohe and Traber, 1999). Synthetic vitamin E (all-rac(racemic)-α-tocopherol) consists of the 8 possible stereoisomers, or their esters, in equal amounts. The bioavailability and bioequivalence of different forms of vitamin E differ because optical isomerism affects activity. Different isomers may have different modes and/or rates of metabolism, and they may interact with specific receptors. There are also selective processes for vitamin E absorption (Brigelius-Flohe and Traber, 1999). The international standard of activity for vitamin E is all-rac-α-tocopherol acetate which has been designated as having a biologic activity of 1.0 IU/mg. Comparatively, the naturally occurring isomer RRR-α-tocopherol has a biologic activity of 1.49 IU/mg and the synthetic all rac-α-tocopherol has a biologic activity of 1.1 IU/mg (Vitamin Deficiency, Dependency, and Toxicity. In The Merck Manual of Diagnosis and Therapy, Seventeenth Edition).

Vitamin E is taken up together with dietary lipids and bile in the proximal part of the intestine (Brigelius-Flohe and Traber, 1999), assembled into chylomicrons and transported to the liver. In the liver, the hepatic protein, α-tocopherol transfer protein (α-TTP), selectively sorts all tocopherols. α-TTP has a preference for 2R-stereoisomers (Hosomi et al., 1997) therefore α-tocopherol is preferentially incorporated into very low density lipoprotein and transported to the

periphery; the other tocopherols are not well retained. Vitamin E isomers are metabolized into CEHCs (2[2'-carboxyethyl]-6-hydroxychroman) by cytochrome p450-mediated ω-hydroxylation followed by β-oxidation before urinary, biliary or fecal elimination (Brigelius-Flohe et al., 2002).

Vitamin E is distributed from the blood into other tissue compartments in a manner similar to fat-soluble drugs; peak levels are reached rapidly in the blood and are redistributed into other compartments. In plasma, vitamin E incorporation is a saturable process (Brigelius-Flohe and Traber, 1999) however tissues are difficult to saturate with vitamin E (Machlin and Gabriel, 1982). In experimental animals there is a linear relationship between the tocopherol content of tissues and the logarithm of the dose administered (Machlin and Gabriel, 1982). This is true over a wide range of intakes for a number of tissues including platelets, liver, muscle, testes, spleen and lung although the rate of accumulation differs depending on the tissue (Machlin and Gabriel, 1982).

In humans, Vitamin E deficiency due to inadequate dietary intake is rare, although vitamin E deficiency anemia is occasionally observed in premature infants (Brigelius-Flohe et al. 2002). Heritable mutations in α-TTP (referred to as familial isolated vitamin E deficiency or ataxia with vitamin E deficiency) or apolipoprotein B deficiencies (abetalipoproteinemia and homozygous hypobetalipoproteinemia) lead to severe vitamin E deficiency. Patients with these syndromes primarily present with neurologic symptoms and myopathies (Ricciarelli et al., 2002). In experimental vitamin E depletion in mature rats, liver, heart and plasma α-tocopherol concentrations were reduced by more than 50% within 1-2 weeks of initiating depletion. In contrast, α-tocopherol concentrations in fat, testis and muscle declined more slowly (Bieri, 1972). In spite of the large sources of α-tocopherol stored in the liver and fat (adipose tissue is thought to contain ~90% of total body α-tocopherol), body stores of α-tocopherol do not maintain plasma levels until they have fallen to 50% or less of normal values (Bieri, 1972).

The toxicity of Vitamin E is very low; it has been demonstrated in animal experiments that vitamin E has no mutagenic, teratogenic or carcinogenic

properties (Kappus and Diplock, 1992). In rats, single doses up to 5000 mg α-tocopherol/kg body weight and up to 2000 mg all-rac-α-tocopherol acetate/kg/day for 104 weeks have been tested with no significant toxicity. In adult humans, 200 to 800 mg/d is generally tolerated without adverse effects, with the exception of gastrointestinal upset. With doses of 800 to 1200 mg/d, antiplatelet effects and bleeding may occur. Doses higher than 1200 mg/d may result in headache, fatigue, nausea, diarrhea, cramping, weakness, blurred vision, and gonadal dysfunction (Fairfield and Fletcher, 2002). High levels of vitamin E are contraindicated in subjects who are receiving vitamin K antagonists as anticoagulant therapy. The current recommended daily intake for vitamin E is 20 mg (30 IU) in the USA and 15 mg of α-tocopherol in Canada. Major dietary sources of vitamin E include vegetable oils, margarine, legumes, and nuts (Stahl et al., 2002).

The antioxidant functions of vitamin E are well known; vitamin E is the most widely distributed antioxidant in nature (Yu, 1994). In cells, Vitamin E partitions into hydrophobic environments where it functions as a chain-breaking antioxidant that prevents the propagation of free radical reactions. Its subcellular localization makes vitamin E particularly important in protecting cells against lipid peroxidation (see above in oxidative damage section). Vitamin E is most important in scavenging lipid peroxyl radicals (figure 6, A) and preventing the propagation of lipid peroxidation (Chaudiere and Ferrari-Iliou, 1999; Wang and Quinn, 1999). The α -tocopheroxyl radical can react with another peroxyl radical (figure 6, B) or be regenerated (reviewed Wang and Quinn, 1999; see below).

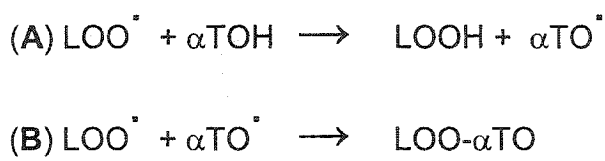

Figure 6. Schematic of reactions involving α-tocopherol. αTOH, α-tocopherol; αTO•, α -tocopheroxyl radical.

Vitamin E will also react with other radical species such as singlet oxygen, alkoxyl radicals, peroxynitrite, nitrogen dioxide, ozone and superoxide although the scavenging of peroxyl radicals is thought to be much more biologically significant. The efficacy of Vitamin E against peroxyl radicals stems from the fact that the rate constant of the reaction of vitamin E with a peroxyl radical is considerably faster ($k \sim 8 \times 10^4$ $m^{-1}s^{-1}$, Buettner, 1993) than the rate at which a peroxyl radical will react with a lipid substrate ($k_{propagation}$, depends on the strength of the C-H bond being broken, $k_p \sim 10$ $m^{-1}s^{-1}$, Porter et al., 1995). The resulting tocopheroxyl radical ($\alpha TO^{\bullet}$) is considerably stabilized through extended aromatic delocalization and therefore relatively unreactive, but it is a weak oxidant and so must be removed (Chaudiere and Ferrari-Iliou, 1999). The regeneration of α-tocopherol by cellular reducing systems is important in its biological activity. In vitro α-tocopherol can be regenerated from its tocopheroxyl radical form by vitamin A, vitamin C and coenzyme Q (Wang and Quinn, 1999).

Many epidemiological studies have reported that populations with higher intake and higher blood levels of vitamin E have lower risks of coronary heart disease, even after corrections have been made for other known risk factors. In prospective cohort studies, where data on dietary intake and vitamin status are collected before the onset of disease, there was an inverse association between dietary intake of vitamin E and coronary heart disease risk. Individuals in the top fifth of vitamin E consumption have 30–40% lower risk of cardiovascular disease (Reviewed in Clarke and Armitage, 2002; original studies: Knekt et al., 1994; Rimm et al., 1993; Stampfer et al., 1993 ; Salonen et al., 1985). These studies, in conjunction with experimental evidence in animals and the recognized potency of vitamin E as an antioxidant, generated a lot of interest in the potential ability of vitamin E to prevent chronic diseases. In particular, diseases and conditions believed to have an oxidative stress component, such as cardiovascular and neurodegenerative diseases and cancer were the primary focus of clinical research in this area. Many clinical trials have been completed and others still

are ongoing. They are summarized in table 2. The effect of vitamin E on these different disease states is inconsistent. In particular, there has been extensive debate and discussion about the contradictory (conflicting) effects of vitamin E supplementation on cardiovascular diseases. The debate and potential reasons for the conflict are reviewed in Meagher, 2003 and Riley and Stouffer, 2003. It is worth mentioning however, that while some of the cardiovascular disease risk trials measured vitamin E concentrations and reported several-fold rises in plasma vitamin concentrations (ATBC Cancer Prevention Study Group [No authors listed], 1994; Heart Protection Study Collaborative Group, 2002; Stephens et al. 1996), no trial assessed the efficacy of antioxidant supplementation by measuring markers of antioxidant action such as lipid peroxidation. This leaves open the possibility that the vitamins used might have been inadequately dosed, given to patients with low-level oxidative stress, or given too late in the course of cardiovascular disease to achieve a clinically relevant impact from inhibition of oxidation (Vivekanantha et al., 2003). The controversy surrounding vitamin E therefore, pertains to its role in cardiovascular disease prevention, not its efficacy as an antioxidant.

1.7 The Aging Male

The idea that women undergo reproductive senescence, or menopause, with age, and that this profoundly impacts female health and psychology is unquestionable. The concept that the aging male might experience a similar endocrine senescence is more controversial. Initial cross sectional studies on testosterone levels in aging men were conflicting. However, two large longitudinal studies, the Massachusetts Male Ageing Study (Feldman et al., 2002) and the Baltimore Longitudinal Study of Ageing (Harman et al., 2001) provided conclusive evidence that there is a gradual decline in testosterone levels with age. A concomitant increase with age in plasma binding protein (sex hormone binding globulin) levels translates into even larger decreases in free (bioavailable) testosterone with age. The term 'andropause' was coined to refer

to this phenomenon, and while it conveys the notion of the physiological and psychological changes that accompany declining hormone levels, it is biologically incorrect. Androgen decline in the aging male, unlike menopause in women, is gradual and not universally experienced.

Under normal circumstances in the adult male, testosterone production is regulated by the hypothalamic–pituitary axis (HPA). Pulsatile gonadotropin-releasing hormone (GnRH) released from the hypothalamus stimulates the pituitary gland to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH acts on Leydig cells in the testis to stimulate testosterone production. Testosterone released into the blood-stream provides negative feedback on the hypothalamus to inhibit GnRH and gonadotropin secretion. Decreased testosterone levels in the aging male can be attributed to changes in both the HPA and Leydig cells in the testis. Hypothalamic dysfunction results in decreased GnRH secretion, which in turn affects LH secretion. With age, LH pulse frequency is increased and pulse amplitude is decreased. At the level of the testis, there is decreased Leydig cell mass and function; for every LH pulse less testosterone is produced (reviewed in Yialamas and Hayes, 2003).

The clinical consequences of hypogonadism in the aging male are thought to be many and include: decreased libido, general well-being, cognitive performance, muscle mass and strength, as well as increased insomnia and abdominal fat. While few large-scale, long term, well-controlled studies have been done, there are many smaller scale studies on the effects of testosterone replacement in older males. The results indicate that testosterone replacement had positive or neutral effect on mood and sexual functioning and a variable effect on cognitive function (Gruenewald and Matsumoto, 2003). The effects of testosterone replacement on bone mineral density and body composition are the best studied and the results indicate that testosterone supplementation improves bone mineral density, increases lean body mass and decreases abdominal fat but has no effect on muscle strength (Gruenewald and Matsumoto, 2003). The beneficial effects of testosterone replacement therapy in these studies have generated a lot of attention. However, along with other side effects, testosterone

supplementation may increase the risk of benign prostatic hypertrophy and prostate cancer. Therefore, more large scale randomized controlled trials need to be done before testosterone supplementation is routinely advocated for controlling the symptoms of endocrine senescence in aging males.

Increasing recognition of endocrine senescence in the male has generated much interest in this field of research. The need to better understand aging of the male reproductive tract has resulted in the search for appropriate animal models in which to study different aspects of male reproductive aging.

1.8 Animal Models for the Study of Male Reproductive Aging

In aging studies, it is crucial that the animal model used is both appropriate for the physiological system under study, and free of age-related pathologies that may confound interpretation of the studies. In order to study aging of the male reproductive tract, the ideal model would demonstrate the following characteristics: have long, disease-free survival rates, be free of confounding age-related tumors, hormonal changes and systemic illnesses; show age-related changes in circulating levels of trophic and metabolic hormones, and alterations in body composition, food intake and body weight regulation similarly to those observed in aging men. Of all the available species a non-human primate model would clearly be the closest approximation of aging in humans. There are rhesus monkey colonies (M. mulatta) maintained by the National Institute on Aging (http://www.nia.nih.gov/research/resources.htm) that are available to researchers for aging studies, however, these monkeys are only approved for non-invasive research thus making their use quite limited.

Numerous rodent models are available that fulfill most of the above criteria. Male reproductive physiology is best understood in the rat. This fact, combined with the inter-strain variability and smaller size (smaller amount of material to work with) of mice, makes the rat a more appealing species to study. However, the biggest problem with almost all aging rat models is that the mechanism of reproductive senescence is different from that in aging men.

Nearly all rats (including Sprague-Dawley and Fischer 344 [F344] rats) exhibit predominantly secondary testicular failure with aging; LH, FSH, and testosterone concentrations progressively decline with age (Kaler and Neaves, 1981; Gruenewald et al., 1992; Pirke et al., 1978). In contrast, aging men exhibit both primary testicular and secondary hypothalamic-pituitary gonadotropin dysfunction (Yiamalas and Hayes, 2003). Furthermore, secondary testicular failure in aging rodent models may be associated with pituitary adenomas (e.g. Sprague-Dawley and F344 rats), often hyperprolactinemia (e.g. F344, Long-Evans, and Wistar rats) (Bethea and Walker, 1979; Cohen et al., 1978; Demarest et al., 1980; Sandusky et al 1988; Simpkins and Gabriel, 1984).

The inbred barrier-reared, specific pathogen-free rat models available from the National Institute on Aging and used in studies of aging are the inbred F344/Nnia (F344) and BN/BiRijNia (brown Norway, BN) and the F1 hybrid F344BN/Nia (F344BN). Old F344 male rats uniformly develop testicular Leydig cell tumors that secrete excessive amounts of progesterone and other steroids (Amador et al., 1985; Gruenewald et al., 1992; Turek and Desjardins, 1979). Like other rat models, F344 male rats exhibit secondary testicular failure with aging, characterized by a progressive decline in serum gonadotropin and testosterone levels, and decreased expression of GnRH (Gruenewald et al., 1992). Orchidectomy (removal of the testis) in these aged animals reduces serum levels of progesterone and other testicular steroids and results in gonadotropin levels and hypothalamic GnRH gene expression that are similar to that in young animals. (Gruenewald et al.,1992). This suggests that the age-related decline in gonadotropin levels and GnRH expression are dependent upon testicular feedback, most likely the high circulating concentrations of progesterone secreted by Leydig cell tumors in the old F344 male rats, not hypothalamic dysfunction.

The F344/BN F1 hybrid rat has a much lower incidence of age-related pathology and a longer disease-free survival rate than either of the pure bred parental rat strains (Sprott, 1991). However, old F344/BN 1 hybrid male rats also develop Leydig cell hyperplasia and tumors, although less frequently and at older

ages than in F344 old male rats (Bronson, 1989; Masoro, 1991;Thurman, 1995). It is not clear whether these animals develop both primary and secondary testicular dysfunction with age because age-related changes in sex steroid, gonadotropin and prolactin secretion have not been characterized fully in this strain. In order to use this strain for aging studies, testis histology and hormone levels would need to be evaluated in all gonadally intact animals to be certain that confounding effects of tumors and/or excessive secretion of progesterone or other hormones such as prolactin are not contributing to changes being attributed to aging per se.

In contrast to other aging rodent models, BN male rats demonstrate both primary and secondary testicular failure with aging (Chen et al., 1994; Gruenewald et al., 1994; Wang et al., 2002; Zirkin et al., 1993). Compared to young animals, old (~24 month old) and senescent (~30 month old) BN male rats have decreased Leydig cell testosterone production and serum testosterone levels, and histological spermatogenesis and Sertoli cell inhibin B production also decrease (Wang et al., 1993). These age-related changes in testis function occur in association with stable serum LH and a progressive increase in serum FSH concentrations in intact animals, and a progressive decrease in both LH and FSH levels in orchidectomized rats. These hormonal changes are consistent with an etiology of both primary and secondary testicular failure (Gruenewald et al., 1994). BN male rats also have a relatively long disease-free survival (Bronson, 1989), and do not develop hyperprolactinemia, hyperprogesteronemia, pituitary adenomas or Leydig cell tumors (Gruenewald et al., 1994). The most common age-related disease that affects male BN rats is bladder carcinoma which occurs in ~35% of animals at about 27 months of age (Burek and Hollander, 1977).

1.9 The Epididymis

The epididymis, a single, highly convoluted tubule that links the efferent ducts to the vas deferens, is a component of the male excurrent duct system and functions in the transport, maturation (acquisition of fertilizing potential), and

storage of spermatozoa. The epididymal duct is made up of two major compartments: the epithelium and the lumen. Anatomically and histologically the tissue is divided into four segments: the initial segment, caput, corpus and cauda.

The epithelium is composed of four major cell types: principal, basal, clear and halo cells (Hermo and Robaire, 2002). The principal cell is the primary cell type of the epididymis; these cells are tall, columnar cells that appear to be very active in the transport and secretion of small organic molecules, protein synthesis and secretion, and the absorption of fluid and particulate matter (Robaire and Hermo, 1988). Basal cells are small elongated cells found throughout the epididymis; their function is unknown, but it has been suggested that they play a role in detoxification because they have high levels of enzymes that detoxify ROS (Nonogaki et al., 1992; Veri et al., 1993). Clear cells are large cells that span the epithelium and are thought to actively remove materials from the lumen (Hermo et al., 1988; Moore and Bedford, 1979). Finally, halo cells are found throughout the epididymis in various positions within the epithelium, but not spanning it; these cells are the immune cells of the epididymis (Serre and Robaire, 1999).

The lumen of the epididymis is filled with spermatozoa and a fluid that changes in composition as one moves along the tissue from the initial segment to the cauda epididymdis. Spermatozoa therefore, are exposed to a continually changing environment as they traverse the epididymis (Olson et al., 2002; Robaire and Hermo, 1988; Turner, 1991). Spermatozoa enter the epididymis as immature germ cells; the process of sperm maturation is an active one and can only occur by exposure to the epididymal environment (Olson et al., 2002; Orgebin-Crist and Olson, 1984; Robaire and Hermo, 1988). By the time spermatozoa reach the cauda epididymidis they are mature and have acquired the ability to fertilize an egg.

In addition to histological and anatomical differences, the different segments of the epididymis have also been shown to be biochemically and functionally distinct (Hermo and Robaire, 2002). For example, there are numerous instances of region specific gene and protein expression along the

tissue (Cornwall et al., 2002; Dacheux et al 2003; Kirchhoff, 2002). Moreover, the different segments are also distinct in that they respond differently to experimental manipulation as segment specific responses under different conditions have been reported (Brooks, 1976; Ezer and Robaire, 2002; Hinton et al. 1998). Different regions of the tissue are functionally distinct and exhibit differential gene expression; the regulation of gene expression is essential in establishing the environment required for sperm maturation. This makes the epididymis an interesting model tissue in which to study gene expression and the regulation of gene expression.

With age, the epididymal epithelium undergoes dramatic morphological changes including the acquisition of morphological hallmarks of aging, such as the accumulation of lipofuscin, lysosomes and vacuoles and the thickening of the basement membrane (Serre and Robaire, 1998b). These changes are independent of the presence or absence of spermatozoa and occur in a segment-specific manner. With age there is also a segment-specific increase in the number of immune cells (halo cells) in the epididymal epithelium (Serre and Robaire, 1998b). The activation of the immune system may result from age related changes in the structure and function of the blood-epididymis barrier; the expression of junctional proteins is affected by age through the length of the tissue but most notably in the corpus (Levy and Robaire, 1999). Age-related, segment-specific changes in the expression of transcripts for selected markers of epididymal function, such as 5α-reductase isozymes, types 1 and 2, proenkephalin, androgen receptor, epididymal proteins B/C and D/E, and sulfated glycoprotein-2 have also been demonstrated (Viger and Robaire, 1995). These results indicate that both the structure and function of the tissue are affected by age. Impaired epididymal function with age directly impacts the production of spermatozoa; sperm from older animals have altered morphology, motility and shedding of the cytoplasmic droplet. These age-related changes in sperm affect progeny outcome; progeny fathered by older males have higher incidence of pre-implantantion loss, a significant decrease in the average fetal weight and a significant increase in neonatal deaths (Serre and Robaire, 1998a).

The epididymis is a particularly attractive model in which to study aging. The epididymis ages and is clearly affected by aging; however this does not compromise the overall health of the animal. Aging in the epididymis can therefore be studied without the confounding influence of disease. Additionally, gene expression in the epididymis is highly complex with tightly regulated region, and cell specificity. Thus, the epididymis is a particularly interesting tissue in which to study gene expression and the effects of age on gene expression. For these reasons we undertook to study the effects of aging on gene expression in the epididymis.

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Table 1. Genetic pathways of longevity that converge on oxidative stress/ stress resistance or susceptibility.

Gene/Pathway	Gene Function	Organism	Effect on Lifespan	Effect	Notes
Ras2 deletion[4, 14]	protein kinase	s.cerevisiae	doubling	↑ resistance to paraquat	requires stress-resistance proteins Msn2/Msn4 and SOD2
SCH9 deletion[3, 14]	serine/threonine protein-kinase	s.cerevisiae	300% increase	↑ resistance to H2O2 and superoxide/H2O2-generating agent menadione	parallel signalling pathway to RAS; mediates the glucose/nutrients-dependent response incl. down-regulation of stress resistance; requires SOD2, protein kinase Rim15, stress-resistance proteins Msn2/Msn4
DAF-2 mutant[8, 11]	Insulin Receptor homologue tyrosine kinase	c. elegans	100% increase	↑ resistance to paraquat; ↑ levels of SOD3(MnSOD)	requires daf-16
AGE-1 mutant[12, 25]	PI(3)K homologue	c. elegans	65% increase	↑ SOD and catalase; ↑ resistance to H2O2 and paraquat	requires daf-16
DAF-16 mutant[11]	Forkhead TF	c. elegans	suppresses longevity conferred by age-1 and daf-2	suppresses stress resistance by daf-2 age-1	regulates the stress response
isp-1 mutant[5]	iron sulfur protein	c. elegans	19.6 to 33%	↑ resistance to paraquat; ↑ levels of SOD3	component of mitochondrial complex III
mev-1 mutant[9, 27]	cytochrome B subunit of succinate dehydrogenase	c. elegans	34% decrease	hypersensitive to oxygen, paraquat; ↓SOD levels	complex II of the mitochondrial electron transport chain; overproduce superoxide
clk-1 mutant[16, 17]	involved in UQ biosynthesis	c. elegans	40% increase	↑ resistance to UV	requires daf-16; UQ is a lipophilic, redox-active molecule that functions as an electron carrier in the mitochondrial respiratory chain

spe-26 mutant[10, 20]	a gene specifying proper segregation of cellular components affecting sperm activation	c. elegans	65% increase	↑ resistance to UV	requires daf-16 a gene specifying proper segregation of cellular components affecting sperm activation
old-1 mutant[21]	transmembrane tyrosine kinase formerly TKR-1	c. elegans	30% decrease	↑ sensitivity to heat , UV	requires daf-16 transmembrane tyrosine kinase formerly TKR-1
methuselah homozygotes[13, 26]	seven transmembrane domain receptor-like molecule	drosophila	35% increase	↑ resistance to heat, paraquat	homologous to the secretin receptor family
InR (InR^{E19}/InR^{p5545}) mutant[24]	member of the insulin receptor family	drosophila	85% longer	2x ↑ concentration Cu/Zn-SOD	females only; homologous to mammalian insulin and insulin-like growth factor-1 (IGF-1) receptors
chico null[2]	insulin receptor substrate	drosophila	48% increase mean 41% max lifespan	↑ SOD activity; ↑ resistance to paraquat	
$p66^{SHC(-/-)}$ [15]	cytoplasmic signal transducer	mice	30% increase	↑ resistance H2O2, UV, paraquat	$p66^{shc}$ is a splice variant of $p52^{shc}$/$p46^{shc}$ involved in the transmission of mitogenic signals from activated receptors to Ras[3]
$Igf1R^{(+/-)}$ [7]	insulin-like growth factor type 1 receptor	mice	ave 26% longer females 33% males 16%(NS)	↑ resistance H2O2, paraquat (cultured embryonic fibroblasts)	insulin-like growth factor type 1 receptor (IGF-1R)
snell dwarf[6, 22]	pit-1	mice	40% increase	↑ resistance to H2O2, paraquat (skin derived fibroblasts)	pituitary transcription factor (Pit)-1
ames dwarf[1]	prop-1	mice	50-65% increase	↑ activity of catalase (CAT) and Cu–ZnSOD in the liver, kidney, and hypothalamus;	spontaneous loss of function mutation at prop-1 locus

MsrA$^{(-/-)}$ [19]	methionine sulfoxide reductase	mice	40% decrease	↓oxidative DNA damage and protein carbonyl content hypersensitive to O_2	(MsrA) anti oxidant enzyme and repairs oxidized methionine residues
SAMP[18,23]		mice	27% shorter	mitochondrial dysfunction, ↓GSH levels	senescence accelerated mice strain, various strains

Table 1 References

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Table 2. Clinical Trials Involving Vitamin E.

Clinical Trial	Dose of vitamin E	number of patients	Duration (years)	Patient Population Characteristics	Primary Outcome Measure	Effect of Vitamin E on Primary Outcome Measure
Alzheimer's Disease Cooperative Study[11]	2000IU racemic dl-α-tocopherol	341	2 total	patients with moderately-severe AD	disease progression	signifiant delay
Mild Cognitive Impairment study[5]	2000IU α-tocopherol	289	3 total	patients with mild cognitive impairment	rate of conversion to AD	ongoing
DATATOP[12]	2000IU racemic dl-α-tocopherol	800	2 total	minimally disabled patients with Parkinson's disease	time to anti-PD (levodopa) therapy	none
ALS Riluzole-Tocopherol Study Group[4]	1000mg α-tocopherol	289	1 total	ALS patients for <5yrs	survival and motor function	none
Primary Prevention Project[3]	300mg synthetic α-tocopherol	4495	mean 3.6	patients had cardiovascular risk factors,age>50	cardiovascular death, nonfatal MI, and nonfatal stroke	inconclusive
ATBC[1,14]	50mg synthetic dl-α–tocopheryl acetate	29133	median 6.1	male smokers 50-69yrs	incidence of lung and other cancers	34% decrease in prostate cancer
subgroup of ATBC[10]	50mg synthetic dl-α–tocopheryl acetate	1862	median 5.3	patients had MI before randomization	nonfatal MI or fatal coronary heart disease	reduction in incidence of nonfatal MI
CHAOS[13]	800 or 400 IU RRR α-tocopherol	2002	median 1.4	patients with angiographically proven coronary atherosclerosis	cardiovascular death and non-fatal MI	reduced risk of non-fatal MI
GISSI-Prevenzione trial[6]	300mg synthetic α-tocopherol	11,324	3.5 total	patients surviving recent (< or = 3 months) MI	death, non-fatal myocardial infarction, and stroke	no effect
HOPE[8,15]	400IU natural source α-tocopherol	9541	mean 4.5	high-risk patients >=55 years of age with previous cardiovascular disease or diabetes plus 1 risk factor	myocardial infarction, stroke, and death from cardiovascular causes	no effect
SELECT[9]	400 mg of dl-α-tocopheryl acetate	32,400	planned 7 to 12	healthy men >50-55y with a digital rectal examination (DRE) not suspicious for cancer and a serum prostate specific antigen (PSA) 4 ng/ml	clinical incidence of prostate cancer	Ongoing; phase III data suggests potential efficacy in prostate cancer prevention.
SPACE[2]	800IU of natural source α-tocopherol	196	median 1.4	Haemodialysis patients with pre-existing cardiovascular disease	myocardial infarction (fatal and non-fatal), ischaemic stroke, peripheral vascular disease	reduced risk

					and unstable angina	
Heart Protection study[7]	600mg all-rac-αtocopherol	20536	5 total	patients with coronary disease, other occlusive arterial disease, or diabetes	major coronary events and fatal or non-fatal vascular events	no effect

DATATOP, Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism; ATBC, alpha-tocopherol, beta-carotene lung cancer prevention study; CHAOS, Cambridge Heart Antioxidant Study; HOPE, Heart Outcomes Prevention Evaluation Study; SELECT, selenium and vitamin E cancer prevention trial; SPACE, Secondary Prevention with Antioxidants of Cardiovascular Disease in End Stage Renal Disease; GISSI, Gruppo Italiano per lo Studio della Sopravvivvenza nell'Infarto Miocardico.

Table 2 References

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Summary

The population is aging. While aging is a universal process, the biological mechanisms that underlie it are poorly understood. The preceding literature review describes the predominant hypotheses that have been proposed to explain why aging occurs. At the commencement of this thesis the preeminent theory was that oxidative stress and the ensuing accumulation of damaged cellular components is what drives the aging process.

The epididymal epithelium is profoundly affected by aging, and, as a result, sperm maturation processes are impaired. The epididymis is a particularly interesting model in which to study aging in that, while the epididymis ages and is affected by aging, the effects of age do not compromise the overall health of the animal. Aging in the epididymis can therefore be studied without the confounding influence of disease. Additionally, gene expression in the epididymis is highly complex with tightly regulated region and cell specificity. Thus, the epididymis is a particularly interesting tissue in which to study gene expression. For these reasons we undertook to study the effects of aging on gene expression in the epididymis.

Previous results from our lab had indicated that oxidative stress was occurring in the aging of the epididymis. Therefore, what we set out to assess is the role that oxidative stress plays in the aging of the epididymis. Our hypothesis was that oxidative stress, and the ensuing damage it causes, are a major contributor to the aging of the tissue. To test this hypothesis, in addition to simply observing the effects of age on the epididymis, we wanted to perform manipulations that would help dissect out the contribution of oxidative stress to the aging of this tissue. We decided to use two manipulations: caloric restriction and long term vitamin E supplementation and deficiency. Caloric restriction had been shown to reduce the production of ROS and decrease the accumulation of oxidative damage, and, therefore, it was a way to affect oxidative stress load. Vitamin E is a potent antioxidant and thus was a means by which to affect oxidative stress defenses. In order to use these manipulations, the studies had

to be designed and started concurrently, before the results of the baseline gene expression studies were analyzed. As with most experiments, the results of these studies and the baseline gene expression experiments, generated as many new interesting questions as were answered. As a result, there are many directions of follow up that beg to be addressed. This will be discussed in greater detail in the general discussion section.

Objectives of the Thesis

The objectives of this thesis were to:

1. To establish the longitudinal distribution along the epididymis of major families of genes

2. To determine the effects of age on gene expression in the epididymis.

3. To determine the consequences of caloric restriction on gene expression in the epididymis.

4. To determine how long-term Vitamin E deficiency and supplementation affect gene expression in the epididymis.

Chapter 2

Dynamic Changes in Gene Expression Along the Rat Epididymis

Abstract

In the epididymis a series of complex, sequential events transform immature, spermatozoa into mature, motile sperm with fertilizing ability. These events are not intrinsic to germ cells but rather are a direct result of exposure to, and interaction with, the environment created by the epididymal epithelium. Regional differences along the epididymis are essential in the establishment of the environment required for sperm maturation. Although parts of this process have been identified, the molecular basis for the segment-specific differences, and how they contribute to the process of sperm maturation are not yet resolved. The identification of genes expressed in a region-specific manner will provide valuable insight into the functional differences between the regions. To characterize gene expression in the different regions of the epididymis, microarrays containing 1198 rat cDNAs were used to examine gene expression in the initial segment, caput, corpus and cauda epididymidis of the adult Brown Norway rat. Overall, the cauda epididymidis expressed the most genes and the corpus epididymidis the fewest. A small percentage of genes (3%) were expressed highly along the tissue. Segment-specific gene expression for genes expressed at high levels was observed in all epididymal segments except the corpus epididymidis. Of the genes on the array, 36% were expressed in all four epididymal segments; expression changes that were a minimum of two-fold in either direction between adjacent segments are discussed. The expression of cathepsins and oxidative stress related genes was investigated. Six of the eight cathepsins on the array (B, C, E, H, L, K) were expressed above two-fold background and showed different levels of expression along the duct with cathepsin K showing the most dramatic change, i.e., a decrease of 87% between the initial segment and the corpus epididymidis. There was also differential expression along the epididymis of many genes associated with oxidative stress defenses. Using the power of expression array technology, we have identified novel transcripts expressed in a segment specific manner and been able to

assess how the expression of several selected gene families is modulated along the epididymis.

Introduction

The epididymis, a component of the testicular excurrent duct system, is a highly specialized tissue that functions in the transport, maturation and storage of spermatozoa [30, 33]. The maturation of spermatozoa and the acquisition of motility and fertilizing ability do not occur as a function of passage of time, but rather as a consequence of exposure to the luminal environment of the epididymis [27]. The composition of the luminal fluid that bathes spermatozoa as they transit through the epididymis is highly complex and changes progressively along the tissue [43,14]. The secretory and absorptive activities of the epididymal epithelium mediate the changes in the luminal fluid and thus determine the microenvironment in which spermatozoa are able to become fully mature.

The epididymal epithelium is composed of four major epithelial cell types and can be divided anatomically into four segments: the initial segment, caput, corpus and cauda epididymidis. While the division between regions may be anatomical, it is now established that discrete functions take place in the various segments. On a physiological level, this is evidenced by studies of sperm populations taken from discrete regions of the adult epididymis where developing spermatozoa exhibit segment related acquisition of motility and fertilizing ability [20]. The increase in the concentration of spermatozoa occurs primarily between the rete testis and the proximal epididymis and, although the bulk of the fluid secreted by the rete testis is absorbed by the efferent ducts, the cells of the proximal epididymis play a role in the reabsorption of fluid and endocytosis of proteins [33]. The extensive modifications associated with sperm maturation occur while spermatozoa traverse the caput and corpus epididymidis, and are a result of the activities of the cells in these segments. Spermatozoal motility parameters change from immotile or vibratory to direct forward movements and membranes are remodeled; the protein composition and location of specific

proteins changes, glycoproteins are acquired or altered, phospholipids are removed or utilized and the lipid composition of the membrane changes [27, 33, 34, 43]. Spermatozoa are mature; that is motile and able to fertilize, upon reaching the cauda epididymidis, where the cells provide a milieu suitable for their maintenance and storage.

The functional segmentation of the epididymis is also represented at a molecular level by regional differences in gene expression [6,19]. Moreover, the differential response of the segments to androgen withdrawal, aging and stress indicates that each region represents discrete regulatory units [19]. The epididymis, therefore, provides a unique opportunity to study regionalized gene expression along a duct where functional changes are occurring in germ cells. Using microarray technology, it is feasible to examine the expression of multiple genes and gene families simultaneously, thus providing a comprehensive picture of the gene expression profile in a tissue or cell under a given condition [44]. Major advantages of microarray techniques over other available techniques to examine the transcriptional profile of a biological sample include the increased sensitivity of the arrays, the ability to work with smaller amounts of starting material, and the extended scale of gene expression analysis possible.

Elucidation of the transcriptional profiles of the different epididymal segments is a crucial step towards uncovering the regulatory and functional differences between them. To this end, we used gene array technology to analyze gene expression in the initial segment, caput, corpus and cauda epididymidis of the rat. In addition, particular attention was paid to the expression of cathepsins and genes related to oxidative stress. Cathepsins are cysteine proteases that play a role in protein processing and intracellular protein degradation. The epididymal epithelium is highly active in the uptake, secretion and processing of proteins and, although these processes are crucial to sperm maturation, the molecular players involved have not yet been fully characterized. Furthermore, given the susceptibility of spermatozoa to oxidative damage and the deleterious effects that this type of damage can have on fertilizing ability [2,

8], we examined epididymal expression of genes associated with oxidative stress defenses.

Materials and Methods

Animals

Adult male Brown Norway rats (3 months) were purchased from the National Institute on Aging (Bethedsa, MD) and supplied by Harlan Sprague Dawley Inc. (Indianapolis, IN). Rats were housed at the McIntyre Animal Resources Centre, McGill University, under controlled light (14L:10D) and temperature (22C); animals had free access to food and water. All animal studies were conducted in accordance with the principles and procedures outlined in the *Guide to the Care and Use of Experimental Animals* prepared by the Canadian Council on Animal Care.

Rats were sacrificed by decapitation. Epididymides were collected, sectioned into initial segment, caput, corpus, and cauda regions, immediately frozen in liquid nitrogen and stored at -80°C until used for RNA extraction.

RNA Extraction

Total RNA was extracted with guanidine thiocyanate (Sigma, St. Louis, MO). Briefly, tissues were ground to a fine powder in pre-chilled (-80°C) ceramic mortars on dry ice and dissolved in 10 vol (ml/g) guanidine thiocyanate solution (4M guanidine thiocyanate, 100mM TrisHCL pH 7.6, 0.5% Sarcosyl, 0.1M β-mercaptoethanol, 50mM EDTA) to which 1 volume saturated phenol (Sigma, St. Louis, MO) and 0.1 volume 2M sodium acetate, pH 4 had been added. The resulting dissoluate was placed in a 2.0ml microfuge tube and 0.1 volume chloroform:isoamyl alcohol (49:1) was added. Following vortexing, the tubes were left on ice for 20 minutes and then centrifuged for 20 minutes (14,000 rpm, 4°C). The upper phase was transferred into a 1.5ml microfuge tube to which an equal volume of isopropanol was added. After vortexing, tubes were placed at -20°C for one hour and then centrifuged for 20 minutes (14,000 rpm, 4°C). The resulting pellet was washed twice with 80% ethanol and resuspended in a

minimal volume of double distilled water (ddH2O). RNA samples were DNase treated (refer to Atlas RNA Pure Isolation Kit TM user manual, section IV, Clontech, Palo Alto, CA) and the concentration determined by absorbance at 260nm (Beckman DU7 spectrophotometer, Montreal, QC). To verify the quality of the sample, 5μg RNA was run on a denaturing gel containing 1% agarose-formaldehyde. Each sample consisted of a single epididymal segment obtained from individual rats; no tissues were pooled.

cDNA Arrays and Hybridization

RNA samples were used to probe cDNA arrays (Clontech, Atlas Rat 1.2K) according to the manufacturers instructions. Five arrays per epididymal segment per age group were probed and are referred to as replicates. Arrays were exposed to phosphorimager plates (Molecular Dynamics, Sunnyvale, CA) for 24h before scanning (Storm, Molecular Dynamics). Analysis of array images with Atlas Image (Version 1.5, Clontech) was done to quantify the intensity of each cDNA spot, which reflects the relative abundance of the RNA in the sample. The raw data for each gene (intensity of each spot on the array minus the background) were imported into GenespringTM (Silicon Genetics, Redwood, CA) for further analysis. To minimize experimental variation, data were normalized by defining the median level of expression on each array as 1 and normalizing the expression of each gene relative to 1; this value was calculated for all five replicates and averaged to generate the relative intensity for any given gene. A gene was considered as expressed if its intensity was at least two fold the average background of all the replicates in that experiment. Changes in gene expression were considered only when the difference in expression level was at least either doubled or suppressed by 50% and consistent in at least three out of five replicate experiments.

Examination of the expression of genes that have a well established expression profile along the epididymis provided a means to validate this approach. These genes were 5α-reductase type 2, a-raf, angiotensin converting enzyme (ACE), clusterin, androgen receptor and c-ros and served as positive

controls (figure 1). Clusterin and ACE had expression patterns that peaked dramatically in the caput epididymidis (6,37). The expression of 5α-reductase type 2, a-raf and c-ros decreased from the initial segment, reaching lowest levels (undetectable in the case of c-ros) in the corpus and cauda epididymidis (45, 46, 48); androgen receptor expression was unchanged along the duct (33)

Results

The number of genes detected in each segment of the epididymis varied widely. While 44% or 46% of the genes studied were expressed in the initial segment (517of 1185) and caput epididymidis (541 of 1198), respectively, there were only 37% of the genes expressed in the corpus epididymidis (463 of 1185); the maximum of genes expressed, 53%, was in the cauda epididymidis (626 of 1185).

Of the 1185 genes on the array, approximately 3% (36) were expressed highly, at a relative intensity of five times or greater than the average expression of all genes, in all segments of the epididymis (figure 2). Not surprisingly, genes that code for cytoskeletal elements (cytoplasmic β actin, ezrin, cofilin), protein translation (ribosomal proteins, elongation factor 2), glyceraldehyde 3-phosphate dehydrogenase and polyubiquitin were included in this group. Other abundantly expressed genes included oxidative stress related genes (glutathione S-transferase subunit 8, glutathione S transferase subunit 4mu, copper-zinc superoxide dismutase 1), cytochrome oxidase subunit 1, cytochrome c oxidases (subunits IV, Vb and VIa), cathepsin L, a-raf, macrophage migration inhibitory factor, β2 microglobulin, heat stable antigen (CD24) and HSP90β a chaperone associated with steroid receptor function.

The initial segment, caput and cauda epididymidis all showed segment specific expression of highly expressed genes. In the initial segment, the genes for c K ras, 2b proto oncogene, cathepsin K, proliferating cell nuclear antigen (PCNA), sodium/ dicarboxylate cotransporter are expressed exclusively and at high levels. The only gene expressed at high levels in the caput epididymidis

alone was the gene for heat shock 70 kDa protein (HSP70). There were no genes expressed at high levels exclusively in the corpus epididymidis. In the cauda epididymidis, the genes that were highly expressed in a segment specific manner were DNA binding protein inhibitor ID1, extracellular signal regulated kinase 1(ERK1), cyclin D2 (CCND2), I kappa B α-chain, insulin receptor-related receptor-α, and STAT3.

Another subset of genes was highly expressed in more than one, but not in all, segments (figure 3). In the initial segment, caput and cauda epididymidis, the genes for clusterin, DNA-binding protein inhibitor ID2, glutathione s-transferase (GST) subunit 7 pi and an ATP synthase, were expressed highly. Insulin-like growth factor binding protein-6 (IGFBP-6) and microsomal GST were expressed above a relative intensity of five in the initial segment, corpus and cauda epididymidis. One gene, fibroblast growth factor activating protein was expressed at high levels in the caput, corpus and cauda epididymidis. Finally, in the corpus and cauda epididymidis, the genes for serotonin receptor subtype 5B (HTR5B), ATPase F, DNA-binding protein inhibitor ID3, sodium/potassium-transporting-ATPase β1, ornithine decarboxylase and platelet derived growth factor-associated protein were expressed highly.

Segment Specificity of Gene Expression

Genes that were expressed above two fold background in all four segments of the epididymis make up approximately 36% (425 of 1185) of all the genes on the array. In order to provide more insight into the selective regional gene expression along the epididymis, genes that were expressed at least twice or at half the levels in one segment as compared to the adjacent segment, are discussed (figure 4)

Initial Segment and Caput Epididymidis

Between the initial segment and the caput epididymidis, more genes decreased in expression than increased (21 and 11 respectively). Interestingly, transcripts for three genes involved in glutathione metabolism (epididymal

secretory glutathione peroxidase, glutathione synthetase, glutathione S transferase subunit 7 pi) decreased between these two regions. The expression of 3β hydroxysteroid dehydrogenase (3βHSD), an essential enzyme in androgen biosynthesis, and cytochrome P450 IB1, an enzyme that catalyzes the 4-hydroxylation of 17β-estradiol, also decreased in the caput epididymidis. Other genes that decreased in expression included cysteine rich protein 2 (CRP2), neuropeptide Y5 receptor, and cyclin D1 (CCND1). The genes that increased in the caput epididymidis from the initial segment included clusterin, a well characterized glycoprotein, angiotensin converting enzyme (ACE), an enzyme that plays a role in the regulation of ion flux in the epididymis, HSP70, and the adenosine A1 receptor.

Caput and Corpus Epididymidis

Between the caput and corpus epididymidis, a similar number of genes increased (15) and decreased (13) in expression. In the caput, the genes for steroid 5α-reductase 2, cathepsin K, HSP70, prostaglandin F2 receptor and the DNA binding protein inhibitor ID2 were expressed at greater levels than they were in the corpus. In contrast, expression of c-neu, cysteine rich protein 2, and ornithine decarboxylase, a rate limiting enzyme in polyamine synthesis, increased between these two regions.

Corpus and Cauda Epididymidis

The vast majority of genes that changed by two-fold or more between the corpus and cauda epididymidis increased in expression (37 increase, 5 decrease). The genes expressed at higher levels in the corpus epididymidis were the cAMP dependent protein kinase inhibitor (testis form), ACE, glycerol kinase, and the interleukin receptor 2Aα chain. Interestingly all of the DNA binding protein inhibitors on the array (ID1, ID2, ID3) and many of the insulin-like growth factor-binding proteins (IGFBP-1, IGFBP-3, IGFBP-5, IGFBP-6) had increased expression in the cauda epididymidis. Other genes that had increased expression in the cauda epididymidis were ornithine decarboxylase, cyclin D2

and the receptors for 5-hydroxytryptamine (serotonin receptor 5B) and neuropeptide Y (NPY receptor 5Y).

Gene Families

Besides revealing the expression of novel genes, microarray technology can add to our understanding of the expression of gene families known to be important in the epididymis. Specifically, we examined the expression of cathepsins and genes involved in oxidative stress defenses.

Cathepsins

Of the eight cathepsins on the array (cathepsins B, C, D, E, H, L, K, S), all but cathepsin D and S were expressed above two-fold background in all four segments of the epididymis (figure 5). Cathepsin E expression increased (1.9 fold) between the initial segment and the caput epididymidis and was relatively unchanged along the rest of the tissue. Cathepsin C expression decreased (slightly less than 50%) between these two regions but increased by two-fold between the corpus and cauda epididymidis. Cathepsin H expression increased by 1.5 fold between the caput and the corpus epididymidis. Cathepsin K expression decreased dramatically (87%) between the initial segment and corpus epididymidis and remained constant in the cauda. Cathepsin L and B expression were relatively constant along the epididymis.

Oxidative Stress Related Genes

The expression of glutathione synthetase (GSH synthetase), one of the enzymes involved in glutathione biosynthesis, decreased dramatically (71%) between the initial segment and the caput epididymidis and increased (1.5 fold) between the corpus and the cauda epididymidis. Epididymal secretory glutathione peroxidase (GPX5) was expressed in the initial segment of the epididymis but decreased to undetectable levels in the caput epididymidis and along the rest of the tissue. Phospholipid hydroperoxide glutathione peroxidase (GPX4) was also expressed along the epididymis; its expression did not change

proximal regions of the tissue but increased by almost two-fold between the corpus and cauda epididymidis. Another peroxidase, thioredoxin peroxidase, which in its monomeric form will scavenge oxidants to protect cellular components, was also expressed to a similar extent along the epididymis, but at very low levels. Copper-zinc superoxide dismutase (CuZn SOD) was expressed very highly at relatively unchanging levels along the epididymis (figure 6). Eight glutathione S-transferases (GSTs) were present on the array, five of which were expressed above background along the epididymis (figure7). GST subunit 13 expression changed only between the corpus and cauda epididymidis, where it increased by 2.3 fold. The expression of GST subunit 7 pi (GST7 7) decreased by 82% between the initial segment and corpus epididymidis, then increased by 1.7 fold in the cauda epididymidis. Similarly, microsomal GST (GST12; MGST1) expression decreased (37%) between the initial segment and caput, then increased by 1.7 fold in the corpus and cauda epididymidis. The expression of GST subunit 4 mu (GSTM2) and GST subunit 8 remained relatively unchanged throughout the length of the tissue.

Discussion

Microarray technology is a powerful and efficient way to investigate region specific gene expression along the epididymis. Many genes have been reported to be expressed exclusively, or at their highest levels, in the proximal epididymis [6,19]. By comparison, few examples of regional specificity have been described in the distal epididymis [6,19], thus leaving gene expression in the cauda region relatively unexplored. Based on histological evidence, one would expect the caput epididymidis, the segment that has a very extensive supranuclear Golgi complex and endoplasmic reticulum [33] to be the most active segment in terms of protein synthesis; yet studies in which total protein synthesis by 35S methionine incorporation was assessed along the epididymis [5, 16] suggested that the highest rate of protein synthesis was in the cauda epididymidis. Our results are consistent with the protein synthesis studies in that the cauda epididymidis expressed more genes overall, and more genes at high levels, than

the caput epididymidis. Interestingly, in the corpus epididymidis, the adjacent segment, gene expression was the lowest in both proportion and level than in any other epididymal region.

Highly Expressed Genes

Genes expressed at high levels in the epididymis, particularly in a segment specific manner, were of particular interest. In the initial segment, cathepsin K was one such gene. Several members of the cathepsin family of lysosomal cysteine proteases have been localized in the rat epididiymis. Cathepsins are known to be involved in intracellular protein degradation [25], however the discovery of new family members with tissue specific expression has led to the speculation that certain cathepsins have more specialized functions than simple housekeeping enzymes [42]. Cathepsin K, one of the more recently discovered cathepsins, plays a central role in bone reabsorption and acts extracellularly in both osteoclasts [48] and thyrocytes [39]. The identification of transcripts for proteases such as cathepsin K that are expressed in segment specific manner supports the hypothesis that pro-protein processing is an important event in sperm maturation and that different proteins may be processed in different epididymal regions [17, 41].

Two-fold or Greater Changes in Gene Expression Between Adjacent Segments

Analysis of those genes that changed by a minimum of two-fold between adjacent segments is one way to highlight the relative importance of different genes in different regions. The initial segment, when compared to the caput epididymidis, expressed higher levels of cytochrome P450 IB1 and 3β-HSD, genes involved in steroid metabolism [9, 24]. The initial segment is the first epididymal segment exposed to testicular factors and hormones via the luminal fluid and is the most dramatically affected by their withdrawal [13, 32, 45]. The activity and the messages for 5α-reductases, enzymes that metabolize testosterone to dihydrotestosterone, the active androgen in the epididymis, are most abundant in the proximal epididymis [34]. Aside from endocrine factors and

direct testicular input, factors that act in a paracrine manner are also thought to play a role regulating epididymal epithelial function [21, 27, 31]. Neuropeptide Y, a peptide that modulates the central gonadotropic axis via the Y5 receptor subtype in the rat hypothalamus [29], is expressed in the epididymis [18] and may be one such factor. Interestingly, we found that NPY Y5 receptor expression also decreased from the initial segment to the caput epididymidis. In the caput epididymidis, the gene for ACE was expressed at a higher level than in the adjacent segments. The regulation of fluid and ion secretion is crucial in order to develop the optimal environment for sperm maturation and storage [33, 43]. Several humoral factors, including angiotensins and prostaglandins, are thought to act locally in the epididymis to regulate anion secretion [23]. Interestingly, prostaglandin F2 receptor expression, like that of ACE, decreased between the caput and the corpus epididymidis. Only a few genes, such as ornithine decarboxylase (ODC) for example, were expressed at two fold higher levels in the corpus epididymidis than in the caput epididymidis. ODC is a rate limiting enzyme in the biosynthesis of polymines, such as spermine, spermidine and putrescine [37].

In the cauda epididymidis, two groups of genes, insulin-like growth factor binding proteins (IGFBPs) and DNA binding protein inhibitors (IDs), were expressed at two-fold or greater levels than in the corpus epididymidis. Insulin-like growth factors, small peptides that regulate cellular growth, differentiation and metabolism, act by binding to high affinity cell surface receptors. IGF-I is known to modulate Leydig and Sertoli cell function and IGF-I null mutant mice are infertile [4]. IGF-I has been localized in the caput and cauda epididymal regions of the postnatal epididymis [22]. IGFs are bound in serum and other biological fluids by binding proteins (IGFBPs) that compete with binding of IGF receptors and thus modulate IGF action [35]. That four of the six known IGFBPs were expressed in the cauda epididymidis at levels two-fold higher than those observed in the corpus indicates that IGFs may play a role in the cauda epididymidis.

ID proteins, members of the basic helix-loop-helix (bHLH) family of transcription factors that lack DNA binding domains [26], were expressed at least two-fold greater levels in the cauda than any other epididymal region. These proteins act as dominant negative regulators of bHLH transcription factors by forming inactive heterodimers with them and inhibiting their DNA binding and transcriptional activities [26]. While the profile of transcriptional activators expressed in the cauda epididymidis is relatively unknown, the expression of such high levels of transcriptional repressors is suggestive of bHLH activity in this epididymal segment.

Gene Families

Cathepsins are cysteine proteases that play a role in the degradation and processing of proteins [25]. One of the primary activities of the epididymal epithelium is the endocytosis and degradation of luminal contents [11, 33]. Additionally, it is now thought that the epididymis produces proteases that participate in pro-peptide processing and sperm maturation [17, 41]. Cathepsins A, B, D, H, and L have been localized immunohistochemically in the rat epididymis [3, 15, 40] and enzyme assays have been done for cathepsins B, H and L [40]. In addition to cathepsin B, H and L, which were detected along the entire tissue, cathepsin E, C and K were expressed in all four segments of the epididymis. Cathepsin L expression is much higher than the other cathepsins, however expression did not fluctuate much between the different epididymal regions. In contrast, cathepsin K was expressed highly in a segment specific manner; between the initial segment and corpus epididymidis its expression decreases by almost 90%. Since cathepsins have different substrate specificities, mechanisms of activation and endogenous inhibitors and are regulated differently by pH [42], it is thus not surprising to observe that most cathepsin transcripts had different expression patterns along the epididymis and, in any given segment, were expressed in varied abundance.

It is widely recognized that epididymal spermatozoa are highly susceptible to damage resulting from reactive oxygen species (ROS) [2, 8]. Spermatozoa

have little in the way of intracellular antioxidant enzymes and high concentrations of membrane lipids (polyunsaturated fatty acids) that are particularly vulnerable to peroxidative damage [1]. Spermatozoa also produce ROS (hydrogen peroxide and superoxide) that are essential for capacitation and chromatin condensation [1]. Thus. the epididymal epithelium must be able to protect spermatozoa and itself from oxidative damage. The epididymis is a rich source of antioxidant enzymes [36, 50]. Several of the antioxidant related genes on the array we used were expressed along the tissue with relatively little regional differences. Strikingly, the expression of GSTpi, a class of GST that has been immunolocalized in the epididymis of the rat [12], decreased remarkably (82%) between the initial segment and the corpus epididymidis. GSTpi functions in the metabolism of xenobiotics and has been implicated in carcinogenesis and the acquisition of anti-neoplastic drug resistance [10]. Such high expression in the initial segment of the epididymis may indicate the presence of a preferred substrate in the proximal epididymis.

In this study, we have exploited the power of microarray technology to investigate region specific gene expression along the epididymis. We have identified the segment-specific expression of several genes never before described in the epididymis. Moreover we describe the expression of new members of gene families known to be important in epididymal physiology.

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Figure 1. Expression of 5α-reductase type II, a-raf, angiotensin converting enzyme (ACE), clusterin, c-ros and androgen receptor (AR) in the initial segment (solid bars), caput (hatched-right bars), corpus (cross-hatched bars) and cauda (hatched-left bars) of the epididymis. Gene expression is expressed as relative intensity. n=5 replicates per segment.

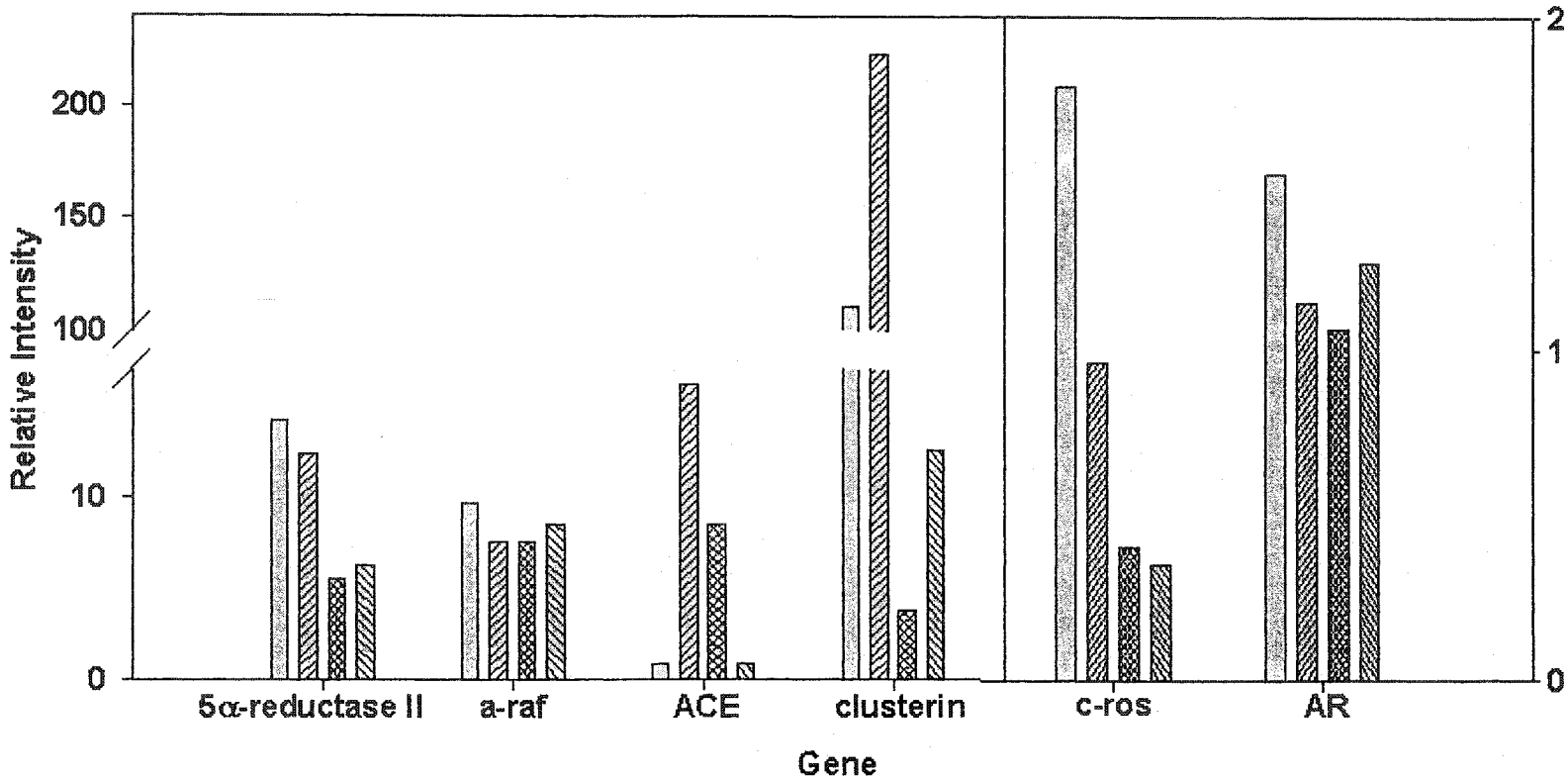
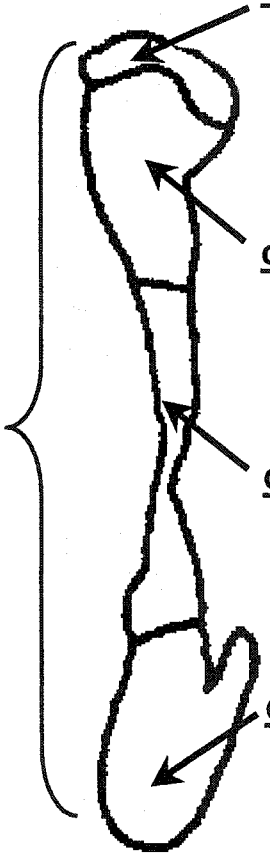

Relative Intensity
200
150
100
10
0
2
1
0
5α-reductase II
a-raf
ACE
clusterin
c-ros
AR
Gene

Figure 2. Genes expressed at a relative intensity of five or greater along the epididymis. *Only reported in human epididymis. ^Only reported in murine epididymis. Both italicized and underlined genes are genes that have not been reported previously in the epididymis; the former are those that were expected due to their ubiquitous distribution, whereas the latter have not been previously described in the epididymis and are not ubiquitous. Ribosomal proteins, L11, L12, L13; 40S ribosomal proteins, S11, S12, S3A; 60S ribosomal protein, L19, L21, L44. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione S- transferase; Cu-Zn SOD1, copper-zinc superoxide dismutase 1; cytochrome oxidase, subunit I; cytochrome c oxidases, subunits IV, Vb, and VIa; MIF, macrophage migration inhibitory factor; HSP, heat shock protein; ANT2, adenine nucleotide translocator 2; CABP2, calcium binding protein 2; PCNA, proliferating nuclear cell antigen; Na1/dicarbox. cotransporter, sodium/dicarboxylate cotransporter; ID1, DNA binding protein inhibitor 1; ERK1, extracellular signal-regulated kinase 1; IRRα, insulin receptor-related receptor alpha.

Highly Expressed Genes

cytoplasmic βactin
ezrin*
cofilin
ribosomal proteins
elongation factor 2
GAPDH
Polyubiquitin

GST8-8
GSTM2
Cu-Zn SOD1
cytochrome oxidase
cytochrome c oxidases
cathepsin L
a-raf proto-oncogene^
MIF
β2-microglobulin
heat stable antigen
HSP90β
ANT2
CABP2
prothymosinα
aldehyde reductase
GTP-binding protein
sterol carrier protein 2
transducin β2 subunit
14-3-3 protein zeta/delta



initial segment

c-K-ras 2b
cathepsin K
PCNA
Na^+/dicarbox. cotransporter

caput

HSP70

corpus

none

cauda

ID1
ERK1
cyclin D2
I-kappa B αchain
IRRα
Stat3

Figure 3. Genes expressed at a relative intensity of five or greater in more than one but not all segments of the epididymis. Both italicized and underlined genes are genes that have not been previous reported in the epididymis; the former are those that were expected due to their ubiquitous distribution, whereas the latter have not been previously described in the epididymis and are not ubiquitous. FGFR-activating protein, fibroblast growth factor receptor activating protein; IGFBP-6, insulin-like growth factor binding protein 6; HTR5B, 5-hydroxytryptamine receptor 5B; ID3, DNA-binding protein inhibitor ID3; PDGF-associated protein, platelet derived growth factor-associated protein; ID2, DNA-binding protein inhibitor ID2.

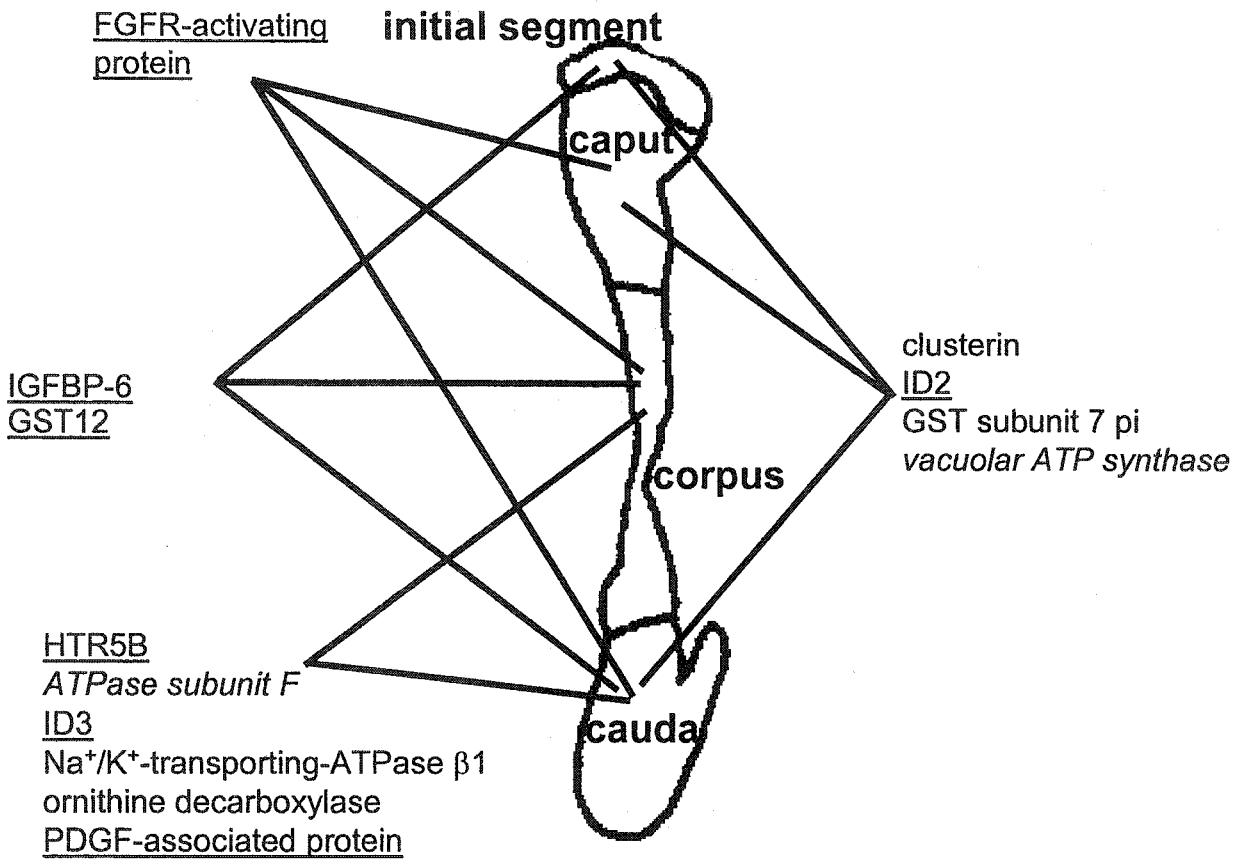

FGFR-activating protein
initial segment
caput
IGFBP-6
GST12
clusterin
ID2
GST subunit 7 pi
vacuolar ATP synthase
corpus
HTR5B
ATPase subunit F
ID3
Na+/K+-transporting-ATPase β1
ornithine decarboxylase
PDGF-associated protein
cauda

Figure 4. Gene expression changes along the epididymis. Changes of a minimum of two fold in either direction between adjacent segments are included.

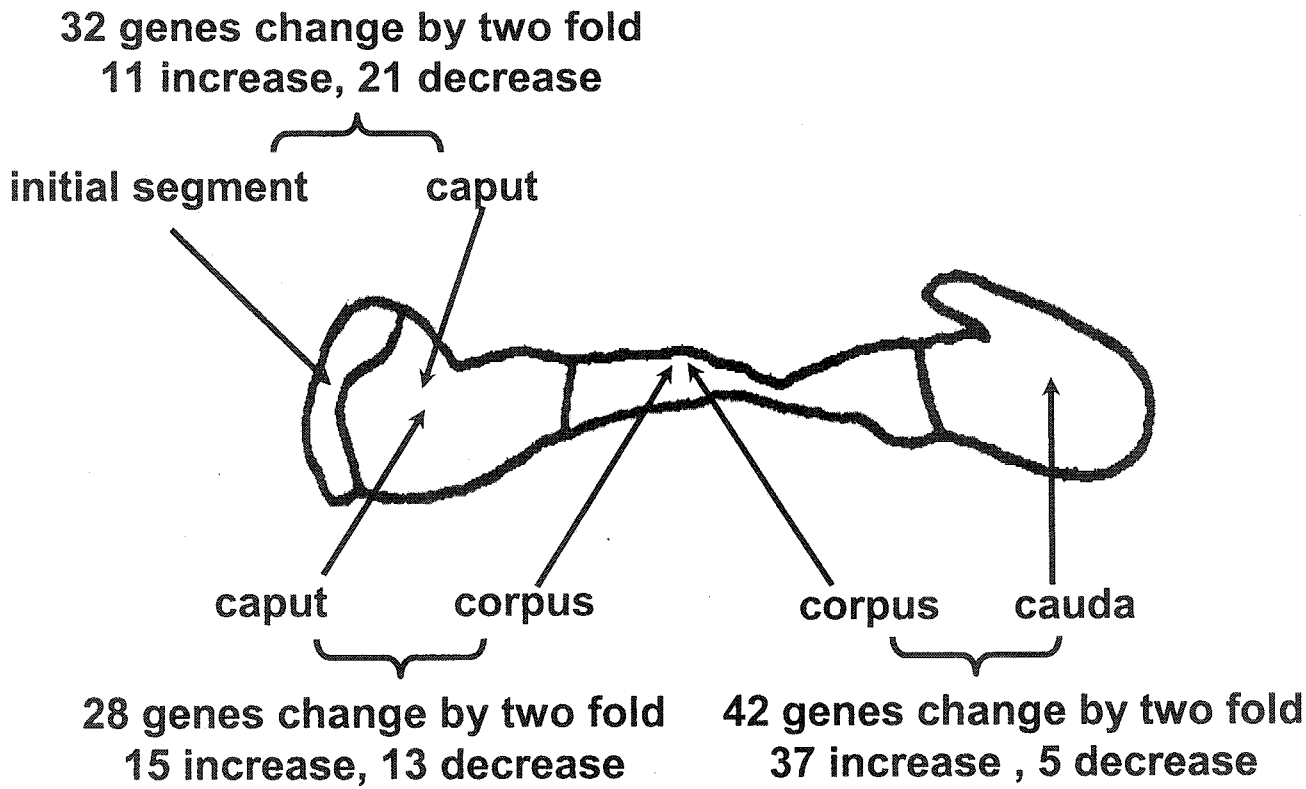

32 genes change by two fold
11 increase, 21 decrease
initial segment
caput
caput
corpus
corpus
cauda
28 genes change by two fold
15 increase, 13 decrease
42 genes change by two fold
37 increase , 5 decrease

Figure 5. Expression of cathepsins E, C, H, B, L and K in the initial segment (solid bars), caput (hatched-right bars), corpus (cross-hatched bars) and cauda (hatched-left bars) of the epididymis. Gene expression is expressed as relative intensity. n=5 replicates per segment.

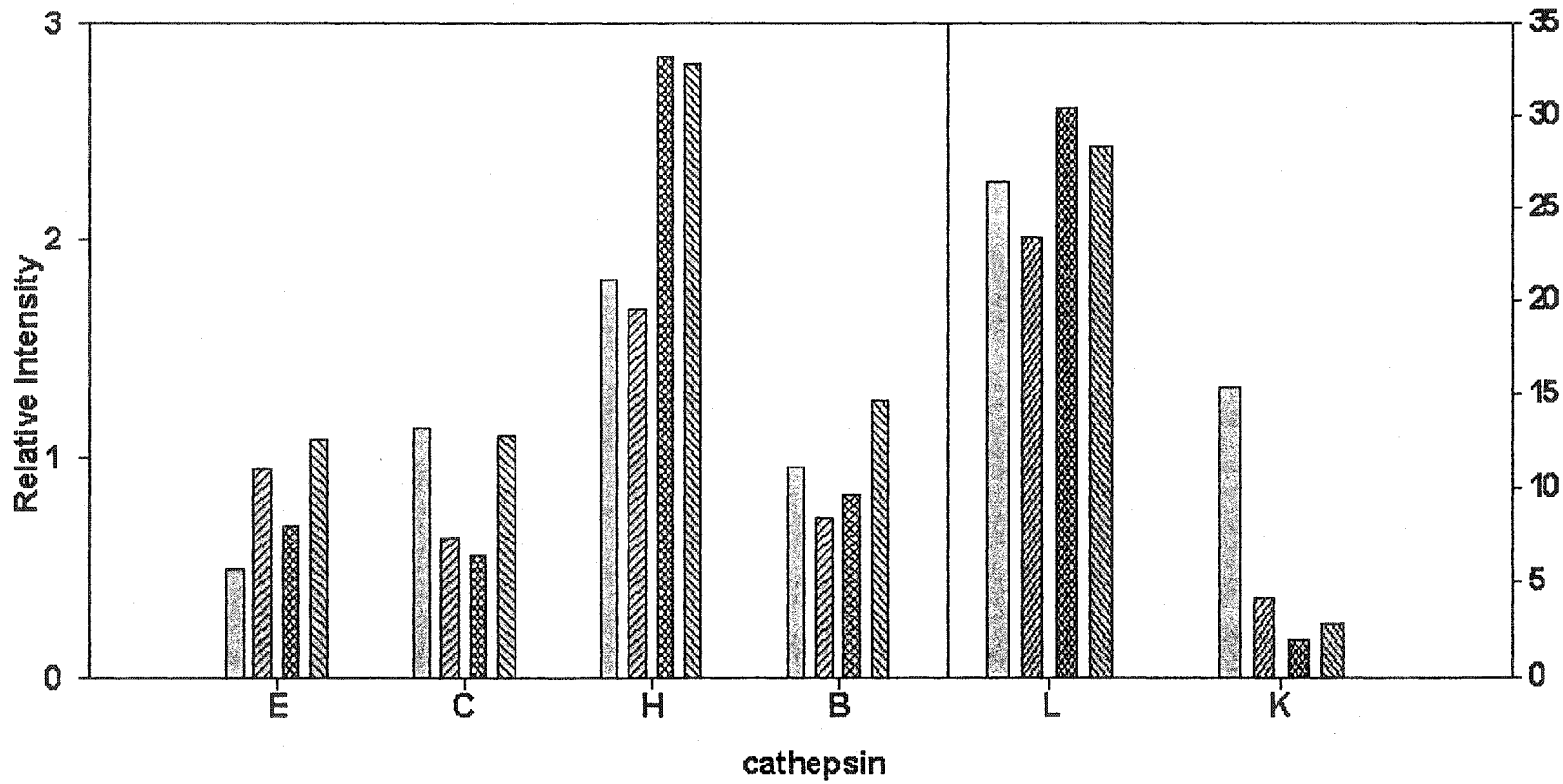

Relative Intensity
3
2
1
0
35
30
25
20
15
10
5
0
E
C
H
B
L
K
cathepsin

Figure 6. Expression of Oxidative stress related transcripts in the epididymis. Expression of glutathione synthetase (GSH synthetase), epididymal secretory glutathione peroxidase (GPX5), phospholipid hydroperoxide glutathione peroxidase (GPX4), thioredoxin peroxidase (TDPX1) and copper-zinc superoxide dismutase (CuZn SOD) in the initial segment (solid bars), caput (hatched-right bars), corpus (cross-hatched bars) and cauda (hatched-left bars) of the epididymis. Gene expression is expressed as relative intensity. n=5 replicates per segment.

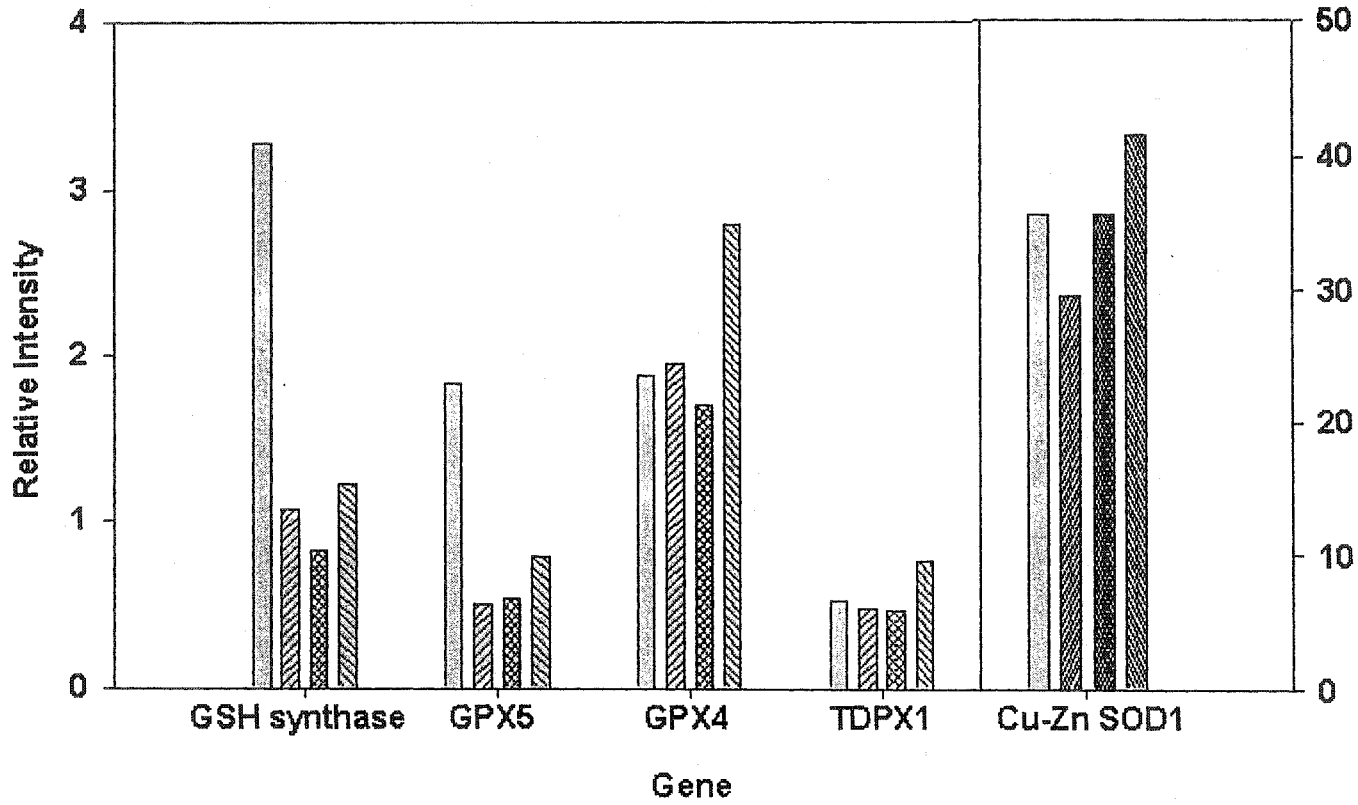
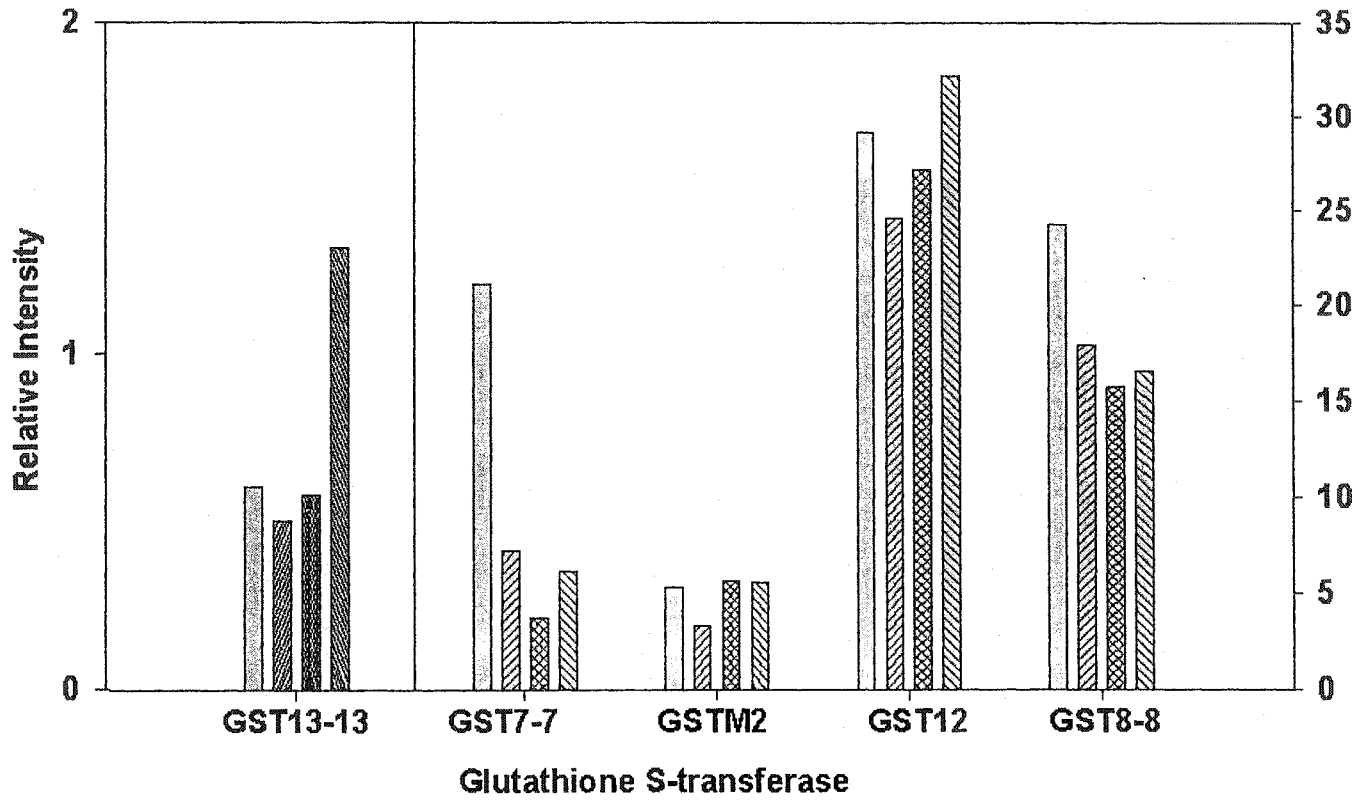

Relative Intensity
4
3
2
1
0
50
40
30
20
10
0
GSH synthase
GPX5
GPX4
TDPX1
Cu-Zn SOD1
Gene

Figure 7. Glutathione S-transferase expression in the initial segment (solid bars), caput (hatched-right bars), corpus (cross-hatched bars) and cauda (hatched-left bars) of the epididymis. Glutathione S transferase subunit 13 (GST 13 13), glutathione S transferase subunit 7 pi (GST7 7), glutathione S transferase mu (GSTM2), microsomal glutathione S transferase (GST12), glutathione S transferase 8 (GST8 8). Gene expression is expressed as relative intensity. n=5 replicates per segment.


2
1
0
35
30
25
20
15
10
5
0
Relative Intensity
GST13-13
GST7-7
GSTM2
GST12
GST8-8
Glutathione S-transferase

Connecting Text for Chapter Two to Three

The epididymal epithelium is profoundly affected by aging. In order to better understand the aging process in this tissue we wanted to undertake large scale gene expression analysis at the RNA level. However, in order to understand the transcriptional response of the epididymal epithelium to aging we first had to characterize the transcriptome of the tissue in the young condition. The region-specific expression of genes and secretion of proteins in the epididymis is essential in order to create the fluid environment required for sperm maturation and segment specific gene expression has been reported for several genes; however, at this point in time no large scale analysis of gene expression at the RNA level had been reported. Once gene expression in the young had been characterized, the changes that occur with age along the tissue could be explored in order to better understand aging of the epididymis.

Chapter 3

Changes in Gene Expression During Aging in the Brown Norway Rat Epididymis

Abstract

Aging of the Brown Norway rat is associated with a decline in reproductive function. With advancing age, serum testosterone and sperm production decrease and sperm morphology is altered. In the epididymis, the site of sperm maturation and storage, dramatic histological and biochemical changes occur with age; these changes occur in a region-specific manner. In order to provide insight into the process of aging in the epididymis, we employed cDNA microarrays to analyze changes in gene expression with age in the initial segment, caput, corpus and cauda epididymidis. The overwhelming effect was a decrease in the relative intensity of gene expression during aging. In the initial segment, corpus and cauda epididymidis, more genes had decreased relative intensity with age than did not change. Interestingly, the magnitude of the decreases in relative intensity was considerably larger in the corpus and cauda epididymidis, where expression of 83% (211 of 254) and 62% (157 of 254), respectively, of the genes decreased by greater than 50% with age. This is in contrast to the initial segment, in which only 31% of genes (78 of 254) had relative intensities that decreased by at least 50%, and the caput epididymidis, the only segment where the largest proportion of genes did not change with age (less than 33% changed between young and old). No genes had increased relative intensity with age throughout the tissue, however the expression of four transcripts was increased in a segment-specific manner. The expression of oxidative stress related genes, heat shock proteins and components of intracellular degradation pathways were examined in particular detail. We found that the relative intensity of some gene families decreased with age in a segment-specific manner; these decreases correlated well with the histological changes that occur in the aging epididymis.

Introduction

The Brown Norway (BN) rat has been established as a valuable model for the study of aging of the male reproductive system (Zirkin et al., 1993; Wang et al., 1993; Gruenewald et al., 1994; Robaire et al., 2000; Syed and Hecht, 2001). In the aging BN rat, as in man, serum testosterone levels decline (Zirkin et al., 1993) and spermatogenesis decreases (Wright et al., 1993). These age-related changes in reproductive function occur in otherwise healthy animals; BN rats are long lived, gain little weight and rarely develop pituitary, testicular or other tumors (Zirkin et al., 1993). Therefore aging in the reproductive tract can be studied without the confounding influence of pathology. We have shown that progeny outcome, sperm morphology and motility are altered in aged BN rats (Serre and Robaire, 1998; Syntin and Robaire, 2001).

The epididymis, the site of sperm maturation and storage, is composed of four major epithelial cell types and can be divided anatomically into four segments: the initial segment, caput, corpus and cauda epididymidis. With age, the epididymal epithelium undergoes dramatic morphological changes, including some characteristic signs of aging, such as the accumulation of lipofuscin, lysosomes and vacuoles and thickening of the basement membrane (Serre and Robaire, 1998). Interestingly, these changes are independent of the presence or absence of spermatozoa and occur in a segment-specific manner. Age-related, segment-specific changes in the expression of transcripts for selected markers of epididymal function, and the expression of junctional proteins have been reported (Viger and Robaire, 1995; Levy and Robaire, 1999). That the different regions of the epididymis are differentially affected by aging makes this tissue a particularly interesting one in which to study aging. Elucidation of the molecular basis underlying the segment-specificity of the effects of age may provide valuable insight into the process of aging.

There is a wealth of information in the literature that implicates oxidative stress in the aging process (Cadenas and Davies, 2000; Finkel and Holbrook, 2000). Indeed, some of the changes observed in the aging epididymis, such as the accumulation of lipofuscin (Terman and Brunkt, 1998) and vacuolization (Wilt

et al., 2000), are indicative of oxidative stress. If, under periods of oxidative stress, the proteolytic machinery of the cell is unable to cope with the amount of oxidatively damaged molecules, or the capacity of the proteolytic machinery declines below a critical threshold of activity, the oxidized cellular components will accumulate and be detrimental to the cell (Sitte et al., 2000; Keller et al., 2000). The proteasome plays a major role in the recognition and degradation of oxidized proteins (Grune et al., 1997; Friguet et al., 2000). In order to assess the effects of age on gene expression in the different epididymal regions, we used microarrays containing 1176 cDNAs to analyze expression in the initial segment, caput, corpus and cauda epididymides of young (3 month) and old (21 month) BN rats. We paid particular attention to the expression of genes involved in oxidative stress defenses, including heat shock proteins, and the proteolytic machinery of the cell. Additionally, since the accumulation of lysosomes in some epididymal regions is suggestive of abnormalities in lysosomal degradation pathways, the effects of age on cathepsin gene expression were examined.

Material and Methods

Animals

Male BN rats (4, 22 months) were purchased from the National Institute on Aging (Bethedsa, MD) and supplied by Harlan Sprague Dawley Inc. (Indianapolis, IN). Rats were housed at the McIntyre Animal Resources Centre, McGill University, under controlled light (14L:10D) and temperature (22C). The animals were kept in a pathogen free environment and had free access to food and water. All animal studies were conducted in accordance with the principles and procedures outlined in *A Guide to the Care and Use of Experimental Animals* prepared by the Canadian Council on Animal Care.

Rats were sacrificed by decapitation. No gross abnormalities were observed in any of the tissues examined. Epididymides were sectioned into initial segment, caput, corpus, and cauda regions, immediately frozen in liquid nitrogen, and stored at -80oC until used for RNA extraction.

RNA Extraction

Total RNA was extracted with guanidine thiocyanate (Sigma, St. Louis, MO) as reported previously (Jervis and Robaire, 2001). Briefly, tissues were ground to a fine powder in pre-chilled (-80oC) ceramic mortars on dry ice and dissolved in 10 vol (ml/g) guanidine thiocyanate solution (4M guanidine thiocyanate, 100mM TrisHCL pH 7.6, 0.5% Sarcosyl, 0.1M β-mercaptoethanol, 50mM EDTA) to which 1 volume saturated phenol (Sigma, St. Louis, MO) and 0.1 volume 2M sodium acetate, pH 4 had been added. The resulting dissoluate was placed in a 2.0ml microfuge tube and 0.1 volume chloroform:isoamyl alcohol (49:1) was added. Following vortexing, the tubes were left on ice for 20 minutes and then centrifuged for 20 minutes (14,000 rpm, 4oC). The upper phase was transferred into a 1.5ml microfuge tube to which an equal volume of isopropanol was added. After vortexing, tubes were placed at -20oC for one hour and then centrifuged for 20 minutes (14,000 rpm, 4oC). The resulting pellet was washed twice with 80% ethanol and resuspended in a minimal volume of double distilled water (ddH2O). RNA samples were DNase treated (refer to Atlas RNA Pure Isolation Kit TM user manual, section IV, Clontech, Palo Alto, CA) and the concentration determined by absorbance at 260nm (Beckman DU7 spectrophotometer, Montreal, QC). To verify the quality of the sample, 5µg RNA was run on a denaturing gel containing 1% agarose-formaldehyde. Each sample consisted of a single epididymal segment obtained from individual rats; no tissues were pooled.

cDNA Arrays and Hybridization

RNA samples were used to probe cDNA arrays (Clontech, Atlas Rat 1.2K) according to the manufacturer's instructions. Five arrays per epididymal segment per age group were probed and are referred to as replicates. Arrays were exposed to phosphorimager plates (Molecular Dynamics, Sunnyvale, CA) for 24h before scanning (Storm, Molecular Dynamics). Analysis of array images with Atlas Image (Version 1.5, Clontech) was done to quantify the intensity of each

cDNA spot, which reflects the relative abundance of the RNA in the sample. The raw data for each gene (intensity of each spot on the array minus the background) were imported into GeneSpringTM (Silicon Genetics, Redwood, CA) for further analysis. To minimize experimental variation, data were normalized by defining the median level of expression on each individual array as 1; the genes on that array were then normalized relative to 1. This was done for every single array individually. Relative expression values, once calculated for all five replicate arrays, were averaged. This average is referred to as the relative intensity for any given gene. A gene was considered as expressed if its relative intensity was at least two fold the average background of all the arrays in that age group. The importance of replication in gene array expression studies has been documented (Lee et al., 2000), therefore, changes in the relative intensity of a gene were considered only when consistent in at least three out of five replicate experiments (Jervis and Robaire, 2001).

The effects of age on the expression of selected markers of epididymal function has been reported previously (Viger and Robaire, 1995). Of these genes, transcripts for 5α-reductase type II, clusterin and androgen receptor are present on the array; the effects of age on their relative intensities follows the trends expected, as previously described (Viger and Robaire, 1995; Jervis and Robaire, 2001).

Results

Overall, the number of genes that were detected above two-fold background along the epididymis decreased with age (figure 1). This effect of age was more than four fold greater in the corpus and cauda epididymidis, where the number of genes detected decreased by 44% and 37%, respectively, than in the initial segment or caput epididymidis, where the decreases in number of detected genes were only 8% and 10%.

The number of genes detected above background in all four segments of the young epididymis was 425. With age, this number decreased by 40% to 254. Interestingly, the only transcript that was detected above two fold background in

all four segments of the old, but not the young epididymis, was growth hormone receptor.

Decreasing Relative Intensity of Gene Expression with Age

The different regions of the epididymis were differentially affected by aging with respect to the number of genes detected, and the magnitude of their decreases in relative intensity with age (figure 2). In the initial segment, of the 254 genes detected above background in all four epididymal segments, 155 transcripts had relative intensities that decreased between 33% and 80%. For 97 genes, age had no effect on their relative intensity (less than 33% change). In the caput epididymidis, 43 genes had relative intensities that decreased between 33% and 50% and 57 genes decreased by greater that 50% with age. The caput epididymidis is the only region where the majority of detected genes (152) did not change with age. Of all the epididymal segments, the corpus epididymidis had the fewest transcripts (12) that did not change. Only 30 genes had relative intensities that decreased by 33% to 50%. The vast majority (211) of the genes in this region had relative intensities that decreased by 50% or greater. In the cauda epididymidis, roughly the same number of genes had relative intensities that decreased by 33% to 50% (54), as did not change (43). As in the corpus epididymidis, most genes (157) in the cauda epididymidis had relative intensities that decreased by 50% or greater.

Since there are numerous age related decreases in the relative intensity of gene expression along the epididymis, we will examine in detail only those genes for which relative intensities decrease by a minimum of 50% with age.

Genes That Had Decreased Relative Intensities with Age in All Four Segments of the Epididymis

A small proportion of detected genes (23 of 254 genes, 4%) had relative intensities that decreased with age by a minimum of 50% in all four segments of the epididymis. The largest decreases in relative intensity were for ribosomal

protein L12 (average decrease of 88% across the four segments), β2 microglobulin (87% decrease) and insulin receptor related receptor α (sIRRα 1, 84% decrease). Other genes that decreased in relative intensity in all four segments with age included genes involved in ATP production (cytochrome c oxidases, ubiquitous mitochondrial creatine kinase), protein translation (ribosome components, elongation factors), ion channels (potassium channel Kir6.2, sodium channel SCNB2), glutathione S-transferase pi, C type natriuretic peptide precursor (CNP), DNA binding protein inhibitor ID2 and cyclin D3 (CCND3). For all of these genes, the percentage decreases in relative intensity with age were larger in the distal segments of the epididymis than in the proximal segments.

Genes That Decreased in Relative Intensity with Age in a Segment Specific Manner

Only one gene, a calcium ATPase, had decreased relative intensity with age uniquely in the initial segment. In the caput epididymidis, the expression of 5 hydroxytryptamine receptor 5B (5HTR5B) was the only transcript that had decreased relative intensity uniquely in this region. The corpus epididymidis had by far the most genes, 56, that were affected by age in a segment specific manner. This included the genes for transcriptional activators and repressors (Myc Max interacting tumor suppressor, Stat3, NF kappa B transcription factor, cyclin G, proliferating cell nuclear antigen, maspin, c jun proto oncogene, proto oncogene c crk, A raf proto oncogene, c H ras proto oncogene, p130), proteases and protease inhibitors (carboxypeptidase E, insulin regulated membrane aminopeptidase vp165, proteasome component C3, 26S protease regulatory subunit 7, tissue inhibitor of metalloproteinase 3), components of intracellular trafficking pathways (rab8, rab13, synaptotagmin XI), cytoskeletal elements (ezrin, cofilin), heat shock 70 kDa protein, GST subunit 4 mu (GSTM2), NADPH cytochrome P450 reductase, heat stable antigen, calcium binding protein 2, and clusterin. The cauda epididymidis had segment specific age related decreased relative intensity of seven transcripts, namely, neuropeptide Y5 receptor, platelet derived growth factor alpha receptor, insulin like growth factor binding protein 5,

Na,K ATPase a1 subunit, survival of motor neuron protein, sodium/potassium transporting ATPase β-1 subunit and c src kinase.

Increasing Relative Intensity of Gene Expression with Age.

There were no transcripts that had increased relative intensity with age in all four segments of the epididymis, however some genes exhibited segment specific increased relative intensity. In the initial segment, the relative intensity of angiotensin converting enzyme (ACE) and fatty acid binding protein (FABP) increased by 3.0 and 1.7 fold respectively. The aged caput epididymidis exhibited 2.2 fold increased relative intensity of syntaxin 3, the protein product of which is involved in vesicle transport, and a 2.1 fold increase in relative intensity of tissue inhibitor of metalloproteinase-2. The corpus epididymidis, like the initial segment, had age related increased relative intensity of fatty acid binding protein, which increased by 2.3 fold. No genes had increased relative intensity in the cauda epididymidis.

Gene Families

Oxidative Stress Related Genes

Transcripts for copper-zinc superoxide dismutase, glutathione synthetase, epididymal secretory glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase, thioredoxin peroxidase and several glutathione s-transferases (subunit 4mu, subunit 8, subunit 7pi, subunit 13, microsomal glutathione S-transferase) were detected along the epididymis (figure 3A, B; table 1). With age, the relative intensity of copper-zinc superoxide dismutase (Cu-Zn SOD1) decreased; the smallest decrease occurred in the initial segment and the largest in the corpus epididymidis. Glutathione synthetase relative intensity decreased dramatically with age in the initial segment, corpus and cauda epididymidis. Epididymal secretory glutathione peroxidase (GPX5) and phospholipid hydroperoxide glutathione peroxidase (GPX4) transcript levels decreased in relative intensity with age in all four segments of the epididymidis. Interestingly, the largest decrease in GPX5 relative intensity occurred in the initial

segment, in contrast to GPX4 for which the largest decrease in relative intensity was in the cauda epididymidis. Transcript levels for another peroxidase, thioredoxin peroxidase (TDPX1), decreased in relative intensity to below the level of detection with age in all epididymal segments. The relative intensities of two glutathione S-transferases (GSTs), GSTsubunit 8 and GSTsubunit 7 pi, decreased by more than 50% with age in all four segments of the epididymis. The largest decrease in the relative intensity of GST subunit 8 occurred in the corpus epididymidis, whereas GST subunit 7pi relative intensity exhibited the largest decrease in the cauda epididymidis. The relative intensities of GST subunit 13 and microsomal GST decreased by more than 33% with age in every segment except the caput epididymidis. GST subunit 4mu relative intensity was affected by age only in the corpus and cauda epididymidis.

Heat Shock Proteins

All of the five heat shock proteins (HSPs) included on the array (HSP90β, HSP70, HSP60, HSP27, HSPA5) were detected above background in all four segments of the young epididymis (figure 4; table 1). With age, the relative intensity of HSP90β was unchanged in the initial segment and caput epididymidis, however in the corpus and cauda epididymidis, HSP90β relative intensity decreased. Similarly, HSP27 relative intensity was affected by age only in the corpus and cauda epididymidis; transcript levels decreased to below the level of detection in the aged corpus epididymidis. The relative intensity of HSP70, decreased with age in the caput, corpus and the cauda epididymidis, but was unaffected in the initial segment. HSP60 and HSPA5 transcript levels decreased with age in all four segments of the epididiymis; the largest decreases occurred in the corpus epididymidis.

Cathepsins

Of the six cathepsin transcripts detected above background in the epididymis from young animals, three, cathepsin E, cathepsin B and cathepsin H had decreased relative intensities with age in all four epididymal regions.

Cathepsin H relative intensity decreased most dramatically in the corpus and cauda epididymidis (figure 5A; table 1). For cathepsin B the largest decrease in relative intensity occurred in the corpus epididymidis (figure 5B; table 1). Cathepsin E transcripts decreased to below the level of detection in all epididymal regions (figure 5B; table 1). Relative intensity of the other three cathepsin transcripts, cathepsin C, cathepsin K and cathepsin L decreased in all epididymal regions except the caput epididymidis (figure 5A; table 1). The relative intensity of Cathepsin K was affected by age to the greatest extent in the initial segment and corpus epididymidis. Transcript levels for cathepsin L decreased most in the corpus and cauda epididymidis. Similarly, in aged animals, the relative intensity of cathepsin C decreased in the cauda epididymidis and was below the level of detection in the corpus epididymidis.

Proteasome components

The effect of age on proteasome components was most dramatic in the distal epididymis (figure 6 A, B; table 1). In the initial segment and caput epididymidis, four of the seven proteasome components detected above background (proteasome component C3, proteasome subunit RC6 1, proteasome activator rPA28 subunitα, proteasome β subunit) did not change by more than 33% with age. For the remaining three components (proteasome subunit C5, proteasome subunit R delta, proteasome component C2), relative intensity changes ranged from 49% to 62%. This is in sharp contrast to the corpus and cauda epididymidis, where five of the seven components (proteasome subunit RC6 1, proteasome βsubunit, proteasome subunit C5, proteasome subunit R delta, proteasome component C2) had relative intensities that decreased by more than 67% with age. The other two components, proteasome component C3 and proteasome activator rPA28 subunitα, decreased most strikingly in the corpus epididymidis.

Discussion

In this study we have used cDNA microarrrays to analyze gene expression during aging in the epididymis. Age had a profound effect on overall relative intensity of gene expression in this tissue; this effect was remarkably different in the proximal and the distal epididymis. While fewer genes were detected with age in all regions of the tissue, the discrepancy between old and young was much greater in the corpus and cauda epididymidis than in the initial segment and caput epididymidis.

A small subset of genes had decreased relative intensity with age in all four segments of the epididymis. It was not surprising that genes coding for ribosome components and ATP synthetic machinery were among those genes that decreased along the entire tissue, since both protein synthesis and cytochrome c oxidase activity have been shown to decrease with age (Moldave et al., 1979; Rattan, 1991; Shikama and Brack, 1996).

Very few genes decreased in relative intensity with age exclusively in the initial segment, caput and cauda epididymidis. In contrast, many genes were affected by age uniquely in the corpus epididymidis. That the corpus epididymidis is the region most severely affected by aging in terms of relative intensity of gene expression correlates quite well with histological observations where the most extensive changes occurred in the corpus epididymidis (Serre and Robaire, 1998)

There were no transcripts that had increased relative intensity with age in all four segments, however some genes had segment specific increased relative intensity with age. One of these genes was tissue inhibitor of metalloproteinase-2 (TIMP-2), an inhibitor (in tissue) of matrix metalloproteases, the principal matrix degrading proteases involved in extracellular matrix remodeling. Basement membrane thickening is a characteristic of aging and is observed along the aged epididymis (Serre and Robaire, 1998).

Oxidative stress damage is implicated in the aging process. In the epididymal epithelium, age had a profound effect on the relative intensity of transcript levels for the oxidant defense systems that we examined; all relative

intensities decreased with age in all epididymal segments. Interestingly, while some HSPs decreased in all four epididymal segments, there were several that decreased exclusively in the distal epididymis. HSP transcription is activated, in order to protect the cell, by numerous stressors, including exposure to heat, oxygen free radicals, heavy metals, fever, inflammation and infection (Morimoto, 1998). Decreased expression of HSPs may result in decreased cellular capacity to repair damaged protein and aging has been associated with a decrease in chaperone mediated autophagy (Cuervo and Dice, 2000). The segment-specific, age related accumulation of lysosomes in the different epididymal regions (Serre and Robaire 1998) may be a reflection of decreased activity in this pathway.

In addition to increased production of damaged protein, aging is associated also with decreased cellular capacity to degrade proteins (Sitte et al., 2000; Friguet et al., 2000). In order to assess the protein degradative capabilities of the aged epididymal epithelium, we examined the expression of both lysosomal cathepsin enzymes, and components of the proteasome. All of the cathepsins we examined had decreased relative intensity with age. This is interesting given that the extent of lysosome accumulation is much more pronounced in the corpus and cauda epididymidis (Serre and Robaire, 1998). Like the cathepsins, the relative intensity of proteasome components in the aged epididymis decreased, however for the proteasome components the age effect was highly segment specific; the corpus and cauda epididymidis had dramatic decreases in relative intensity of transcript levels while relative intensity in the initial segment and caput epididymis was relatively unaffected.

In conclusion, aging in the BN rat epididymis is associated with profound changes in the relative intensity of gene expression. While many genes and gene families were affected along the entire tissue, we have identified two gene families, heat shock proteins and proteasome components, that are affected by age in a segment specific manner. The relative intensity of these gene families is affected most dramatically in the distal regions of the epididymis and may account for the high extent of damage previously observed in these segments at the histological level.

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Table 1. Relative expression of genes in four families found in epididymal segments in young (4 months) and old (22 months) Brown Norway rats (abbreviations: SOD, superoxide dismutase; GPX, glutathione peroxidase; TDPX, thioredoxin peroxidase; GST, glutathione S-transferase; HSP, heat shock protein)

	initial segment		caput		corpus		cauda	
GENE	YOUNG (4 mo)	OLD (22 mo)	YOUNG (4 mo)	OLD (22 mo)	YOUNG (4 mo)	OLD (22 mo)	YOUNG (4 mo)	OLD (22 mo)
Cu-Zn SOD1	35.6±3.5	19.0± 6.7	29.5± 6.9	14.6± 5.1	35.7± 5.9	13.1± 6.9	41.6± 14.1	16.6± 5.5
glutathione synthase	3.3± 0.7	0.7± 0.3	1.1± 0.2	0.7± 0.2	0.8± 0.1	0.1± 0.1	1.2± 0.3	0.4± 0.1
GPX 5	1.8± 0.3	0.5± 0.2	0.5±0.1	0.3± 0.1	0.5± 0.1	0.2± 0.1	0.8± 0.1	0.4± 0.1
GPX 4	1.9± 0.3	1.2± 0.3	2.0± 0.6	1.3± 0.4	1.7± 0.2	0.6± 0.2	2.8± 1.0	0.9± 0.3
TDPX1	0.5± 0.1	0.1± 0.04	0.5± 0.1	0.2± 0.1	0.5± 0.1	0.04± 0.02	0.8± 0.4	0.1± 0.02
GST subunit 4mu	29.2± 3.8	20.3± 5.9	24.6± 5.8	19.3± 6.0	27.2± 4.0	9.7± 4.8	32.1± 8.1	17.6± 6.3
GST subunit 8	24.3± 4.4	10.8± 4.1	18.0± 4.7	9.5± 4.6	15.8± 4.1	2.9± 1.8	16.6± 5.6	5.8± 1.8
GST subunit 7pi	21.1± 1.7	10.1± 5.2	7.2± 1.9	2.6± 0.7	3.7± 0.7	1.2± 0.6	6.2± 2.1	1.6± 0.6
GST subunit 13	0.6± 0.1	0.4± 0.1	0.5± 0.1	0.5± 0.1	0.6± 0.1	0.3± 0.1	1.3± 0.6	0.4± 0.1
microsomal GST	5.4± 0.7	3.2± 1.0	3.4± 0.9	3.2± 1.1	5.7± 1.3	1.9± 0.9	5.6± 0.9	2.6± 0.9
HSP90-β	45.0± 5.6	35.8± 10.7	31.3± 5.3	35.3± 10.5	28.7± 5.4	17.3± 6.5	31.8± 3.3	19.5± 7.4
HSP70	2.4± 0.7	2.7± 1.0	5.2± 1.2	2.9± 0.9	2.5± 0.5	0.9± 0.4	4.0± 0.4	2.5± 0.8
HSP60	2.6± 0.7	1.1± 0.3	2.3± 0.8	1.5± 0.7	3.1± 0.8	0.9± 0.4	2.5± 0.7	1.0± 0.2
HSP27	0.8± 0.2	1.0± 0.3	0.9± 0.2	0.7± 0.2	0.7± 0.1	0.4± 0.1	1.0± 0.1	0.6± 0.2
HSPA5	3.0± 0.5	1.3± 0.8	1.6± 0.4	0.6± 0.2	1.3± 0.5	0.2± 0.1	2.1± 0.9	0.4± 0.2
cathepsin E	0.5± 0.1	0.2± 0.1	0.9±0.04	0.3± 0.1	0.7± 0.1	0.1± 0.05	1.1± 0.3	0.1± 0.03
cathepsin B	1.0± 0.2	0.7± 0.2	0.7± 0.2	0.5± 0.2	0.8± 0.2	0.3± 0.2	1.3± 0.3	0.8± 0.2
cathepsin H	1.8± 0.4	0.5± 0.2	1.7± 0.5	0.7± 0.2	2.8± 0.3	0.2± 0.1	2.8± 0.8	0.4± 0.1
cathepsin C	1.1± 0.2	0.4± 0.1	0.6±.0.01	0.5± 0.2	0.5± 0.1	0.1± 0.1	1.1± 0.2	0.4± 0.1
cathepsin K	15.5± 2.5	3.9± 1.3	4.2± 0.9	3.9± 2.2	2.0± 0.3	0.5± 0.4	2.8± 0.4	1.2± 0.3
cathepsin L	26.5± 3.7	17.2± 5.7	23.4± 5.0	27.5± 9.3	30.5± 2.4	6.9± 4.9	28.4± 6.0	13.2± 4.1
proteasome component C3	4.0± 1.0	3.4± 0.9	3.7± 1.3	3.6± 0.9	4.7± 0.6	1.1± 0.5	5.2± 1.1	3.1± 0.7
proteasome subunit RC6-1	2.3± 0.4	2.0± 0.5	2.1± 0.4	2.5± 0.7	2.7± 0.5	0.6± 0.3	3.6± 1.1	1.2± 0.3
proteasome activator rPA28 subunit α	8.5± 1.9	7.3± 2.7	7.7± 1.7	7.6± 2.7	7.6± 1.9	2.2± 1.6	7.3± 1.8	3.7± 1.0
proteasome beta subunit	1.6± 0.4	1.2± 0.4	1.3± 0.3	0.9± 0.3	2.4± 0.4	0.4± 0.3	3.5± 1.4	0.9± 0.2
proteasome subunit C5	2.0± 0.6	1.0± 0.3	1.5± 0.4	1.1± 0.5	2.5± 0.5	0.3± 0.2	3.3± 1.1	0.9± 0.2
proteasome subunit R-delta	1.7± 0.2	0.7± 0.2	1.8± 0.8	0.8± 0.3	1.6± 0.3	0.5± 0.2	1.9± 0.7	0.6± 0.2
proteasome component C2	2.3± 0.3	1.1± 0.4	1.9± 0.6	1.4± 0.7	2.7± 0.4	0.4± 0.3	3.5± 0.8	0.7± 0.1

Figure 1. Number of genes expressed above two fold background in the epididymis. All, all four epididymal segments. Black bar, number of genes expressed in the young epididymis. Light grey bar, number of genes expressed in the aged epididymis.

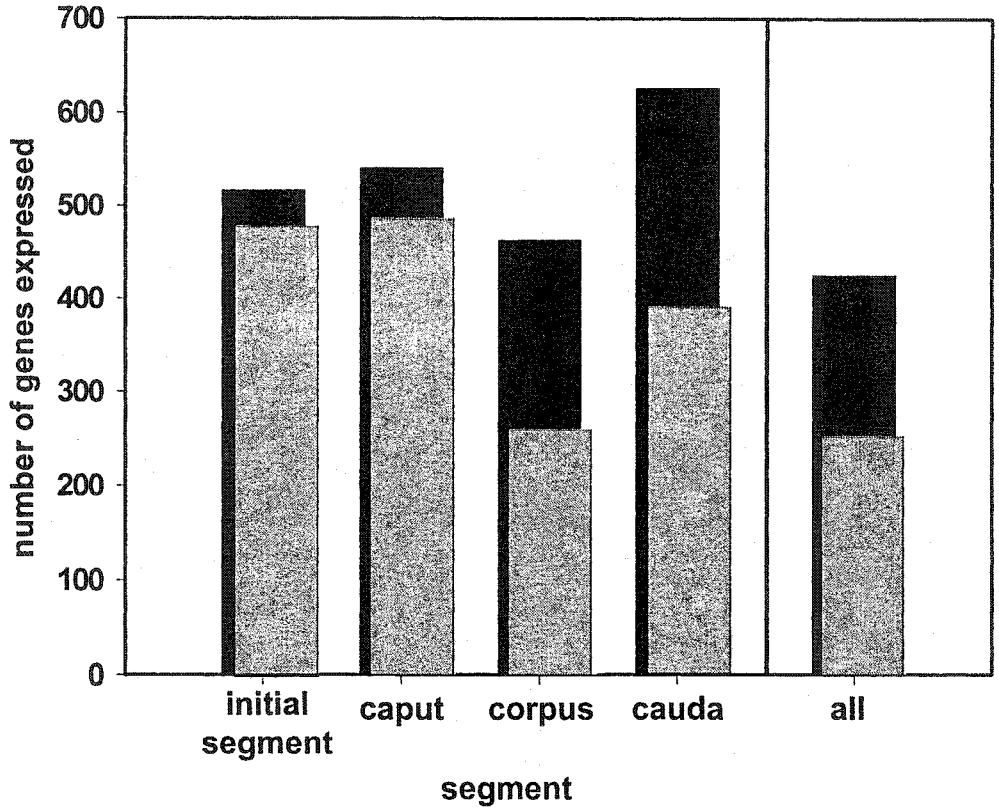

700
600
500
400
300
200
100
0
number of genes expressed
initial segment
caput
corpus
cauda
all
segment

Figure 2. Gene expression decreases with age along the epididymis. White, less than 33% decrease with age. Light grey, 33% to 50% decrease with age. Dark grey, 50% or greater decrease with age.

GENE EXPRESSION DECREASES WITH AGE

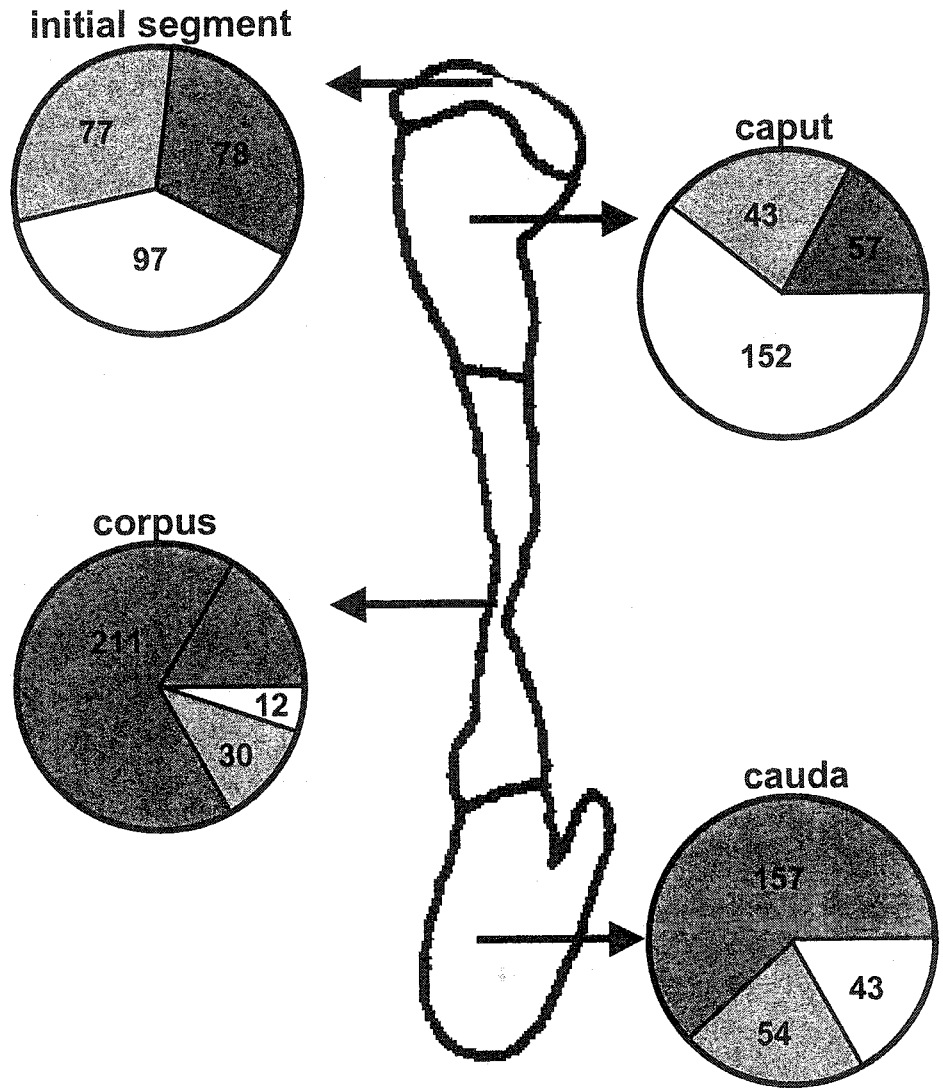
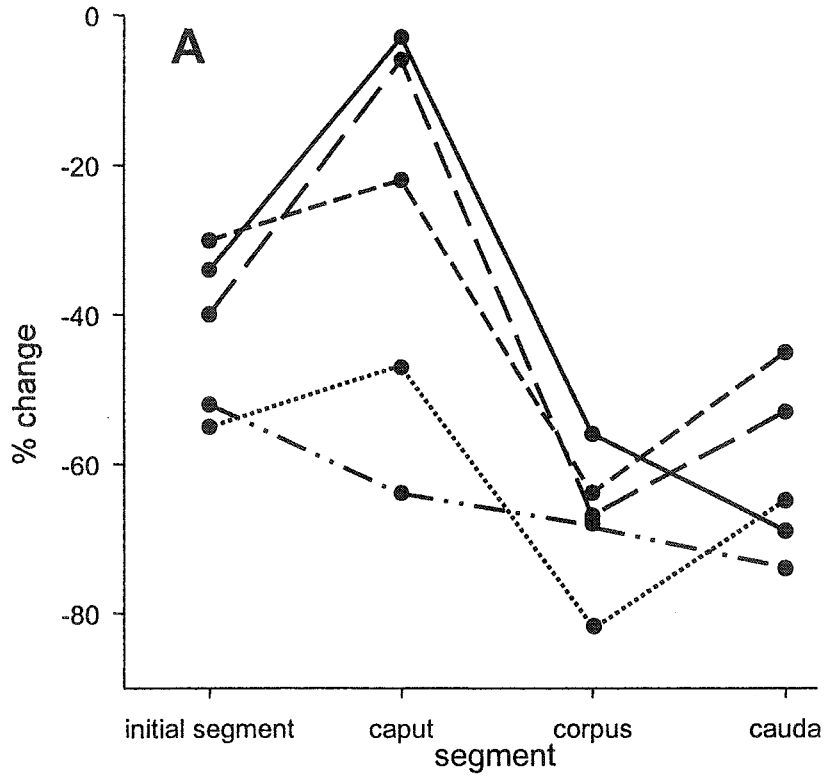


Figure 3. Ratio of expression (old to young) of oxidative stress related genes along Brown Norway rat epididymides. A) Phospholipid hydroperoxide glutathione peroxidase (GPX4), dashed line-one dot. Epididymal secretory glutathione peroxidase (GPX5), dashed line. Glutathione synthetase, solid line. Copper-zinc superoxide dismutase (Cu-Zn SOD1), dotted line. Thioredoxin peroxidase (TDPX1), dashed line-two dots. B) Glutathione s-transferase (GST) subunit 13, solid line. Microsomal GST, long dash. Glutathione s-transferase (GST) subunit 4mu, short dash. GST subunit 8, dotted line. GST subunit 7pi, dashed line-two dots.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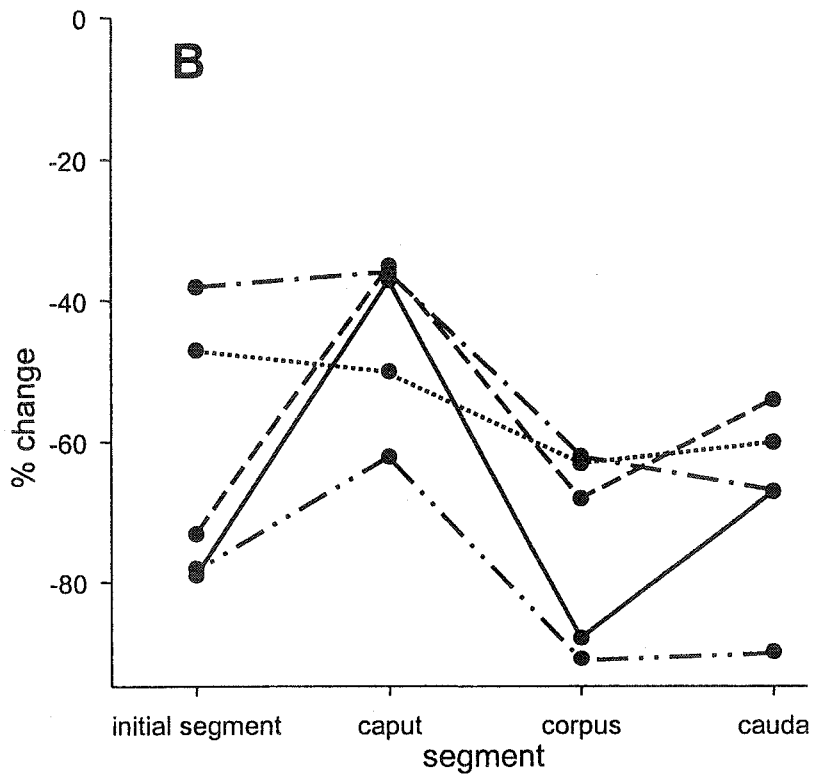
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Figure 4. Ratio of expression (old to young) of heat shock protein (HSP) along Brown Norway rat epididymides. HSP90b, solid line. HSP27, dashed line-two dots. HSP60, dotted line. HSP70, short dash. HSPA5, long dash.

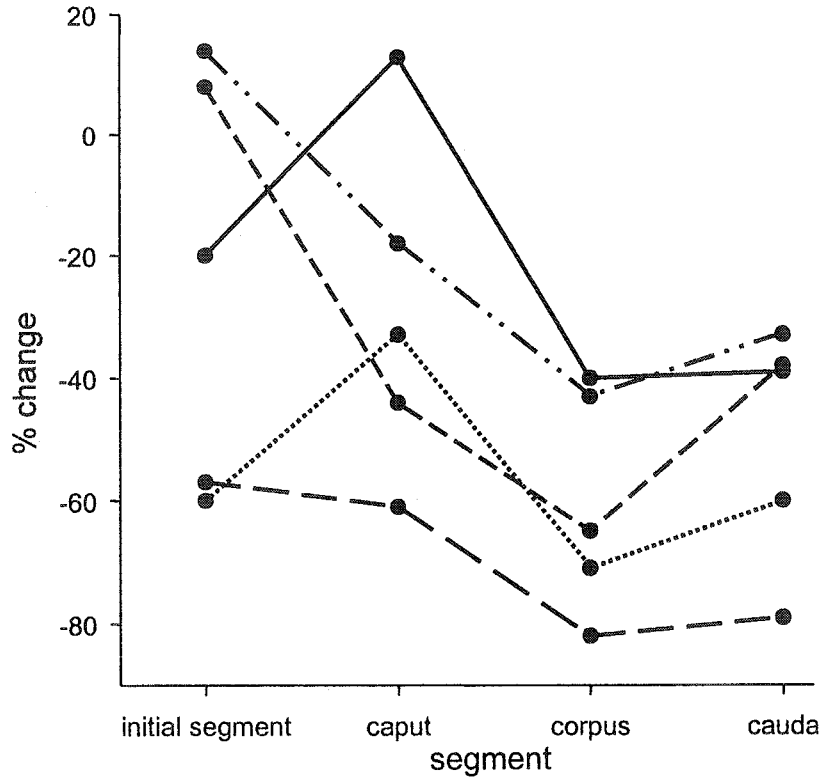
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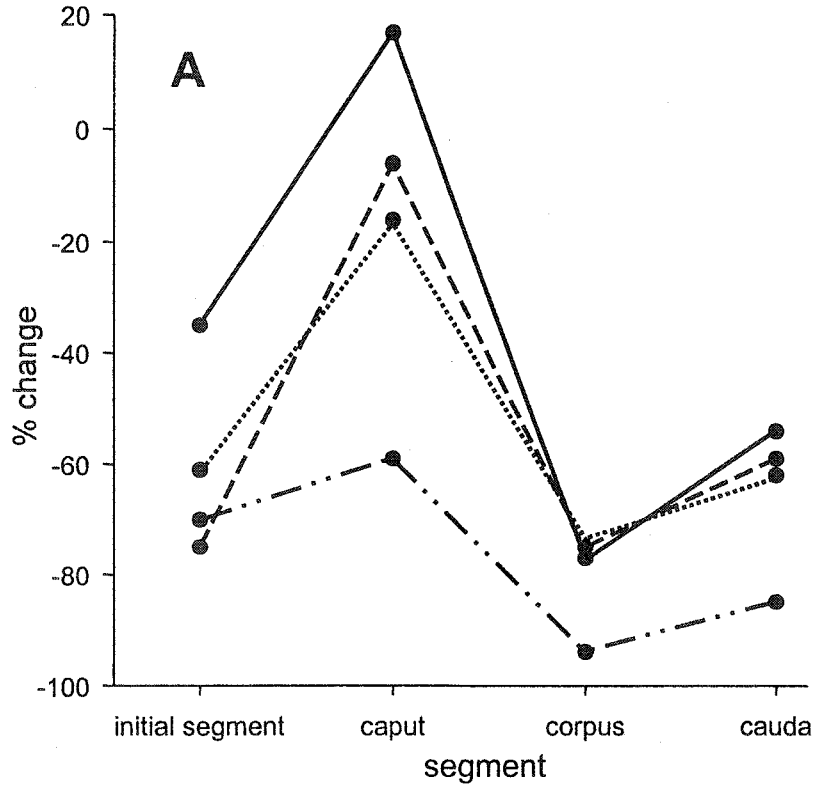
Figure 5. Ratio of expression (old to young) of cathepsins along Brown Norway rat epididymides. A) Cathepsin L, solid line. Cathepsin K, dashed line. Cathepsin C, dotted line. Cathepsin H, dashed line-two dots. B) Cathepsin B, dotted line. Cathepsin E, solid line.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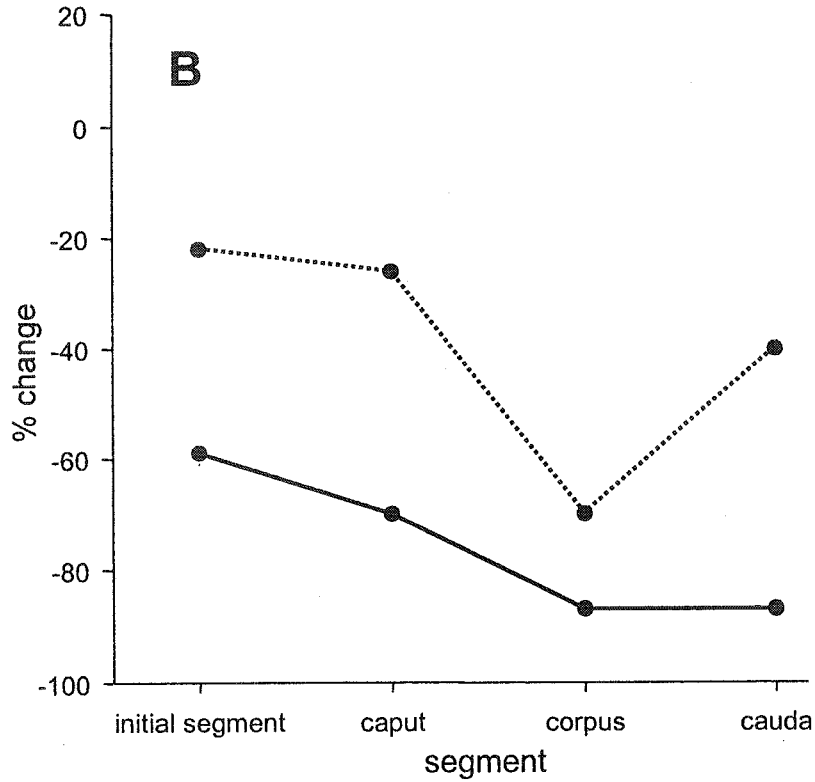
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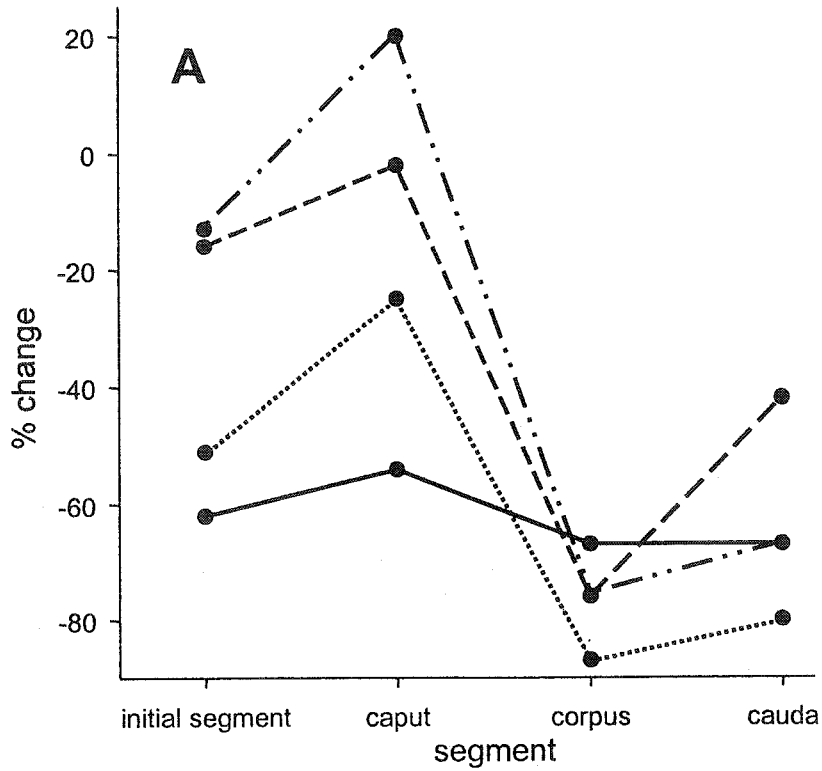
Figure 6. Ratio of expression (old to young) of proteasome components along Brown Norway rat epididymides. A) Proteasome subunit RC6-1, dashed line-two dots. Proteasome component C3, dashed line. Proteasome component C2, dotted line. Proteasome subunit R-delta, solid line. B) Proteasome activator rPA28 subunit, dashed line. Proteasome beta subunit, solid line. Proteasome subunit C5, dotted line.


A
20
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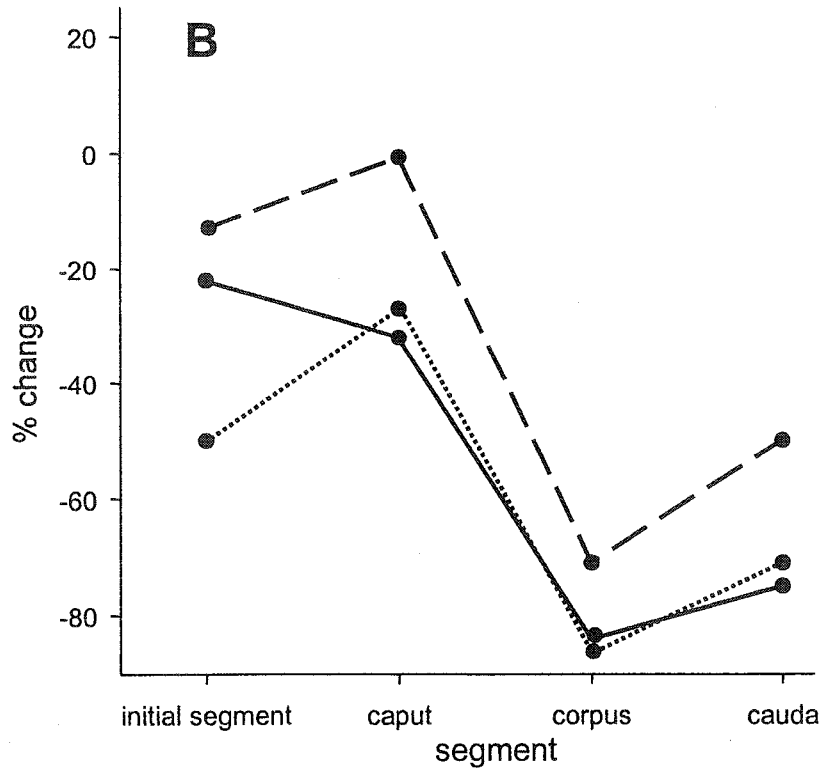
-60
-80
% change
initial segment
caput
corpus
cauda
segment


B
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-80
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initial segment
caput
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segment

Connecting Text for Chapter Three to Four

Once we had characterized the transcriptional response of the epididymal epithelium to aging, we wanted to dissect out the molecular mechanisms that underlie these gene expression changes. Specifically, we wanted to assess the contribution of oxidative stress to aging of the epididymal epithelium. The most powerful tool available to aging researchers is caloric restriction, the only manipulation known to consistently attenuate the aging process. Caloric restriction is known to modulate oxidative stress, and genetic pathways that mediate the effects of caloric restriction converge on oxidative stress response genes. We wanted to assess the effects of caloric restriction on gene expression in the aging epididymis as one way of dissecting out the contribution of oxidative stress to this process.

Chapter 4

Effects of Caloric Restriction on Gene Expression Along The Epididymis Of The Brown Norway Rat During Aging

Abstract

Aging in the Brown Norway (BN) rat, is associated with changes in the male reproductive tract. In the epididymis, the site of sperm maturation and storage, aging has profound effects on the histology, biochemistry and gene expression of the tissue. Caloric restriction consistently slows aging and maintains health in aging animals. The goal of this study was to assess the effects of caloric restriction on aging-related changes in gene expression in the BN rat epididymis using cDNA microarrays. Caloric restriction attenuated or reversed age-related gene expression changes throughout the epididymis. In the distal regions of the tissue (corpus and cauda epididymidis), greater than 80% of the gene expression decreases were attenuated by caloric restriction. In the most proximal region of the tissue (initial segment), caloric restriction affected the expression of genes associated with lipid and carbohydrate metabolism. In all regions of the epididymal epithelium, caloric restriction had a dramatic effect on the age-related decreased expression of genes associated with protein synthesis and mitochondrial function. These results indicate that caloric restriction lessens age-related mitochondrial dysfunction and improves protein synthetic capability throughout the epididymis.

Introduction

The Brown Norway (BN) rat is an established model for the study of aging of the male reproductive system (Zirkin et al., 1993; Gruenewald et al., 1994; Zirkin and Chen, 2000). Aging in the male BN rat, as in man, is associated with decreased serum testosterone levels (Gruenewald et al., 1994) and decreased spermatogenesis (Wright et al., 1993) in otherwise healthy animals. BN rats are long lived, do not become obese, and rarely develop pituitary, testicular or other tumors (Zirkin et al., 1993); thus ,aging in the reproductive tract can be studied without the confounding influence of pathology.

The epididymis, the tissue where spermatozoa are matured and stored, is composed of four major epithelial cell types and can be divided anatomically into four segments: the initial segment, caput, corpus and cauda epididymidis. With age, the epididymal epithelium acquires morphological hallmarks of aging. For example, there is a region specific accumulation of lipofuscin, lysosomes and vacuoles and the basement membrane thickens (Serre and Robaire, 1998). Gene expression along the tissue has also been shown to be altered with age in a segment specific manner (Viger and Robaire, 1995; Jervis and Robaire, 2002). These changes in gene expression are analogous to those observed in other aging tissues and suggest that an altered stress response, including oxidative stress, and decreased protein turnover and degradation, play a role in the pathology of the aging epididymal epithelium (Jervis and Robaire, 2002).

Caloric restriction, the under but not malnutrition of an organism, is the only intervention conclusively and reproducibly shown to slow aging and delay the appearance of age-associated pathologic and biologic changes (Weindruch and Sohal, 1997; Nicolas, 1999). Caloric restriction has life extending action in both genders and across many species, including laboratory rodents and non-mammalian species such as fish and flies (Masoro, 2000). This robustness and reproducibility make caloric restriction a commonly employed methodology in aging studies. Caloric restriction induces a plethora of biological effects, but the mechanisms underlying the anti-aging effects of caloric restriction are unknown; the many hypotheses that have been proposed include attenuation of oxidative stress/damage, increased resistance to stress, altered intermediary metabolism and improved glucoregulation (Weindruch, 2001; Yu and Chung, 2001). In order to determine the effects of caloric restriction on aging of the epididymal epithelium, we used microarray technology to explore how the changes that take place in the epididymal epithelium with age are affected by caloric restriction.

Materials and Methods

Animals and diets

Male BN rats and their age matched caloric restricted controls (5, 25 months) were obtained through the National Institutes on Aging (Bethesda, MD) from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Caloric restriction was as follows: caloric restriction was initiated at 14 weeks of age at 10% restriction, increased to 25% restriction at 15 weeks, and to 40% restriction at 16 weeks of age. From 16 weeks, caloric restriction was maintained at 40% for the life of the animal. Access to water was ad libitum. The diet of caloric restricted rats was the same as that of the ad libitum fed animals (NIH31) except that it was supplemented with vitamins and minerals in order to assure proper intake of essential nutrients (NIH31/NIA fortified). Both caloric restricted and ad libitum animals were individually housed (22°C, humidity 40-55%, 12L:12D) in a Specific Pathogen Free (SPF) environment. This calorie restricted diet has been used successfully to increase life span and to study the effect of caloric restriction on longevity and disease; survival curves, food intake curves and body weights of the NIA strains have been published (Turturro et al., 1999).

Rats were sacrificed by decapitation. No gross abnormalities were observed in any of the tissues examined. Epididymides were sectioned into initial segment, caput, corpus, and cauda regions, immediately frozen in liquid nitrogen, and stored at -80°C until used for RNA extraction. All animal studies were conducted in accordance with the principles and procedures outlined in A Guide to the Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care (McGill protocol #206).

RNA Extraction

Total RNA was extracted and DNase treated as reported previously (Jervis and Robaire, 2001). The concentration was determined by absorbance at 260nm (Beckman DU7 spectrophotometer, Montreal, QC). To verify the quality of the sample, 5µg RNA was run on a denaturing gel containing 1% agarose-formaldehyde. Each RNA sample was extracted from a single epididymal segment obtained from an individual animal.

cDNA Arrays

RNA samples were used to probe cDNA arrays (Clontech, Atlas Rat 1.2K, Palo Alto, CA) according to the manufacturer's instructions and as reported previously (Jervis and Robaire, 2001). Five arrays per epididymal segment per age group were probed and are referred to as replicates. Arrays were exposed to phosphorimager plates (Amersham Biosciences, Piscataway, NJ) for 24h before scanning (Storm, Amersham Biosciences, Piscataway, NJ).

Data Analysis

Analysis of array images with Atlas Image (Version 1.5, Clontech, Palo Alto, CA) was done to quantify the intensity of each cDNA spot, which reflects the relative abundance of the RNA in the sample. The raw data for each gene (intensity of each spot on the array minus the background of that array) were imported into GeneSpring (Silicon Genetics, Redwood, CA) for further analysis. A specific cutoff value was defined in order to ensure that the median expression on an array was not anomalous; the median expression on an array had to fall above this specified cutoff in order to be normalized and thus analyzed in the data set.

Data were normalized as follows: the median level of expression on each individual array was defined as one, and all genes on that array were normalized relative to one. The resulting relative expression values for each gene were calculated for all five replicate arrays and averaged. This average is referred to as the relative intensity for any given gene. A gene was only considered expressed if its relative intensity was at least two fold greater than background in at least three of the five replicate arrays (Jervis and Robaire, 2001). Replication in gene array expression studies has been documented to result in more consistent and reliable findings (Lee et al., 2000; Herwig et al., 2001). In this study, changes in the relative intensity of a gene were considered only when consistent in at least three out of five replicates (Jervis and Robaire, 2001, 2002). Gene expression changes of two fold or greater are focused on throughout the

text except for genes belonging to specific gene families; the data for these genes are presented regardless of the extent of the change in expression.

Results

The Effects of Caloric Restriction on Epididymal Gene Expression

Caloric restriction resulted in gene expression changes at all ages examined. Since caloric restriction induced changes in gene expression at 25 months of age cannot be dissociated from those changes in gene expression that take place with age, in this section we will focus only on the changes that take place at 5 months. The elapsed time between the establishment of caloric restriction (at 16 weeks) and the age at which these animals were sacrificed (20 weeks) is short, therefore, these changes are presumed to be caloric restriction-specific. Of the genes expressed in tissues from both ad libitum and caloric restricted animals (average for all segments at 5 months, 222 genes), the number of genes that exhibited at least two fold changes in relative intensity with caloric restriction was relatively small in all four segments of the epididymis (figure 1). In general, more genes decreased in relative intensity with caloric restriction at 5 months than increased. Of the genes that did increase (Table1), few increased in more than one region of the tissue. Clusterin, a ubiquitously expressed heterodimeric glycoprotein, was increased in relative intensity in the corpus and cauda epididymidis, and insulin like growth factor binding protein-3 (IGFBP-3) was increased in the caput and cauda epididymidis.

With respect to gene expression decreases with caloric restriction (Table 2), the corpus epididymidis had by far the largest number of genes that decreased by two fold or more. These genes included cell cycle associated genes (G1/S-specific cyclin D1, D2 and D3, serine/threonine-protein kinase PCTAIRE 1), members of the insulin-like growth factor family (IGFBP-1, IGFBP-5), oncogenes and tumour suppressors (ovalbumin upstream promoter gamma nuclear receptor rCOUPg, c-jun, a-raf) and genes associated with immune function/immune cells (β2-microglobulin, interleukin (IL) 6 receptor β chain, CD25, tumor necrosis factor (TNF) receptor 1, calcineurin A). Similarly, genes that had a decreased

expression with caloric restriction in the initial segment were cell cycle associated genes (G1/S-specific cyclin D1, and D3), members of the insulin-like growth factor family (IGFBP-5) and genes associated with immune function/immune cells (β2-microglobulin, CD24. Interestingly, the genes that decreased in relative intensity in the cauda epididymidis are completely unrelated to those that decreased in the other segments and include neural related genes and MMP-2 (gelatinase A). Since the cauda region of the epididymis has contractile capabilities for moving mature spermatozoa along to the vas deferens, the identity of these genes may reflect the large proportion of smooth muscle and nerve cells required to perform this function.

The Effects of Caloric Restriction on Epididymal Gene Expression During Aging

Of the genes that are expressed at both 5 and 25 months in tissues from ad libitum and caloric restricted animals (average for all segments, 169 genes), the number of genes that changed by at least two fold with age was remarkably fewer in caloric restricted animals (39 genes changed by two fold or greater) as compared to ad libitum controls (119 genes changed by two fold or greater; figure 2). In ad libitum animals, the initial segment had a large number of genes that decreased expression with age however no genes increased expression with age. In contrast, in the caput epididymidis no genes decreased expression with age but many increased. In the corpus and cauda epididymidis, more genes decreased expression with age than increased. With respect to gene expression increases with age, in the distal regions of the tissue (corpus and cauda epididymidis), more genes increased in tissues from caloric restricted animals than in the tissues from ad libitum animals. In order to better understand the effects of caloric restriction on this tissue, the transcripts that changed by at least two fold with age will be discussed in further detail in the subsequent two sections.

Age-Dependent Increases in Gene Expression

In general, the expression of more genes increased with age in caloric restricted animals than in ad libitum animals, although in both groups many of these genes were those affected by caloric restriction at 5 months (e.g., gastric inhibitory polypeptide precursor (GIP), IGFBP-1 and 3, CCND2, CD24 and clusterin). In ad libitum animals, the caput epididymidis had the largest number of mRNA's with increased relative intensity with age including those related to the cytoskeleton (cofilin, cytoplasmic β-actin, ezrin), cathepsins (cathepsin L and K) and ribosomal proteins (60S ribosomal protein L21, ribosomal protein S29 40S subunit (RPS29)). K-ras 2b, a proto-oncogene, increased 123% with age in the caput epididymidis of ad libitum animals but decreased 51% in relative intensity with age in caloric restricted animals.

Several of the genes that had increased relative intensity with age in tissues from caloric restricted animals are involved in the control of cell growth/proliferation, e.g., transforming growth factor-β1 (TGF-β1), Myc-Max-interacting tumor suppressor (MXI1) and I-kappa B (I-κB). Notably, I-κB and one other gene, ribosomal protein S4, both increased with age in tissues from caloric restricted animals but decreased with age in tissues from ad libitum animals. Genes that increased with age in tissues from both ad libitum and caloric restricted animals were few but included tissue inhibitor of metalloproteinase-2 (TIMP-2) and glutathione S-transferase P subunit (GST subunit 7 pi).

Age-Dependent Decreases in Gene Expression

Caloric restriction was able to reverse or attenuate many of the gene expression changes that occurred in tissues from ad libitum animals with age; genes that decreased with age in tissues from ad libitum animals but did not decrease in tissues from caloric restricted animals are referred to as "rescued" (Table 3). Of the genes that decreased by at least 50% with age in the initial segment, corpus and cauda epididymidis in tissues from ad libitum animals, 56%, 95% and 83% (respectively) were rescued in tissues from caloric restricted animals. There was little overlap in the identity of these rescued genes between the different regions of the tissue except in the initial segment and corpus

epididymidis where five genes, sertoli cell cytochrome c oxidase polypeptide I (COX1), ATP synthase lipid-binding protein P1 (ATP5G1), mitochondrial ATP synthase D subunit (ATP5H), calmodulin, calcium binding protein 2 and inositol 1,4,5-triphosphate 3-kinase receptor 2, were in common. Genes that were rescued by caloric restriction exclusively in the initial segment were numerous and included mRNA's involved in energy, lipid and carbohydrate metabolism, ribosomal proteins and oncogenes/tumour suppressors. In the corpus epididymidis, many of the mRNAs that decreased in tissues from ad libitum but not in tissues from caloric restricted animals were ribosomal proteins. Interestingly, the message for steroid 5α-reductase type 2, a critical enzyme for the maintenance of epididymal physiology was also rescued in this segment of caloric restricted animals. In the cauda epididymidis, since only very few genes decreased with age in tissues from ad libitum animals, fewer genes were rescued in this segment than in the initial segment or corpus epididymidis and many of them were affected by caloric restriction at 5 months of age.

The initial segment was the only region of the tissue that had a subset of mRNAs (10) that decreased by at least 50% with age in tissues from both ad libitum and caloric restricted animals and therefore were not rescued by caloric restriction. Of these, 3 were affected by caloric restriction in other segments at 5 months (PCTAIRE1, ribosomal protein L12, LIM domain protein CLP36). Those that were not affected by caloric restriction include prothymosin-α (PTMA), synaptobrevin-2, GTP-binding protein (G-α-8), Casein kinase I δ, glutathione S-transferase Yb subunit; GST subunit 4 mu (GSTM2), ras-GTPase-activating protein (p120GAP) and polyubiquitin.

There were genes in all four segments of the epididymis that decreased with age in tissues from caloric restricted animals that did not in tissues from ad libitum animals (Table 4). One gene, polyubiquitin, showed age related decreased relative intensity of expression in the initial segment, caput and corpus epididymidis from caloric restricted animals. The caput epididymidis had the largest number of genes that decreased with age in caloric restricted animals and

interestingly, these included glutathione S-transferases (subunit 8, GST subunit 4 mu).

Gene Families

Caloric restriction attenuated the age related changes in expression for many genes representing a variety of gene families. However, two gene families, ribosomal protein subunits and energy related transcripts, were particularly noteworthy as caloric restriction affected the expression of multiple members of these families in more than one epididymal region. In order to further assess the effects of caloric restriction on the epididymal epithelium, we examined the expression of members of these gene families in detail.

Ribosomal Protein Subunits

Of the many ribosomal subunits present on the microarray, we examined the expression of subunits purported to be involved in different facets of ribosomal function, including translation initiation (40S ribosomal subunits S3a and S19), fidelity or accuracy of translation (ribosomal subunits S4 and S12), RNA binding (ribosomal subunits L11 and S11) and elongation or peptidyl transfer (60S ribosomal subunits L44 and L12). For the transcripts of those subunits involved in translation initiation and accuracy (figure 4, A and B) caloric restriction attenuated, and in some regions of the tissue reversed, the age related decreases in gene expression observed in tissues from ad libitum animals. This effect was particularly dramatic for ribosomal subunit S3a, where in the body of the tissue (caput and corpus epididymidis), age related gene expression decreases of 27 and 36% (respectively) in tissues from ad libitum animals were 51 and 66% increases in tissues from caloric restricted animals. Similarly, for ribosomal subunit S4, in some regions of the tissue (initial segment and corpus epididymidis) age related gene expression decreases of 67% and 41% (respectively) in tissues from ad libitum animals were attenuated in tissues from caloric restricted animals. In another region of the tissue (caput epididymidis) an

increase in gene expression of 49% with age in ad libitum animals was further increased in caloric restricted animals to 110%.

Transcripts of ribosomal subunits involved in RNA binding and elongation/peptidyl transfer (figure 4, A and B) also showed age related decreased expression in tissues from ad libitum animals and again, marked attenuation of these decreases in tissues from caloric restricted animals. For example, ribosomal subunit L11 expression decreased by 50% and 53% in the initial segment and corpus (respectively) of ad libitum animals; in caloric restricted animals these age related decreases were attenuated to 11 and 13% respectively.

Energy Related Transcripts

To better understand the impact of caloric restriction on the expression of transcripts related to energy metabolism, we examined in detail the expression of genes involved in electron transport (cytochrome c oxidase (COX) polypeptide Vb, COX5B; cytochrome b5, CYB5; Sertoli cell COX polypeptide I, COX1; COX subunit IV, COX4) and components of mitochondrial ATP synthase (mitochondrial ATP synthase D subunit, ATP5H; mitochondrial ATP synthase subunit C isoform 1, ATP5G1; mitochondrial ATP synthase B subunit, ATP5F1; mitochondrial ATP synthase β subunit, ATP5B). For transcripts involved in electron transport (figure 5, A and B), caloric restriction attenuated, and reversed in some cases, the effects of age on gene expression decreases. Interestingly however, caloric restriction also attenuated any age related increases in gene expression for these genes. Most dramatically, changes in COX5 gene expression with age (50% decreases in the initial segment and corpus epididymidis and 94% increases in the caput epididymidis) in tissues from ad libitum animals were all reduced to less than 15% changes with age in tissues from caloric restricted animals. Expression of components of the Fo portion of the F1Fo ATP synthase (ATP5H, ATP5G1, ATP5F1; figure 5) decreased dramatically in ad libitum tissues with age but in caloric restricted tissues the decreases were substantially reduced. The F1 component of ATP synthase

(ATP5B) increased in one region of the tissue (caput epididymidis) and decreased to a lesser extent in other regions of the tissue in ad libitum animals; caloric restriction attenuated and reversed the age related decreases in expression but also diminished the age related increased expression of this transcript.

Discussion

The robustness and reproducibility of caloric restriction have made it a commonly used experimental paradigm for the study of aging (Nicolas et al., 1999). As a result, the effects of caloric restriction on different tissues and how they age, is becoming well documented (Kayo at al., 2001; Cao et al., 2001; Weindruch et al., 2002). We found that short term caloric restriction (5 weeks) induced changes in gene expression along the epididymis. These gene expression changes include genes and gene families that were affected by caloric restriction in other species and tissues. For example, the effects of caloric restriction on the insulin-like growth factor signaling pathway (Kari et al., 1999; Sonntag et al., 1999) and immune function (Frame et al, 1998; Pahlavani, 2000) have been recognized. Other gene expression changes we observed, such as the decreases in androgen receptor and clusterin expression, are more particular to the effects of caloric restriction on the epididymal epithelium. Additionally, the four different regions of the epididymis responded quite differently to caloric restriction. The different regions of the epididymis are histologically, functionally and biochemically distinct (Hermo and Robaire, 2002). The response of the tissue in a region specific manner to short term caloric restriction is consistent with our knowledge of gene expression in the epididymis; there are numerous examples of region specific gene expression in this tissue (Cornwall et al., 2002; Kirchoff et al., 2002), and many instances of segment specific responses to various experimental manipulations have been reported (Ezer and Robaire, 2002).

In the epididymis of aged rats, long term caloric restriction had profound effects on gene expression. Age related increases and decreases in expression

were reversed or attenuated by caloric restriction for many different genes and gene families. Many genes and gene families were affected by caloric restriction in a segment specific manner. In the initial segment of the epididymis, for example, the region of the tissue with the largest number of genes that decreased in expression by two fold or more with age, there was a specific effect of caloric restriction on transcripts involved in carbohydrate and lipid metabolism. Caloric restriction can affect aspects of carbohydrate and lipid metabolism (Weindruch et al., 2001a, b), and metabolic enzymes in this region of the tissue have been shown to respond more profoundly than the rest of the tissue in other experimental manipulations (Brooks, 1979).

The effects of age on the epididymis have been shown to be highly region specific (Viger and Robaire, 1995; Serre and Robaire, 1998; Jervis and Robaire, 2002). In a previous study (Jervis and Robaire, 2002), we demonstrated that age caused a reduced expression of several oxidative stress related genes, heat shock proteins and components of intracellular degradation pathways. A similar conclusion may be drawn from the present study; however, there are differences between region-specific expression described in our former study and the present results. The primary reasons for some of the observed differences are likely differences in the control group (controlled diet vs. ad lib feeding of rat chow) and a changed method for normalization of data. Many of the effects of caloric restriction were region specific as well, it was particularly interesting that the expression of genes for ribosomal components and transcripts involved in energy metabolism was rescued by caloric restriction in all regions of the epididymal epithelium. Protein turnover, the balance between protein synthesis and degradation, has long been recognized to decrease with age (reviewed in Richardson and Cheung, 1982; Rattan, 1991) and has been proposed to play a role in the aging process (Rattan, 1996; Jazwinski, 2000; Ryazanov and Nefsky, 2000). The mechanism(s) by which protein turnover decreases with age are unclear and may be numerous, however, any imbalance in the protein synthetic machinery will impair the capacity of a cell to synthesize protein. Our results indicate that caloric restriction is able to attenuate and even reverse the age

associated decreased transcript levels of several ribosomal components; this effect occurred across all of the regions of the tissue for ribosomal components involved in many different facets of ribosomal function. This result may signify improved protein synthetic capability in caloric restricted tissues as compared to their ad libitum counterparts.

As we observed for ribosomal components, age related changes in the expression of energy related transcripts in ad libitum animals were reversed or reduced in caloric restricted animals in all regions of the epididymal epithelium. Mitochondria produce ATP through the process of oxidative phosphorylation and as a by-product of this process, reactive oxygen species (ROS) are generated. ROS are required for a variety of cellular processes; however, in excess they can react with DNA, protein and lipids and ultimately damage the cell if repair capabilities are inadequate (Martindale and Holbrook, 2002). Mitochondria are the primary cellular source of ROS: mitochondrial dysfunction leading to excess production of ROS is the basic tenet underlying the free radical theory of aging (Harman, 1956; reviewed in Harman, 2001), a hypothesis that has gained considerable attention in aging research. The observation that age related changes in components of oxidative phosphorylation are ameliorated in caloric restricted animals as compared to ad libitum controls suggests that there is less dysfunction at the level of the mitochondria, therefore resulting in decreased production of ROS.

In conclusion, our results show that in the epididymal epithelium, age related alterations in two aspects of cellular metabolism, protein synthesis and energy production, are partially or fully reversed with caloric restriction. The effect of caloric restriction on these gene families was not limited to one region of the tissue but rather occurred throughout its length. This suggests that caloric restriction may improve biosynthetic capabilities and reduce mitochondrial dysfunction in the aging epididymis.

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Table 1. Increased Gene Expression with Caloric Restriction

Epididymal Segment	Gene	Genbank Acession #	Ad libitum 5 months ±SEM	Caloric Restricted 5 months ±SEM	↑ by (%)
initial segment	carboxypeptidase D	U62897	3.6±1.20	7.34±1.34	103.89
caput	PKI-β	M64092	2.15±0.48	7.0±3.7	225.58
	androgen receptor	M20133	1.07±0.23	2.64±0.48	146.73
	retinoblastoma protein	D25233	1.16±0.29	2.82±0.64	143.10
	RAB8	M83675	0.97±0.26	2.18±0.50	124.74
	IGFBP-3	M31837	1.28±0.35	2.75±0.93	114.84
	ACE	U03734	11.99±3.00	24.4±4.65	103.50
	EDDR1	L26525	2.21±0.36	4.53±0.73	104.98
	EIF-2-α	J02646	7.73±1.18	15.83±3.61	104.79
corpus	clusterin	M64723	3.37±0.99	6.97±1.77	106.82
cauda	clusterin	M64723	9.93±3.44	36.83±5.86	270.90
	IGFBP-3	M31837	2.42±0.33	5.07±0.75	109.50

PKI-β, cAMP-dependent protein kinase inhibitor 2; RAB8, ras-related protein; IGFBP-3, insulin-like growth factor binding protein-3; ACE, angiotensin-converting enzyme; EDDR1, epithelial discoidin domain receptor 1; EIF-2-α, eukaryotic translation initiation factor 2α subunit.

Table 2. Decreased Gene Expression with Caloric Restriction

Epididymal Segment	Gene	Genbank Acession #	Ad libitum 5 months ±SEM	Caloric Restricted 5 months ±SEM	%↓
initial segment	proteasome β	L17127	10.29±8.80	2.55±0.46	75.22
	CCND3	D16309	8.61±1.77	3.51±1.15	59.23
	CD24 antigen	U49062	23.04±11.77	9.59±3.51	58.38
	IGFBP-5	M62781	12.95±4.96	5.54±1.85	57.22
	β-2-microglobulin	X16956; U26663	156.52±30.55	69.92±10.46	55.32
	CCND1	D14014	7.61±1.90	3.58±0.4	52.96
caput	PCNA	Y00047	2.21±0.40	0.95±0.30	57.01
	CCND1	D14014	4.13±0.67	1.91±0.44	53.75
corpus	CLP36	U23769	4.73±1.49	0.91±0.51	80.76
	IGFBP-1	M89791	9.08±5.98	2.48±1.38	72.69
	CCND3	D16309	4.26±0.79	1.32±0.55	69.01
	CCND1	D14014	7.89±1.81	2.60±0.76	67.04
	interleukin-6 receptor βchain	M92340	7.17±1.54	2.60±0.66	63.74
	rCOUPg	AF003926	2.13±0.84	0.80±0.37	62.44
	elongation factor SIII P15	L29259	2.40±0.53	0.95±0.29	60.41
	CD25 protein	M55049	4.45±1.22	1.77±0.67	60.22
	CCND2	D16308	4.76±0.94	1.95±0.45	59.03
	BTG1 protein	L26268	3.00±0.43	1.28±0.47	57.33
	TNF-R1	M63122	2.70±0.43	1.16±0.40	57.03
	GIP	L19660	17.05±7.84	7.35±3.45	56.89
	ATPase	U11760	8.92±1.45	3.86±0.77	56.73
	IGFBP-5	M62781	4.38±1.03	2.02±0.59	53.88
	β-2-microglobulin	X16956; U26663	113.17±37.34	52.50±12.65	53.61
	PCTAIRE1	U36444	5.98±2.07	2.79±1.62	53.34
	calcineurin A subunit α	M29275	1.71±0.55	0.80±0.36	53.21
	HSP70	Z27118	3.51±0.97	1.69±0.51	51.85
	a-raf	X06942	10.19±1.45	4.91±1.32	51.82
	ribosomal protein L12	X53504	21.49±8.45	10.51±4.35	51.09
	c-jun	L27129	1.58±0.47	0.78±0.30	50.63
cauda	heparin-binding growth protein	M55601	2.61±0.80	0.75±0.21	71.26
	UNC5H2	U87306	5.06±0.91	1.64±0.68	67.59
	gelatinase A	U65656	5.80±1.21	2.78±0.55	52.07

CCND3, G1/S-specific cyclin D3; IGFBP-5, insulin-like growth factor binding protein-5; CCND1, G1/S-specific cyclin D1; PCNA, proliferating cell nuclear antigen; CCND1, G1/S-specific cyclin D1;CLP36, LIM domain protein CLP36;

IGFBP-1, insulin-like growth factor binding protein-1; CCND3, G1/S-specific cyclin D3; CCND1, G1/S-specific cyclin D1; rCOUPg, ovalbumin upstream promoter gamma nuclear receptor; CCND2, G1/S-specific cyclin D2; TNF-R1, tumor necrosis factor receptor 1; GIP, gastric inhibitory polypeptide; ATPase, transitional endoplasmic reticulum ATPase; IGFBP-5, insulin-like growth factor binding protein-5; PCTAIRE1, serine/threonine-protein kinase; HSP70, heat shock protein 70; UNC5H2, transmembrane receptor unc5 homolog 2.

Table 3. Age-Dependent Decreases in Gene Expression

ididymal egment	Gene	Genbank Accession #	Ad libitum 5 months ±SEM	Caloric Restricted 5 months ±SEM	%↓	Ad libitum 25 months ±SEM	Caloric Restricted 25 months ±SEM	% change
initial egment	sodium/dicarboxylate cotransporter	U51153	10.51±3.20	1.87±0.94	82.24	7.16±1.97	5.86±1.82	-18.08
	CCND1	D14014	7.62±1.90	2.03±0.46	73.35	3.58±0.40	3.65±0.83	1.92
	ATP5F1	M35052	6.85±2.87	1.86±0.68	72.90	5.16±2.30	5.57±2.17	7.80
	proteasome β	L17127	10.29±8.80	2.79±0.95	72.88	2.55±0.46	3.42±0.88	34.30
	CCND3	D16309	8.61±1.77	2.41±0.35	71.95	3.51±1.15	3.18±0.74	-9.23
	Na+/H+ exchange protein 1	M85299	3.72±0.98	1.04±0.36	71.93	2.59±0.36	1.81±0.46	-29.99
	LCAD	J05029	3.20±1.22	0.99±0.41	68.99	3.47±1.08	2.67±0.12	-23.05
	LACS2	M55642, D90109, J05439	4.41±1.79	1.40±0.52	68.31	4.00±1.05	4.60±1.52	15.02
	Neurodegeneration associated protein 1	D32249	4.24±0.87	1.36±0.40	67.81	3.72±0.90	2.53±0.40	-31.95
	ribosomal protein L44	M19635	32.72±6.91	10.69±2.00	67.32	25.17 ±3.01	16.96±2.32	-32.63
	ribosomal protein S4	X14210	9.91±2.08	3.32±0.83	66.55	5.84±1.96	5.92±1.19	1.39
	aldehyde reductase	D10854	67.90±11.16	22.81±5.63	66.40	81.55 ±22.32	62.88±20.91	-22.89
	IGFBP6	M69055	11.95±2.92	4.11±1.11	65.60	13.09 ±2.84	9.25±1.99	-29.32
	ALDOA	M12919	10.54±0.72	3.86±1.18	63.39	7.66 ±1.29	7.37±0.51	-3.68
	adenylate kinase 3	D13062	10.89±3.47	4.02±0.85	63.10	9.41±0.75	9.09±1.22	-3.37
	dipeptidyl peptidase IV	J02997	4.28±2.15	1.59±0.56	62.79	5.60±1.97	3.87±0.65	-30.91
	Rb2/p130	D55627	5.58±0.98	2.12±0.20	62.07	3.09±0.75	2.93±0.49	-5.12
	INSP3R	U38665	3.13±0.62	1.26±0.27	59.74	3.41±0.16	2.80±0.12	-17.95
	elongation factor 2	K03502	75.00±13.60	32.23±4.24	57.04	57.36±9.62	60.39±5.65	5.29
	LAR	L11586	8.66±1.97	3.72±0.70	57.02	10.19±2.57	6.92±1.44	-32.11
	BMPR1A	D38082	7.45±1.66	3.24±0.42	56.52	7.09±2.11	5.78±0.68	-18.54
	ribosomal protein S3A	M84716	34.59±7.24	15.07±3.23	56.44	26.66±3.94	27.34±2.81	2.56
	β–2microglobulin	X16956, U26663	156.52±30.55	68.22±19.57	56.41	69.92±10.46	87.35±22.82	24.93
	Sertoli cell COX1	S79304	203.75±47.72	89.13±21.80	56.26	196.87±46.56	186.61±40.80	-5.21
	ATP5G1	D13123	5.68±0.93	2.50±0.38	56.03	4.71±0.52	3.42±0.36	-27.50
	CREBP1	U38938	5.36±1.17	2.36±0.60	55.89	3.69±0.58	2.70±0.55	-26.81
	PTP1B	M33962	4.02±1.04	1.80±0.51	55.22	3.50±0.35	2.61±0.79	-25.37
	NDKB	M91597	10.79±2.19	4.91±0.58	54.55	6.36±0.75	4.72±0.91	-25.83
	RL/IF1	X63594	8.02±1.40	3.67±0.71	54.30	5.58±0.57	3.86±0.43	-30.78
	calmodulin	X13817	8.75±1.67	4.00±0.99	54.29	8.73±2.52	6.91±1.36	-20.79
	phosphatidate phosphohydrolase 2	U90556	4.52±0.63	2.11±0.74	53.32	3.67±0.52	4.09±0.77	11.35
	PDGFassociated protein	U41744	13.82±5.10	6.53±2.72	52.74	14.53±4.98	10.01±1.98	-31.10
	CABP2	M86870	82.74±13.20	39.20±5.11	52.61	67.53±11.23	65.73±8.32	-2.66
	c-H-ras	M13011	6.16±0.89	2.93±0.42	52.46	3.45±0.89	3.92±0.46	13.50
	PMCA1AB	J03753	3.37±0.81	1.61±0.53	52.20	2.31±0.50	3.13±0.25	35.38
	IGF2R	U59809	3.52±1.42	1.69±0.54	51.99	2.36±0.88	3.21±0.38	35.94
	ATP5H	D10021	11.48±1.50	5.58±1.09	51.39	10.09±2.72	7.58±0.91	-24.87

	IGFBP3	M31837	5.44±2.23	2.65±1.04	51.21	4.66±1.23	4.74±0.86	1.84
corpus	ribosomal protein L12	X53504	21.49±8.45	4.675	78.24	10.51±4.35	7.69±3.17	-26.80
	FRAG1	U57715	11.16±3.52	2.86±0.73	74.40	6.79±1.40	5.81±1.83	-14.47
	5αreductase 2	M95058	8.97±2.21	2.36±0.78	73.70	6.26±1.51	5.15±2.94	-17.74
	HMG1	M64986	15.70±4.92	5.08±1.15	67.63	9.52±2.24	7.82±1.22	-17.82
	ACE	U03734	10.21±1.84	3.38±1.15	66.85	14.54±3.61	9.98±2.04	-31.35
	Sertoli cell COX1	S79304	206.10±56.18	73.16±19.26	64.50	128.64±23.61	163.99±53.48	27.48
	Prothymosin α	M20035, M86564	16.36±5.88	6.26±1.74	61.75	8.64±3.24	5.82±2.41	-32.67
	CLP36	U23769	4.73±1.49	1.90±0.54	59.83	0.91±0.51	1.39±0.53	52.75
	COX5A	X15030	4.59±1.01	1.91±0.68	58.44	3.82±1.00	3.57±0.85	-6.54
	PI3K p85α	D64045	5.45±1.04	2.32±0.35	57.51	4.10±0.90	4.46±0.97	8.99
	INSP3R	U38665	4.42±0.52	1.90±0.17	57.04	3.50±0.49	3.66±0.62	4.84
	ribosomal protein S19	X51707	15.05±1.96	6.55±0.85	56.48	10.44±2.06	11.27±2.70	7.98
	ATP5G1	D13123	7.45±0.94	3.28±0.80	55.92	4.38±1.01	4.14±0.33	-5.61
	IGFBP1	M89791	9.08±5.98	4.16±0.64	54.20	2.48±1.38	10.28±5.70	314.49
	ribosomal protein L11	X62146, S37517	33.77±7.41	15.65±2.04	53.65	33.48±8.57	29.24±4.32	-12.66
	ATP5H	D10021	10.13±0.83	4.73±0.89	53.27	7.09±1.52	6.65±0.64	-6.14
	CABP2	M86870	58.80±9.13	28.33±2.61	51.82	55.04±10.53	41.28±2.63	-24.99
	SCP-2	M34728	15.21±1.94	7.41±0.70	51.30	13.07±2.17	11.28±2.16	-13.67
	ribosomal protein S12	M18547	66.04±13.77	32.54±5.93	50.73	68.27±13.12	53.89±14.91	-21.06
cauda	rCOUPg	AF003926	2.64±0.92	1.2173	53.84	2.62±0.33	1.98±0.74	-24.5146
	UNC5H2	U87306	5.06±0.91	2.35±0.91	53.57	1.64±0.68	3.10±1.06	89.43437
	proteasome β	L17127	4.91±1.59	2.29±0.35	53.43	3.08±0.44	2.69±0.71	-12.466
	GNAS	M17525	9.56±1.71	4.46±1.16	53.34	8.03±0.88	5.47±1.80	-31.8508
	IGFBP5	M62781	7.52±1.39	3.57±0.88	52.54	3.95±1.15	3.20±0.34	-18.9971

CCND1, G1/S specific cyclin D1; ATP5F1, mitochondrial ATP synthase B subunit precursor; CCND3, G1/S specific cyclin D3;LCAD, long chain specific acylCoA dehydrogenase precursor; LACS2, long chain acylCoA synthetase 2; IGFBP6, insulin like growth factor binding protein; ALDOA, fructose bisphosphate aldolase; INSP3R, inositol 1,4,5 triphosphate 3 kinase receptor 2; LAR, leukocyte common antigen related tyrosine phosphatase; BMPR1A, bone morphogenetic protein type IA receptor; Sertoli cell COX1, cytochrome oxidase, subunit I, Sertoli cells; ATP5G1, ATP synthase lipid binding protein P1; CREBP1, cAMP response element binding protein 1; PTP1B, protein tyrosine phosphatase PTPase; NDKB,

nucleoside diphosphate kinase B; CABP2, calcium binding protein 2; PMCA1AB, brain calcium transporting plasma membrane ATPase 1; IGF2R, insulin like growth factor receptor; ATP5H, mitochondrial ATP synthase D subunit; IGFBP3, insulin like growth factor binding protein 3; FRAG1, fibroblast growth factor receptor activating protein; HMG1, high mobility group protein 1; ACE, angiotensin converting enzyme; COX5A, cytochrome c oxidase, subunit Va, mitochondrial; PI3K p85α, phosphatidyl inositol 3 kinase regulatory subunit, phosphoprotein p85; IGFBP1, insulin like growth factor binding protein 1; SCP2, sterol carrier protein 2; rCOUPg, ovalbumin upstream promoter gamma nuclear receptor; UNC5H2, transmembrane receptor UNC5H2; GNAS, guanine nucleotide binding protein alpha stimulating activity polypeptide; IGFBP5, insulin like growth factor binding protein 5.

Table 4. Decreased Gene Expression with Age in Caloric Restricted Animals

Epididymal Segment	Gene	Genbank Accession #	5 months (±SEM)	25 months (±SEM)	%↓
initial segment	prothymosinα	M20035; M86564	37.84± 8.89	11.99± 4.09	68.3
	synaptobrevin2	M24105	4.91±1.14	1.56± 0.31	68.22
	GNAS	M17525	9.75±2.10	3.38± 1.44	65.33
	GAPDH	M17701	19.49±3.58	7.80± 1.25	59.98
	Casein kinase I δ	L07578	6.29± 0.99	2.79± 0.11	55.64
	GST subunit 4 mu	J02592	88.72± 21.64	41.20± 5.09	53.56
	p120GAP	L13151	4.66± 2.62	2.24± 0.20	51.93
	polyubiquitin	D16554	798.66± 250.03	394.79± 82.90	50.57
caput	ID2	D10863	16.18± 3.75	6.16± 1.13	61.93
	GST subunit 8	X62660	36.78± 6.46	14.13± 4.74	61.58
	polyubiquitin	D16554	763.27± 144.00	337.99± 60.47	55.72
	Stat3	X91810	8.82± 2.10	3.91± 1.11	55.67
	GST subunit 4 mu	J02592	64.68± 17.37	30.43± 8.68	52.95
	K-ras 2B	U09793	9.46± 1.91	4.62± 1.22	51.16
corpus	polyubiquitin	D16554	468.40± 156.50	177.71± 31.53	62.06
	Na,K-ATPase α1	M28647	14.39± 4.70	6.33± 1.65	56.01
	glycerol kinase	D16102	3.78± 1.68	1.68± 0.34	55.56
cauda	HMG2	D84418	2.72±.017	1.22± 0.53	55.15

GNAS, guanine nucleotide-binding protein α stimulating activity polypeptide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST subunit 4 mu, glutathione S-transferase subunit 4 mu; p120GAP, ras p21 protein activator; ID2, inhibitor of DNA binding 2; GST subunit 8, glutathione S-transferase subunit 8; Stat3, signal transducer & activator of transcription 3; Na,K-ATPase α1, sodium/potassium-transporting ATPase α 1 subunit; HMG2, high mobility group protein 2.

Figure 1. Effects of Caloric Restriction. A. Number of genes that had increased or decreased relative intensity by at least two fold with caloric restriction at 5 months of age. Black bars, gene expression decreases. Grey bars, gene expression increases. N=5.

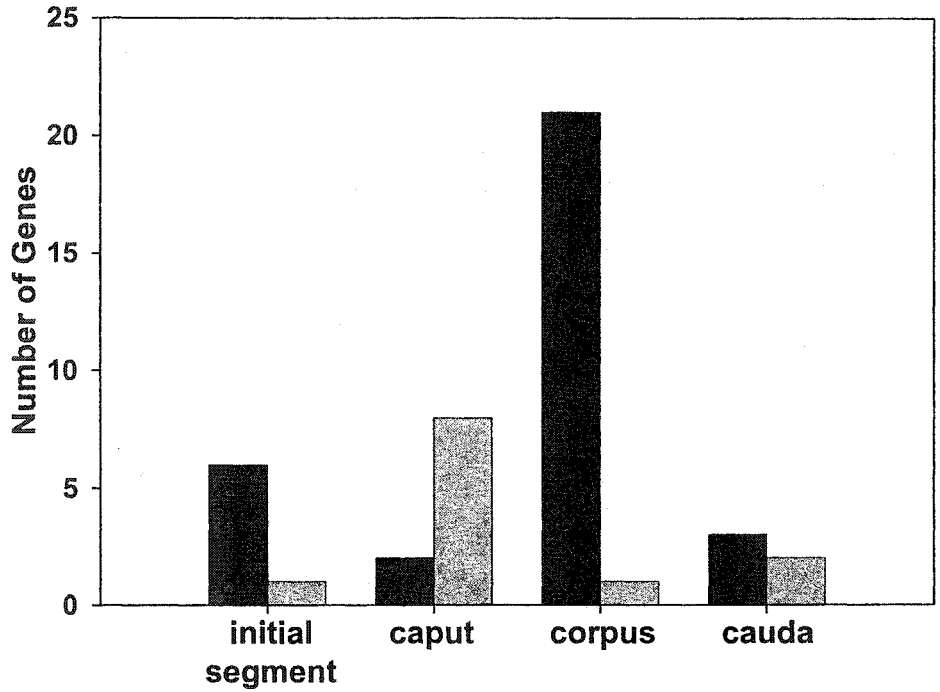
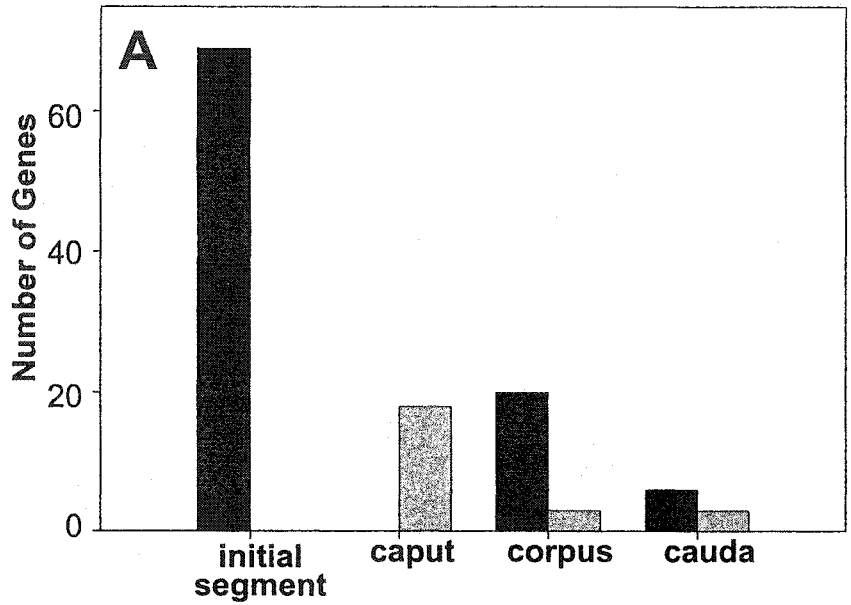

25
20
15
10
5
0
Number of Genes
initial segment
caput
corpus
cauda

Figure 2. Effects of Age on Gene Expression. A, Number of genes that had increased or decreased relative intensity by at least two fold with age in epididymides from ad libitum fed animals. Black bars, gene expression decreases. Grey bars, gene expression increases. B, Number of genes that had increased or decreased relative intensity by at least two fold with age in epididymides from caloric restricted animals. Black bars, gene expression decreases. Grey bars, gene expression increases. N=5.


A
Number of Genes
60
40
20
0
initial segment
caput
corpus
cauda

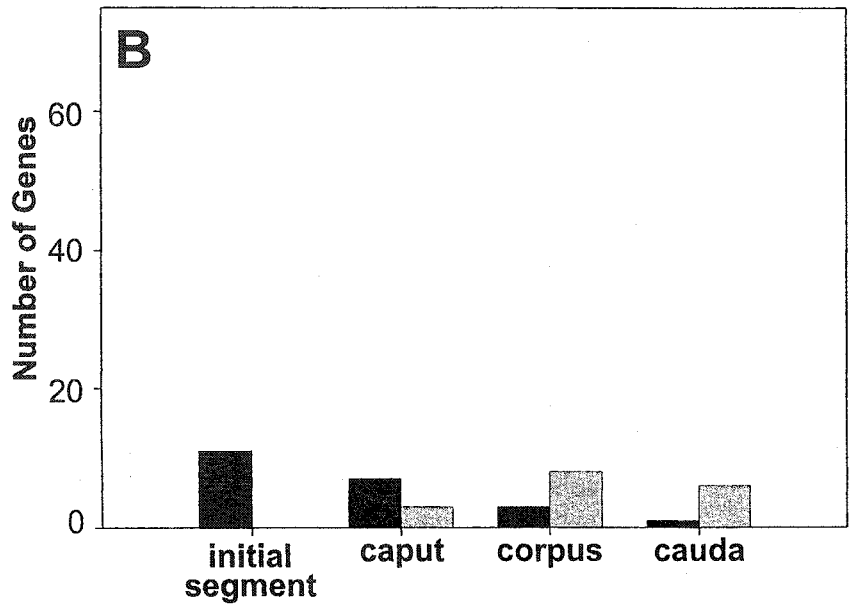

B
Number of Genes
60
40
20
0
initial segment
caput
corpus
cauda

Figure 3. Percent change in expression with age (5, 25 months) of ribosomal components in the epididymis of ad libitum and caloric restricted animals. A, ad libitum tissues. B, caloric restricted tissues. A and B: ribosomal subunits S19 (RPS19), S3a (RPS19), S12 (RPS12), S4 (RPSX4), L11, S11 (RPS11), L44 (RPL44) and L12. Black bars, initial segment. Dark grey bars, caput epididymidis. Cross-hatched bars, corpus epididymidis. Light grey bars, cauda epididymidis. N=5.

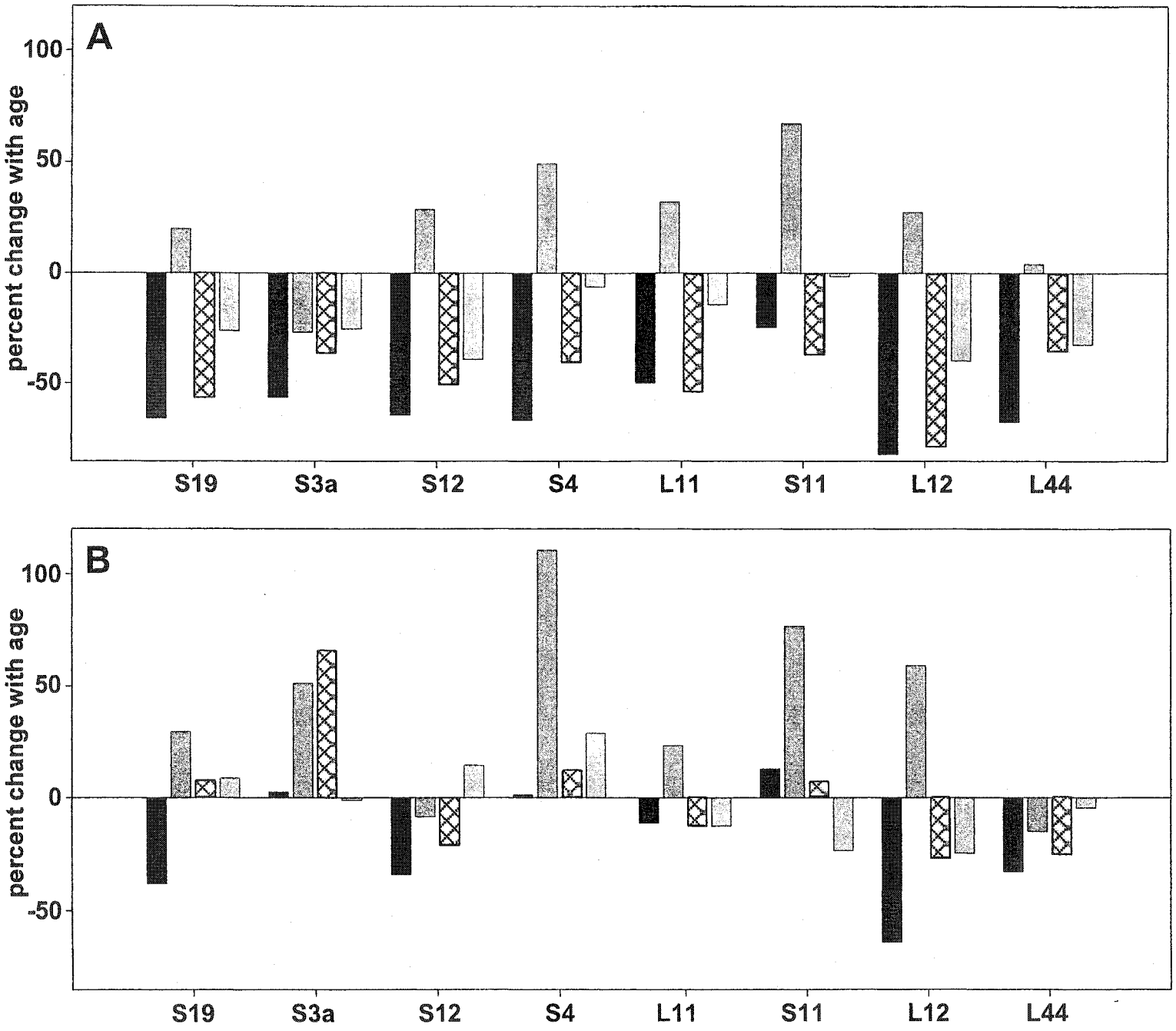
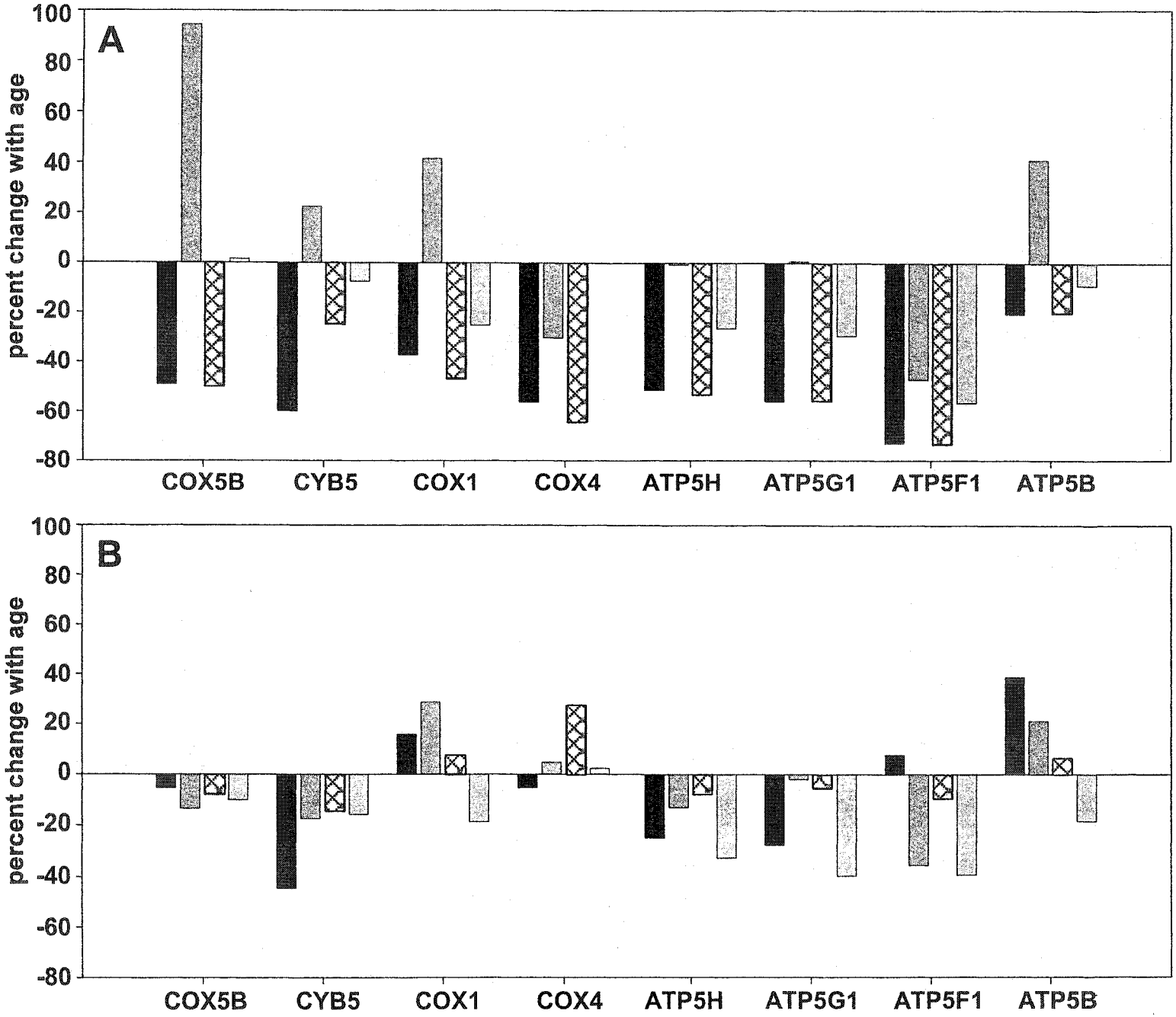

A
B
100
50
0
-50
percent change with age
S19
S3a
S12
S4
L11
S11
L12
L44

Figure 4. Percent change in expression with age (5, 25 months) of energy metabolism related genes in the epididymis of ad libitum and caloric restricted animals. A, ad libitum tissues. B, caloric restricted tissues. A and B: COX5B, cytochrome c oxidase polypeptide Vb; CYB5, cytochrome B5; COX1, Sertoli cell cytochrome c oxidasepolypeptide I; COX4, cytochrome c oxidase subunit IV; ATP5H, mitochondrial ATP synthase D subunit; ATP5G1, mitochondrial ATP synthase subunit C isoform 1; ATP5F1, mitochondrial ATP synthase B subunit; ATP5B, mitochondrial ATP synthase β subunit. Black bars, initial segment. Dark grey bars, caput epididymidis. Cross-hatched bars, corpus epididymidis. Light grey bars, cauda epididymidis. N=5.


A
B
percent change with age
100
80
60
40
20
0
-20
-40
-60
-80
COX5B
CYB5
COX1
COX4
ATP5H
ATP5G1
ATP5F1
ATP5B

Connecting Text for Chapter Four To Five

In order to manipulate oxidative stress load, one can either reduce the production of free radical species or augment the cellular defenses against them. Vitamin E is a potent lipid soluble chain breaking antioxidant that functions to protect cellular lipids from oxidative damage. Vitamin E is very safe, well tolerated, and highly efficacious as an antioxidant thus making it an appealing means by which to augment cellular antioxidant defenses. In order to further assess the role of oxidative stress in the aging of the epididymis, we analyzed the effects of long-term vitamin E supplementation and deficiency on the transcriptional response of the tissue and the accumulation of oxidative damage within the epithelium.

Chapter 5

The Effects of Long Term Vitamin E Treatment on Gene Expression and Oxidative Stress Damage in the Aging Brown Norway Rat Epididymis

Abstract

The male reproductive tract of the Brown Norway rat is profoundly affected by aging. In the epididymis, the site of sperm maturation and storage, aging results in histological and biochemical changes that are suggestive of oxidative stress. Vitamin E is a potent lipid soluble antioxidant that ameliorates the oxidative stress load associated with some chronic disease conditions. To determine the effects of long term (18 month) vitamin E deficiency and supplementation on aging in the epididymis, we assessed gene expression changes using cDNA microarrays and lipid peroxidation using immunohistochemical detection of 4-hydroxynonenal (4-HNE) in 24 month old rats. Plasma vitamin E levels were significantly lower in vitamin E deficient animals, and higher in vitamin E supplemented animals, compared to age matched controls. Vitamin E deficiency resulted in increased expression of oxidative stress related transcripts along the epididymis. This effect was most marked in the corpus epididymidis where expression of glutathione S-transferases pi, 8 and mu as well as superoxide dismutase increased by over 50%. The effect of vitamin E supplementation on the expression of oxidative stress related transcripts was primarily decreased expression; however, the magnitude of the gene expression changes was smaller than that observed for vitamin E deficiency. 4-HNE immunostaining was present throughout the epididymis in control animals. Vitamin E deficiency both increased the intensity and altered the distribution of 4-HNE staining, while vitamin E supplementation had no observable effect. In summary we found that long-term vitamin E treatment alters the expression of oxidative stress related transcripts. Moreover long term vitamin E deficiency exacerbates the effects of age on the accumulation of oxidative stress damage in the epididymis.

Introduction

Aging profoundly affects the reproductive tract of the male Brown Norway rat. With advancing age, serum testosterone levels and spermatogenesis decline [1,2] and sperm morphology and motility parameters are altered [3]. We have

demonstrated that in the epididymis, the site of sperm maturation and storage, both the histology and biochemistry of the tissue are affected by age [4,5].

The epididymis is a complex tissue that is anatomically and histologically separated into four different regions; the initial segment, caput, corpus and cauda epdididymidis. These regions are biochemicallly distinct and exhibit differential gene expression [6; reviewed in 7, 8]. The four regions of the epididymis also respond to the aging process in a segment specific manner [4,5,9].
Some of the age-related changes, for example the accumulation of lipofuscin [4], the altered distribution of components of antioxidant defense systems [10], and the decreased expression of gene products involved in antioxidant defenses [9] suggest that oxidative stress may play a role in the aging of the epididymal epithelium. Oxidative stress has long been associated with the aging process. Evidence that demonstrates the age-related accumulation of oxidative stress damage is abundant [11], and there are many examples of increased longevity with the attenuation of oxidative stress [12-15].

Vitamin E is a term that refers to a group of lipid soluble, chain breaking antioxidant molecules, the most potent of which is α-tocopherol. Vitamin E is well known for its antioxidant properties [reviewed in 16]; it functions as a chain-breaking antioxidant that prevents the propagation of free radical reactions and thus protects cells from oxidative damage. Vitamin E is particularly important in protecting cells against lipid peroxidation, where free radical attack on fatty acids causes structural damage to membranes and results in the formation of cytotoxic secondary products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) [17].

In prospective cohort studies, dietary vitamin E intake has been shown to be inversely associated with coronary heart disease risk. Individuals in the top fifth of vitamin E consumption have 30–40% lower risk of cardiovascular disease [reviewed in 18; original studies: 19-22]. This observation, in conjunction with experimental evidence in animals and the recognized potency of vitamin E as an antioxidant, stimulated interest in the potential ability of vitamin E to prevent chronic diseases. In particular, diseases and conditions believed to have an

oxidative stress component, such as cardiovascular and neurodegenerative diseases and cancer have been a primary focus of clinical research. Several large-scale clinical trials have been completed and indicate that vitamin E supplementation can decrease the risk of non-fatal myocardial infarction [23], the incidence of prostate cancer [24] and non-fatal myocardial infarction [25]. Other chronic conditions associated with oxidative stress have also been shown to be ameliorated by vitamin E supplementation. For example, chronic hemodialysis and cystic fibrosis patients have reduced acute oxidative stress load and in vivo lipid peroxidation and lung inflammation, respectively [26].

In order to assess the role of oxidative stress in the aging epididymal epithelium we analyzed the effects of long-term vitamin E supplementation and deficiency on gene expression in the epididymis. Since vitamin E is particularly important in protecting cells against lipid peroxidation, we also analyzed the effects of vitamin E on the accumulation of a marker of lipid peroxidation, 4-hydroxy, 2-nonenal, in the epididymal epithelium.

Materials and Methods

Animals and Diets

Six month old male Brown Norway rats were randomly assigned to one of 3 experimental diets: vitamin E deficient (8.36 IU/Kg all-rac-α Tocopheryl Acetate), control (25.4 IU/Kg all-rac-α Tocopheryl Acetate) or vitamin E supplemented (106 IU/Kg all-rac-α Tocopheryl Acetate). All other constituents of the diet were identical. Diet was added to the cage feeders weekly and weighed seven days later to determine the amount consumed. Access to water was ad libitum. Animals were housed at Harlan Sprague Dawley Inc. (Indianapolis, IN) under controlled light (12L/12L) and temperature (22-24C). Rats were weighed weekly.

Six month old animals (n=6) were sacrificed by cardiac puncture at the start of the study; blood was collected into heparinized tubes, centrifuged at 1,600 x g for 15min, and the resulting plasma was frozen at -80C for analysis of

vitamin E levels. Epididymides were collected, sectioned into initial segment, caput, corpus, and cauda regions, frozen in liquid nitrogen and stored at -80C for subsequent analysis of gene expression at the RNA level. After 18 months on the experimental diets, at 24 months of age, 6 animals for each of the three treatment groups were sacrificed and tissues and blood were collected as described above. No gross abnormalities were observed in any of the tissues examined. All animal studies were conducted in accordance with the principles and procedures outlined in A Guide to the Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care.

RNA Extraction

Frozen epididymal segments were ground to a powder on dry ice using a mortar and pestle. The resulting powders were then used for RNA extraction according to the RNeasy method (RNeasy Midi Kit, Qiagen, Mississauga, ON) following the manufacturers instructions. The RNA was then DNase treated using the RNase-Free DNase Set (RNeasy Midi Kit, Qiagen, Mississauga, ON). Concentration were determined spectrophotometrically (Beckman DU7 spectrophotometer, Montreal, Que.), and the quality of the RNA was assessed by denaturing gel electrophoresis on a 1% agarose gel. RNA was extracted individually from each of the four different epididymal segments (n=5 for each segment); each segment represents a different animal and no tissues were pooled.

cDNA Arrays and Normalization

cDNA arrays (Clontech, Atlas Rat 1.2K) were used to analyze RNA expression. Five arrays for each epididymal segment for each treatment group and the 6 month control group were completed following the manufacturers instructions. Each individual array is referred to as a replicate; it represents the RNA profile of an individual epididymal segment taken from an individual rat. Arrays were exposed to phosphorimager plates (Molecular Dynamics, Sunnyvale, CA) for 24 h before scanning (Storm, Molecular Dynamics). Array

images were first analyzed with Atlas Image (version 1.5, Clontech) to quantify the intensity of each cDNA spot, and the raw data for each gene (intensity of each spot on the array minus the background of that array) were imported into GeneSpring™ (Silicon Genetics, Redwood, CA) for further analysis.

Data were normalized by array normalization and gene normalization. Array normalization controls for array-wide variations in intensity that may be due to technical issues such as inconsistent washing, or inconsistent sample preparation. In array normalization, the expression of each gene on an array is normalized to the median expression on that array. Only gene intensities that are above a specified cutoff value are included in the determination of the median expression of an array. This was done for every single array individually. Gene normalization accounts for the difference in detection efficiency between spots. In gene normalization the signal strength of a gene is divided by the median expression of every measurement taken for that gene throughout the experiment. If the median of the gene's measurements is below the specified cutoff value, the cutoff is used instead. This is done for every gene on every array. The resulting value of each spot on all five replicate arrays was calculated and averaged and this average is referred to as the relative intensity for any given gene. A gene was only considered to be expressed if its relative intensity was at least twofold the average background of all the arrays in that treatment group. Replication is important in gene array studies [27] and therefore, changes in the relative intensity of a gene were considered only when consistent in at least three out of five replicate experiments [6].

Vitamin E Analysis

Plasma Vitamin E (n=6 per group) levels were assessed using HPLC by a modification of the procedure by Widicus and Kirk [28] (Laboratory Services, Guelph, ON).

Perfusion and 4-Hydroxy-2-nonenal Immunohistochemistry

Animals (6 animals/ treatment group and the 6 month control group) were perfused for immunohistochemical analysis of the presence of 4-Hydroxy-2-nonenal (4-HNE). Briefly, animals were anesthetized with a cocktail (20:10:1) of Vetalar (ketamine hydrochloride 115.4mg/ml, Vetrepharm London, ON), Anased (xylazine hydrochloride 20 mg/ml, Novopharm Toronto, ON) and Atravet (Acepromazine Maleate 10mg/ml, Ayerst Montreal, PQ). Epididymides were then fixed with Bouin's solution via perfusion through the abdominal aorta. After perfusion, epididymides were post fixed for 24 hours with the same fixative, dehydrated, and embedded in paraffin. Thin sections (5μm) were cut on a microtome and mounted on glass slides.

For immunohistochemical detection of 4-HNE, sections were deparaffinized with xylene and then rehydrated in graded alcohol solutions. Endogenous peroxidase activity was neutralized by a15 min incubation in 70% alcohol containing 1% hydrogen peroxide. After hydration, free aldehydes were blocked by incubation in 300 nM glycine for 5 min. In order to minimize nonspecific binding, sections were blocked with 10% normal horse serum for 30 min at room temp prior to incubation with the primary antibody (mouse Anti-4-Hydroxy-2-nonenal Antibody, OXIS International Inc. Portland, Oregon). Immunohistochemical staining was done using a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Sections were incubated for 1.5h at room temp with the primary antibody (12.5μg/ml or 8.3μg/ml) in the blocking serum. Bound antibodies were observed through the use of biotinylated goat anti-rabbit IgG secondary antibodies and the Avidin:biotinylated horseradish peroxidase complex. Sections were counterstained with a 0.075% methylene blue solution. Negative controls were processed in exactly the same manner except for the omission of the primary antibody. The immunohistochemical results were analyzed by light microscopy.

Statistics

The effects of vitamin E on body weight, tissue weights and plasma vitamin E levels were analysed by Bonferroni adjusted t-tests. For all analyses, values were considered statistically significantly different at $p < 0.05$.

Results

The Effects of Vitamin E on Body and Tissue Weights

Vitamin E deficiency or supplementation did not result in any significant change in body weight, or in the weights of reproductive tissues (testis, or epididymis ($p > 0.5$) (figure1).

Effects of Vitamin E on Plasma Vitamin E Levels

Age had no significant effect on plasma vitamin E levels in animals fed the control diet ($p=0.066$) (figure 2). In contrast, at 24 months of age, plasma vitamin E levels were 52% lower in animals on the vitamin E deficient diet ($p=0.001$), and 106% higher ($p=0.003$) in animals on the supplemented diet, then animals on the control diet.

Effects of Vitamin E on Gene Expression in the Epididymis

The predominant effect of vitamin E deficiency on gene expression in the epididymis was an increase in the relative intensity of gene expression compared to that in control animals (table 1). Interestingly, the expression of many ribosomal proteins increased with vitamin E deficiency, particularly in the caput and cauda epididymidis (table 2A). In the corpus epididymidis vitamin E deficiency increased the expression of copper-zinc superoxide dismutase 1 (Cu-Zn SOD1) by 77%. The initial segment was the only segment where there were more genes with decreased relative intensity in vitamin E deficient animals compared to controls. The identity of these genes is diverse (table 2B) and includes several growth factors/growth factor receptors (basic fibroblast growth factor receptor, platelet derived growth factor, heparin binding growth factor, f-

spondin) and channels/transporters (P2X purinoceptor 1, voltage-gated potassium channel protein 2.1, antigen peptide transporter 1)

The effects of vitamin E supplementation on gene expression in the epididymis were more variable. In the proximal regions of the tissue (initial segment and caput), few genes increased or decreased in vitamin E supplemented animals as compared with controls (table 2, A and B). In the initial segment of the epididymis, the expression of several proto-oncogenes (c-ros, sky, p21) decreased with vitamin E supplementation. In the cauda epididymidis, however, vitamin E supplementation resulted only in increased gene expression. Gene families with increased expression in the cauda epididymidis include proteases (amonipeptidase B, cathepisn L, presenilin-1), ATPases (brain calcium-transporting ATPase, sodium/potassium-transporting ATPase $\beta 1$) and translation associated transcripts (elongation factor 2, elongation initiation factor 2α). Gene expression in the corpus epididymidis was largely unaffected by vitamin E supplementation.

Effects of Vitamin E on Oxidative Stress Related Genes

Of the oxidative stress genes on the array, only four (glutathione-s-transferase pi, glutathione-s-transferase 8, glutathione-s-transferase mu and superoxide dismutase) were expressed above the level of detection in all four segments under all three conditions (figure 3). Vitamin E deficiency predominantly increased the relative intensity of expression of oxidative stress related genes in the epididymis. This effect was most pronounced in the corpus epididymidis where the relative intensity of expression of all four transcripts increased in vitamin E deficient animals as compared to controls. Superoxide dismutase expression was the most dramatically affected of all four transcripts; the relative intensity of expression increased by over 200% in the corpus and cauda epididymidis. Interestingly, the initial segment was the only region of the tissue where the expression of oxidative stress related genes was largely unaffected (<30% change in either direction) by vitamin E deficiency. In contrast, vitamin E supplementation had a variable effect on the expression of oxidative

stress related transcripts in the epididymal epithelium. Gene expression in the caput epididymidis was the most profoundly affected; expression of three of the four transcripts decreased by more than 50% as compared to controls. The magnitude of gene expression changes in vitamin E supplemented animals was also much lower than that observed in vitamin E deficient animals. The largest percent change in expression was 63% (GSTpi in the caput epididymidis) in supplemented animals, compared to 334% (SOD in the cauda epididymidis) in deficient animals.

Effects of Vitamin E on 4-Hydroxy-2-nonenal Immunoreactivity.

All regions of tissues from control animals at 24 months of age exhibited 4-HNE immunoreactivity distributed throughout the cytoplasm of principal cells (figure 4, A and D) The intensity of the immunoreactivity was light throughout the cytoplasm but increased towards the apical plasma membrane and appeared to form a dark line just under the plasma membrane (arrow figure 4). Other epididymal cell types (basal and clear cells) were unstained (arrowhead, figure 4, D).

The pattern of 4-HNE immunoreactivity was maintained in tissues from both vitamin E deficient and vitamin E supplemented animals. However, in vitamin E deficient tissues the intensity of immunoreactivity in the cytoplasm of principal cells was increased along the epididymis (figure 4, B and E and data not shown). This effect was most dramatic in the corpus epididymidis (figure 4, B and E) where the 4-HNE immunoreactivity was much more intense. In addition, vacuoles in the corpus epididymidis from vitamin E deficient animals exhibited intense 4-HNE immunoreactivity along their periphery (figure 5, B). This staining of the inside aspect of vacuoles was not observed in tissues from the control or vitamin E supplemented group (figure 5, A and C). In tissues from vitamin E supplemented animals 4-HNE immunoreactivity was equivalent in intensity to that observed in control animals (figure 4, C and F). When the primary antibody was omitted there was an absence of immunoreactivity (figure 6).

Discussion

In addition to its well documented role as an antioxidant, vitamin E exhibits biological functions not related to its antioxidant properties. The effects of vitamin E on gene transcription and translation are known to result in the inhibition of cell proliferation, platelet aggregation and monocyte adhesion [29]. Vitamin E is thought to exert a number of its effects on gene expression by acting on protein kinase C activity [30]. Protein phosphatase 2A, which dephosphorylates and inhibits protein kinase C, is activated by vitamin E [31]. Other effects of vitamin E on gene expression, such as the expression of the adhesion molecule ICAM-1 [32] and some integrins [33], are protein kinase C independent [34]. For example, vitamin E is known to activate gene expression via the pregnane X receptor (PXR), a nuclear receptor that regulates the expression of a variety of drug metabolizing enzymes [35]. In the epididymis, vitamin E treatment affected the expression of known regulators of protein kinase C activity, such as of phosphatase 2Aα and 14-3-3 proteins [31, 36]. We also found that epididymal expression of a number of cell adhesion molecules decreased and expression of cytochrome P450's increased in response to vitamin E treatment. This suggests that both protein kinase C dependent and protein kinase C independent mechanisms are mediating the effects of vitamin E on gene expression in the epididymis.

Vitamin E treatment also affected the expression of oxidative stress related transcripts in the epididymis. In particular, vitamin E deficiency resulted in increased relative intensity of glutathione S-transferases and superoxide dismutase along the tissue, most notably in the corpus epididymidis. Interestingly, the corpus epididymidis was also the most profoundly affected region of the tissue at the level of 4-HNE immunoreactivity, exhibiting both increased intensity and a differential staining pattern in vitamin E deficient animals as compared to age matched animals on the control or vitamin E supplemented diet. 4-HNE, the most abundant product of lipid peroxidation, is a highly reactive molecule that forms stable adducts with cellular proteins [37]. The stability and abundance of 4-HNE make it a commonly used indicator of oxidative

stress in tissues. Increased immunoreactivity towards 4-HNE in the corpus epididymidis of vitamin E deficient animals is thus suggestive of increased oxidative stress in this region of the tissue. Vacuoles are an indicator of oxidative stress, and the accumulation of oxidatively damaged cellular components around the inside aspect of vacuoles has been reported for other oxidative stress markers in other tissues [38,39].

The increase in 4-HNE immunostaining in the corpus epididymidis occurs in conjunction with dramatically increased expression of oxidative stress related transcripts. Together these data suggest that the corpus epididymidis is the most sensitive region of the epididymis to the effects of vitamin E deficiency. This is in keeping with our previous results that demonstrate that the corpus epididymidis is the region that is the most dramatically affected with age at the histological and gene expression level [4,9].

The effects of vitamin E supplementation on the expression of oxidative stress related transcripts and 4-HNE immunoreactivity were less pronounced than vitamin E deficiency. In the caput epididymidis, vitamin E supplementation did result in decreased expression of oxidative stress transcripts, however the effects on the rest of the tissue were more variable. While the reasons for this are unclear, it is possible that the dose used for supplementation was not large enough to generate an effect of great magnitude.

In conclusion, these data show that vitamin E treatment has a profound effect on epididymal gene expression. Moreover, we show that vitamin E deficiency impacts oxidative stress in the epididymis, with a pronounced effect on the corpus epididymidis, suggesting that this region of the tissue is the most vulnerable to oxidative stress with age.

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Table 1. Number of genes that increase or decrease in relative expression with vitamin E deficiency or supplementation compared to controls.

initial segment			caput			corpus			cauda		
	D	S		D	S		D	S		D	S
# of genes that ↑ 50%	1	5	# of genes that ↑ 50%	41	7	# of genes that ↑ 50%	8	0	# of genes that ↑ 50%	27	34
# of genes that ↑ 75%	0	1	# of genes that ↑ 75%	20	2	# of genes that ↑ 75%	1	0	# of genes that ↑ 75%	9	14
	D	S		D	S		D	S		D	S
# of genes that ↓ 50%	24	18	# of genes that ↓ 50%	0	2	# of genes that ↓ 50%	0	2	# of genes that ↓ 50%	0	0
# of genes that ↓ 75%	11	5	# of genes that ↓ 75%	0	1	# of genes that ↓ 75%	0	0	# of genes that ↓ 75%	0	0

D, vitamin E deficient; S, vitamin E supplemented.

Table 2A. Gene Expression Increases with Vitamin E Treatment

initial segment

control	deficient	%change	gene
0.45	1.54	70.55	polyubiquitin
control	**supplemented**	**%change**	**gene**
0.21	0.97	77.94	aldolase C
0.14	0.42	66.94	ADORA1
0.56	1.68	66.72	PTP-PS
0.81	2.32	65.27	phosphatase 2Aα
0.78	1.89	58.56	rab16, ras related GTPase

caput

control	deficient	%change	gene
0.29	2.99	90.28	ribosomal protein L10
0.61	4.62	86.75	ribosomal protein L12
0.44	3.24	86.34	40S ribosomal protein S11
0.25	1.82	85.97	sterol carrier protein 2
0.58	3.90	85.23	ribosomal protein L13
0.26	1.68	84.78	40S ribosomal protein S17
0.37	2.23	83.58	ribosomal protein S4
0.45	2.75	83.54	14-3-3 protein ε
0.85	5.06	83.25	14-3-3 protein ζ/δ
0.43	2.52	83.06	ribosomal protein L11
0.35	1.97	82.41	ATP6C
0.78	4.34	81.98	A-FABP
0.93	4.67	80.05	40S ribosomal protein S29
0.81	3.82	78.71	cathepsin L
0.32	1.47	78.49	calmodulin
0.42	1.92	78.15	60S ribosomal protein L21
0.56	2.51	77.84	40S ribosomal protein S19
0.57	2.56	77.80	60S ribosomal protein L19
4.76	19.28	75.29	clusterin
0.59	2.37	75.27	neural plasticity-related protein
0.98	3.80	74.08	elongation factor 2

cauda

control	deficient	%change	gene
0.34	2.43	86.18	elongation factor 2
0.35	2.30	84.71	cathepsin L
0.53	3.31	84.10	14-3-3 protein ε
0.29	1.75	83.19	Mak
0.40	2.33	82.87	ras-related protein RAB1A
0.71	3.68	80.71	40S ribosomal protein S29
0.43	1.81	76.32	HNRNP K
0.50	2.05	75.40	40S ribosomal proteinS3A
0.49	1.92	74.51	ANT2
0.86	3.31	73.91	Cu-Zn SOD1
0.63	2.17	71.19	PDGF-associated protein
0.52	1.80	70.93	ribosomal protein L11
1.25	4.30	70.83	heat shock 70-kDa protein
0.44	1.47	70.07	Calcineurin B subunit
0.33	1.10	69.55	c-K-ras 2b
0.86	2.80	69.14	ADP-ribosylation factor 3
0.74	2.37	68.71	clusterin
0.49	1.53	68.34	60S ribosomal protein L19
0.43	1.37	68.33	PMCA1AB
0.41	1.18	65.61	MIF
0.29	0.83	64.74	Prothymosin-α
0.39	1.10	64.39	rat CD1 antigen precursor
0.97	2.64	63.48	ATP1B1
0.46	1.17	60.50	proteasome subunit 5
0.65	1.61	59.81	60S ribosomal protein L21
0.39	0.91	56.72	calcium binding protein 2
0.56	1.28	55.90	40S ribosomal protein S11
control	**supplemented**	**%change**	**gene**
0.29	2.13	86.38	PCNA
0.43	2.89	84.99	PMCA1AB

0.93	3.56	73.96	presenilin 1
0.87	3.26	73.29	EIF-2α
0.98	3.66	73.19	COX5B
0.48	1.74	72.72	40S ribosomal protein S3A
1.10	4.03	72.65	HMG-1
0.56	1.97	71.80	P26S4
0.64	2.23	71.37	aldehyde reductase
0.66	2.30	71.35	BMPR-IA
0.58	2.02	71.29	40S ribosomal protein S12
0.79	2.73	70.97	ADP-ribosylation factor 5
1.05	3.60	70.94	proteasome rPA28α
0.76	2.61	70.92	amonipeptidase B
0.81	2.77	70.87	adenylate kinase 3
0.85	2.48	65.79	COX1
0.61	1.77	65.34	ezrin; cytovillin; villin 2
0.56	1.57	64.20	60S ribosomal protein L44
1.00	2.56	60.78	HNRNPK
1.05	2.54	58.79	PRKAR1A
0.93	2.20	57.76	steroid 5α-reductase 2
0.71	1.51	52.80	LAR
control	**supplemented**	**%change**	**gene**
0.25	1.88	86.42	sterol carrier protein 2
0.45	1.91	76.33	14-3-3 protein ε
0.61	2.32	73.55	ezrin; cytovillin; villin 2
0.23	0.84	72.70	interferon induced protein
0.29	0.96	69.64	ribosomal protein L10
0.48	1.47	67.70	40S ribosomal protein S3A
0.43	1.20	64.50	ribosomal protein L11

corpus

control	**deficient**	**%change**	**gene**
0.81	3.50	76.99	Cu-Zn SOD1
0.71	2.79	74.50	cytoplasmic β-actin
0.45	1.67	73.27	BMPR-IA
0.96	6.09	84.27	ras-related protein RAB12
0.34	2.15	83.99	β-2-microglobulin
0.29	1.69	82.64	GNAI2
0.29	1.62	81.84	prothymosin-α
1.29	6.72	80.81	c-kit proto-oncogene
0.83	3.78	78.16	cytochrome P450 2C11
0.34	1.51	77.82	elongation factor 2
0.35	1.55	77.33	cathepsin L
0.39	1.67	76.42	calcium binding protein 2
0.29	1.21	75.67	GNAS
0.71	2.77	74.39	40S ribosomal protein S29
0.97	3.63	73.43	ATP1B1
0.70	2.62	73.42	EIF-2α
0.74	2.74	72.86	amonipeptidase B
0.56	1.97	71.80	HMG-1
0.73	2.39	69.33	presenilin 1
0.52	1.70	69.11	ribosomal protein L11
0.63	1.99	68.55	PDGF-associated protein
0.74	2.33	68.19	clusterin
0.40	1.24	67.93	40S ribosomal protein S12
0.91	2.76	67.19	ezrin; cytovillin; villin 2
0.43	1.32	66.99	aldehyde reductase
1.56	4.73	66.95	ornithine decarboxylase
0.33	0.96	65.20	c-K-ras 2b
1.65	4.61	64.20	RCL
0.49	1.33	63.28	cytochrome B5
0.49	1.32	63.24	60S ribosomal protein L19
0.93	2.47	62.19	BMPR-IA
0.56	1.43	60.97	cofilin
0.86	2.17	60.16	ADP-ribosylation factor 3
0.46	1.11	58.28	proteasome subunit 5
0.67	1.47	54.77	PDGFRα

0.54	1.88	71.24	ANT2
0.86	2.74	68.50	NDKB
0.55	1.59	65.35	FRAG1
1.02	2.83	63.86	14-3-3 protein ε
1.08	2.90	62.59	PDGF-associated protein

ADORA1, adenosine A1 receptor; PTP-PS,Receptor-linked protein tyrosine phosphatase; ATP6C, vacuolar ATP synthase 16-kDa proteolipid subunit; A-FABP, adipocyte fatty acid-binding protein; COX5B, cytochrome c oxidase polypeptide Vb; HMG-1, high mobility group protein 1; P26S4, 26S protease regulatory subunit 4; BMPR-IA bone morphogenetic protein type IA receptor; COX1, Sertoli cell cytochrome c oxidase polypeptide; HNRNPK, heterogeneous nuclear ribonucleoprotein K; PRKAR1A, cAMP-dependent protein kinase type α regulatory subunit; LAR, leukocyte common antigen-related tyrosine phosphatase; Cu-Zn SOD1, copper-zinc-containing superoxide dismutase 1; ANT2, adenine nucleotide translocator 2; NDKB, nucleoside diphosphate kinase B; FRAG1, fibroblast growth factor receptor-activating protein 1; Mak, male germ cell-associated kinase; PMCA1AB, brain calcium-transporting ATPase plasma membrane 1; MIF, macrophage migration inhibitory factor; ATP1B1, sodium/potassium-transporting ATPase β1 subunit; GNAI2, adenylate cyclase-inhibiting Gα; GNAS, guanine nucleotide-binding proteinα stimulating activity polypeptide; RCL, growth-related c-myc-responsive protein RCL.

Table 2B. Gene Expression Decreases with Vitamin E Treatment

initial segment			
control	deficient	%change	gene
2.04	0.25	-87.67	EIF-5
4.13	0.53	-87.21	integrin-associated protein form 4
3.44	0.58	-83.22	myelin protein MVP17
2.79	0.50	-82.07	inositol polyphosphate 5'phosphatase
1.18	0.24	-79.67	Fas ligand
3.08	0.70	-77.33	COX7A
3.62	0.83	-77.18	CD53
1.79	0.41	-76.86	PDGFβ
1.64	0.40	-75.58	inhibin, βA subunit
1.93	0.47	-75.39	DRK1;KCNB1
1.59	0.39	-75.30	40S ribosomal protein S17
1.38	0.35	-74.82	14-3-3 protein β/α
1.06	0.27	-74.20	amphotropic murine retrovirus R
4.42	1.20	-72.88	F-spondin
1.03	0.30	-70.95	heparin-binding growth factor 1
2.99	0.93	-68.89	p27Kip1
1.61	0.52	-68.04	PDE4D; DPDED
2.13	0.71	-66.50	dipeptidyl peptidase IV
3.44	1.16	-66.19	cyclin-dependent kinase 2α
0.83	0.29	-65.47	proteasome subunit RC6-1
2.75	0.99	-63.79	BFGF-R1
2.27	0.90	-60.43	proteasome subunit 5
2.25	0.93	-58.44	antigen peptide transporter 1
0.78	0.36	-53.85	P2X purinoceptor 1

initial segment			
control	supplemented	%change	gene
1.76	0.28	-84.30	COX1
3.08	0.59	-80.91	COX7A
3.93	0.83	-78.97	GAPDH
4.42	1.00	-77.36	F-spondin
0.65	0.16	-75.87	steroid 5-α-reductase 2
3.62	0.95	-73.74	CD53
2.19	0.58	-73.64	ATP5F1
3.37	0.92	-72.64	Sky proto-oncogene
2.27	0.74	-67.23	GPX4
1.15	0.39	-66.26	COX5A
2.69	0.91	-66.19	p21; cip1; waf1
1.84	0.63	-65.98	cathepsin B
0.98	0.34	-65.88	PCTAIRE1
10.54	3.64	-65.44	c-ros-1 proto-oncogene
2.53	0.91	-63.99	HMG-CoA synthase
4.13	1.51	-63.32	integrin-associated protein 4
2.08	0.77	-63.16	integrin, 1α
3.44	1.45	-57.97	myelin protein MVP17

caput			
control	supplemented	%change	gene
0.85	0.14	-83.03	COX1
0.70	0.24	-65.72	β-2-microglobulin

corpus			
control	supplemented	%change	gene
6.86	1.85	-72.99	A-FABP
1.43	0.43	-69.82	60S ribosomal protein L44

EIF5, eukaryotic translation initiation factor 5; COX7A, liver/heart cytochrome c oxidase polypeptide VIIa; PDGFβ, platelet-derived growth factor β–chain; DRK1;KCNB1, voltage-gated potassium channel protein 2.1; PDE4D; DPDE3, cAMP-dependent 3',5'-cyclic phosphodiesterase 4D; BFGF-R1, basic fibroblast growth factor receptor 1 precursor; COX1, Sertoli cell cytochrome c oxidase polypeptide ; GAPDH, glucose-6-phosphate dehydrogenase; ATP5F1, mitochondrial ATP synthase B subunit precursor; GPX4, phospholipid hydroperoxide glutathione peroxidase; COX5A, mitochondrial cytochrome c oxidase polypeptide Va; PCTAIRE1, cdc2-related serine/threonine-protein kinase; HMG-CoA synthase, mitochondrial hydroxymethylglutaryl-CoA synthase precursor; A-FABP, adipocyte fatty acid-binding protein.

Figure 1. Effects of Vitamin E deficiency or supplementation on body weight and tissue weights. Body weight (A), testis weight (B) and epididymis weight (C) in grams of animals on control diets at 6 months of age and vitamin E deficient, control or vitamin E supplemented diets at 24 months of age. White bar, control at 6 months of age; horizontal striped bar, deficient at 24 months of age; black bar, control at 24 months of age; diagonal striped bar, supplemented at 24 months of age.

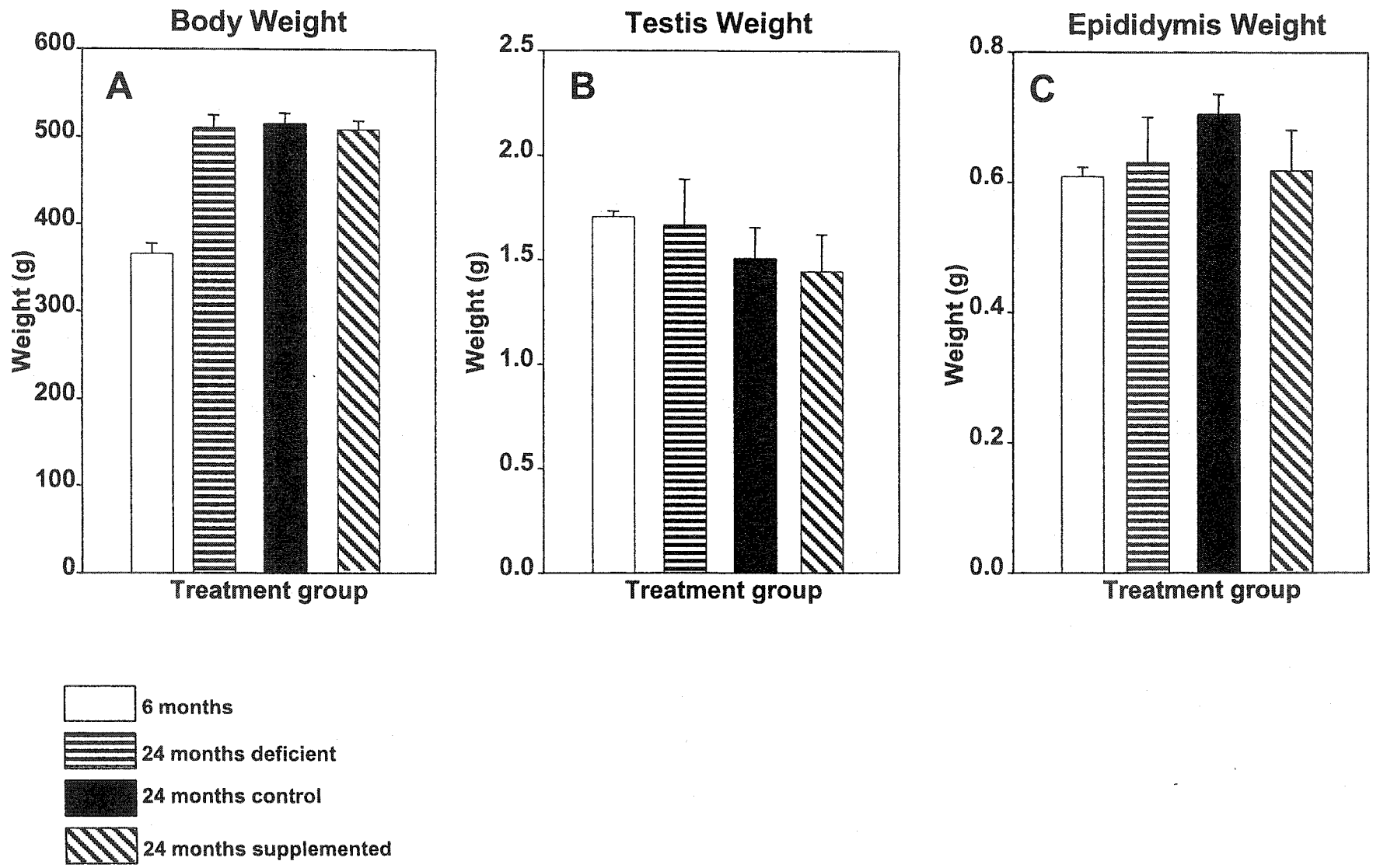

Body Weight
A
600
500
400
300
200
100
0
Weight (g)
Treatment group
Testis Weight
B
2.5
2.0
1.5
1.0
0.5
0.0
Weight (g)
Treatment group
Epididymis Weight
C
0.8
0.6
0.4
0.2
0.0
Weight (g)
Treatment group
6 months
24 months deficient
24 months control
24 months supplemented

Figure 2. Effects of Vitamin E deficiency or supplementation on plasma vitamin E levels. Plasma vitamin E in µg/ml in animals on control diets at 6 months of age and vitamin E deficient, control or vitamin E supplemented diets at 24 months of age. White bar, control at 6 months of age; horizontal striped bar, deficient at 24 months of age; black bar, control at 24 months of age; diagonal striped bar, supplemented at 24 months of age.

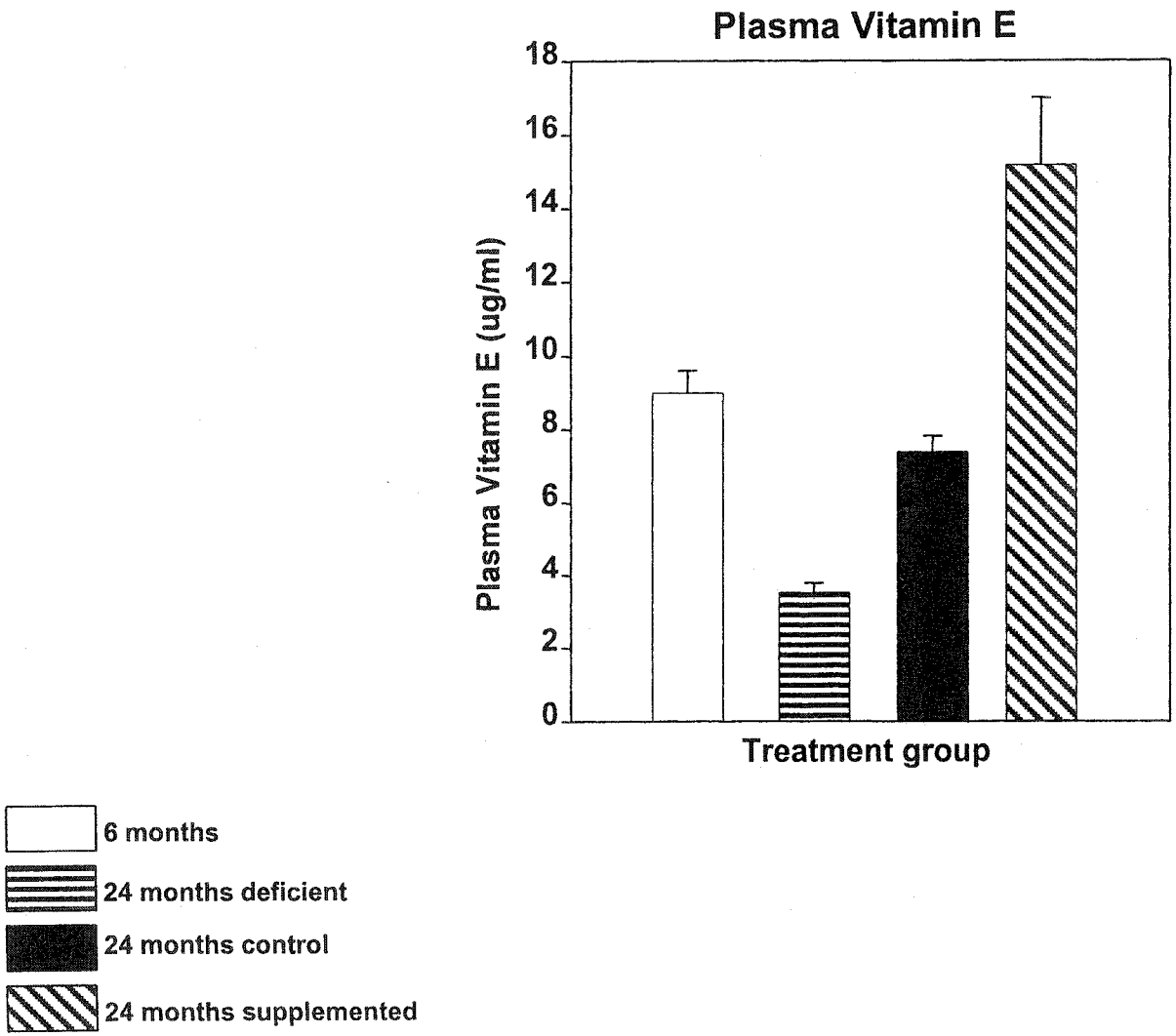

Plasma Vitamin E
Plasma Vitamin E (ug/ml)
18
16
14
12
10
8
6
4
2
0
Treatment group
6 months
24 months deficient
24 months control
24 months supplemented

Figure 3. Effects of Vitamin E on the Expression of Oxidative Stress Related Genes. Percent change from control in the relative intensity of expression in the four segments of the epididymis of vitamin E deficient (A) and supplemented animals (B) at 24 months of age. GSTpi, glutathione-s-transferase pi; GST8, glutathione-s-transferase 8; GSTmu, glutathione-s-transferase mu; SOD, superoxide dismutase. Black bar, initial segment; white bar, caput epididymidis; dark grey bar, corpus epididymidis; light grey bar, cauda epididymidis.

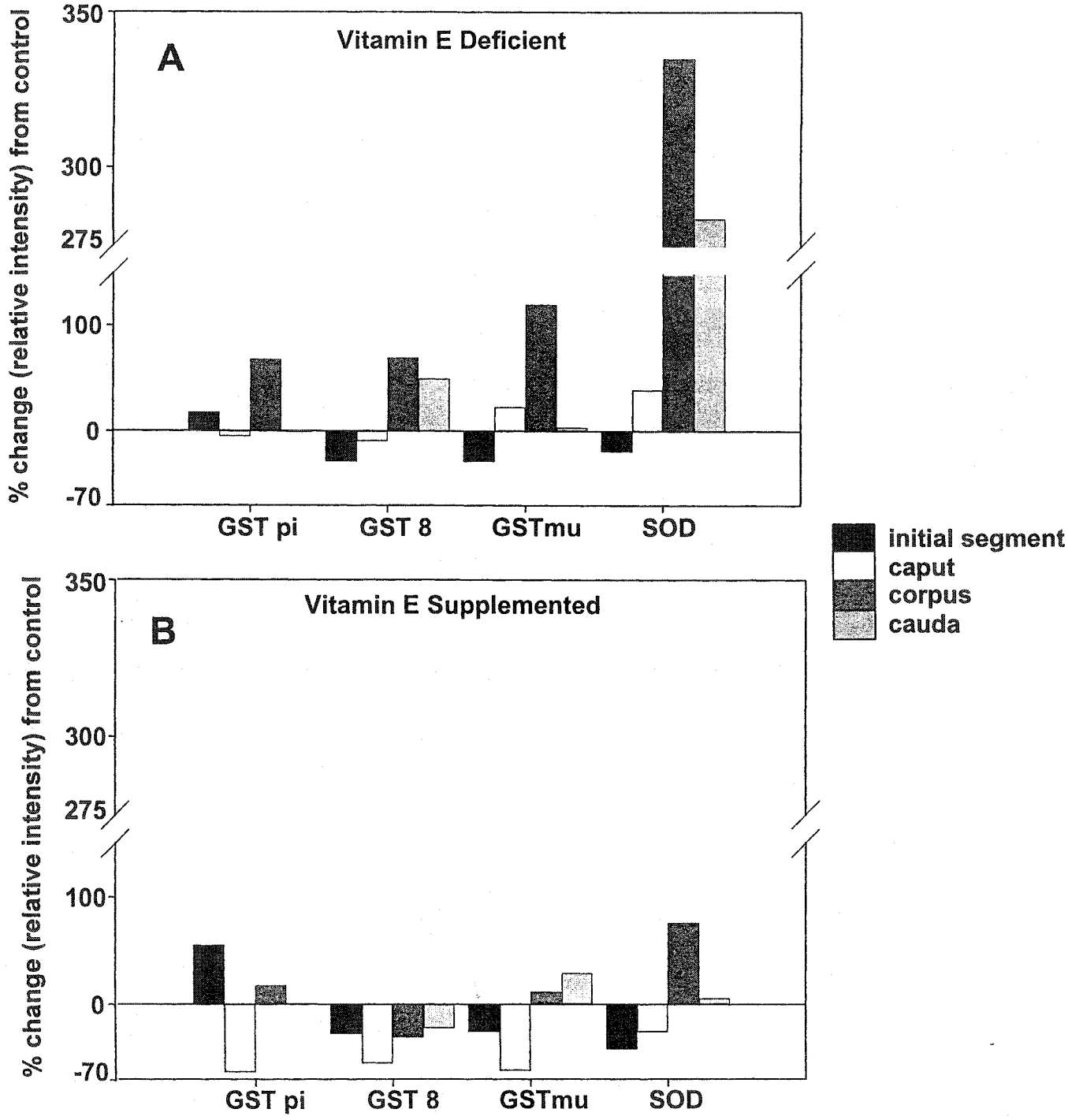
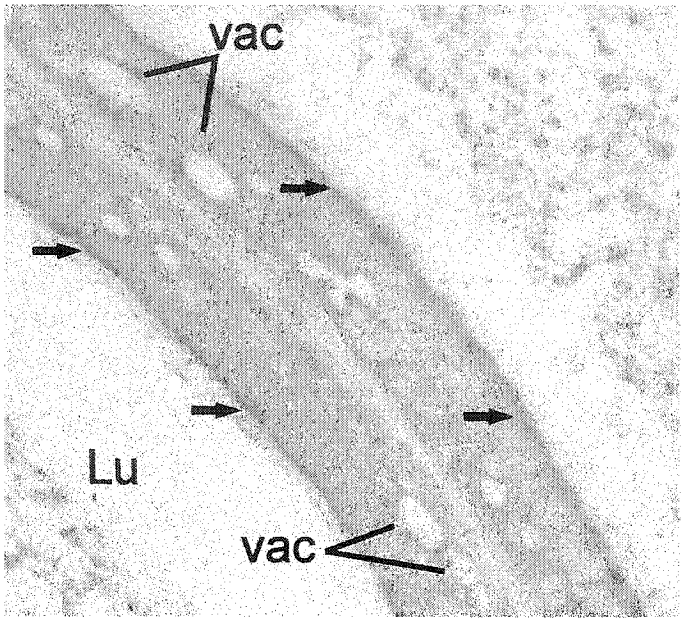
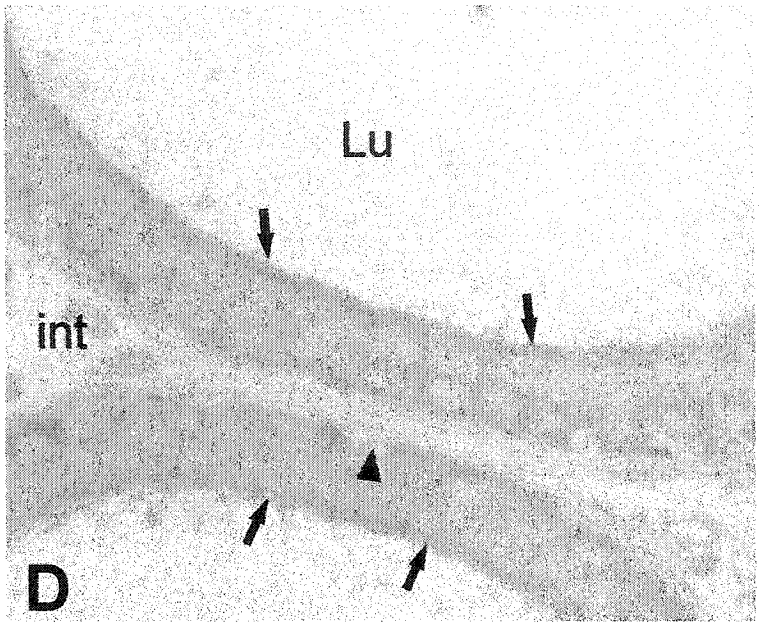
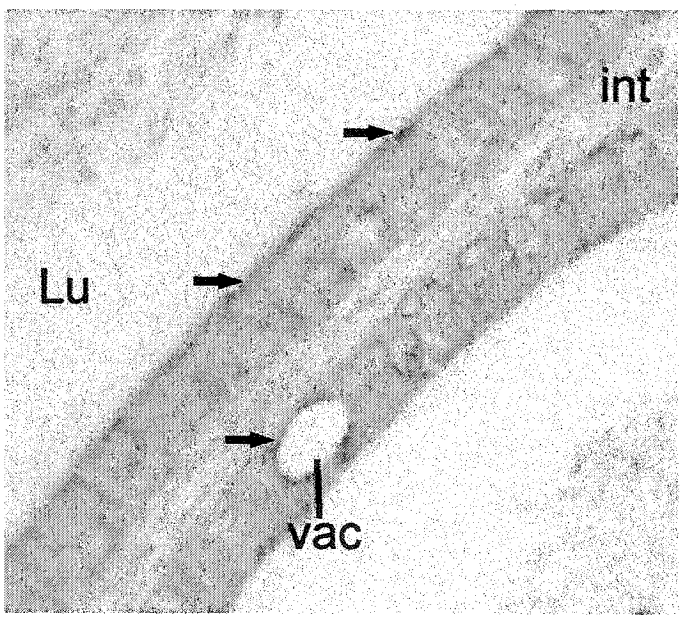
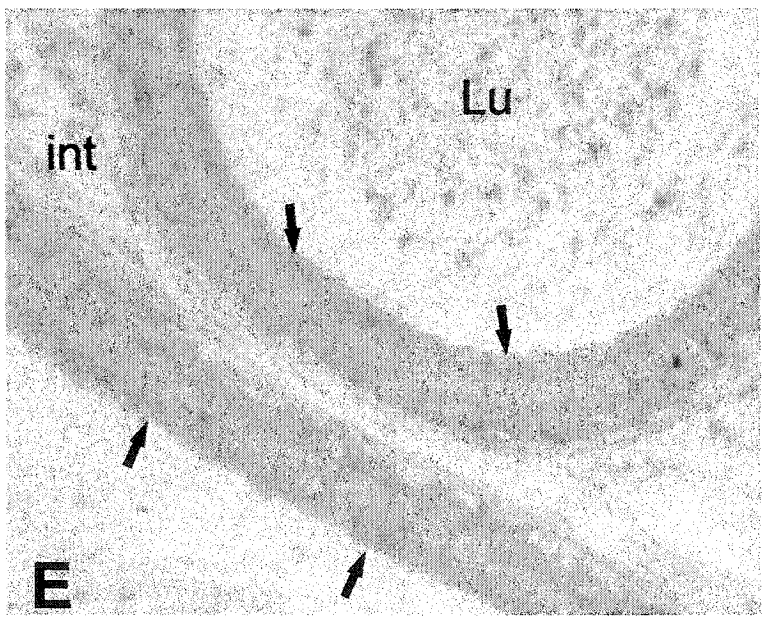

A
Vitamin E Deficient
% change (relative intensity) from control
350
300
275
100
0
-70
GST pi
GST 8
GSTmu
SOD
B
Vitamin E Supplemented
% change (relative intensity) from control
350
300
275
100
0
-70
GST pi
GST 8
GSTmu
SOD
initial segment
caput
corpus
cauda

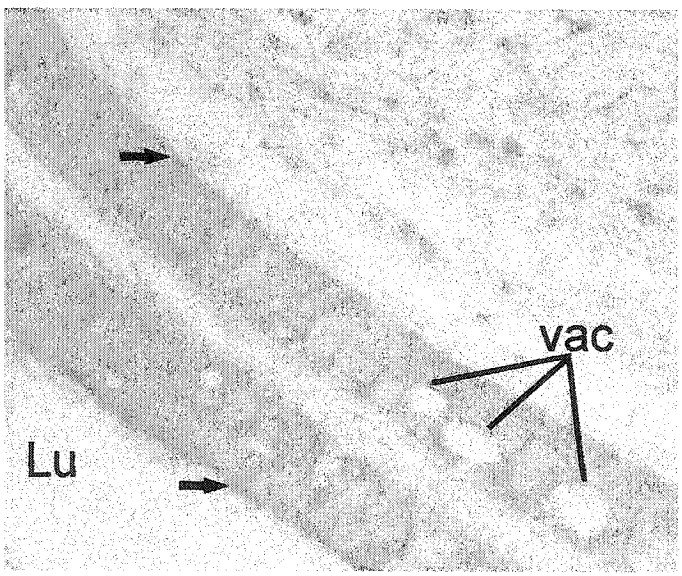
Figure 4. Epididymal sections demonstrating anti-4-HNE immunostaining. A-C, proximal corpus epididymidis. D-F, caput epididymidis. A and D, tissue sections from animals on the control diet at 24 months of age. B and E, tissue sections from animals on the vitamin E deficient diet at 24 months of age. C and F, tissue sections from animals on the vitamin E supplemented diet at 24 months of age. Int, interstitium; Lu, lumen; vac, vacuole; arrowhead, basal cell; arrows indidcate anti-4-HNE immunoreactivity. Magnification 40X.


vac
Lu
vac


Lu
int
D


int
Lu
vac


Lu
int
E


vac
Lu

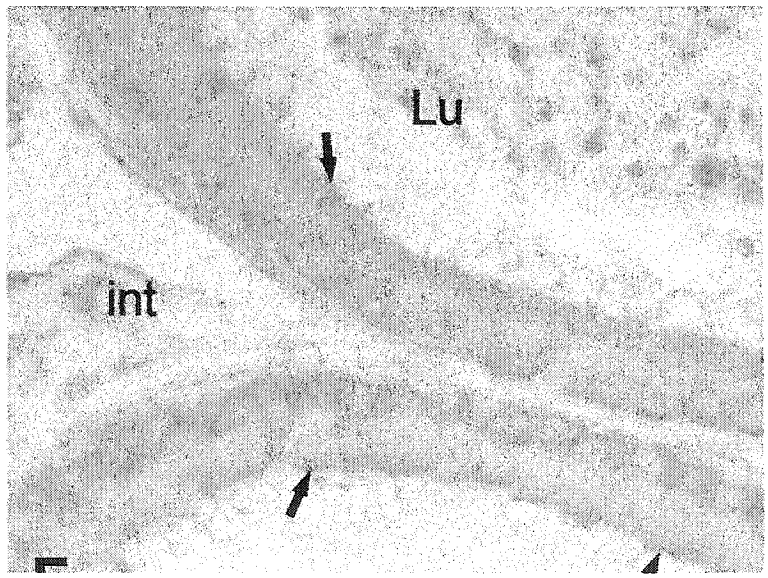

Lu
int

Figure 5. Epididymal sections demonstrating anti-4-HNE immunostaining of vacuoles in the corpus epididymidis. A, tissue section from animals on the control diet at 24 months of age. B, tissue section from animals on the vitamin E deficient diet at 24 months of age. C, tissue section from animals on the vitamin E supplemented diet at 24 months of age. Int, interstitium; Lu, lumen; arrows indidcate anti-4-HNE immunoreactivity. Magnification 100X.

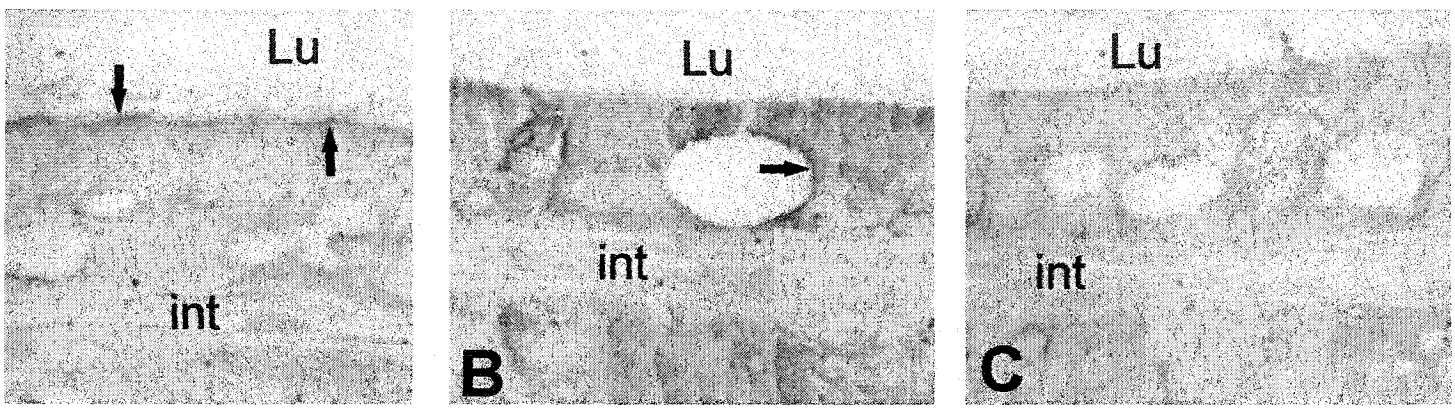

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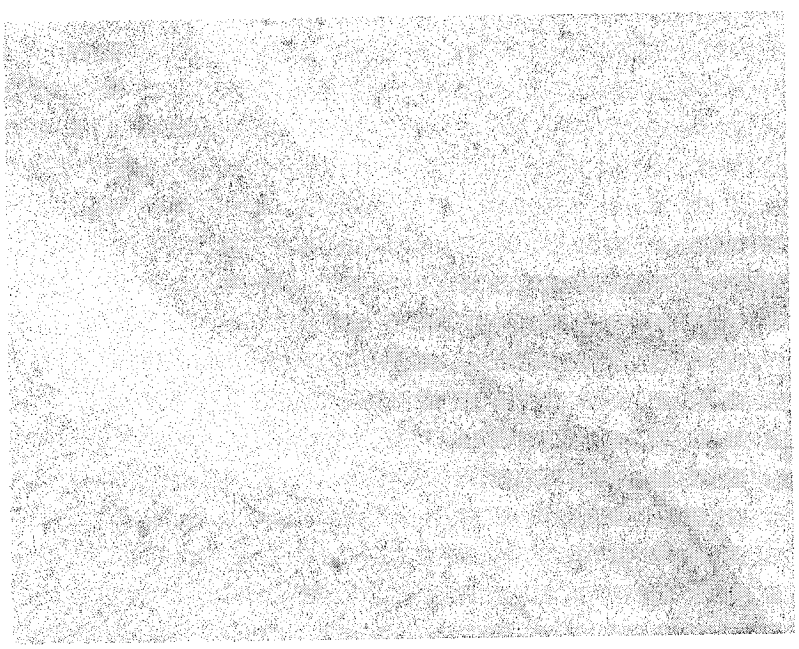
Figure 6. Negative control for 4-HNE immunoreactivity. Epididymal section demonstrating the absence of 4-HNE immunoreactivity in the caput epididymidis when the primary antibody is omitted.



Appendix 1

cDNA Micorarrays

A variety of techniques, including serial analysis of gene expression (SAGE) (Velculescu et al.,1995), differential display (Liang and Pardee, 1992), oligonucleotide arrays (Lockhart et al., 1996), and cDNA microarrays (Schena et al., 1995), have been developed to assess mRNA expression on a global scale, allowing the simultaneous analysis of gene expression of hundreds or even thousands of genes in a single experiment. The most common use of these technologies is to determine patterns of differential gene expression, comparing differences in mRNA expression levels in different cells and tissues under different conditions.

Microarray expression analysis (Schena et al., 1995) has a number of features that have made it the most widely used method for profiling mRNA expression. There are many different formats and processing systems available; many of these are commercially available, while others are custom-made. Methodological differences aside, all microarray technologies present biological researchers with the same conundrum, vast amounts of data. How to handle these vast data sets, make biological sense of the results and apply appropriate statistics has turned into a field of research on its own. Over the past three years a growing number of volumes have been published dealing only with microarray technology (for example Nature Genetics, 2002, 32 Suppl: 461-552). While these publications provide researchers with a survey of the available methods, the analysis and statistics of array data continue to evolve.

Our experience with microarrays began when the technology was still in its infancy and few labs were routinely using microarrays to analyze gene expression. The first and, until very recently, the only company to manufacture microarrays for the rat was Clontech. The first format they manufactured was a nylon microarray to which 588 rat cDNAs had been fixed. Fluorescence-based glass array systems were available for other species however nylon arrays were also appealing to us because at the time they tended to be more sensitive (approx 100-fold) and require less RNA than most glass chips (Bertucci, F et al., 1999). Additionally, nylon microarrays are significantly cheaper to use and to

analyze, are reusable and use 32(33) P as an inexpensive, highly efficient, widely available label. Several publications have addressed the sensitivity and reproducibility of microarrays. Microarrays provide very similar results to those obtained by Northern analysis, the "gold standard" for analysis at the RNA level (Taniguchi et al., 2001; Bartosiewicz et al., 2000). Many of the initial reasons that led us to use nylon microarrays are still valid today, however with the completion of the rat genome and the reduction in price of glass chips, the use of nylon microarrays is likely to diminish.

Initially, investigators using the glass chip platform were performing one replicate per experiment because of the cost of the arrays. With nylon arrays, it became feasible to perform more than one replicate per experimental condition and so the literature erupted with controversy over how many replicates were appropriate and debate over the concept of pooling samples vs. performing more than one replicate ensued. Since there was no resolution to this issue when we began using the arrays we decided to err on the side of caution and used 5 replicates per experimental condition. We decided against pooling samples because we did not want to minimize or eliminate the biological variation between samples. The importance of replication in microarray experiments has since been recognized (Chuaqui et al., 2002; Herwig et al., 2001; Lee et al., 2000) however there is still no consensus on the ideal number of replicates. When we began to use the nylon microarrays there were no software programs specifically designed to analyze the kind of data generated by microarray experiments. 588 data points per experimental endpoint multiplied by 5 replicates made for an enormous amount of data. Additionally, we switched from the 588 array to an array with 1,176 rat cDNA's that Clontech had developed. The analysis of our array data was greatly streamlined by using software that was specifically designed to handle array data. The most comprehensive software package and the only one that allowed for direct importation of Clontech data was Genespring (Silicon Genetics). This is the only product line of that company and programmers and biostatisticians are continuously updating components of the data loading, organization, normalization, and analysis. Genespring has become

one of the more commonly used commercial products for array/chip data analysis around the world.

The first problem we encountered when analyzing array data was how to normalize the data. Arrays are scanned after hybridization and independent grayscale images are generated for each array. These images must then be analyzed to identify the arrayed spots (representing individual cDNAs) and to measure intensities for each spot. In order to compare the data from two independently scanned images the data must be 'normalized' where the individual hybridization intensities are adjusted to balance them appropriately so that meaningful biological comparisons can be made. There are several reasons why data must be normalized, including unequal quantities of starting RNA, differences in labeling efficiencies, and systematic biases in the measured expression levels (Quackenbush, 2002). Expression levels measured by Northern blot are routinely adjusted relative to the expression of one or more reference genes whose levels are assumed to be constant between samples; normalizing array data is conceptually similar. There are many ways to normalize data and the method chosen depends on the data set in question (reviewed in Quackenbush, 2002). Some of the more popular methods include normalizing relative to a reference gene or genes, normalizing to an internal positive control gene and normalizing relative to the overall expression on the array. We decided to use the latter because there were no genes on the array that were constant under all experimental conditions and there were no control genes on the array.

After the data had been normalized we had to define when a gene was 'expressed'. We chose a cutoff value in order to minimize the occurrence of false positives; a gene was considered expressed if its relative intensity was 2 fold the background of the array. Background was determined for each array independently and the same area on the array was used for all arrays.
Once we had defined an expression threshold we then had to determine what constituted a change in gene expression between experimental conditions. Investigators commonly used a minimal fold change in intensity to define whether

gene expression changed between experimental conditions. This particular issue generated a great deal of controversy in the literature. While fold changes were the most popular device to assess gene expression changes, the numerical fold change that appropriately achieved a balance between false positives and false negatives was hotly debated. We defined a two fold or greater change in relative intensity as a change in gene expression. It must be made clear that fold changes in expression are used simply as a selection criterion to sort data; it does not imply biological or statistical significance. It must also be stated that while we used fold change to sort our data, there are many other more sophisticated methods to analyze array data, for example principal component analysis, clustering, class prediction, singular value decomposition etc. (reviewed in Slonim, 2002). Genespring supports several of these methods and they were used when we felt they were appropriate for the analysis of interest.

Clearly the next issue that arose was how to apply statistics. When we began publishing array data, since replication was so rare, statistics were not done nor asked for by reviewers. As the field evolved, researchers wanted a way to assess the quality and reproducibility of the data in order to have confidence in the biological significance of the results. The appropriate way to statistically analyze array data has not yet been resolved and clearly the method applied will depend on the data set in question. More and more researchers are using variants of common statistical tests (Slonim, 2002), although their value versus the use Bayesian statistics is still highly controversial. Since there was, and is, so much controversy surrounding this issue we opted to provide the mean and SEM values for our data as a measure of confidence but we did not use statistical tests.

Since the methods used in the analysis of array data are constantly evolving, during the time between the analysis of the data from my first experiment (chapter one) and the analysis of the data from the subsequent experiments (chapters two, three and four), the software program used to analyze the data was upgraded. This meant that different normalization procedures were applied to the data in each experiment. The methods used for the analysis are explicitly

stated in the methods section in all manuscripts; the analyses of the data were done to the best of anyone's ability using the tools available at the time of the study.

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Appendix 2

Effects of Long term Vitamin E treatment on Retention of the Sperm Cytoplasmic Droplet

Introduction

Passage through the epididymis is essential for sperm maturation and the acquisition of motility and fertilizing ability (Orgebin-Crist, 1967). Under the influence of the epididymal luminal environment, many features/elements of spermatozoa, including the nucleus, acrosome, cytoskeleton, and the plasma membrane, are extensively modified (Toshimori, 2003). One well established hallmark of sperm maturation in the epididymis is the shedding of the cytoplasmic droplet (Toshimori, 2003).

When released from the germinal epithelium, spermatozoa contain only small amounts of residual cytoplasm, as most of the cytoplasm and cell organelles are discarded and phagocytosed by Sertoli cells in the testes. However, after spermiation, a small amount of cytoplasm is generally retained as the cytoplasmic droplet. The droplet is located near the proximal region of the flagellum. In the rat, the cytoplasmic droplet is displaced from the sperm neck along the mid-piece to the tip of the mitochondrial sheath prior to bring shed (Hermo et al., 1988). The majority of spermatozoa in the cauda epididymidis are reported to lack droplets (Cooper and Yeung, 2003). The mechanism by which droplets migrate along the flagellum within the epididymis, and the role of the components of the droplet are unknown.

Shedding of the cytoplasmic droplet is a recognized hallmark of sperm maturation, and thus the retention of the droplet by cauda epididymal or ejaculated sperm is often used as an indicator of altered sperm maturation processes. Previous results from our lab have shown that in aging BN rats the proportion of spermatozoa that retained their cytoplasmic droplet was markedly elevated (Syntin and Robaire, 2001). In order to assess a functional endpoint of the effects of vitamin E supplementation and deficiency on the aging of the epididymal epithelium, cauda epididymal sperm were collected, fixed for electron microscopy and analyzed for the presence or absence of the cytoplasmic droplet.

Material and Methods

Animals and Diets

Six month old male Brown Norway rats were randomly assigned to one of 3 experimental diets: vitamin E deficient (8.36 IU/Kg all-rac-α Tocopheryl Acetate), control (25.4 IU/Kg all-rac-α Tocopheryl Acetate), or vitamin E supplemented (106 IU/Kg all-rac-α Tocopheryl Acetate). All other constituents of the diet were identical. Diet was added to the cage feeders weekly and weighed seven days later to determine the amount consumed. Access to water was ad libitum. Animals were housed at Harlan Sprague Dawley Inc. (Indianapolis, IN) under controlled light (12L/12L) and temperature (72-77F). Rats were weighed weekly. All animal studies were conducted in accordance with the principles and procedures outlined in *A Guide to the Care and Use of Experimental Animals* prepared by the Canadian Council on Animal Care.

Sample Preparation

Spermatozoa were collected (Hank's Minimum Essential Medium, Invitrogen Canada Inc., Burlington, Ontario, Canada) from the cauda epididymides of 24 month old animals only from epididymides where the testis was non regressed. Spermatozoa were washed in Hanks media, fixed in the same media containing 1% glutaraldehyde (Mecalab Ltd., Montréal, Québec, Canada), and embedded for electron microscope analysis. Briefly the tissues were washed three times in sodium cacodylate buffer (0.1M) containing 3% sucrose, pH 7.4, postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide and embedded in epoxy resin. N=three animals for each treatment group.

Analysis and Statistics

Spermatozoa were analyzed on the electron microscope. A minimum of 100 mid-piece sperm tail (flagellum) cross sections per animal were photographed, and the number of sperm tails with cytoplamsic droplets recorded. The effect of vitamin E treatment on the proportion of sperm tails with a

cytoplasmic droplet was analysed by Chi squared analysis. For all analyses, values were considered statistically significantly different at $p<0.05$.

Results

The proportion of spermatozoa retaining a cytoplasmic droplet (table 1) was greater in animals on the vitamin E deficient diet than in animals on the control diet ($p=0.002$). There was no effect of the vitamin E supplemented diet as compared to controls ($p=0.193$) and there was no difference between animals on vitamin E deficient versus vitamin E supplemented diets ($p=0.087$).

In cross section, the cytoplasmic droplet appears as a membrane bound structure that surrounds the sperm tail (figure 1, B-D). The droplet often contains other membraneous elements (figure 1, C, D). There was no effect of vitamin E treatment on the phenotype (nature or appearance) of the cytoplamsic droplets (figure 1).

Discussion

The migration and eventual shedding of the cytoplasmic droplet is a recognized feature of sperm maturation processes in the epididymis. The function of the droplet in sperm maturation, and what regulates the timing of its loss, are not yet understood; however one hypothesis is that spermatozoa develop the ability to adjust to osmotic changes during maturation through the droplet (Yeung et al., 1999).

Treatment with a variety of agents, such as insecticides, aflatoxin, cyproterone acetate, cyclosporine A, and gossypol acetate, has been shown to interfere with the shedding of the cytoplasmic droplet (Agnes and Akbarsha, 2003; Akbarsha et al., 2000; Kaur et al., 1990; Seethalakshmi et al., 1987; Shi and Friend, 1985). Additionally, c-ros tyrosine kinase receptor knockout animals and animals with a partial Y chromosome deletion also display defective shedding of the cytoplasmic droplet (Styrna et al., 2002; Yeung et al., 2000). The

mechanism by which these agents and gene mutations affect shedding of the droplet is unclear. There is some evidence to demonstrate that sperm with retained cytoplasm have impaired function (Keating et al., 1997; Gomez et al., 1996), and it was recently shown that retention of the cytoplamsic droplet positively correlates with defective DNA integrity (Fischer et al., 2003).

In this study, Vitamin E deficiency had a significant effect on the proportion of sperm retaining their cytoplasmic droplet. Vitamin E is a potent antioxidant molecule that functions primarily to protect cellular lipids and membranes from oxidative damage. The sperm plasma membrane contains a high proportion of polyunsaturated fatty acids (Jones, 2002) that are particularly susceptible to lipid peroxidative damage. Lipid peroxidation of membrane lipids causes a range of effects including a loss of membrane fluidity (Beckman and Ames, 1998). Although the mechanism behind the shedding of the cytoplasmic droplet is unclear, it is thought that the process is dependant on the physical restraints of the cell membrane (Cooper and Yeung, 2003). The changes in sperm plasma membrane composition that occur between the caput and cauda epididymidis are well known (Jones, 1998) and in the rat, result in an increase in membrane fluidity (decrease in sterol:phospholipid ratio) from caput to cauda epididymidis (Jones, 2002). Increased lipid peroxidation of the sperm plasma membrane as a result of vitamin E deficiency would therefore reduce membrane fluidity and may result in increased retention of the cytoplasmic droplet. Interestingly, long term vitamin E supplementation did not reverse the age related increased retention of the cytoplasmic droplet in cauda epididymal sperm. The reasons for this are unclear but may relate to the dose of Vitamin E supplementation used in the study; larger doses of Vitamin E may be required to observe an effect.

In conclusion, long term vitamin E deficiency impacts epididymal sperm maturation processes and results in increased retention of the cytoplasmic droplet over the effect observed by aging alone. This suggests that oxidative processes may impact sperm maturation in the epididymis.

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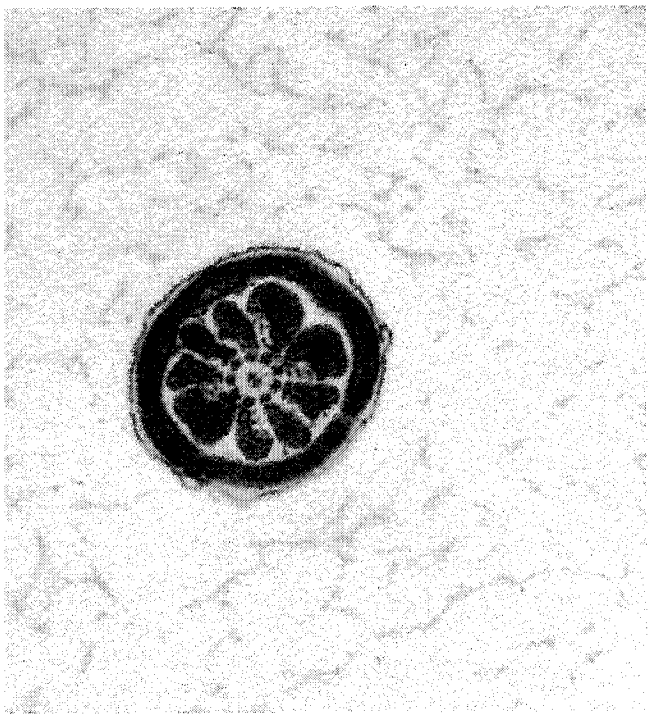
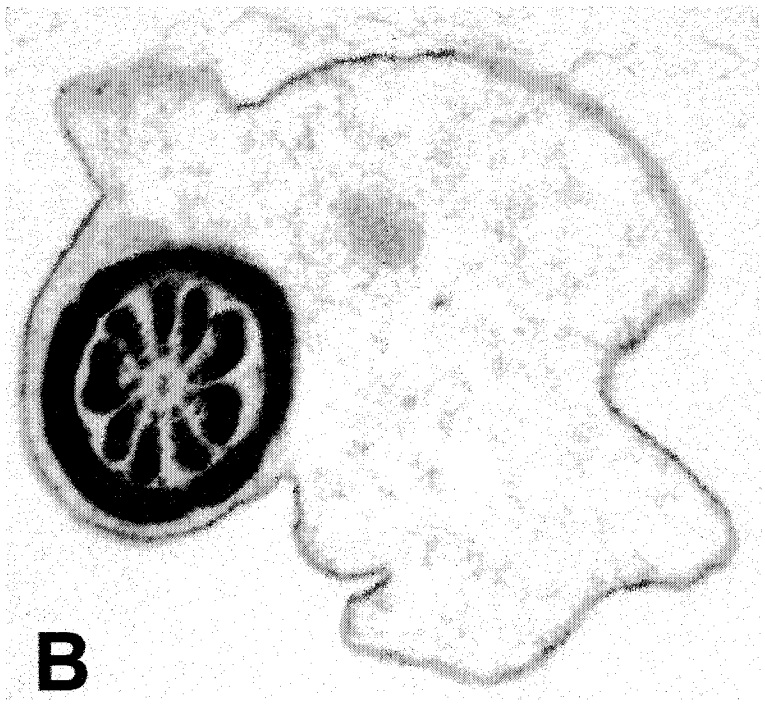
Table 1. Effects of Vitamin E treatment on Retention of the cytoplasmic droplet

	Control	Deficient	Supplemented
# of sperm	305	305	310
# of sperm with droplet	12	35*	21

*P=0.002, versus control.

Figure 1.Representative electron micrographs of cauda epididymal sperm from 24 month old animals. **A.** Sperm flagellum cross-section without a cytoplasmic droplet. **B-D.** Sperm flagellum cross-sections with cytoplasmic droplets; the different phenotypes of the retained cytoplasmic droplets are represented and were not affected by treatment.

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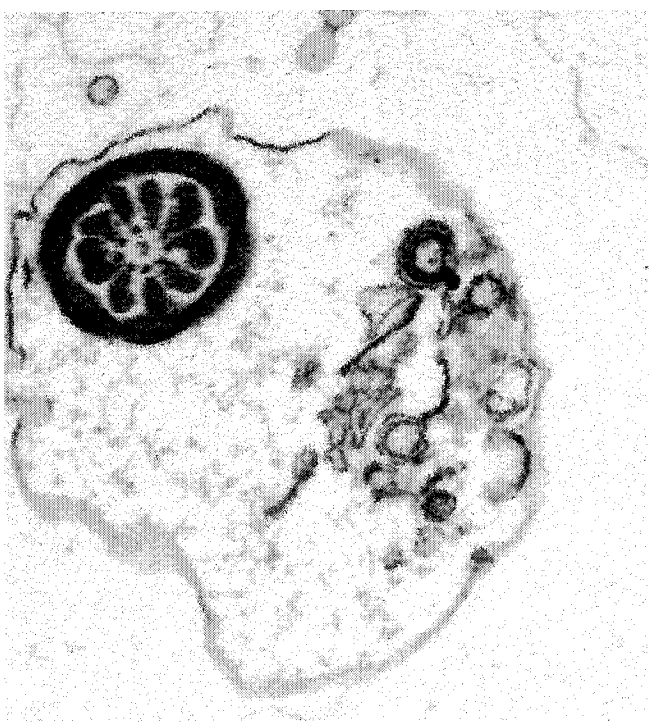

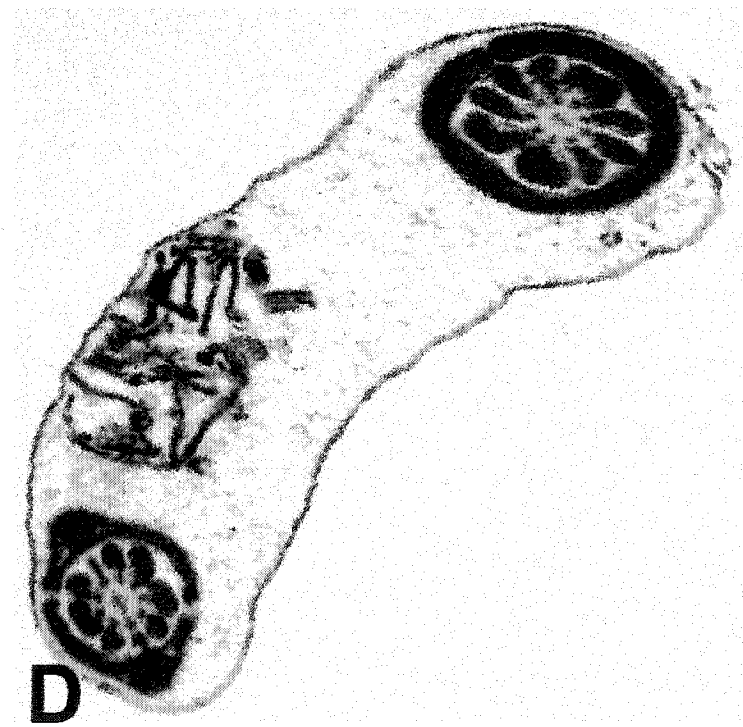
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Chapter 6

Discussion

The primary objective of this thesis was to ascertain how oxidative stress contributes to the aging process of the epididymis of the BN rat. Chapters 2-4 address this question through large scale gene expression analysis and manipulations that affect oxidative stress. At the commencement of this thesis, aging in the epididymis had been well characterized at the histological level but little was known about how these changes were reflected at the level of gene expression. However, before using large scale gene expression analysis to assess how aging affects gene expression in the epididymis, we first had to characterize gene expression in the epididymis of young animals. At the time, gene expression in the epididymal epithelium had never been studied at the RNA level on such a large scale. Chapter two details the results of this fundamental study.

The goal of the following discussion is to summarize the overall contribution of oxidative stress to the aging of the epididymis as assessed in this thesis and place it in the context of our understanding of the aging process as it stands today. Further, the avenues for further study subsequent to this thesis will be addressed.

Gene Expression in the Epididymis

As is a recurring theme of all of these studies, the results of Chapter two could provide the basis for the development of an entire thesis on their own. Gene expression regulation in the epididymis is particularly complex with highly organized region and cell specificity. Although gene expression patterns in the epididymis continue to be characterized, the molecular mechanisms that underlie this organization are unresolved. The results presented in chapter two are a valuable contribution to this end. By using microarray technology to study gene expression, we identified many transcripts with interesting expression patterns that have never before been described in the epididymis. Following up on any of these genes or gene families would further our understanding of the tissue. Two

groups of genes that I found particularly interesting were the transcription factors and the transcripts related to immune function.

Transcription factors act as activators or repressors of transcription and their localization is one means by which eukaryotic cells regulate gene expression. The identification of transcription factors that are expressed in a segment specific manner along the epididymis gives insight into the regulation of regionalized gene expression. In all of the gene expression studied in this thesis we extracted RNA from entire epididymal segments. While this approach is physiologically relevant and gives us a snapshot of the expression in the segment, it does not identify the cell type from which the transcript originated. By following up gene expression results of interest at the protein level using immunohistochemistry, we could determine the cellular localization of these transcription factors. Moreover, identifying the transcriptional targets, binding partners and activation patterns of these transcription factors would start to address the molecular basis of gene expression regulation in the epididymis. This kind of approach to the follow up of gene expression studies is being pursued by a student in our lab for the ID family of transcription factors that were identified using the array technology.

The gene expression study presented in chapter two also identified many immunological markers never before reported as expressed in the epididymis. Several of these markers were expressed at high levels throughout the length of the duct. The need to understand and treat male infertility, and the desire to devise alternative methods of contraception has generated a lot of interest in the normal immune function of the male reproductive tract. Six percent of men presenting with infertility have sperm-reactive antibodies (SpAb) attached to sperm or present in serum or seminal fluid (Beagley, 1998). SpAb have been shown to interfere with sperm motility and/or sperm-egg interaction, and the epididymis is the most likely site of their generation. Understanding the immune function of the epididymis would provide insight into immunological infertility and could potentially identify novel avenues of immuno-contraception.

In chapter three we clearly illustrate that the aged epididymal epithelium has decreased expression of transcripts associated with oxidative stress defenses. This suggests that there is decreased ability of the tissue to withstand oxidative stress with age. In general, oxidative stress defense systems are stress responsive and will react to stressful stimuli with increased transcription. Since there is no evidence in the literature to suggest that oxidative stress load *decreases* with age (Cadenas and Davies, 2000; Inoue et al., 2003; Van Remmen and Richardson, 2001; Wei and Lee, 2002), these results suggest that with age, the epididymal epithelium has decreased capacity to respond to oxidative stress. Even in the event of no change in the production of reactive oxygen species with age, a decrease in the transcription of oxidative defense system genes would result in increased vulnerability of the tissue to oxidative stress. One way to determine whether oxidative defenses are indeed impaired would be to assess the accumulation of oxidative stress damage in aged epididymides in the presence or absence of an exogenous oxidative stressor. Treating with a chemical that has limited epididymal toxicity and induces oxidative stress, would be one approach. A student in our lab is exploring this question with buthionine sulfoximine (BSO), a chemical that induces oxidative stress by inhibiting gamma-glutamylcysteine synthetase, the rate limiting enzyme in glutathione synthesis (Anderson, 1998).

It would have also been interesting to directly measure the production of reactive oxygen species in aged epididymidis; however, this latter study presents methodological challenges. Reactive oxygen species are very short lived and are present in low to very low concentrations (from 10^{-4} to 10^{-9} M); therefore, analytical techniques to assess their production in situ need to be highly sensitive (Batandier et al., 2002). The reactive oxygen species $O_2^{\bullet}$ and H_2O_2 are also linked by spontaneous or enzymatic dismutation and which species to measure as appropriate indicators is not entirely obvious. For these reasons oxidative stress is generally assessed by measurement of secondary products such as

such as oxidized DNA, proteins or lipids (Frank et al., 2000a). However, ROS can be measured directly; the most common methods are with the compound 2',7'-dichlorofluorescin diacetate (DCF-DA) and the chemiluminescent probes Luminol and lucigenin. DCF-DA is a fluorescent dye that exhibits a dramatic increase in fluorescence in the presence of oxidizing agents (Frank et al., 2000b). The specificity of DCF-DA is controversial (Myhre, 2003), and its ability to detect the superoxide anion has been questioned (Bantandier, 2002; Frank et al., 2000b). Moreover, artifactual amplification of DCF fluorescence is also problematic (Frank et al., 2000b). Lucigenin and Luminol are chemiluminescent probes that react with $O_2^{\bullet-}$ to generate an unstable intermediate that emits light as it decomposes. Like DCF-DA, there is controversy over the specificity of lucigenin (Myhre, 2003). Luminol is reported to be made chemiluminescent only by HOCl (Myhre, 2003), and so is useful as a detector only under specific circumstances. In addition to the issues surrounding the specificity of these probes, the other technical concern is that these probes work best in isolated cells and cell suspensions; using them in intact tissues is challenging.

Epididymal cell lines derived from immortalized mouse principal cells have recently become available and they will be a useful tool for this kind of methodology. However, other issues will arise with the use of these cells. These include the fact that these cells represent only one of the four major epididymal cell types, that they represent only one segment of the epididymis, that they are removed from the epididymal environment and all endocrine and paracrine signaling, and, perhaps most importantly, that they are immortalized. Immortalized cells in culture undergo replicative senescence, a phenomenon that is very different from chronological aging. Using these cell lines would be useful methodologically, but they would be likely to produce misleading results if used to answer questions about aging.

The results of chapter three also clearly illustrate that the age-related decrease in oxidative stress transcript levels is not happening in isolation. Rather, this was accompanied by a profound change in the degradative capacity of the tissue at both the lysosomal and proteasomal level. These results illustrate

the utility of the microarray approach in that we were able to assess the effects of age on oxidative stress related transcripts in the context of other changes occurring simultaneously in the tissue. The value of the microarray method would be borne out further by examining how these changes in lysosomal and proteasomal transcripts affect the tissue. The proteasome is largely responsible for the degradation of oxidatively damaged cellular components (Carrard et al., 2002), and there is a body of literature to suggest that defects in degradation of oxidatively damaged cellular components is a pathophysiological consequence of aging (Szweda et al., 2003). It would be interesting to see to what extent the decrease in degradative capacity exacerbates the increased vulnerability to oxidative stress and contributes to the phenotype of the aging epididymis. One way to address this would be to use inhibitors of enzymes of the lysosomal or proteasomal enzymes in young epididymides. Histological examination of the resulting tissues would shed light onto what changes can be accounted for by defects in cellular degradative capacity in isolation of other changes occurring in the tissue with age.

Caloric Restriction and Aging of the Epididymis

Caloric restriction is the most powerful manipulation in aging research and working firsthand with caloric restricted animals is a remarkable experience. Anecdotally, the behavioral differences between caloric restricted animals and their age matched ad libitum fed counterparts is astounding and makes the efficacy of the treatment as an anti-aging intervention unquestionable to the observer. The effects of caloric restriction on gene expression in the epididymis are described in chapter four. Over the course of this thesis, research into caloric restriction increased exponentially. Although the way in which caloric restriction slows aging remains a mystery, what has become apparent is that anti-aging effects of caloric restriction are not due to a single mechanism. Rather, it appears as though caloric restriction slows aging through concerted (coordinated) effects on multiple pathways, including oxidative stress. The effect

of caloric restriction on the transcriptional profile of the aged epididymis is a good illustration of such concerted effects.

Caloric restriction has profound effects on gene expression in the epididymis, including the expression of some oxidative stress related genes. At the same time however, transcripts involved in multiple aspects of cellular function are affected as well. These changes are summarized in schematic 1. The most dramatic effect of caloric restriction on the transcriptome of the aging epididymis was the striking reversal or attenuation of the gene expression changes that occurred with age in transcripts involved in energy production and biosynthetic processes. This effect of caloric restriction has been observed in other tissues such as muscle (Lee et al., 1999; reviewed in Weindruch et al., 2001). The attenuation of the effects of age on mitochondrial bioenergetics by caloric restriction may directly impact the production of ROS. Any defect in the components of the mitochondrial electron transport chain can result in impaired electron transfer and increased production of ROS. In muscle, where caloric restriction also affected energy related transcripts, it has been shown that caloric restriction prevents the age associated increase in mitochondrial protein or lipid oxidative damage and superoxide anion radical generation (Lass et al., 1998). It would have been interesting to determine if, in fact, the effects of caloric restriction on mitochondrial transcripts carry over to an effect on the production of ROS. One approach to get at this question would have been to assess the production of ROS in isolated mitochondria. Assessing the accumulation of oxidative stress damage in epididymides of calorically restricted animals would also have been interesting but would not directly address the effect of caloric restriction on mitochondrial bioenergetics.

The effects of caloric restriction on gene expression have now been studied in a number of different tissues in a number of different species (Cao et al., 2001; Kayo et al., 2001; Lee et al., 1999; Prolla, 2002; Sreekumar et al., 2002; Weindruch et al., 2001). It is clear that the identity of the transcripts affected by caloric restriction differs depending on the tissue and species; however, in every instance caloric restriction is able to attenuate or reverse age

related changes in gene expression. The obvious next question to ask would be 'how does the attenuation or reversal of these age related gene expression changes affect the aging of the tissue?' Prior to the commencement of this thesis, aging in the epididymis had been assessed by both histological and functional endpoints such as sperm maturation. To address whether the gene expression changes that occur with caloric restriction affect aging of the tissue, one could need to assess either of these parameters. Tissues were collected for analysis of the effects of caloric restriction on the histology of the epididymis and the analysis was completed by a postdoctoral fellow in our laboratory. The results (unpublished) suggest that caloric restriction does not reverse or attenuate the effects of aging on the histology of the epididymis. The discrepancy in the results may reflect the sensitivity of the parameters we used to analyze the effects of caloric restriction. Gene expression is highly sensitive to perturbations of the system while histology is comparatively less sensitive. Even endpoints such as the effects of caloric restriction on sperm maturation may be too blunt a measure. Therefore, it would have been interesting to assess the affects of caloric restriction on other aspects of epididymal function. Biochemical endpoints, such as enzyme activity or the production and secretion of protein, known to be vital to epididymal physiology, might be a more telling measure of how caloric restriction affects aging of the epididymis.

Vitamin E Treatment

Vitamin E is recognized as a promising compound, both as an antioxidant and more recently, as a regulator of gene expression (Ricciarelli et al., 2002). Its antioxidant activity coupled with its low toxicity made the long-term treatment and deprivation of vitamin E an ideal means by which to modulate oxidative stress during aging. The results of this study are presented in chapter five and summarized in schematic 2. At the commencement of this thesis, vitamin E was generating a great deal of interest as a pharmacologic antioxidant intervention for chronic diseases with an oxidative stress component. More recently, the ability

of vitamin E to act as a modulator of gene expression and the role that this plays in the beneficial effects of vitamin E is garnering attention (Azzi et al., 2003; Rimbach et al., 2002).

In the epididymis, both antioxidant and non antioxidant aspects of vitamin E action are evident after long term vitamin E treatment. The expression of oxidative stress related transcripts along the epididymis was altered by vitamin E treatment, most dramatically by vitamin E deficiency. Interestingly, in spite of increased expression of oxidative stress related transcripts, the accumulation of a marker of oxidative stress damage to lipids, 4-HNE, was also increased in vitamin E deficient animals. This suggests that levels of enzymatic antioxidant defenses are increased by vitamin E deficiency, but evidently this is not sufficient to prevent oxidative stress damage from occurring.

Unexpectedly, vitamin E deficiency also affected the proportion of sperm that retain their cytoplasmic droplet. Shedding of the cytoplasmic droplet is an often used marker of sperm maturation processes; therefore, this observation suggests that vitamin E deficiency has a detrimental effect on epididymal sperm maturation. Aging alone increases the number of sperm that retain their cytoplasmic droplet (Syntin and Robaire, 2002); therefore, vitamin E deficiency is exacerbating this effect of age. Although we cannot rule out the non-antioxidative effects of vitamin E on sperm maturation processes, the observed effect is likely to be related to the antioxidant actions of vitamin E. Sperm are known to be particularly sensitive to oxidative stress (Sikka, 2001), and there is evidence in the literature to suggest that vitamin E protects sperm from damage induced by chemicals and toxicants that induce oxidative stress (reviewed in Hsu et al., 1998; Latchoumycandane et al., 2002). Moreover, some chemicals that are known to cause increased retention of the cytoplasmic droplet induce oxidative stress (Akhgari 2003; Shen et al., 1996; Yamano, 1996). In spite of having an effect on gene expression in the epididymis, long term Vitamin E supplementation had no effect on the age-related increased retention of the cytoplasmic droplet.

Vitamin E supplementation had no effect on the age related accumulation of oxidative stress damage to lipids as assessed by 4-HNE abundance. This may be related to the dose we used for supplementation. At the commencement of this study no other long term study had assessed the effects of vitamin E supplementation or deficiency on oxidative stress markers. Due to the length of this study, we were conservative and chose a dose unlikely to cause any long term adverse health affects. Long term studies on the effects of Vitamin E supplementation on oxidative stress in animal models of disease have been done and vitamin E has had a beneficial effect (Bauersachs et al., 2001; Gokkusu and Mostafazadeh, 2003). In healthy aging animals, where the oxidative stress load is presumably lower, a larger dose of Vitamin E may have been needed to have had a more pronounced effect on the parameters we assessed. On the other hand, since vitamin E supplementation did affect gene expression in the aging epididymis, it would have been interesting to see what affects this may have had on other aspects of epididymal function during aging. One aspect of Vitamin E function that is particularly interesting is its effects on immune function and inflammation (De la Fuente and Victor, 2000; Grimble, 2003; Meydani, 1999; Serafini, 2000). For example, vitamin E has anti-inflammatory properties (Grimble, 2003) and vitamin E supplementation has been shown to improve cell-mediated immunity in mice and in humans and modulate immune/endothelial cells interactions (Meydani, 1999). Immune cells have been shown to infiltrate the aging epididymidis (Serre and Robaire, 1998; Serre and Robaire, 1999), thus it would be interesting to further investigate the effects of Vitamin E treatment on immune parameters in the aging epididymis.

Oxidative stress, and the damage that it causes, is clearly implicated in the aging process. This thesis is the first step towards assessing the contribution that oxidative stress plays in the aging of the epididymis, and, to this end, it has opened many avenues of exploration. Together, the results presented in this thesis indicate that oxidative stress is playing a role in the aging of the

epididymidis. Moreover, results show that increased oxidative stress load worsens some of the effects of age on the epididymis.

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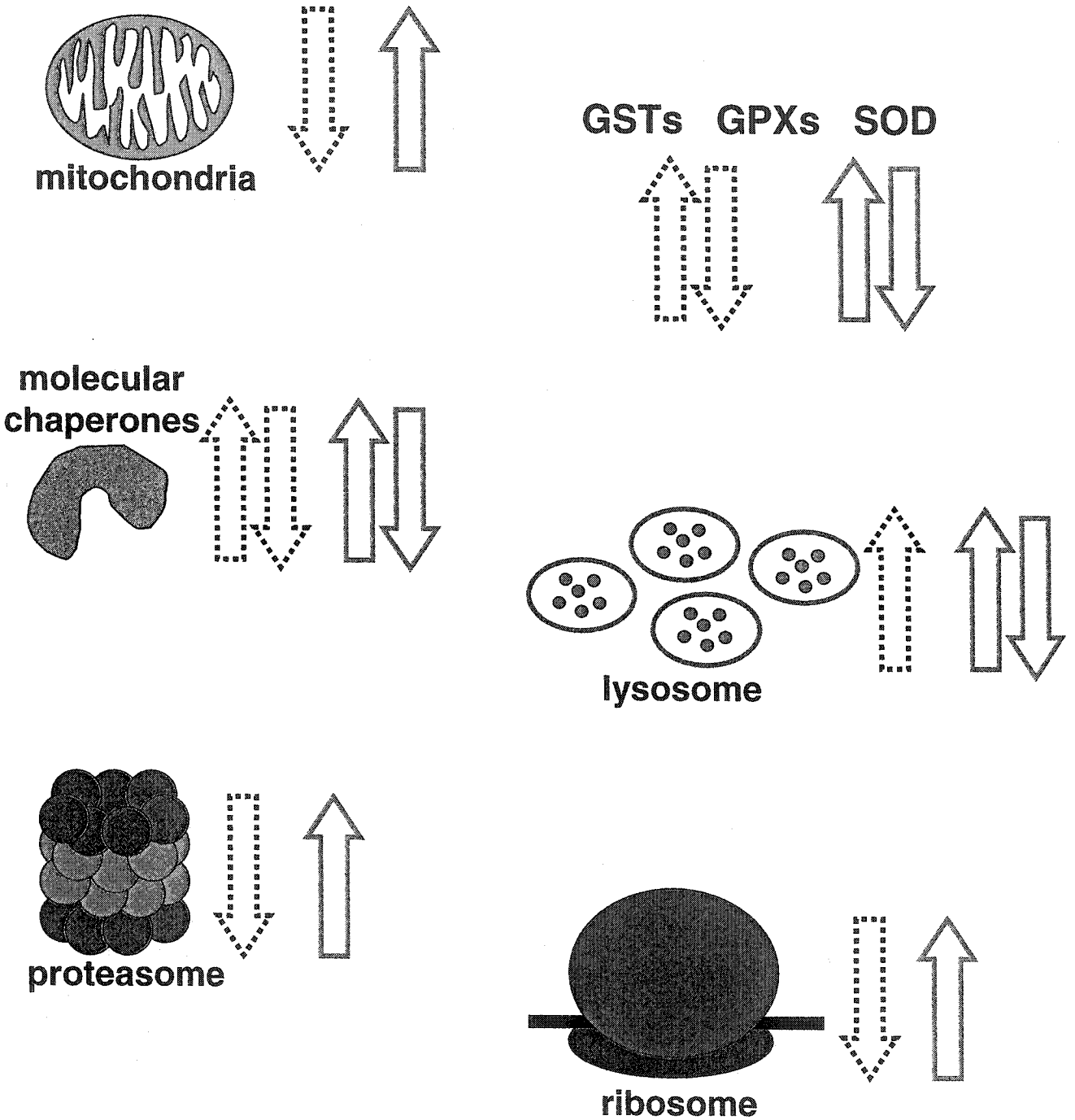
Schematic 1. The effects of Caloric Restriction on gene expression changes in the epididymis

The Effects of Caloric Restriction on Aging of the Epididymis

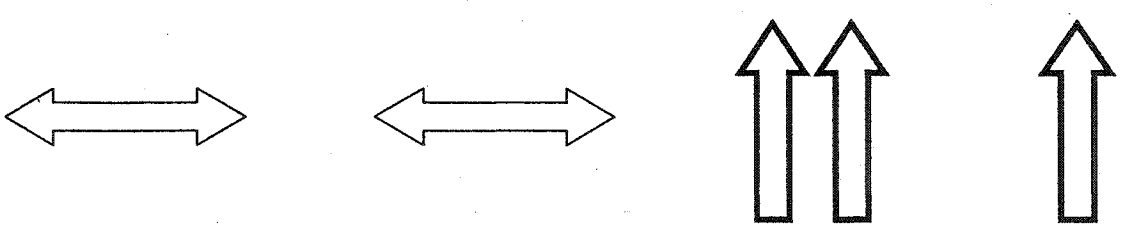


Schematic 2. The Effects of Vitamin E on the Expression of Oxidative Stress Related Genes and lipid peroxidation in the epididymis

Effects of Vitamin E on the Expression of Oxidative Stress Related Genes and lipid peroxidation in the epididymis

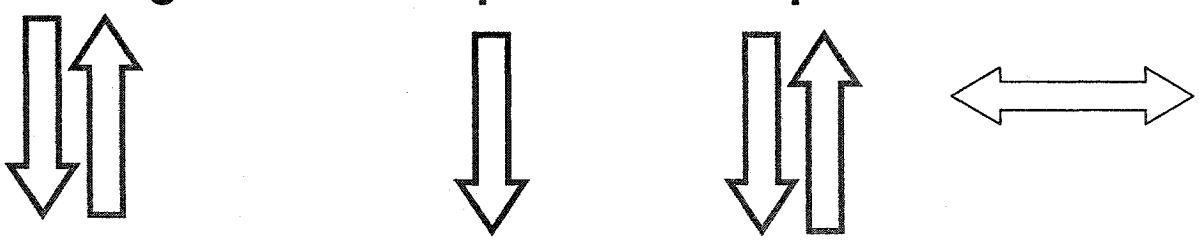
Vitamin E deficient

initial segment caput corpus cauda



Vitamin E supplemented

initial segment caput corpus cauda



Lipid Peroxidation

Vitamin E deficient

Vitamin E supplemented