



Journal of Biological Sciences

ISSN 1727-3048

science
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New Prospects in the Understanding of Molecular Basis of Ageing

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Abstract: Many of us would like to experience a healthy old age. It might now be possible to achieve this goal by protecting our immune system from the damage that accumulates with time. The story starts with the explorer, Ponce de Leon, who set forth from Spain in 1513 in search of the 'Fountain of Youth'. Both before and since that time, claims have been put forth, often without the benefit of scientific testing, that certain interventions may increase lifespan or promote sustained health in old age. Age-associated reductions in circulating levels of peroxisome proliferator activated receptor α (PPAR α) activators such as dehydroepiandrosterone- 3β (DHEAS) may compromise normal cellular responses that involve PPAR α activation, thereby hindering the essential maintenance of cellular redox balance. Excesses in reactive oxygen species can lead to the activation of nuclear factor-kappa β (NF- κ B) and the downstream expression of some NF- κ B controlled inflammatory genes. This imbalance in redox state appears to be intimately linked to many age-associated alterations in immune function and other ageing-related tissue damage. Supplementing aged mice with PPAR α activators has proven to be helpful in re-establishing cellular redox balance, thereby promoting the reacquisition of immune competence and possibly alleviating a number of age-associated pathophysiologicals. An understanding of the ageing at the molecular level offers hope for strengthening the aged immune system; thereby preventing some of the diseases commonly linked to advanced age.

Key words: Ageing, oxidative stress, immune system, dehydroepiandrosterone (DHEAS), Peroxisome Proliferator Activated Receptor α (PPAR α), Nuclear Factor-Kappa β (NF- κ B)

INTRODUCTION

Ageing is a luxury that most individuals in industrialised countries hope some day to experience. In general, this process involves a decline in the efficiency of various cells and tissues and systems. The real question is then what precipitates this decline in efficiency and can it be avoided. Thanks in large part to the development of vaccination and antibiotics, the average life expectancy of humans has increased dramatically over the past century. One of the assumptions in biology is that normal cells can go through only a fixed number of divisions before they die, a process called senescence. The assumption leads to the conclusion that this accounts for the ageing process. It is the damage to cells over a lifetime that stimulates the effects of ageing, which induces a gradual loss of differentiated function of the cells and growth rate. This stress (e.g. biochemical damage) on the cells reduces its capacity to multiply. While many physiologic changes

take place as we grow older, ageing does not involve alterations in the genes per se. It is their regulated expression that goes awry. As the age of an individual advances beyond adolescence, undesirable changes in gene expression or function become increasingly prominent, altering normal physiological processes and imposing challenges to a healthy and independent lifestyle. Consequently, it is important to understand the mechanisms underlying the ageing process and to initiate treatment and lifestyle changes that can extend optimal organ system function.

The oxidative stress theory of ageing: There are many theories relating to the mechanisms of ageing. One such theory is centred on shortening telomeres, the ends of chromosomes, which were once considered to simply protect against DNA breakdown. However, the theory that currently has the most evidence supporting it and the least number of apparent contradictions, is the 'oxidative stress theory'^[1]. This theory is based on observations of

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progressive accumulation of molecular damage in many tissues, the theory suggests that excesses of reactive oxygen and nitrogen species determine maximum lifespan and also account for many of the diseases of advanced age.

Normally, oxidation involves the transfer of a pair of electrons from one atom to another. When an unpaired electron escapes, called a free radical, it can cause damage to the molecules in nearby cell membrane. These single electrons, the free radicals, are highly reactive, seeking to capture another electron to complete a pair and in doing so they damage or destroy the function of another molecule. This type of damage could be a major contributing factor to ageing and to infectious diseases.

Free radical damage is cumulative, building up with age. The cell membrane lipids, an important role player in nerve function, are oxidized, resulting in impaired cell-to-cell communication and transmission. The immune alert systems are slow to react and the memory to deal with the invading pathogen is impaired.

This oxidative damage changes the local cellular environment, disrupts cellular signals, changes metabolic pathways and interferes with immunological functions, with resulting potential for infection. A struggle results, with the body repair system trying to repair the damage. The strength of this repair system is compromised with age and the infection takes over. Oxidative damage has stimulated interest in antioxidants in preventing the development of infectious diseases.

It is well accepted that the cellular levels of oxidatively modified macromolecules increase with age in all species studied, especially in the later part of the lifespan^[2]. In general, oxidative modifications to proteins decrease their structural or enzymatic activities. Oxidative damage also accrues in DNA, altering both the cellular and mitochondrial genomes, which are not always effectively repaired. Finally, cellular and subcellular membranes become oxidatively damaged, stimulating the propagation of a free radical generating cascade that catalyses the production of additional Reactive Oxygen Species (ROS). The increased generation of ROS with ageing is often accompanied by a reduction of endogenous antioxidant activities.

There is strong evidence that damage to the immune system (including the decline in the ability to be effectively vaccinated, respond appropriately to pathogens and suppress cancer cell growth) is linked to a reduced capacity to control oxidative stress. Excesses in oxidative stress affect many distinct cell-signaling pathways and cause numerous changes in gene expression. One important example involves nuclear factor-kappa B (NF- κ B). NF- κ B is a transcription factor

that modulates the expression of many genes associated with inflammatory processes a number of which become aberrantly regulating in ageing.

Ageing in the immune system: During ageing, protective responses to infectious agents are reduced, as are antibody responses and cell-mediated immune responses in a process known as 'immunosenescence'. This is accompanied by an increased susceptibility to infection, a lowered ability to be successfully vaccinated and a diminished natural response to tumour development^[3]. Some of these age-associated changes are even observed in the stem cells that serve as progenitors that develop into cells comprising the immune system^[4]. As will be explained, however, it is too simplistic to define immune responsiveness of elderly individuals as being only diminished.

Macrophages: The innate immune system is the first line of host defense against infection. The role of the macrophage in innate immunity is to remove and consume materials, including bacteria and other microbes, foreign- and self-macromolecules and dead or injured host tissues. Upon stimulation, macrophages produce cytokines that recruit other inflammatory cell types, contributing to the systemic effects of inflammation. Macrophages can also function as Antigen-Presenting Cells (APC) and may assist in tumour killing.

DHEA and the immune system: While long appreciated to be a major steroid hormone within the circulation, the exact physiological role(s) of DHEAS remains unclear. It possesses weak androgenic activities and serves as a reservoir precursor for the production of sex steroids. In addition, numerous reports ascribe anti-obesity, anti-diabetogenic activities to DHEA, the non-sulphated derivative of DHEAS. Moderate dose DHEA or DHEAS supplementation has also been found to exert a number of beneficial effects upon the immune systems of experimental animals and humans. Supplementation with orally administered DHEA results in a rapid metabolism to other steroids or conversion to DHEAS. Therefore, most of the effects of DHEAS *in vivo* are also manifest subsequent to DHEA administration. In our studies, mice are simply supplemented with DHEAS in their drinking water.

DHEA pretreatment increases resistance to viral, bacterial or protozoan pathogens in experimental mice. In these models of infection DHEA was able to modulate cytokine production by lymphoid cells and macrophages. Aged experimental animals given DHEA had reduced age-associated elevation in circulating IL-6 levels. In addition,

the production of cytokines by activated T lymphocytes was effectively restored to normal and the titres of autoantibodies were reduced. DHEAS supplementation also enabled aged mice to be effectively vaccinated^[5]. Following these successes in animals models, limited human trails have also been conducted. These studies have reported some success in enhancing protective vaccination responses in the elderly, although conflicting reports do exist.

Age-associated changes in the function of macrophages have not been extensively studied. Macrophages from aged mice have a reduced ability to engage in tumour killing compared to similar cells from young mice. These cells also possess a decreased ability to present certain antigens to antigen-responsive T cells. Work performed in the Daynes laboratory has further established that macrophages isolated from various lymphoid organs of aged mice overproduce a number of inflammatory cytokines (TNF- α , interleukin (IL)-1, IL-12) *in vitro*, in the absence of any additional exogenous stimulation.

The constitutive production of IL-6 by cells from aged experimental animals and humans is of such a magnitude that it can be readily measured in the blood plasma. As a result, IL-6 has been termed a 'cytokine for gerontologists'^[6]. Although IL-6 is required for a number of normal cellular processes, excessive or unregulated production of this cytokine has been associated with many inflammatory diseases, such as B cell lymphomas, Alzheimer's disease and acute phase responses. Elevated IL-6 might also contribute to the development of breast cancer, an increased susceptibility to stroke and other vascular diseases and the development of osteoporosis.

T and B cells: T lymphocytes are important components of adaptive immunity that are generated from the maturation of bone-marrow-derived progenitors within the thymus, hence the name 'T cell'. One of the earliest descriptions of age-associated alterations in the immune system was the marked reduction in mass and cellularity of the thymus with ageing. In aged mammals, over 90% of the thymus is lost. In addition, secretion of thymus-derived hormones, some of which are essential for the development and differentiation of T cells, can no longer be detected in humans over age 60, or in mice over six months of age.

The parallels between thymic involution and decreases in immune function that occur with ageing have fostered many studies. T cell-mediate responses to novel antigens, tumour rejection, anti-viral response, the helper activities necessary for essential B cell functions and the generation of graft-versus-host responses are all impaired in aged mice and elderly humans. These functions are

dependent upon the adequate production of the cytokine, interleukin (IL)-2, by T lymphocytes, which is significantly reduced from aged subjects compared to younger individuals. Likewise, the generation of cytotoxic CD8⁺ T lymphocytes, essential for the killing of virally infected or tumour cells, is temporally delayed, reduced in overall cytotoxic activity and of a shorter duration in aged mice. Ageing is additionally accompanied by increased numbers of 'memory' T cells, believed to be a result of clonal expansion subsequent to encountering specific antigens. Consequently, there are decreased numbers of 'naïve' T cells in aged individuals.

Memory T lymphocytes should be inherently responsive to secondary encounters with specific stimulating antigens. Whether the expanded pool of memory T cells observed with ageing results from responses to previously encountered antigens, or arises from the aberrant activation of a signaling cascade that increases expression of memory cell markers, remains controversial. One consequence, however, is an elevated capacity of T cells from the aged to produce the cytokines IL-4 and IL-10 upon stimulation.

Production of IL-4 increases with age and favours the development of humoral (antibody based) immunity. The inflammatory cytokine IL-6 can also stimulate the production of IL-4 by activated T cells. This illustrates the intricacy and interconnectedness of the immune system—changes in the synthesis of one cytokine will affect the production of other immune signals.

The specific repertoire of cytokines that T cells produce following stimulation helps to orchestrate the generation of humoral immune responses-mediated by B-cells. In fact, some of the age-associated alterations in the functions of B lymphocytes have now been attributed to the inability of T cells to appropriately modulate B cell activation and differentiation. This may partly explain the inability of aged individuals to be effectively vaccinated with protein antigens.

T cells from aged animals and humans also show and increased incidence of autoreactivity (where the immune system inappropriately responds to 'self'). It is of interest to note that healthy centenarians, a select population of physiologically exceptional individuals over 100 years of age, have dramatically low levels of autoreactive antibodies in their circulation. Characterization of the molecular and biochemical processes that differentiate healthy centenarians from 'unselected' elderly individuals should prove useful in the design of intervention strategies to aid the 'average' ageing individual.

The long-life immune system: Many of the diseases that affect elderly individuals have an associated pro-oxidant or inflammatory component. Age-associated

pathophysiologies may be ameliorated, or even prevented, by taking antioxidants or anti-inflammatories. Agents capable of modulating cellular levels of antioxidant enzymes, as well as enzymes capable of breaking down inflammatory molecules, might also be useful in the treatment of age-associated diseases and as therapies for eliminating or preventing the alterations responsible for the aged immune system. It may, thus, be possible to alleviate some of the symptoms of advanced age with proper intervention.

Caloric restriction, although imposing significant lifestyle hindrances, is nevertheless able to extend the maximum and mean lifespan of experimental animals, including worms, flies, mice and, according to anecdotal accounts, humans^[7]. Caloric restriction appears to work by decreasing oxidative stress and increasing internal antioxidant defenses and repair. The onset of late life illnesses such as autoimmune disorders, certain cancers, cataracts, diabetes, hypertension and kidney failure are delayed in their occurrence in calorically restricted rodents and non-human primates.

Mice maintained on a calorically restricted dietary regimen remain immunocompetent well into old age. Furthermore, calorically restricted mice do not spontaneously overproduce IL-6, unlike age-matched control mice. Self-imposed caloric restriction of the magnitude and duration necessary to produce these effects, however, is nearly impossible to achieve in normal human populations. Therefore, proper caloric restriction studies have never been performed with human subjects.

Appropriate dietary supplementation has been reported to induce some immunorestorative effects. Of the therapeutic interventions that have been reported to produce beneficial effects in aged experimental animals, one involves the dietary supplementation with the natural steroid hormone dehydroepiandrosterone-3 β -sulphate (DHEAS).

Role of dehydroepiandrosterone (DHEAS): Another substance that has been implicated in immune system deterioration is DHEA, the most abundant adrenal steroid in young healthy individuals. It is released from the zona reticularis of the adrenals after birth, increasing throughout puberty until maximum serum levels are reached in the third decade of life. Then a slow, steady decline commences of about 2% per year in circulating blood levels. By the time one reaches the eighth decade of life, the levels are at 10-15% of the maximum. This information is arrived at by doing a challenge test (adrenocorticotrophic hormone challenge test) on different age groups which indicates that the decline is not the result of a change in the metabolism of DHEA, but instead appears to be affected by a diminished adrenal secretory

rate. This is contrasted with the cortisol secretion, which is maintained throughout life. The resulting increase in cortisol/DHEA ratio in the blood may be another culprit partially responsible for the vulnerability that develops during ageing. This is further confounded by the loss of receptors for DHEA that arise with ageing such that there is an irreversible process rapidly overcoming any attempts to enhance the immune system.

Nevertheless, there are clear signs of hormonal changes that signify ageing in the male. The process is called "andropause" and can be seen in the age-related hormonal changes in plasma levels of testosterone, dehydroepiandrosterone (DHEAS) and melatonin which, as the levels of these hormones change in the body, have there consequences on the individual. Other hormonal changes may be subtler and may involve such concepts as hemodynamic shear stress, which may regulate endothelial function and gene expression and give rise to atherosclerosis via enhancing the inflammatory components of the body^[8]. Atherosclerosis is the leading cause of death in the developed world. Administering DHEA may protect against development of atherosclerosis, but no robust relationship has been demonstrated between plasma DHEAS and coronary artery disease or myocardial infarction or any other chronic disease associated with the ageing process.

The levels of DHEAS, which are high in healthy young humans and other primates, decline with age; the levels found in a 70-year-old represent only 10-20% of the levels found in a 20-year-old. This decline has been linked with decreases in the normal physiology of many organ systems and has been correlated with an increased frequency of cardiovascular disease, neurologic dysfunction, osteoporosis, hypothyroidism and immune dysfunction. Intriguingly, age-associated reductions in circulating DHEAS levels are statistically correlated to the parallel age-associated increases in circulating IL-6 levels.

Despite many reports attributing remarkable immunomodulatory activities to DHEAS, a second literature also exists in which high dose supplementation with DHEA caused liver pathology and even hepatic tumours following chronic administration to rodents. This has been extensively researched and is linked to overexpression of peroxisomes and peroxisomal enzymes in rodent livers. DHEAS functions as a Peroxisome Proliferator (PP), increasing both the number and activity of peroxisomes. It now appears that the activation of a PP activated nuclear hormone receptor, termed the peroxisome proliferator activated receptor α (PPAR α), physiologically regulates production of the many enzymes and proteins that reside in peroxisomes, plus numerous mitochondrial proteins involved in fatty acid metabolism. Additionally, activated PPAR α can

effectively inhibit the transcription factor activities of NF- κ B^[9]. Present studies indicate that DHEAS elicits much of its anti-inflammatory and immunorestorative effects in aged mice.

Gene transactivation by PPAR: PPAR α activation results in the transcriptional upregulation of many peroxisome-associated and non-peroxisome-associated genes^[10]. PPAR α is implicated in the regulation of; fatty acid metabolism; nearly all cell-associated catalase activity; copper-zinc superoxide dismutase (Cu, Zn-SOD) and certain mediators in the glutathione pathway. Catalase catalyses the conversion of hydrogen peroxide (which is formed during the peroxisomal β -oxidation of fatty acids), to water and oxygen ($2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$). Clearly, PPAR α activation can result in a plethora of bio-chemical changes, many of which alter the functions of both responsive and bystander cells through the elimination of inflammatory molecules and reactive oxygen species.

The best evidence to date in support of an important role for PPAR α in mediating some of the biological effects of DHEAS comes from work using mice bearing a null mutation for PPAR α . This strain of mouse is unresponsive to a number of peroxisome proliferating agents, including DHEAS. We have used the PPAR α knockout mouse to demonstrate that the immunorestorative effects of DHEAS absolutely require a functional PPAR α ^[11]. In addition, cellular oxidative stress becomes apparent at a much younger age in PPAR α knockout mice. Genetically normal mice undergo an age-associated decline in the mRNA levels of PPAR α ^[12]. This suggests that PPAR α may play a functional role in the complex regulation of the intracellular redox balance, which becomes compromised during the ageing process.

Present studies have determined that DHEAS exerts its immunomodulatory effects in aged experimental animals through the activation of PPAR α . Dietary supplementation of aged mice with low doses of DHEAS or another known PPAR α activator reverts many characteristics of their ageing immune systems to those commonly observed in younger control animals. Modest doses of PPAR α activators were able to reduce the dysregulated nuclear activity of NF- κ B in the spleens of aged mice. Furthermore, it also resulted in a correction of the dysregulated constitutive expression of various cytokines.

Similar beneficial changes were achieved following the administration of the dietary antioxidant, vitamin E (α -tocopherol), to aged animals and humans. The fact that similar 'immunocorrective' effects were observed by supplementing with PPAR α activators or an antioxidant, supports our hypothesis that PPAR α activation regulates

cellular redox state. In fact, we have demonstrated that the rate of aged mice with PPAR α activators. This may be partially due to the upregulation of genes encoding endogenous antioxidant and lipid-metabolising enzymes, thus removing reactive oxygen species, oxidized lipids and other inflammatory lipid mediators more effectively. Additionally, activated PPAR α may be 'buffering' the cellular activities of various transcription factors linked to inflammatory processes^[13] and certain types of immune responses^[14].

Nf- κ b and PPAR α in ageing: NF- κ B normally exists in the cytoplasm as a protein complex, comprised primarily of p65/p50 heterodimers or p50/p50 homodimers bound to an inhibitor of kappa B (I κ B). Various types of cell stimuli (e.g., inflammatory mediators, physical stress and oxidative stress) can induce NF- κ B activation. In fact, hydrogen peroxide (H_2O_2) itself can cause the activation of NF- κ B^[15]. Through a yet to be elucidated mechanism(s), ROS cause the recruitment and activation of the multi-subunit I κ B kinase (IKK) complex. Activated IKK phosphorylates I κ B, which is subsequently degraded. The free NF- κ B dimer then travels to the nucleus and binds to the promoter region of genes possessing a κ B motif, the necessary transcriptional machinery is then recruited to the NF- κ B-DNA complex and gene transcription begins.

NF- κ B is active (present in the nucleus bound to DNA) in the heart, liver, kidney, brain and cardiac muscle of aged experimental animals. We have demonstrated that aged mice show a markedly elevated NF- κ B activity in many of their lymphoid organs when compared to young adult controls^[16]. Furthermore, NF- κ B is also present in an active state in macrophages, B lymphocytes and T lymphocytes that reside in the spleens of aged mice. Present studies suggest that excesses in oxidative stress are responsible for constitutive activation of the NF- κ B system in cells from aged animals. Supplementation of aged animals with the dietary antioxidant α -tocopherol (vitamin E) reduces NF- κ B activity to levels seen in young controls. Dysregulated cytokines and proteins under NF- κ B control could be responsible for changes in immune competence and may also contribute to other diseases that accompany ageing.

NF- κ B controls the production of many inflammatory mediators such as cytokines, prostaglandins and acute phase proteins. Activation of PPAR α can effectively elicit an 'anti-inflammatory' effect. The mechanism of this effect is not yet known; however, adding PPAR α specific ligands to human aortic smooth muscle cells *in vitro* can effectively inhibit the activation of NF- κ B and consequently represses the transcriptional upregulation of IL-6. Equally impressive results come from the

therapeutic administration of the PPAR α activator, fenofibrate, to patients with coronary artery disease. This supports the role of PPAR α activators as anti-inflammatories *in vivo* and reveals their potential in certain clinical applications. Interestingly, the fenofibrate-treated 'healthy' control population also responded positively.

Therapeutic interventions: In addition to the PPAR α activators already described, a number of nutritional fatty acids are also able to inhibit inflammatory reactions *in vivo* and enhance average healthy lifespan. For example, the n-3 polyunsaturated fatty acids, such as docosahexaenoic acid (22: 6n-3) and eicosapentaenoic acid (20: 5n-3), appear to represent natural PPAR α is mechanistically involved in their anti-inflammatory activities remains a controversial subject at the present time.

Despite enthusiasm for the therapeutic use of PPAR α activators in the treatment of certain inflammatory conditions, it is important to proceed slowly when attempting to translate results obtained from experimental animal studies or *in vitro* cell culture systems to the development of human therapies. It is first necessary to consider different responses of rodent and human cells to PPAR α activators. In addition, a better understanding is needed of the mechanisms that control PPAR α expression at the transcriptional and translational levels and the breadth of functional capabilities of this recently described transcription factor. Finally, the role(s) of endogenous PPAR α activators, including various naturally occurring fatty acids, their derivatives and DHEAS must be further explored if we are to effectively manipulate the extent of cellular PPAR α activation through therapeutic intervention.

ACKNOWLEDGMENT

We thank to Mr. Ghulam Rasool for expert editorial assistance.

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