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Apolipoprotein D:

An overview of its role in aging and age-related diseases

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Abstract

"*It's got to be doing something important!*"

—Seymour Benzer, reflecting on the observation that ApoD mRNA levels increase 500-fold following neuronal crush injury.

Seymour Benzer's curiosity was legendary and seemingly limitless.[1-5] Towards the end of his life, one of the (many) questions that kept him awake at night concerned the emerging role of Apolipoprotein D (ApoD) in aging and neurological disease. In this perspective, we will discuss the clinical and biochemical data on ApoD, and the input from the recent genetic studies in model systems, including those from the Benzer lab.

Keywords

ApoD; *GLaz*; *NLaz*; drosophila; aging; p53; cancer

Molecular Biology of Apolipoprotein D (ApoD)

Apolipoprotein D (ApoD) is a secreted glycoprotein assigned with many putative functions including lipid transport. Human ApoD was first formally identified in plasma High Density Lipopoproteins (HDL) in 1973.[6] The cDNA is 855 bp long,[7] processed from a gene region with 5 exons spanning 20 kbp, typical of the superfamily.[8] In silico promoter analysis identified multiple steroid response elements (RE) upstream of the ApoD gene.[9] Further analysis in cell culture showed the gene was also under the primary control of serum-responsive elements during cellular growth arrest, and has response elements for oestrogen, glucocorticoids, progesterone, and vitamins A and D.[10]

In humans, the protein is widely expressed, unlike other apolipoproteins, which are mainly produced by the liver, hinting at fundamental cellular functions.[11] However, its main sites of expressions are the brain and the testes. In the central nervous system, it is mainly expressed in glial cells (both astrocytes and oligodendrocytes) and their precursors,[12] but can also be expressed by neurons in pathological situations. The protein itself is predicted to be small (18 kD), soluble and secreted and has no homology to other apolipoproteins (such as the

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well-known ApoE).[11] Early studies showed diverse levels of glycosylation, sometimes specific to expression site.[11] Based on its primary sequence, it was predicted to be a member of the lipocalin family of proteins. These are cup-shaped molecules, presenting 8 beta-pleated sheets arranged in a calyx structure, capable of binding a single hydrophobic ligand in the pocket they form. This was recently confirmed by the 1.8A structure of a hydrophilized form of recombinant ApoD.[13,14] These studies highlighted the presence of hydrophobic residues outside of the binding pocket, which may allow the molecule to interact with membranes or HDL, and favor ligand exchange, while the base of the calyx could provide a docking site for an unidentified cellular receptor. Both the crystallization studies and earlier affinity assays, point to arachidonic acid (AA) and progesterone (PG) as putative physiological ligands for ApoD.[15,16]

A complicated picture of ApoD biology has emerged from these biochemical studies. The protein is expressed in many different tissues, with different glycosylation profiles. It can bind with micromolar affinity several ligands, and interact with membranes and circulating lipid particles and its expression appears to be under the control of a complex array of regulatory elements. ApoD is potentially a protein with multiple ligands and interacting partners, through which it can exert multiple different functions in different tissues.

ApoD and Neurological Disorders and Nerve Injury

ApoD has been described in association with various neurological disorders, including Alzheimer's Disease (AD), Parkinson's disease (PD) and stroke.[11] Interestingly, oxidative stress has been implicated in the etiology of each of these pathologies.[17,18] For example, an imbalance in reactive oxygen species (ROS) production and clearance in the dopaminergic neurons of the substantia nigra (SN) has long been linked to neuronal cell death in PD. Recently, it was reported that high levels of ApoD could be found in the SN of patients with PD.[19] In this case, the neurons that are the target of the pathology do not express ApoD, but the surrounding glial cells display an increased immuno-reactivity. ApoD has also been found to be elevated in the brains of patients with Alzheimer's disease (two-fold elevation over age-matched controls).[20,21] In these patients, expression can be seen in oligodendrocytes and astrocytes, but also in neurons affected by neurofibrillary tangles, and in the vicinity of amyloid plaques. It is also worth noting that a particular allele of ApoD was enriched in AD patients, in a small African American sample,[22,23] while another allele was enriched in a Finnish population with early onset AD.[24]

Since ApoD is a lipid carrier, it is particularly interesting to observe its upregulation in disorders of the myelin sheath. Cerebrospinal fluid levels and plasma ApoD levels are significantly elevated in multiple sclerosis and other inflammatory diseases of the central nervous system.[25] As mentioned in our introduction, one of the most striking increases is observed in regenerating and remyelinating sciatic nerve, in the rat. After 3 weeks post-crush injury, ApoD increases 500-fold at the site of the lesion, and remains elevated while attempts at regeneration take course.[26]

More recently, in a rat model of stroke, ApoD was found to increase up to a week after reperfusion, in the penumbra of the injury.[27,28] It is important to note that ApoD protein levels, but not its mRNA, increased in the dying neurons. On the contrary, in the zone bordering the infarct, oligodendrocytes were shown to upregulate ApoD mRNA expression, thereby suggesting secretion and recruitment in the dying areas.

ApoD and Psychiatric Disorders

ApoD has also been associated with psychiatric disorders such as Schizophrenia and bipolar disorder.[29] For example, circulating ApoD is elevated in the serum of schizophrenic patients

receiving no medication, and in the brains of treated patients.[30,31] Similarly, a two-fold increase in ApoD can be found in the prefrontal cortex of patients with either schizophrenia or bipolar disorder, but a concurrent two-fold increase in the parietal cortex appears to be specific of bipolar disorder patients.[32] It is possible that ApoD's elevation in these specific regions is related to the membrane pathology observed in these disorders. It is therefore interesting to find that high ApoD levels correlate with membrane AA in schizophrenic patients' erythrocytes.[33]

Interestingly, antipsychotic drugs that work best in treating these psychiatric disorders—atypical antipsychotics such as clozapine—further elevate the levels of ApoD.[34] One may therefore suggest that ApoD elevation in these disorders, and a further boost by drugs, can act to prevent deleterious events, stabilizing membrane bound AA.

ApoD, Cancer and the p53 Family

In recent years, a number of studies have reported alterations in ApoD expression in relation to cancer.[35-37] In general, ApoD expression correlates inversely with aggressive behavior of several different types of malignant tumors. Although the underlying mechanism has not yet been elucidated, ApoD has been shown to function in cell growth inhibition.[38-41] Recently, it was reported that ApoD is a direct transcriptional target of p53 family members p73 and p63.[42] p53 is probably the best-studied tumor suppressor gene, and is found to be mutated or inactive in up to 70% of all cancers. p53 protein is present at low levels in normal cells but is activated in response to a variety of environmental stimuli, including DNA damage, hypoxia, viral infection, or oncogene activation, resulting in transactivation of a specific set of target genes. These targets are involved in cell cycle control, apoptosis, DNA replication, repair, proliferation, inhibition of angiogenesis, and cellular stress response.[43] Both p73 and p63, share high homology to p53, especially in the central DNA binding domain. Therefore, these p53 family members may overlap functionally with p53. Interestingly, in the same study it was reported that addition of recombinant human ApoD to culture medium was associated with an inhibition of cancer cell proliferation in vitro.

Age-Related Changes in ApoD Expression

Aging is the greatest risk factor for most cancers and neurological disorders. However, the underlying molecular mechanisms relevant to the aging process remain poorly understood. Many microarray studies have been conducted in several species, including humans, to gain insights into the aging process.[44] A recent study performed a meta-analysis of age-related gene expression profiles using 27 datasets from mice, rats and humans.[45] The objective of the study was to identify genes with the largest number of putatively age-related signals in multiple datasets. The study identified 56 genes that were consistently upregulated as a function of age, with ApoD being identified as the most significant. An independent study set out to compare age-related changes in gene expression in the cortex of humans, rhesus macaques and mice.[46] A major conclusion of the comparison of all three species was that the most robustly age-upregulated gene was ApoD.

These reports clearly identify ApoD as a "suspect at many different crime scenes," including the aging brain. The question is whether ApoD is the perpetrator, an innocent bystander or a 'good Samaritan' trying to help the 'victim' under attack.

Modeling the Role of ApoD Homologs in Longevity and Stress Resistance

Our own interest in ApoD began when we performed a genetic screen to identify genes that protect the fruit fly Drosophila against oxygen stress. As is often the case, there were a relatively large number (~20) of "hits" from the screen. One of these "hits," *Glial Lazarillo*

(*GLaz*), was a fly homolog of human ApoD. After exposing flies to a sub-lethal dose of oxygen stress, we remember showing our mentor, Seymour Benzer, the very active flies that were overexpressing *GLaz* next to the very sickly control flies. From that day on, we would discuss ApoD on an almost daily basis until the end of Seymour's life.

To validate the protective effects of *Glaz*, we generated independent transgenic lines carrying the *GLaz* cDNA under control of the UAS/GAL4 system.[47] Upon doing so, we discovered that overexpression of *Glaz* protected against a range of extrinsic stressors and extended the lifespan of normal flies by ~30%.[48] An independent loss-of-function study revealed that *GLaz* mutants are sensitive to both oxidative and starvation stress.[49] Interestingly, a *GLaz*-GFP reporter line was expressed in glial cells in the adult fly brain. This expression pattern is consistent with the developmental expression pattern of *GLaz* in Drosophila,[50] and with the mainly glial expression pattern of vertebrate ApoD.[51-53] In addition, *GLaz* gene activity was found to protect against neuronal apoptosis as a function of both age and extrinsic oxidative stress.[49] At a physiological level, *GLaz* mutant flies display decreased fat content,[49] highlighting a putative role for *GLaz* in lipid metabolism.

As well as *GLaz*, the Drosophila genome contains another lipocalin gene: *Neural Lazarillo* (*NLaz*), which is expressed in a subset of neuronal cells, and, interestingly, in the developing fat body.[50] Recently, it was reported that *NLaz* plays an important role in regulating metabolic homeostasis and longevity in the fly.[54] Activation of the Jun-N-terminal Kinase (JNK) signaling pathway, which can be induced by a variety of environmental stressors, including oxidative stress, represses insulin/IGF-1 signaling (IIS) activity, extending lifespan but limiting growth.[55,56] In a candidate gene approach, *NLaz* was identified as a downstream transcriptional target of JNK signaling.[54] Moreover, *NLaz* mutant flies were found to display reduced stores and rapid starvation-induced decline of glucose, trehalose, glycogen and triglyceride levels and were sensitive to starvation. In contrast, overexpression of *NLaz* in the fat-body was found to increase starvation resistance, suggesting that JNK-mediated induction of *NLaz* in the fatbody regulates metabolic homeostasis. In support of this idea, fatbody expression of *NLaz* is sufficient to restore starvation resistance in flies mutant for the JNK activating Kinase Hemipterous (JNKK/Hep).

In the same study, it was reported that loss of *NLaz* confers sensitivity to both dietary paraquat and hyperoxia while overexpression of *NLaz* both ubiquitously and in the fatbody, protects against oxidative stress.[54] Finally and importantly, the role of *NLaz* in modulating longevity was also examined. Consistent with the *GLaz* findings,[48,49] *NLaz* mutant flies are short-lived relative to isogenic controls, while overexpression of *NLaz* with a ubiquitous driver increases fly lifespan.

These findings are intriguing and provocative: manipulating the expression of either *Glaz* or *Nlaz* results in changes in resistance to extrinsic stress and longevity. However, there appear to be important differences in the regulation of the two fly lipocalins. While *NLaz* expression is controlled by JNK signaling, *GLaz* expression is not.[54]

Recently, two additional studies have reported characterizing the function of ApoD homologs in mammals[57] and also in plants.[58] An ApoD knockout mouse (ApoD-KO) was generated by replacing the wild-type ApoD gene with a copy interrupted by the insertion of the neomycin resistance gene, which rendered a transcription null mutant. Interestingly, ApoD-KO mice display reduced locomoter and exploratory behavior as well as deficits in learning.[57] Remarkably, murine ApoD appears to protect against oxidative stress also. ApoD null mice display reduced tolerance to the ROS generator paraquat. This result was observed in two different genetic backgrounds, strongly indicating that loss of ApoD gene function is

responsible for the phenotype. Bioinformatic studies have revealed that plants also possess lipocalins, which were classified as temperature-induced lipocalins (TILs), including the ApoD ortholog *AtTIL* identified in *Arabidopsis thaliana*.[59] *AtTIL* knock-out plants are acutely sensitive to oxidative stress, cold and light.[58] Whereas, overexpression of the normal gene confers resistance to extrinsic stress.

The upregulation of human ApoD (hApoD) in various diseases involving chronic stress, predicts that experimentally induced stress may regulate the expression of ApoD orthologs in model systems. To gain insight into this question, we examined the regulation of *GLaz* as a function of extrinsic stress. Quantitative real-time PCR (qRT-PCR) revealed that *GLaz* mRNA levels were dramatically increased in response to dietary paraquat, hyperoxia or heat stress.[60] In addition, *NLaz* expression is also induced in response to both oxidative stress and starvation stress.[54] A careful study in mice has revealed the temporal and spatial expression of ApoD in response to oxidative stress.[57] An acute upregulation of ApoD in mouse brain was observed 3 hours after exposure to paraquat, and the expression returns to baseline by 24 hours. No upregulation was observed in the lung or liver. In plants, *AtTIL* is induced by both thermal and water stress.[61]

Understanding the relationship between different stressors and ApoD induction may shed light on the mechanisms underlying neurological disease. Towards this goal, a recent study examined the response of ApoD to a range of stressors in cell culture.[62] Interestingly, stresses that cause an extended growth arrest, such as UV light or hydrogen peroxide, increase ApoD expression. This study also addressed the subcellular localization of ApoD under normal and stressful conditions. Interestingly, under normal conditions ApoD is mainly perinuclear but it accumulates in the cytoplasm and nucleus under stressful conditions. In fact, exogenous ApoD from the medium can be taken up by cells and translocated in this way, making the search for the receptor(s) involved in this trafficking a high priority in ApoD research. These studies in plants, insects and mammals strongly support a conserved function for these lipocalins, responding to and protecting against extrinsic and intrinsic stress.

Manipulating Human ApoD (hApoD) Gene Activity

As outlined above, ApoD homologs in model systems appear to show conservation of both regulation and function. But what about the human gene itself? To examine the role of human ApoD (hApoD) on longevity and stress resistance directly, we generated two independent transgenic fly lines carrying the hApoD cDNA under control of the UAS/GAL4 system.[47] Using this system, we have shown that overexpression of hApoD in flies protects against oxidative stress and extends lifespan under normal conditions.[60] To examine the role of hApoD in mice, a transgenic animal (HApoD-Tg) overexpressing hApoD under the control of a neuronal promoter was generated.[57] Indeed, HApoD-Tg mice display improved survival following exposure to two different concentrations of paraquat. In addition, Do Carmo et al. showed conclusively that ApoD overexpression is sufficient to temper inflammation observed in coronavirus-induced encephalitis.[63] The study of mutant mice in similar conditions would also greatly inform our current understanding of some of ApoD's physiological functions.

The insulin/IGF signaling (IIS) pathway modulates lifespan in several species ranging from yeast to mammals, and thus appears to be evolutionarily conserved.[64] Recently, it was reported that neuronal expression of hApoD in mice results in alterations in glucose and insulin metabolism. More precisely, although they are not obese and have normal lipid concentration in circulation, hApoD mice are glucose intolerant, insulin resistant, and

develop hepatic steatosis.[65] These findings suggest that the interaction between IIS-mediated longevity and ApoD-mediated longevity warrants more attention.

Closing Remarks

ApoD is emerging as a focal point in a number of studies investigating the molecular basis of aging and age-related diseases. For the most part, these studies have been correlative in nature. However, a number of recent genetic studies in flies, mice and plants have demonstrated that manipulating ApoD gene activity can have profound physiological consequences. These studies, whether using ApoD homologs or human ApoD transgenics, have greatly advanced the field: we now know that ApoD can act as a "good Samaritan" helping cells and organisms better cope with both extrinsic and intrinsic stress. We note that whether we consider cancers or neuronal disorders, there is an underlying age-dependency. Therefore, it seems particularly important that both *GLaz* and *NLaz* modulate lifespan in flies. In part, the involvement of ApoD in age-related disorders could be related to a direct scavenging activity against free radical damage. Studies in both mice and flies have shown ApoD's ability to decrease lipid peroxides in membranes.[57,60] Future work should focus on establishing exactly how ApoD decreases lipoxic damage in aging animals.

Over the years, ApoD has been studied from widely different angles, which are today primed to coalesce, and provide a significant new understanding of its various roles. However, the community working on this protein and its homologs has only started to scratch the surface of ApoD's mechanisms of action. The recent elucidation of its crystal structure can only benefit biochemical studies of ligands and interacting partners, and provide educated targets for mutational analysis. If direct ligand interactions are involved in these therapeutic approaches, it is important to note that ApoD will benefit from work to generate anticalins, engineered lipocalins, with customized affinities for lipidic ligands of choice.[66]

Taken together, the studies described in this perspective strongly suggest that ApoD may provide an important therapeutic target for several devastating diseases. In characteristic fashion, Seymour Benzer's contribution to this emerging field was both timely and important. Seymour's overwhelming excitement and enthusiasm for the ApoD projects, both in his own lab and in the labs of his colleagues, was both inspiring and contagious. For this, and all the lessons in science and life that we picked up sitting around the table in the Benzer lunchroom we are deeply grateful.

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