Miscellaneous

Heat shock proteins: new keys to the development of cytoprotective therapies

Michael Tytell[1,2] & Philip L Hooper[3]

[1]*Department of Neurobiology and Anatomy, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA*
[2]*Prosperon Pharmaceuticals, Inc, 2225 New Castle Drive, Winston-Salem, NC 27103, USA*
[3]*University of Colorado Health Sciences, Department of Endocrinology, Denver, Colorado, USA*

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Review

All cells, from bacterial to human, have a common, intricate response to stress that protects them from injury. Heat shock proteins (Hsps), also known as stress proteins and molecular chaperones, play a central role in protecting cellular homeostatic processes from environmental and physiologic insult by preserving the structure of normal proteins and repairing or removing damaged ones. An understanding of the interplay between Hsps and cell stress tolerance will provide new tools for treatment and drug design that maximise preservation or restoration of health. For example, the increased vulnerability of tissues to injury in some conditions, such as ageing, diabetes mellitus and menopause, or with the use of certain drugs,, such as some antihypertensive medications, is associated with an impaired Hsp response. Additionally, diseases that are associated with tissue oxidation, free radical formation, disorders of protein folding, or inflammation, may be improved therapeutically by elevated expression of Hsps. The accumulation of Hsps, whether induced physiologically, pharmacologically, genetically, or by direct administration of the proteins, is known to protect the organism from a great variety of pathological conditions, including myocardial infarction, stroke, sepsis, viral infection, trauma, neurodegenerative diseases, retinal damage, congestive heart failure, arthritis, sunburn, colitis, gastric ulcer, diabetic complications and transplanted organ failure. Conversely, lowering Hsps in cancer tissues can amplify the effectiveness of chemo- or radiotherapy. Treatments and agents that induce Hsps include hyperthermia, heavy metals (zinc and tin), salicylates, dexamethasone, cocaine, nicotine, alcohol, α-adrenergic agonists, PPAR-γ agonists, bimoclomol, geldanamycin, geranylgeranylacetone and cyclopentenone prostanoids. Compounds that suppress Hsps include quercetin (a bioflavinoid), 15-deoxyspergualin (an immunosuppressive agent) and retinoic acid. Researchers who are cognisant of the Hsp-related effects of these and other agents will be able to use them to develop new therapeutic paradigms.

Keywords: *adrenergic, aging, alcohol, atherosclerosis, bimoclomol, cancer, cytoprotection, diabetes, heart disease, heat shock proteins, heavy metals, Hsc70, Hsp70, hypertension, inflammation, ischemia, quercetin, sepsis, steroids, stress, thiazolidenediones, transplant, trauma, wound healing*

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1. Introduction - the importance of Hsps in normal and stressed cell metabolism

1.1 What are Hsps?

The original discovery of this class of proteins is attributed to one of those accidents of research, the significance of which was recognised by Ritossa in 1962 [1]. He noted that in *Drosophila* salivary gland tissue that had been accidentally incubated at an elevated temperature or in the presence of the inhibitor of oxidative metabolism, dinitrophenol, new genes were turned on. Furthermore, he observed that those new genes could be turned off and on simply by lowering and raising the incubation temperature of the tissue. From this observation came the name applied to all the proteins later found to be the products of those temperature-sensitive genes, the Hsps. They might have been called, more appropriately, stress proteins, since the research subsequent to that of Ritossa found that a wide variety of agents and conditions causing metabolic stress stimulated the Hsp response. However, the term Hsp has become entrenched in the literature. It continues to be applied to most of the proteins discovered since 1962 that are synthesised in response to hyperthermic and other forms of metabolic stress. The various Hsps are distinguished from each other by appending to Hsp a number corresponding to the approximate molecular weight in kilodaltons. The one exception to this rule are a set of proteins and their genes that are stimulated by glucose starvation rather than heat stress [2]. These were called glucose-regulated proteins or Grps. **Table 1** lists the Hsps that presently are well-characterised, along with a brief statement of the primary function and intracellular location of each.

For more than a decade after Ritossa's report, the Hsp response remained primarily a tool of Drosphila geneticists, who used them to study gene regulation. Beginning In 1978, a series of reports documented that proteins with molecular weights similar to those in heat-shocked *Drosophila* tissues were produced by vertebrate cells [3-5]. As more reports of the Hsp response appeared in an increasing variety of organisms, including plants [6], it was found that all the 70 kDa Hsps were similar in structure. In fact, human Hsp70s have about 50% homology with those of *E. coli* [7] and 85% with those of *Drosophila* [8]. This high level of sequence similarity across species signified that the Hsp70s were phylogenetically ancient proteins that must have very basic and essential functions in all living organisms. In fact, they are believed to be about 2.5 billion years old [8]. Their importance in basic cell functions is implied by the absence of Hsp knockout organisms. Even in cultured cells, suppression of Hsp70 expression is associated with poor growth and increased cell death [9-11]. Thus, while most medical research seeks to study animal models that are closely related to the human (i.e., the monkey, dog, rat, or mouse), the study of Hsps in plants, bacteria, or insects may be relevant to the human condition because of their structural similarities.

1.2 Overview of how they work

1.2.1 In normal cells

There are several recent reviews that describe in detail our current understanding of the mechanisms by which Hsps facilitate the folding of other newly synthesised proteins as they emerge from the ribosome and how they promote the translocation of certain cytoplasmic proteins into mitochondria and lysosomes [12-15]. Therefore, only a brief description will be presented here for the constitutive and stress-induced forms of the 70 kD Hsps, the primary foci of this review.

Hsc70 and Hsp70 are 90% identical with regard to their amino acid sequences [16] and, in situations in which the two have been compared directly, they appear to be functionally interchangeable as well [16]. However, there is indirect evidence that they do have some distinctive functions. For example, in the normal rat retina, the inner segments of the photoreceptors contain readily detectable levels of Hsp70, whereas the rest of the retinal cells contain abundant amounts of Hsc70 [17]. Since most normal cells contain little if any Hsp70 [18], it is not known what special function Hsp70 might be serving in retinal photoreceptors which causes them to have higher levels of Hsp70 even though they contain Hsc70 in abundance.

Hsc70 has a protein-binding region in its carboxyl half that has an affinity for exposed hydrophobic amino acids on other proteins and binds to them when its ATP-binding portion, the amino half of the molecule, is either empty or contains ADP. This bound Hsc70 prevents inappropriate interactions of hydrophobic residues within the growing polypeptide chain before it is completely synthesised and released from the ribosome. After the nascent polypeptide chain is completed and released, the bound Hsc70 promotes its interaction with a barrel-shaped complex formed from the small molecular weight Hsps, Hsp25/27 and

Table 1: Members of the heat shock protein family, with synonyms and primary functions.

Name	Other names and relationships	Major Function
Small molecular weight Hsps		
Ubiquitin		Becomes convalently bound to cytoplasmic proteins to mark them for proteolysis by the proteasome.
Hsp10	Cpn10 (chaperonin 10), early pregnancy factor, GroES (*E. coli* form)	Protein folding, control of cell growth and development in the embryo.
Hsp25/27	Structurally related to αA- and αB-crystallins. Hsp25 is the rodent form. Hsp27 is the primate form	Forms large oligomers in normal cells to assist in protein folding and stabilisation of the actin cytoskeleton; can serve as a structural protein (lens).
Hsp30	Found in fish	Unknown
Mid-molecular weight Hsps		
Hsp40	Homologous to bacterial DnaJ and yeast SCJ1, Sec63/NPI1, YDJ1, & SIS1	Modulates the binding and folding activity of Hsp70.
Hsp47	Colligin, gp46	Facilitating folding of procollagen in RER.
Hsp60/65	DnaK, Cpn60	In bacteria, it is the homologue of Hsc/Hsp70. The 65 kD form is found in myocobacteria. In eukaryotes, it functions as a chaperone in the mitochondrial matrix. It is produced constitutively and induced by stress.
Hsp70	Hsp72	Absent or at low levels in normal cells. Synthesis is highly induced in metabolically stressed cells. Prevents protein aggregation and renatures unfolded proteins in the cytoplasm and nucleus.
Hsp70B′		Isoform of Hsp70 that is produced only in stressed cells.
Hsp71		Mycobacterial isoform of Hsp70.
Hsc70	Hsp73, Hsc73	70 kD heat shock cognate protein produced constitutively and moderately induced by stress. Promotes folding of newly synthesised cytoplasmic proteins, removal of clathrin from coated vesicles, refolding of damaged proteins, translocation of cytoplasmic proteins into mitochondria and lysosomes and to the cell surface.
Glucose-regulated protein:		
Grp 58	ERp57	Belongs to the thioredoxin superfamily. Found in the ER. Increases after oncogenic transformation and is associates with MHC class 1 complex. This and other Grp family members induced by glucose deprivation.
Grp75	mtHsp70	Found in mitochondrial matrix and facilitates protein folding there together with Hsp60.
Grp78	BiP (immunoglobulin heavy chain binding protein), Hsp78	Similar to Hsc/Hsp70 in function but found in ER lumen where it facilitates protein and glycoprotein folding.

Table 1: (Cont'd)

Grp94	gp96, Hsp100, ERp99	Found in ER. Structurally related to Hsp90. Function similar to Grp78.
High molecular weight Hsps		
Hsp90	Hsp82, Hsp83, Hsp84, Hsp86, Hsp89	Produced constitutively and is one of the most abundant cytoplasmic proteins. Has two isoforms, Hsp90α and Hsp90β. Forms dimers. Maintenance of steroid hormone receptors in hormone-binding competent state; chaperones signal transduction proteins, 'buffering' minor sequence errors to maintain normal function.
Hsp110	Hsp105, homologue of SSE protein in yeast,	Has partial sequence homology with Hsc70 but does not have ATPase activity. Is found in cytoplasm and nucleus. Little known about function but overexpression confers heat tolerance like Hsp70.

Hsp10 [15]. The interior of the barrel provides the new protein with a protected environment in which it undergoes cycles of folding and unfolding until the appropriate 3D conformation is achieved, whereupon it is released from the Hsp 'foldosome' to move to its appropriate location in the cytoplasm [15]. The cycles of folding occur in conjunction with ATP hydrolysis, which is thought to be the source of energy for the process [19-21]. For proteins synthesised on the RER, a similar process is thought to occur as the growing polypeptide chain emerges in the lumen of the RER, mediated by the luminal homologues of Hsc70, called Grp78 or BiP (see **Table 1**). The folding/unfolding activity of Hsc70 also is used by the cell to disassemble the clathrin polymers of coated vesicles, releasing the vesicle. In fact, the clathrin-uncoating activity was the first function found for Hsc70 [22].

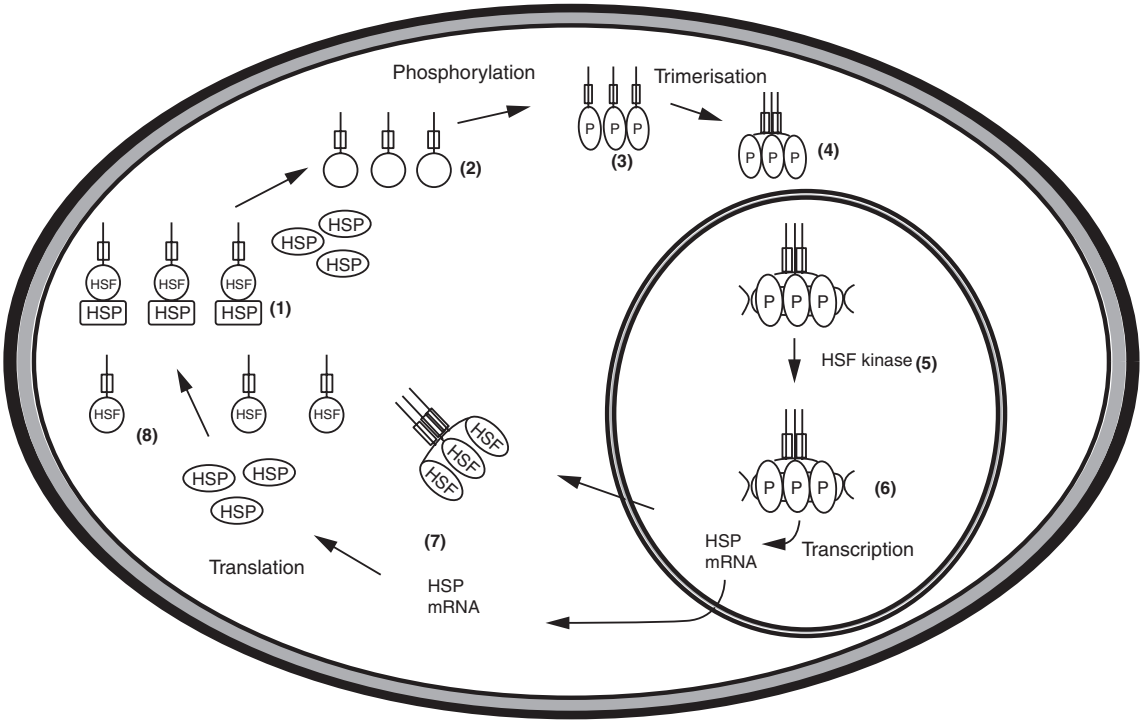
Certain proteins synthesised on cytoplasmic ribosomes but destined for other intracellular organelles require Hsc70 to translocate across the organelle membrane. An intriguing ratchet type of mechanism has been proposed in the case of cytoplasmic proteins imported into the mitochondrion [23,24]. Hsc70 has also been found within lysosomes and proposed to be involved in targeting cytoplasmic proteins for lysosomal degradation [25]. However, the process by which Hsc70 itself is translocated into the mitochondrial matrix and the interior of the lysosomes remains unknown.

1.2.2 In stressed cells

The most notable distinction between the Hsc70 and Hsp70 is in their genes. The expression of Hsp70 is under the control of a promoter called the heat shock element (HSE) [12,26] whereas Hsc70 is not, being produced constitutively in normal cells. HSE is activated by the binding of a transcription factor called heat shock factor (Hsf), of which there are two forms, Hsf1 and Hsf2 [12,27-29]. In a normal cell, the Hsfs are maintained in a monomeric, inactive form by bound Hsc70. When a cell becomes subject to metabolic stress, whether caused by heat or exposure to toxins, changes in the intracellular environment cause a failure of proper protein folding during synthesis and existing proteins begin to unfold. Improperly folded proteins have a greater affinity for Hsc70 than the Hsfs, so the Hsfs lose their bound Hsc70. That event allows them to become phosphorylated and to form trimers. The trimeric Hsfs enter the nucleus and bind to HSE, thereby stimulating the expression of Hsp70 genes. This scheme is summarised in **Figure 1**.

Another distinction between Hsc70 and Hsp70 that facilitates the increased expression of the latter during stress is the absence of introns in the Hsp70 genes, in contrast to Hsc70, which includes introns. This difference means that the former is more easily synthesised during stress because no post-transcriptional processing of the mRNA is required [30]. The preferential accumulation of Hsp70 stimulated by the stress then increases the Hsp70 available for binding to hydrophobic groups on partially unfolded proteins

Figure 1: Hypothesised scheme for the regulation of Hsp70 expression. The heat shock transcription factor (HSF) normally resides in the cytoplasm and is kept inactive by associating with Hsc70 or Hsp70 (HSP, (1)). Under the effects of metabolic stress, competition between other unfolded cytoplasmic proteins and HSF leads to the dissociation of HSP from HSF (2). HSF can then be phosphorylated by protein kinase C and other kinases (3), which promotes it to trimerise (4). The trimers enter the nucleus, where they are further phosphorylated (5), promoting their binding to the heat shock element on the DNA (6) and stimulating transcription of Hsp70 mRNA. Subsequently, the HSF trimers return to the cytoplasm, where they are dephosphorylated, dissociate and, if sufficient additional Hsp70 has accumulated, become re-associated with Hsp70, which maintains them in an inactive state. Adapted from [12].



and prevents irreversible denaturation and aggregation. Thus, a cell that accumulates high levels of Hsp70 becomes more resistant to the deleterious effects of metabolic stress on its proteins than a cell with basal levels of Hsc70 and Hsp70. In fact, without Hsps the cells and tissues of our bodies would be too fragile to survive normal stresses and traumatic injuries of daily life without difficulty. This conclusion is supported by experiments showing that inhibition of Hsp70 synthesis or its function with antibodies greatly increased the death of cells following a mild stress [31]. Furthermore, because the stress-inducibility of Hsp70 declines with age, it has been proposed that the increased fragility of aged cells and organisms is, in part, a result of a deficiency in the Hsp70 response [32-35].

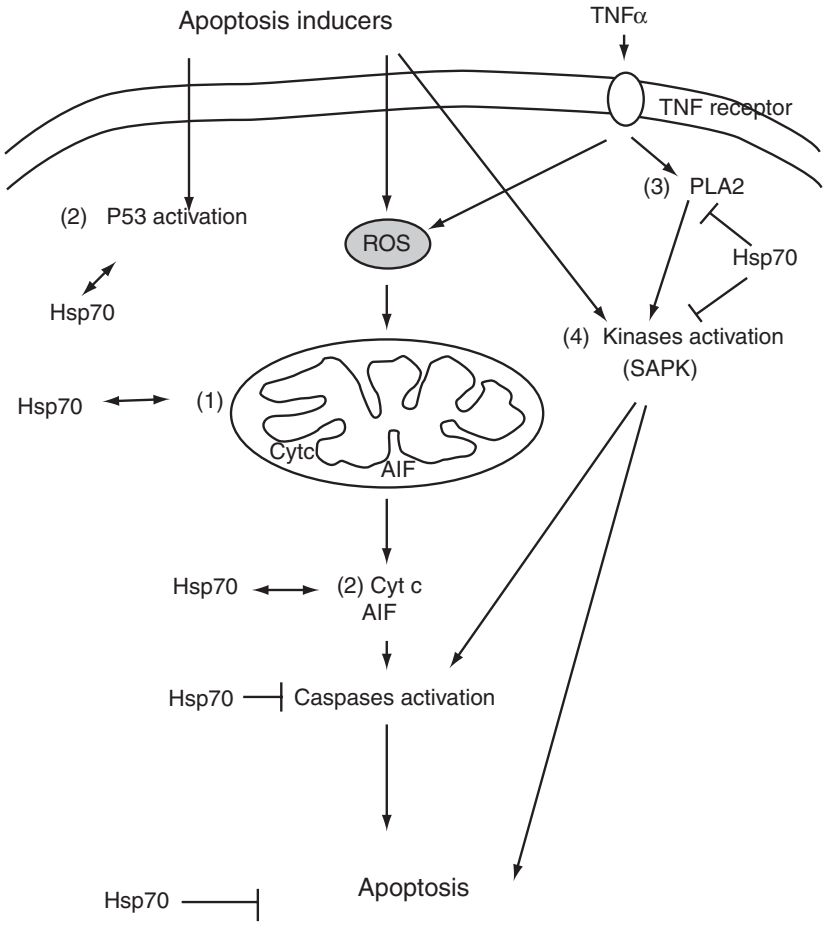
In addition to protecting other cytoplasmic proteins from stress-induced denaturation, there is increasing evidence that Hsp70 can inhibit the cascade of signals leading to apoptosis, the cell death program that often initiated when cells are under metabolic stress [36-40]. A summary of the various points at which Hsp70 may influence the stress-initiated cascade of signals is illustrated in **Figure 2**.

2. The possible consequences of impaired Hsps

2.1 Chronic human disorders and misfolded or abnormal protein

Organs in which there is little or no renewal of cells, like the nervous system, are particularly prone to this problem because misfolded or abnormal proteins have a lifetime to accumulate. Thus, even if the rate of formation of misfolded protein is slow, it may reach levels that significantly disrupt cell function. Two examples are Alzheimer's disease, marked by the accumulations of plaques of abnormal β-amyloid and neurofibrillary tangles of abnormal tau protein and Creutzfeldt-Jakob disease, caused by the accumulation of aggregates of prion proteins. The latter has attracted attention in the popular press recently

Figure 2: Many factors in the extracellular environment can initiate intracellular changes that promote the activation of the apoptotic pathway through either the production of reactive oxygen species (ROS) or by stimulation of stress-activated protein kinases (SAPK). An increasing number of reports suggest that Hsp70 can interact with the components of these pathways in a variety of ways, either protecting them from being affected by ROS (double-headed arrows between Hsp70 the mitochondrion (1)), chaperoning some of the signalling proteins (double-headed arrows between Hsp70 and p53 or cytochrome c (Cyt c) and other mitochondria-derived apoptosis inducing factors (AIF) (2)), or by inhibiting the conversion of caspases or SAPK to active forms. (TNFα, tumour necrosis factor α; PLA2, phospholipase 2). Adapted from [39].



because of the suspected transmission of mad cow disease (bovine spongiform encephalopathy, BSE), the analogous bovine condition, to humans in Great Britain. It is interesting to consider the possibility that one risk factor in individuals who succumb to those diseases may be a deficit in Hsp action. Although Hsps would not prevent the abnormal proteins in these diseases from being produced, they may be able to prevent them from forming aggregates that compromise cell function. Support for this hypothesis is provided by recent work by Bonini and colleagues, who showed in a *Drosophila* model of Huntington's disease that concomitant overexpression of Hsp70 prevented the neurodegeneration seen in that condition [41]. Apparently, the extra Hsp70 was able to prevent the proteins containing polyglutamine repeats from forming toxic aggregates in the neural cells.

The retina, being an extension of the central nervous system, also exhibits age-related degenerative changes that are, in some aspects, analogous to Alzheimer's disease. Age-related retinal conditions, including macular degeneration, are marked by the accumulation in the retina of aggregates of material referred to as drusen, which resemble, to some degree, the accumulation of amyloid plaques in the cortex of an individual with Alzheimer's disease. The drusen arise from a breakdown in the normal turnover of photoreceptor outer segments. As they accumulate, they interfere with the normal interactions between photoreceptor outer segments and the retinal pigment

epithelial cells, which recycle the effete outer segments. This exacerbates the failure of photoreceptors to regenerate their outer segments and causes a loss of photoreceptors in the macula, the region of the retina responsible for fine visual acuity. Recently, it was reported that in the aged monkey and human retina there is a decrease in Hsc70 gene expression [42]. The co-existence of these two changes in the retina raises the possibility that the state of Hsp production plays a role in the occurrence of such age-related retinal degenerative conditions.

The list of diseases associated with alterations in Hsps and/or disturbed protein structure is growing. It includes cystic fibrosis [43], sickle cell disease [44], gingivitis [45], colitis [46], gastric ulcer [47], rheumatoid arthritis [48], atherosclerosis [49], cancer [50], renal disease [51], myocardial ischaemia [52], viral infections [53] including AIDS [54], common cutaneous lesions [55], pulmonary inflammation [56], ageing [34], neurodegenerative disease [57], prion diseases [58], retinal degeneration [59], toxic substance exposure [60] and heat injury [61]. This list and the correlations between other conditions and altered Hsps discussed below are not meant to suggest that Hsps are at the root of all these problems. Rather, the intent is to point out that many diseases may have in common disturbances in protein folding or other cellular malfunctions that may be treatable by Hsp augmentation. Viewing a disease from this perspective may suggest new approaches to treatment that would not have been considered otherwise.

2.2 Other problems that may be related to impaired Hsps

2.2.1 Adaptation to environmental extremes may involve increased Hsps

In nature, species that express high levels of Hsps can survive harsher environments than species that have lower levels of Hsps. In other words, natural selection removes species that are unable to accommodate a particularly stressful environment and selects for the species that can adapt best to that environment. For instance, *Cataglyphi*, an ant living in the Sahara desert, produces more Hsps in response to exposure to high ambient temperatures than does *Formica*, an ant that lives in a more moderate climate [62]. Similarly, desert lizards express more Hsp70 in response to heat shock than non-desert dwelling lizards [63]. The painted turtle, known to be anoxia tolerant, expresses greater levels of Hsps in its myocardium in response to anoxic stress than several other species not tolerant to such stress [64]. In short, individuals or species that are able to express Hsps more readily than others may, indeed, be more resistant to certain disorders than those who do not. On the other hand, organisms with conditions associated with a lower Hsp response may be more prone to metabolic or environmental stresses. Ageing, diabetes mellitus, steroid deficiency or certain pharmaceutical agents can reduce the cellular Hsp response, potentially increasing vulnerability to injury or other complications.

2.2.2 Diabetic complications

Diabetes mellitus is associated with an impaired Hsp response [65,66]. For example, the Hsp response to surgical wounding is impaired in diabetic animal models. In non-diabetic mice, the peak Hsp response after wounding is observed at 24 h post injury; whereas, in diabetic mice the peak response is not seen until day 3. The delayed response correlates with the clinical delay in wound healing observed in diabetic mice [67]. The reason for impaired Hsp response in diabetes is unknown. One explanation may be that Hsps are themselves impaired by the metabolic damage of diabetes. Another explanation is that chronic hyperglycaemia may give the cell a false 'sense of well-being' and thereby downregulate Hsp response [57]. Diabetes mellitus is a disease that is associated with injury to critical organ tissues over time, such as retinopathy, neuropathy, nephropathy, myopathy and premature cardiovascular disease. The metabolic injury of diabetes is thought to be secondary to increased free radical formation, oxidation and glycation of proteins [68]. It is possible that increased Hsps may protect diabetics from such injurious changes, since they are efficacious in protecting and repairing damaged proteins, as may occur in protein glycation [69].

2.2.3 Heart disease and α-adrenergic blockers

Doxazosin, an α-adrenergic blocker used to treat hypertension and urinary problems caused by prostatic hypertrophy, has been recently found to be associated with a two-fold greater incidence of congestive heart disease (CHD) compared to a diuretic drug in a prospective study of 24,335 patients followed for 3.3 years [70]. This result was unanticipated because doxazosin is associated with a beneficial metabolic profile, which includes elevation of HDL, lowering of triglycerides, increasing insulin

sensitivity and improving fibrinolysis, while also reducing left ventricular after-load. There is reason to suspect that the increase in CHD may have been a result of reduced Hsps because prazocin, another α-adrenergic blocker, inhibits Hsp expression in the myocardium following noradrenaline stimulation and furthermore, it abolishes noradrenaline-induced cardio-protection, thus leaving the heart more vulnerable to injury [71]. Additionally, like doxazosin, prazocin has been associated with a higher than expected incidence of mortality compared to other after-load reducing agents [72]. Further support for this hypothesis is suggested by the observation that Hsps are higher in failing or straining hearts, where their elevation is believed to provide compensatory healing [73].

A similar speculation may apply to the association of calcium channel blockers with heart failure. Whereas the physiological effect of calcium channel blockers on the heart seems positive, producing vasodilation with associated anti-ischaemic activity and inhibiting myocyte hypertrophy, the degree of improvement in outcome with their use in congestive heart failure remains limited [74]. Like α-blockers, these agents are associated with reduced Hsp synthesis in cardiac myocytes challenged with a toxin [75].

2.2.4 Steroid hormones and stress tolerance

Hsps and steroid hormones are closely linked systems. While Hsps are the stress-responding agents of cells, steroid hormones are the stress-responsive agents of the organism. Therefore, it is not surprising that the two systems are interrelated. Knowlton and co-workers hypothesised that equilibrium exists between Hsp90 and the various receptors that it binds and that changes in levels of certain steroid hormones will alter the intracellular distribution of HSP90, activating HSF-1 and thus, the production of Hsp70. They have demonstrated that oestrogen, progesterone [76] and dexamethasone [77] activate HSF1 and increase Hsp70 in cardiac myocytes. In short, lower Hsps secondary to glucocorticoid deficiency (Addison's disease) or oestrogen deficiency (menopause) may further explain the increased vulnerability of individuals with those two conditions. Patients with Addison's disease cannot tolerate stress; and women in menopause lose their advantage over men in susceptibility to cardiovascular disease.

3. Benefits of elevated Hsps

3.1 Circumstances under which Hsps have therapeutic value and how such benefit might be achieved

3.1.1 Preconditioning prior to injury

The most common experimental paradigm for testing and evaluating the potential cytoprotective effects of Hsps is the preconditioning experiment. Cells in culture or whole organisms are first conditioned by exposure to a mild or moderate metabolic stress, one that is sufficient to stimulate accumulation of Hsps but does cause significant cell death. A period of hours, up to 24, after the conditioning stress is allowed so that the Hsps have time to accumulate to higher than normal levels. Then the cells or organisms are subjected to a more severe, test stress, one that typically causes significant cell death. The result is that the preconditioned specimen shows significantly improved survival compare to the specimen exposed only to the severe stress. This outcome has been documented in animal models to protect the heart, brain, spinal cord and other organs from ischaemic damage [78-85]. For example, prior heat shock protected the spinal cord and kidney in aortic occlusion animal models. Specifically, whole body hyperthermia consisting of 15 minutes at 42.5°C prevented paralysis in rabbits subjected to a 20 minute aortic occlusion. Pigs made hyperthermic survived a 90 minute period of warm renal ischaemia with minimal renal damage, whereas non-heat-treated animals all experienced renal failure and half of them died [86]. Furthermore, the retina is protected against light damage by hyperthermic preconditioning [87]. More recently, in human coronary bypass patients, a positive correlation has been found between the presurgical Hsp70 levels in the cardiac tissue and improved postsurgical outcome [88].

Generalising from these types of experiments, it is logical to suggest that a patient scheduled for an elective surgical procedure, but who is otherwise in a relatively healthy state, would show an improved rate of recovery and healing if he or she were preconditioned by hyperthermia within a 24-hour period prior to surgery. It could be accomplished *via* a hot water bath or sauna, or some other method known to elevate Hsps. The preconditioning might not even have to be applied to the whole person, if the organ or area to be operated upon could be locally treated.

3.1.2 Treatment after traumatic injury

Using the Hsp response to try to improve recovery in a patient who is already injured is problematical because there is no time to use the preconditioning paradigm. The injured tissue is already compromised and a conditioning stress treatment most likely would only cause more damaged cells to die. The key question in this situation is how to elevate Hsps in injured cells without causing additional metabolic stress. There are two ways to accomplish this, either amplify the natural Hsp response of injured cells, so that they produce more Hsps in response to the injury than they normally would, or administer Hsps directly to the injured cells, raising the intracellular levels of Hsps without having to depend on the cells' synthetic machinery to make it. The latter approach seems to have the greatest potential because it could rescue cells too damaged to make their own proteins but both are being pursued, as described in subsequent sections.

3.1.3 Tissue and organ transplantation

A number of reports described improved wound healing and graft survival, along with lessened surgical damage to organs, in conjunction with prior heat stress. Skin flap survival is improved by prior local or total body hyperthermia and it correlates with increased levels of Hsp70 [89]. With regard to the skin, it is also notable that local hyperthermia applied to human forearm skin reduced damage caused by ultraviolet irradiation administered six hours later [90]. Preconditioning transplanted rat hearts at a high temperature increased functional recovery and decreased cellular necrosis [91]. Similarly, pancreatic islets [92], renal allographs [93] and pulmonary isografts [94] were all protected by prior heat shock. Notable in this regard are human studies that found that low Hsp70 levels in grafts correlated with early rejection [95].

3.1.4 Diseases and chronic degenerative conditions

3.1.4.1 Cardiac surgery

A recent study examined Hsp70 levels in samples of myocardium and found that it was induced during the cardiopulmonary bypass procedure. The level of induction was negatively correlated with the release of enzymes specific to myocardium, suggesting that the hearts of those individuals that showed higher induction of Hsp70 were less damaged by the surgical trauma [88].

3.1.4.2 Septic shock

Hyperthermia improves an organism's tolerance to infection. For instance, raising the body temperature of mice from 37°C to 39-39.5°C prior to injection of *Klebsiella pneumoniae* into the peritoneum improved survival by 50% and decreased bacterial load by 100,000-fold [96]. Acute heat stress also reduced mortality in rats that had cecal ligation and perforation [97]. Eighteen hours after the perforation, 25% of the non-heated animals were dead, whereas none of the heat stressed animals died. At seven days, 79% of the non-heated animals were dead compared to 31% in the heated group. Additionally, less tissue damage was observed in the latter. In a similar study, lower lactic acid levels were found in the septic animals that had been previously heat stressed [98]. Other investigations showed a direct association between the magnitude of Hsp expression following heat shock and resistance to subsequent sepsis [97,99]. Additional studies in diverse animal species support the idea that fever itself may offer protection from infection. Ectothermic vertebrates (lizards, goldfish, salmon, rainbow trout, crickets and grasshoppers) have improved survival when warmed prior to infection with Gram-negative pathogens, compared to unwarmed animals [100]. Furthermore, administration of an antipyretic agent decreases survival during experimental bacterial infections [101]

3.1.4.3 Hsps in diabetes and other chronic conditions

Diseases that are associated with tissue oxidation, free radical formation, protein misfolding, or inflammation may be therapeutically improved by higher expression of Hsps. For example, in a pilot study conducted by one of us (P. Hooper) to examine whether hyperthermia would improve control of blood glucose in type 2 diabetics, patients were immersed in a hot tub at a temperature of 37.8°C - 41.0°C for 30 minutes, six days per week for three weeks, increasing mean oral temperature by 0.8°C during each session. Not only was there an improvement in fasting blood glucose and haemoglobin A1c, but also in subjective assessment of neuropathy [102]. It is intriguing to note in regard to these results that heat shock inhibits cytokine-induced nitric oxide synthase expression by both rat and human pancreatic islets [103].

Hyperthermic treatments have been reported to be of benefit in other chronic diseases. In patients with osteo-arthritis, hot mud pack therapy for three weeks led to falls in the inflammatory mediators, prostaglandins, leukotrienes and cytokines [104]. Additionally, patients with congestive heart failure were able to reduce their heart sizes and improve their heart failure functional classifications with four weeks of daily hot tub immersion [105]

3.1.5 If Hsps are so beneficial, why are they not elevated all the time?

Since Hsps clearly enhance cellular resistance to so many types of metabolic stressors, it seems reasonable to wonder why they are not elevated all the time and furthermore, to suggest that treatments that cause chronic increases in Hsps might be a way to prevent or, at least, delay many stress- and age-related maladies. We suggest that the maintenance of high levels of Hsps may have metabolic costs to cells that eventually cause other problems. There is some support for this hypothesis. For example, it has been observed that in *Drosophila* cells caused to overexpress Hsp70 by transfection with additional copies of the gene, there were accumulations of granular aggregates of the protein in the nucleus and cytoplasm and growth of the cells was markedly inhibited [106]. Conversely, high levels of Hsps may prevent the death of cells that are supposed to be turned over, since Hsc70 and Hsp70 have been shown recently to inhibit the apoptotic signalling cascade [40]. In fact, many types of cancer cells have elevated Hsps [107]. Therefore, caution must be observed in applying Hsp-based treatments that might cause chronic overexpression, such as the introduction of additional Hsp genes not under the normal stress-regulated signalling pathways or the implantation of genetically modified cells chronically overexpressing Hsps.

4. Current and potential approaches to facilitation of the Hsp response for therapeutic purposes

4.1 Overexpression of Hsps by genetic manipulation

The stress conditioning type of experiment provided considerable information about the potential protective activities of Hsps and it continues to do so. However, it did not allow discrimination between the activities of different Hsps and often yielded observations that were correlative, rather than definitive. Transfection of cells and the creation of transgenic organisms that overexpress Hsp70 have permitted the cytoprotective activity of that protein to be unequivocally documented. For example, a variety of cell types overexpressing Hsp70 show enhanced survival compared to controls in response to hyperthermia [108,109], UV damage [110] and oxygen or glucose deprivation [111,112]. At least three independently generated strains of Hsp70 overexpressing mice have been used to show that the cytoprotective activity of high levels of Hsp70 *in vivo*, after cardiac and cerebral ischemia [52,113-117]. Of particular import is the recent observation, referred to in Section 3.1, that Hsp70 overexpression can even suppress the cytotoxic effects of the Huntington's disease mutation [41]. This result suggests that genetically based Hsp70 overexpression has the potential to be used to treat chronic, degenerative conditions. Besides Huntington's disease, these would include Parkinson's and Alzheimer's diseases, amyotrophic lateral sclerosis, age-related macular degeneration, retinitis pigmentosa and the secondary complications of diabetes. Although directed gene transfection of specific tissues *in vivo* has many technical difficulties to overcome, promising results have been reported in the heart of the rat. Sufficient levels of transfection with the Hsp70 gene have been achieved to yield significant protection from ischaemia-reperfusion injury [118,119].

4.2 Augmentation of the Hsp response using various compounds

The major role Hsps have in maintaining cellular integrity during normal growth and during pathological stress is substantiated by a large body of information. Consequently, finding non-toxic agents that can induce Hsps will be of major therapeutic value. Heavy metals, dexamethasone, cocaine, nicotine, alcohol, α-adrenergic agonists, PPAR-γ ligands, geranylgeranylacetone (GGA), bimoclomol, cyclopentenone prostanoids and ascites Hsp72-inducing factor (AHIF), all increase Hsp expression. Some of these agents have coincidental therapeutic benefits that are not readily anticipated, but in the context of their ability to increase Hsps, their positive actions are perhaps explained

The Hsp-inducing effects of zinc, cadmium, tin and arsenic [120-123] have all been reported [124,125]. Zinc duplicates the effect of heat shock in a number of

pathological models. These include improving survival in endotoxemic mice [126], reducing post-ischaemia infarct size of brain and heart [127,128], improving wound healing [129], decreasing symptoms in patients with the common cold [130], protecting mice from irradiation [131] and reducing gastric mucosal injury induced by ischaemia-reperfusion [132]. Similarly, arsenic induces thermotolerance [123] and is being used effectively to treat haematologic cancers [133]. Lastly, tin prevents the development of hypertension in spontaneously hypertensive rats [134] and ameliorates haemorrhage-induced brain injury [135].

Alcohol [123] and nicotine [136] both increase Hsps and have surprisingly good outcome data associated with them. In particular, people who ingest alcohol show a U-shaped or J-shaped curve of reduced relative disease risk when compared to abstainers. Moderate alcohol consumption is associated with improved overall mortality and reduced incidence or severity of arteriosclerotic vascular disease, hypertension, cancers, peptic ulcer disease, pulmonary infection, gall stones, kidney stones, age-related macular degeneration, osteoporosis and impaired cognitive function [137]. Nicotine is associated with reductions in the incidence and symptoms in inflammatory bowel disease [138] and aphthous ulcers [139]

PPAR-γ ligands, thiazolidinediones, have recently been introduced as insulin sensitisers to be used in the management of type 2 diabetes. Surprisingly, these agents have effects that seem unrelated to improved glucose uptake, not only reducing diabetic complications (nephropathy, neuropathy, vascular defects, hyperlipidemia, [140], retinopathy [141]), but also reducing cancer [142-144], improving psoriasis [145] and ameliorating colitis in a mouse model of irritable bowel disease [146]. Another potent PPAR-γ ligand, $15dPGJ_2$, a natural prostaglandin, blocks macrophage activation [147] and induction of inflammatory response genes, including inducible NO synthase and tumour necrosis factor-α [148]. Both PGJ_2 and the PPAR-γ, troglitazone, increase Hsps in pancreatic islet cells, protecting them from cytokine and endotoxin damage [149]. Furthermore, PPAR-γ agonists prolong the survival and function of islet cell transplantations [150]. The protective cellular actions of these PPAR-γ ligands may be a result of their promotion of Hsp expression

To varying degrees, non-steroidal inflammatory drugs (NSAIDs) increase Hsps [151]. Aspirin potentiates Hsp expression in response to heat-stress, thus acting as a co-inducer of Hsps [152]. The therapeutic activity of NSAIDs goes beyond their anti-inflammatory activity, including cardiovascular event improvement [153], colon cancer prevention [154] and reduced Alzheimer's disease incidence [155]. As with the PPAR-γ ligands, some of therapeutic benefits of NSAIDs may be due to increased Hsp expression.

A pharmaceutical research company in Hungary, Biorex [301], has focused its research on agents that increase Hsp expression. Most of their published work has focused on bimoclomol, a hydroxylamine derivative that acts as a co-inducer of Hsp 70. In streptozotoxcin-induced diabetic rats, bimoclomol improves peripheral neuropathy [156], retinopathy [157] and improves wound healing [158]. Preservation of endothelial function in hypertensive rats [159], as well as reduction in subarachnoid haemorrhage brain damage, are other therapeutic targets of bimoclomol [160]. Biorex has just completed a Phase II clinical trial testing bimoclomol for its potential to improve nerve conduction in diabetic patients.

GGA, an acyclic polyisoprenoid, has been used in Japan for the past 15 years as an anti-ulcer drug. The drug induces resistance of gastric mucosal cells from injury from toxins (NSAIDs, ethanol, hydrogen peroxide) and blocks stress ulcer formation [47]. GGA also protects rat livers from warm ischaemic injury [161]. While many inducers of Hsps are themselves toxic, neither GGA nor bimoclomol appear to be associated with major toxicity [162].

A natural extracellular protein found in ascites fluid has been found to induce Hsp70 synthesis [163]. The protein, ascites Hsp70-inducing factor (AHIF) is capable of protecting ageing human cells in culture from a variety of otherwise lethal stresses including heat shock, tumour necrosis factor, UV irradiation, etoposide and menadione. Specific inhibition of Hsp70 expression by antisense RNA abolishes the cytoprotective effect of AHIF. Ageing cells have a reduced Hsp response, making them more vulnerable to injury. Therefore, AHIF may restore stress resistance to old cells and inhibit some aspects of the ageing process.

4.3 Suppression of the Hsp70 response

In contrast to the above agents that induce Hsps, there are agents that inhibit the Hsp response and, therefore, may have a role in cancer chemotherapy. Both quercetin and retinoic acid suppress the

production of Hsps [164,165]. Quercetin, a bioflavonoid, sensitises tumours to hyperthermia and thereby amplifies the effect of hyperthermia therapy on a variety of different tumours [166-168]. Similarly, all-*trans*-retinoic acid has been used to induce remissions in acute progranulocytic leukaemia [169]. Additionally, depletion of Hsp70 with an adenovirus expressing antisense Hsp70 results in cell death of all tumourigenic cell lines tested (carcinoma of breast, colon, prostate and liver as well as glioblastoma) [170]. On the other hand, high levels of Hsp expression have been associated with poorer prognostic outcome in some cancers [171,172].

4.4 The administration of Hsp70 itself

In the middle 1980s, one of us (M. Tytell) made the observation that, in the squid giant axon, Hsps synthesised in the glial cells ensheathing the axon were transferred into the axon [173]. This discovery indicated that one cell could receive Hsps from a neighbouring cell and therefore, did not need to make Hsps itself to utilise them. That deduction led to the hypothesis that a cell might be able to take up and use Hsp70 administered into the extracellular fluid. The idea was first tested in smooth muscle cells in culture using a preparation consisting of mostly Hsc70 (> 90%) purified from bovine brain. It was found that Hsc70 added to the culture medium inhibited the rate of cell death caused by nutrient deprivation stress [174]. However, the Hsc70 appeared only to become associated with the cell surface and no evidence of internalisation was obtained [175]. Thus, although the exogenous Hsc70 improved cell stress tolerance, it was not clear if it was functioning in the same way as Hsc70 or Hsp70 made in the cell. Later work, in a sciatic nerve injury model in the neonatal mouse showed that exogenous Hsc70 applied to the severed end of the nerve was taken up, retrogradely transported into the sensory neurones and spinal cord and prevented the axotomy-induced death of sensory neurones [176]. Subsequently, a mixture of Hsc70 and Hsp70 (Hsc/Hsp70 from bovine skeletal muscle) was shown to be taken up by a cultured monocyte cell line and to inhibit apoptotic cell death [177]. Most recently, administration of Hsc/Hsp70 into the vitreal chamber of the rat eye has been shown to prevent light-induced photoreceptor damage [178] and administration of recombinant human Hsp70 prevented motor neurone apoptotic death in culture and in the mouse after sciatic nerve axotomy [179]. The mechanism by which the exogenous Hsps act remains to be determined but it appears that exogenous Hsp70 is able to pass through cell membranes. The diffuse intracellular distribution shown by Guzhova *et al.* [177] suggests this and Fujihara and Nadler showed that exogenously presented Hsp70 even becomes localised in the nuclei of certain cultured cells [180]. Several reports support the hypothesis that Hsc or Hsp70 may interact with lipid bilayers. For example, Hsp70 can insert into artificial lipid bilayers, forming ion-conducting channels [181,182] and can inhibit lipid peroxidation by H_2O_2 in cultured *Aplysia California* neurones or cardiac myoblasts [183,184]. Furthermore, Vigh and co-workers [185] have suggested that perturbation of the organisation of the plasmalemma during stress serves to initiate signals to the cell to turn on its Hsp expression. The subsequent accumulating Hsps can then interact with the plasma membrane to stabilise its structure during the period of stress. These observations support the potential for Hsps to be effective cytoprotective agents when administered directly to injured tissues. The development of such Hsp-based therapeutic compounds is the focus of planned preclinical research by Prosperon Pharmaceuticals,, Inc. (Winston-Salem, NC, USA).

4.5 Using Hsp70 as a carrier for other proteins

Either or both Hsc70 and Hsp70 serve to facilitate the translocation of other proteins from the cytoplasm into membranous organelles, like the mitochondrion [186] and lysosomes [187] and can insert into and perhaps through, plasma membranes (see previous Section). Therefore, it is reasonable to consider that one could design fusion proteins composed of Hsp70 and another protein of interest to promote cellular uptake of proteins that otherwise do get into cells from the extracellular space. This approach has been applied to the inflammatory response-signalling molecule, NF-κB. A fusion protein comprised of the C-terminal portion of Hsp70 and the p50 subunit of NF-κB was observed to pass from the extracellular fluid into the cytoplasm and then on into the nucleus of the cell, where it elicited the expression of the same genes as would the endogenously produced NF-κB [180]. Though this observation remains to be replicated, it supports the potential of Hsp70 to serve as a carrier for delivery of a variety of substances into the cytoplasmic and nuclear compartments of the cell.

4.6 Hsp70 and the immune response

Some years ago, it was discovered that Hsp70, in its capacity as a chaperone, was involved in antigen uptake and presentation by macrophages and other members of the antigen-presenting cell (APC) group [188-191]. Furthermore, antigens associated with Hsp70 were found to elicit a more pronounced immune response than they did on their own [192-195]. More recently, it has been shown that extracellular Hsp70 can interact with APCs via a receptor and elicit the release of pro-inflammatory cytokines [196,197]. In fact, Asea and co-workers have proposed the term, 'chaperokine,' be applied to the Hsp70 family [196]. Thus, Hsp70 in the context of the immune system seems to facilitate the process of antigen recognition and processing by APCs and to function as a super-adjuvant. Two companies, StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada, [302]) and Antigenics, LLC. (Woburn, MA, [303]), have been pursuing this application of the Hsps, aiming to develop vaccines against cancers and other diseases for which it has not been possible to do so in the past because of poor immune responses. Currently, StessGen has in preclinical testing an Hsp fusion protein-based vaccine against HIV and in clinical trials a vaccine against human papilloma virus. Antigenics has clinical trials underway on a number of cancer vaccines, including renal cell carcinoma, sarcoma, non-Hodgkin's lymphoma and gastric and pancreatic cancer.

5. How can Hsps be cytoprotective and pro-inflammatory?

The older, extensive body of work on the cytoprotective activities of Hsps is difficult to reconcile with the more recent, growing number of reports on the stimulation of immune and inflammatory responses by Hsps. In fact, the picture has recently been made more puzzling by observations indicating that the stimulation of Hsp synthesis can suppress some aspects of the inflammatory response [198-201] and the consequences of septic shock (see previous Section on Septic Shock). As yet, there have been no studies directly addressing these seemingly contradictory functions of the Hsps. Thus, one only can speculate that the choice of which of these responses becomes manifest upon accumulation of Hsps is context-dependent, being determined by the timing and location of Hsp expression, as well as by the particular Hsps which are induced. Furthermore, it is possible that both activities could occur simultaneously. For the present, these observations raise a cautionary flag for researchers pursuing therapeutic uses of Hsps. In developing cytoprotective protocols, one must be on the lookout for potentially destructive inflammatory responses and, conversely, in designing immune system stimulatory protocols, one must avoid protecting the cells being targeted for destruction.

6. Expert opinion

The diverse diseases and tissue injuries that could potentially be treated by an Hsp-modifying pharmaceutical is left only to the limits of our imagination. From the addition of an Hsp-modifying agent to mouthwash, to a topically applied Hspstimulating sunscreen, to the prescription of a Hsp inducer to prevent diabetic complications, to the addition of a PPAR-γ ligand to a newly diagnosed type 1 diabetic in order to limit pancreatic islet cell destruction, to the iv. infusion of Hsp itself to a heart attack or stroke victim in an ambulance on the way to the hospital, we will witness the therapeutic application of Hsp pharmacologic agents in the coming decades.

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Michael Tytell[†,1,2] & Philip L Hooper[3]
[†]Author for correspondence
[1]Department of Neurobiology and Anatomy, Wake Forest University School of Medicine, Winston-Salem, NC 27157
[2]Prosperon Pharmaceuticals, Inc, 2225 New Castle Drive, Winston-Salem, NC 27103
[3]University of Colorado Health Sciences, Department of Endocrinology, Denver, Colorado, USA