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Heat Shock Proteins and Cardiovascular Pathophysiology

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Snoeckx, Luc H. E. H., Richard N. Cornelussen, Frans A. Van Nieuwenhoven, Robert S. Reneman, and Ger J. Van Der Vusse. Heat Shock Proteins and Cardiovascular Pathophysiology. *Physiol Rev* 81: 1461–1497, 2001.—In the eukaryotic cell an intrinsic mechanism is present providing the ability to defend itself against external stressors from various sources. This defense mechanism probably evolved from the presence of a group of chaperones, playing a crucial role in governing proper protein assembly, folding, and transport. Upregulation of the synthesis of a number of these proteins upon environmental stress establishes a unique defense system to maintain cellular protein homeostasis and to ensure survival of the cell. In the cardiovascular system this enhanced protein synthesis leads to a transient but powerful increase in tolerance to such endangering situations as ischemia, hypoxia, oxidative injury, and endotoxemia. These so-called heat shock proteins interfere with several physiological processes within several cell organelles and, for proper functioning, are translocated to different compartments following stress-induced synthesis. In this review we describe the physiological role of heat shock proteins and discuss their protective potential against various stress agents in the cardiovascular system.

I. INTRODUCTION

Since the first report (338) on the heat-induced appearance of chromosomal puffings in salivary gland tissue of *Drosophila busckii* in 1962, a new research domain has been intensively explored. This research resulted in the discovery of a large number of related proteins and their physiological role in many prokaryotic and eukaryotic organisms, tissues, and individual cells and at the level of subcellular structures. These proteins were originally called "heat shock proteins" (356), because they were discovered in salivary glands and other tissues of *Drosophila melanogaster* recovering from a so-called transient sublethal heat shock, during which body tempera-

ture was increased ~5°C above normal body core temperature (400). Such a mild heat shock elicited a heat shock response, characterized by the synthesis of new heat shock proteins normally almost absent in tissues of adult animals and by an increased synthesis of constitutively present or cognate heat shock proteins. This event was followed by a transient increased tolerance to high, normally lethal temperatures (thermotolerance). Later it was found that not only the tolerance to enhanced temperature increases, but also the resistance toward other events like hypoxia, ischemia, inflammation, and exposure to such cellular toxins as heavy metals, endotoxins, and reactive oxygen species (cross-tolerance), all imposing serious stress upon tissues and their composing cells.

The increased resistance toward stressful events has been described in a great variety of organisms, organs, and tissues, including the heart. During the last years, more and more information has become available on the specific role of individual heat shock proteins and on the mechanisms of transient activation of gene transcription, leading to the enhanced cardiac synthesis of these proteins. A better insight has also been acquired in such subcellular targets as molecules or organelles that can be protected by heat shock proteins. Now the phase is reached that upregulation of heat shock protein synthesis is considered as a powerful physiological, endogenous route for protecting crucial cellular homeostatic mechanisms against disturbing external factors. Therefore, it is propagated that they provide a new therapeutic tool to protect the human heart during and after transient ischemic attacks.

Over the last 5 years comprehensive reviews have been published on the chaperone activity of heat shock proteins (34, 150, 248, 283, 430) and on their role in specific tissues and pathological processes (34, 94, 104, 200, 281, 340, 445). The role of such individual heat shock proteins as the 70-kDa heat shock protein, small heat shock proteins and the heat shock factors has also been highlighted (293, 334, 360, 395, 439).

This survey concentrates on the transient protection of the cardiovascular system in various mammalian species when heat shock protein synthesis is upregulated. The present state of the art regarding the stimuli, leading to the heat shock response, the transduction pathways involved in the activation transcription of the various heat shock genes, and the cellular structures protected by heat shock proteins is presented. Such new experimental tools as transgenic animals and transfection techniques to enhance or inhibit the transcription of heat shock genes are discussed. Although the physiological role of heat shock proteins and their protective potential under pathophysiological circumstances have been studied in detail in animal models, knowledge of the role and effects of these proteins in humans is still limited. Where possible the relevance of the observations for the human situation is discussed.

II. NOMENCLATURE AND FAMILIES OF HEAT SHOCK PROTEINS

The applied nomenclature is primarily derived from the trigger leading to the synthesis of these proteins. Because heat shock was the first discovered trigger of the heat shock response leading to enhanced transcription of certain genes, the related products of this transcriptional activity have been called heat shock proteins. On the basis of the adopted nomenclature after the Cold Spring Harbor Meeting of 1996, throughout this survey heat shock genes will conventionally be designated as hsp-genes, while the related proteins are called Hsps (150). Accordingly, proteins the synthesis of which was increased upon glucose starvation are called glucose-regulated proteins (Grps). For the majority of these genes and proteins the name is associated with a molecular mass indication like for instance hsp27 and Hsp27, respectively. Some proteins that were first discovered in a nonrelated domain carry a particular name like the major structural ocular lens protein αB-crystallin or those proteins targeting abnormal proteins for degradation, i.e., the ubiquitins. The name of some Hsps is often preceded by an indication of the compartment in which they reside, like mitochondrial mt-Hsp75. Furthermore, distinction has been made between the proteins almost absent under nonstressed conditions but synthesized immediately after cellular stress, and the proteins that are constitutively synthesized in the tissue. The first are called inducible proteins, while the second class is known as cognate proteins, like for instance Hsc70. This distinction may be arbitrary because in various cell types low but measurable concentrations have been found of so-called inducible Hsps, while on the other hand the synthesis of some cognate proteins is also increased upon stress.

Classification of various Hsps in families is based on their related function and size, which can vary from 10 to 170 kDa. Family names are conventionally written in capitals (150). The HSP70 family can stand for a typical example. The proteins of this family range in weight between 70 and 78 kDa (194). All HSP70 family members bind ATP (284). Constitutive or cognate members are Hsc70, Hsp75, and Grp75 (see below), while the inducible member is Hsp72, commonly called Hsp70.

The nomenclature and classification for related Hsps in prokaryotic and eukaryotic organisms is different, although the sequence of nucleic acids in genes and amino acids in proteins is often highly identical. Various hsp-genes are well conserved during evolution. For instance, among eukaryotic organisms the identity of the nucleotide sequence of the hsp70-gene varies from 60 to 78%

(194). Compared with the human hsp70-gene, the *Drosophila* hsp70-gene and the prokaryotic *Escherichia coli* dnaK-gene reach a 72 and 50% identity, respectively (168).

In the spectrum of Hsps, the so-called Grps form a special group. As mentioned earlier, the synthesis of these proteins increases when extracellular glucose concentrations are low. Other triggers, however, can also lead to enhanced Grp synthesis, like depletion of intracellular calcium stores or inhibition of protein glycosylation. Grps reside in various HSP families and comprise Grp58, Grp78, Grp94, and Grp170. They are all localized in the endoplasmic reticulum.

In Table 1, only the representative eukaryotic Hsps are presented. The functional characterization of the

TABLE 1. *Eukaryotic members of the various heat shock protein families*

Family	Members Name	Alternative Name	Intracellular Location	Characteristics
Ubiquitins	Ubiquitin		Cytoplasm/nucleus	Constitutive; targets abnormal proteins for degradation by the 26S protease (42)
HSP10	Hsp10		Mitochondria	Constitutive; promotes substrate release in conjunction with Hsp60
Small HSPs	αA-crystallin αB-crystallin		Cytoplasm/nucleus	Constitutive; chaperone (55, 91, 387)
	Hsp27	Hsp25 (murine) Hsp27 [human, canine (219) and rat (408)] Hsp28 (hamster)	Cytoplasm/perinucleus Stress: nuclear translocation	Constitutive; phosphorylated upon stress; overexpression protects against heat shock; forms large aggregates
	Hsp32	HO-1	Cytoplasm	Inducible upon hypoxia (113)
HSP40	Hsp40	Hdj-1	Cytoplasm/nucleus Stress: nuclear translocation	Constitutive; guides protein folding in conjunction with Hsp70 and Hsc70
	Hsp47 (161)		ER	Constitutive; binding and transport of collagen from ER to Golgi (301)
HSP60	Grp58		ER	Constitutive
	Hsp60	Hsp58 Hsp65 (100, 259)	Mitochondria	Constitutive; guides mitochondrial protein import in conjunction with Hsp70; ATP hydrolysis and substrate release stimulated by Hsp10
	TRiC	TCP1 (145)	Cytosol (28)	Protein folding (chaperonin)
HSP70	Hsc70	Hsp73; hsc70 (371); ly-hsc73 (3)	Cytoplasm/peroxisome/nucleus Upon stress and mitosis: nuclear translocation	Constitutive; slightly inducible; guides protein synthesis and import for proteins degradation
	Hsp70	Hsp72; Hsx70; Hsp70l; Hsp70-1; 72K; Hsp71; sp71; Hsp68	Cytoplasm/nucleus Upon stress and mitosis: nuclear translocation	Highly inducible; normally almost absent; guides protein synthesis and binds proteins upon stress
		Hsp70-7	?	Inducible; part of Hsp70? (421)
		Hsp70-6	?	Inducible, only inducible at high temperature; more basic than Hsp70 (225)
		Stch		"ATPase core" of Hsp70 (115)
	Hsp75	Grp75; mt-hsp75; PBP74	Mitochondria (40)	Constitutive; guides and folds proteins in mitochondria
	Grp78 (399)	BiP; p78; Grp80 Hsp78	ER (451)	High constitutive expression in gland cells; inducible upon low [glucose]; glycosylation inhibitor; folds proteins
	Mortalin		Cytoplasm/nuclear membrane (118)	
HSP90	Hsp90-α Hsp90-b (431)		Cytoplasm/nucleus Stress: nuclear translocation (67, 105)	Constitutive but upregulated and phosphorylated upon stress; binds cytoplasmic hormone receptors; guides certain protein kinases ($pp60^{src}$) to cell membranes
	Grp94	Grp90; Grp100; Erp99; ERP100; endoplasmin (420)	ER (28)	Inducible in growing satellite skeletal muscle cells; binds calcium
HSP110	Hsp94		Renal medulla	Inducible by heat and osmotic stress
	Hsp104	Hsp105 (139)	Cytoplasm	Constitutive; only in yeast (?); conveys thermotolerance
	Hsp110	Hsp110 (human) Apg-1 (murine)	Cytoplasm/nucleolus Stress: nuclear translocation	Constitutive; increased synthesis upon stress; protein refolding
	Grp170		ER	Constitutive expression

Hsp, heat shock protein; Hsc, heat shock cognate; HO-1, heme-oxygenase-1; Grp, glucose-regulated protein; prp, peptide recognition protein; TriC, T-ring complex; TCP, T-complex protein; ER, endoplasmic reticulum; ly, lysosomal; mt, mitochondrial; BiP, binding protein.

members in each family is indicated in the last column. Members of the various families do have a specific localization within the unstressed cell, which is indicated in the fourth column. Upon cell stress, however, they can be translocated to other cellular compartments. Inducible Hsps are rapidly synthesized after the onset of the stress and can have a time-dependent, and thus dynamic, specific intracellular localization.

According to the animal species in which they have been discovered, Hsps may have different names, although their nucleotide and amino acid sequence can be highly identical. As an example, in Table 2, the various genes of the HSP70 family for mouse, rat, and human tissues as well as the chromosomal locus symbol in humans are presented. The nature of their expression is also indicated (after Refs. 97, 395). The same accounts for Hsp27. Throughout this survey only the terms Hsp27 and Hsp70 will be used to represent the family member.

III. TRANSCRIPTION OF HEAT SHOCK PROTEIN GENES

A variety of physical and chemical factors evoking environmental stress enhance the synthesis of Hsps in cells of various tissues, including those in the vascular wall and the heart. Such a process is initiated by the stimulation of membrane-bound receptors, changes in physical properties of the cell membrane, or in such intracellular changes as temperature and partial oxygen pressure. At present, numerous stimuli are know that lead to enhanced expression or activation of Hsps in cells belonging to the cardiovascular system. These triggers are summarized in Table 3.

Not all stimuli are evenly potent in the induction of expression of heat shock genes. For instance, heat shock is a more potent activator of hsp70-gene transcription than hypoxia (279). Otherwise, some of these stimuli have an additional effect when applied in combination. For instance, in intact rats in various tissues heat shock combined with intraperitoneally administered aspirin results in a significantly higher synthesis of such inducible Hsps as Hsp70 (117).

A. Signal Transduction Pathways for hsp-Gene Transcription

Figure 1 shows the presently known triggers leading to the activation of the transcription of hsp-genes in the eukaryotic cell. Despite the fact that the transduction pathways involved in the activation of the transcription of hsp-genes have been investigated intensively, for some distinct members of the various families the mechanisms underlying these pathways are still matter of debate.

In many organisms the temperature at which they live and the rate of change of this temperature determine the set point and the intensity of the heat shock response, respectively. For instance, it is obvious that the temperature set point for the heat shock response in arctic fishes is totally different from that in bacteria living in hot springs. Furthermore, slow increases in temperature activate hsp-gene transcription less than abrupt hyperthermia.

Although for such stimuli as angiotensin II and phenylephrine membrane receptors have been identified, no conclusions have been reached regarding the nature of the "primary sensor" for abnormal high temperatures in the cell membrane or within the cell. In yeast it has been suggested that the physiological response to changes in environmental temperature is dependent on the lipid composition of the plasmalemma. In yeast cells with different membrane fatty acid composition, more specifically with different ratios of saturated to unsaturated fatty acids, the threshold temperature of transcription of the hsp70-gene was found to be significantly different (54).

In contrast to the lack of understanding of the nature of the primary sensor at the level of the cell membrane, more information is available on the intracellular signaling cascade ultimately leading to the activation of hsp-gene transcription and subsequent protein synthesis. In eukaryotic cells this transcription is effectuated via a transcription factor known as the heat shock factor (HSF; see below). Because the HSF is inactive under nonstressed conditions, binding to DNA occurs only upon stress, implying that the HSF is negatively regulated (290).

Several factors can lead to HSF activation (see Fig. 1). The central process in HSF activation is the equilibrium between the binding of such free Hsp molecules as

TABLE 2. *The Hsp70 family in mammals*

Family Member	Mouse Genes	Rat Homologs	Human Homologs	Human Locus Symbol	Expression
Hsp70	*hsp70-1* (167, 169, 370)	hsp70-1 (234)	hsp70-2 (353)	HspA 1B (285)	Inducible
	hsp70-3 (169, 321, 370)	hsp70ib (279)	hsp70-1 (168, 370)	HspA 2A (344)	Inducible
				HspA 2B (44)	
Hsc70	*hsc70* (126)	hsc70 (371)	hsc70 (102)	HspA 8 (394)	Constitutive
Hsp75	*grp75* (98)		grp75 (98)	HspA 9 (40, 98)	Constitutive
Grp78	*grp78* (209)		grp78 (399)	HspA 5 (144, 399)	Constitutive

TABLE 3. *Stimuli enhancing cardiovascular hsp-gene transcription or hsp synthesis*

Stress	Stressor	Species	Reference Nos.
Acute hypertension	Intravenous injection of angiotensin II, dopamine, phenylephrine, vasopressin, endothelin 1	Rat	276, 288, 406, 443
Acute load change	Artificial stretch ventricular wall	Rabbit	204
	Exercise	Rat	240, 347
Alcohols	Ethyl alcohol	Rat	380
Amino acids analogs	Exposure to canavanine	Rat	151
Anesthetics	Intraperitoneal methoxital sodium	Rat	365
Cardiac hypertrophy	Aortic stenosis	Rat, ovine	92, 135, 365, 378
	Intravenous injection of monocrotalin	Rat	68
Chemotherapy	Exposure to adriamycin	Mouse	164
Development	Cardiac embryogenesis	Mouse	35
Heat	Exposure to hyperthermia (heat shock)	Rat, rabbit, mouse, chicken embryo	71, 72, 77, 79, 82–85, 99, 135, 173, 190, 257, 267, 268, 279, 423, 446
	Intravenous injection of amphetamine	Pig, rat	265
Heavy metals	Exposure to $CdCl_2$	Mouse	244
Hypoxia	Hypoxic environment	Mouse, bovine, rat	162, 177, 191, 271, 279
Immunogen response	Infection with picornavirus	Mouse	164
Ischemia/reperfusion	Regional myocardial ischemia	Rabbit, pig, rat, dog, rabbit	15, 88, 93, 203, 255, 258, 260, 279, 361
	Global myocardial ischemia	Rat	79
	Postischemic reperfusion	Rat	80, 309
	Cellular ischemia	Rat	315
Metabolic stress (ATP depletion)	Exposure to rotenone	Mouse	32
	Exposure to 2-DOG/lactate	Rat	77
	Exposure to 2-DOG/Na-cyanide	Rat	177
	Anaerobic metabolism	Rat	298
Oxidative stress	Exposure to hydrogen peroxide	Rat, human, mouse	185, 212, 244, 443
	Exposure to ebselen (antioxidant)	Rat	159
	Exposure to irradiated rose bengal	Rat	212
	Exposure to X/XO	Rat	212
	Exposure to XO/HX	Bovine	19
	Exposure to endotoxin	Rat, rabbit	358, 420
	Exposure to monophosphoryl lipid A	Rat	306
	Exposure to TNF-α	Cat	304
Physical stress	Physical restraint	Rat	271, 272, 404
Sodium arsenite	Exposure to sodium arsenite	Rat	140
Transplantation	Allografting	Rat	90
Tyrosine kinase inhibition	Herbimycin-A	Rat	292

2-DOG, 2-deoxyglucose; X/XO, xanthine/xanthine oxidase; XO/HX, xanthine oxidase/hypoxanthine; TNF-α, tumor necrosis factor-α.

Hsp70 to the HSF and to stress-mediated unfolding proteins. Any increase in the presence of unfolding proteins shifts this equilibrium to the Hsp-unfolded proteins side, thereby releasing free HSF monomers, which can be activated subsequently. This hypothesis has been forwarded by several authors (2, 291), but contested by others (332). More recently it was found that not only Hsp70 binds to the HSF, but also Hsp90. Hsp90 also acts as a repressor protein of HSF (see below) (6, 455).

Second, HSF can be activated indirectly by some stressors that activate phospholipase C, thereby enhancing intracellular concentrations of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, associated with subsequent activation of protein kinase C (PKC) (111). The fact that PKC-activating drugs like phorbol 12-myristate 13-acetate (PMA) provoke Hsp70 synthesis (111) and that the PKC inhibitor chelerythrine chloride reduces the heat shock-elicited Hsp70 synthesis (446) underlines the central role of PKC in the activation of hsp70-gene transcription.

On the basis of results obtained in cultured neonatal cardiomyocytes, it has been argued that tyrosine kinases are also involved in the signaling pathway of hsp-gene transcription. In these cultures, herbimycin-A, a benzoquinoid ansamycin antibiotic and reputed tyrosine kinase inhibitor, provokes Hsp70 synthesis and increased resistance against a subsequent lethal heat stress (292). This drug, however, does not activate the transcription of such other genes as hsp90, hsp60, hsp27, and grp78. Moreover, another tyrosine kinase inhibitor, genistein, had no effect on hsp70-gene transcription at all. Thus herbimycin-A induces Hsp70 transcription via a tyrosine kinase-independent mechanism, because an even more specific tyrosine kinase inhibitor, tyrphostin 23, also does not affect hsp70-gene transcription (111).

Whether changes in intracellular calcium concentra-

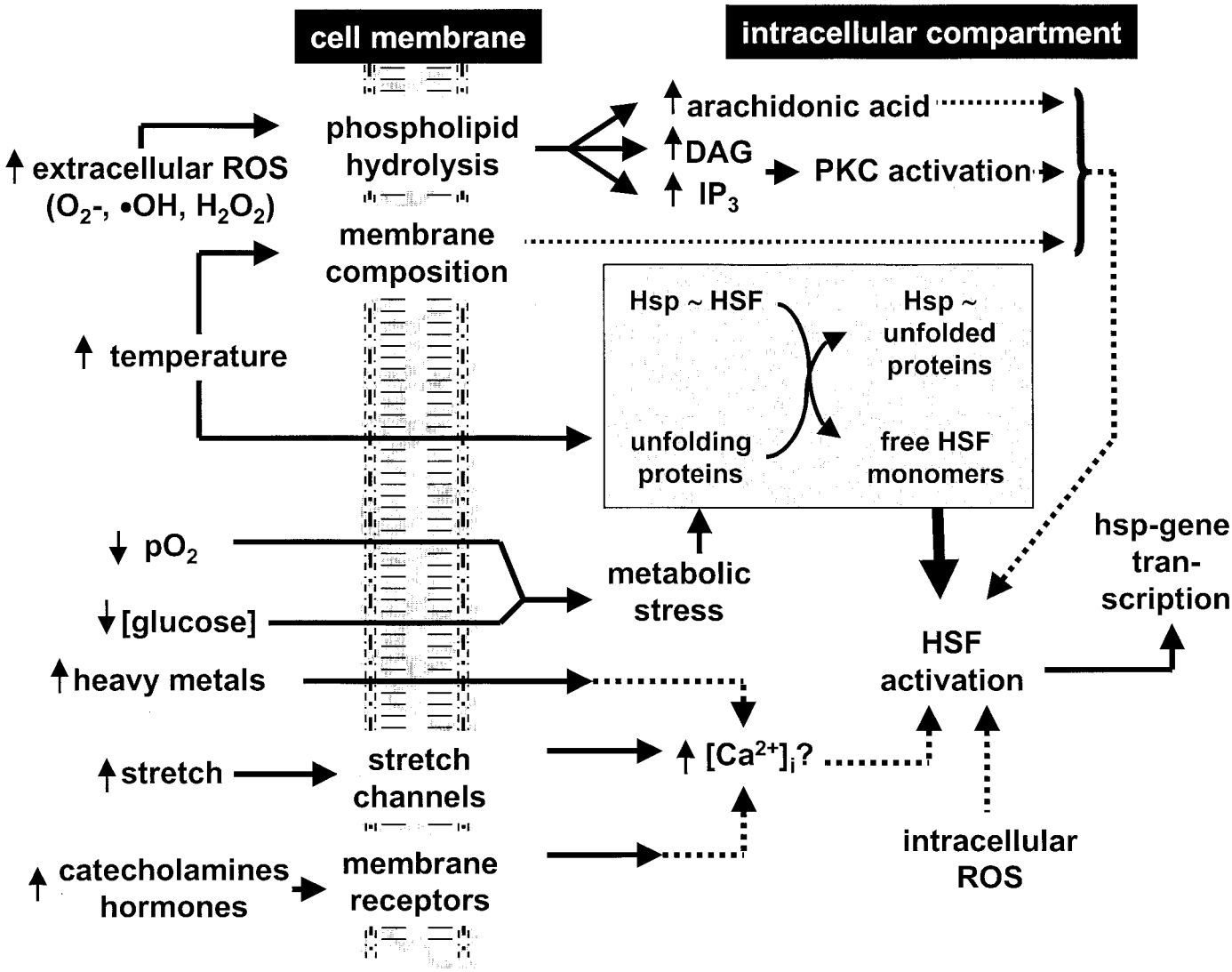

FIG. 1. Extracellular and intracellular stresses that can lead to the activation of the heat shock factor (HSF) and subsequent heat shock protein (hsp)-gene transcription. ROS, reactive oxygen species; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; Hsp~HSF, binding between Hsp molecules and the HSF. Dotted lines indicate that the transduction pathway is not completely known.

tion are involved in the activation of hsp-gene transcription is incompletely understood. In cardiac tissue a transient increase of diastolic intracellular free calcium was found after heat shock (52, 249, 417), increased wall stretch, and α-adrenoreceptor stimulation. All three stresses led to enhanced hsp-gene transcription. Detailed observations during heat shock revealed that the initial rise of the intracellular calcium concentration is due to ion release from intracellular stores, presumably from the endoplasmic reticulum. The rise in calcium concentration is preceded by a rapid release of IP_3, occurring within the first minute after imposition of the heat shock (52). Subsequently, the ion concentration is further increased by a secondary calcium influx from the extracellular space. An interesting observation was made by Löw-Friedrich and Schoeppe (244), who found that a $CdCl_2$-elicited enhanced synthesis of Hsp70 in isolated cardiomyocytes was reduced when the cells were preincubated with calcium antagonists, like nifedipine, diltiazem, or verapamil. The interpretation of these results, however, is complex. Calcium antagonists could interfere directly with the toxic effects of $CdCl_2$, such as blocking its entry into the cell or reducing the $CdCl_2$-elicited calcium release from mitochondria. On the other hand, it cannot be excluded that a long-term incubation with high doses of calcium antagonists has a direct blocking effect on Hsp70 synthesis through its inhibitory effect on protein synthesis.

The mechanism of activation of such small Hsps (sHsps) as Hsp27 and αB-crystallin is likely different from that of the inducible hsp70-gene (16, 360). Because they

are constitutively present in cardiac tissue in appreciable amounts (174), activation occurs mainly through phosphorylation. This process takes place very rapidly in response to heat shock, growth factors, phorbol esters, calcium ionophores, interleukin-1 (IL-1), and tumor necrosis factor-α (TNF-α) (16, 428). With regard to TNF-α-elicited activation of Hsp27, it has been shown that the mitogen-activated protein kinase (MAPK) pathway is involved via the p38 MAP kinase cascade and the MAPKAP kinases 2 and 3 (110, 170). PKC is likely not involved in the activation of sHsps, because TNF-α-elicited phosphorylation of Hsp27 is not inhibited by such kinase inhibitors as staurosporine, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), *N*-(2-[methylamino]ethyl)-5-isoquinolinesulfonamide (H-8), *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA-1004), or chelerythrine chloride (418).

B. Heat Shock Factors

Ultimately, the transcription of several hsp-genes is governed by the so-called HSFs, which are constitutively synthesized cellular transcription factors. At present, four different HSFs have been identified, i.e., HSF1 (330), HSF2 (357), HSF3 (300), and HSF4 (302). HSFs are products of the transcription of four different genes. HSF1, HSF2, and HSF4 have been identified in human tissues. At present, HSF3 has only been described in the chicken, in which it is involved in the development of various tissues (300, 388).

All four factors are thought to be activated differently by various forms of cellular stress (300, 330). HSF1 activation is extremely sensitive to temperature changes, whereas HSF2 is not (74, 95). HSF1 is primarily involved in the stress response, while HSF2 and HSF3 regulate the transcription of hsp-genes in specific tissues, undergoing processes of differentiation and development. HSF2 (72 kDa) is present in two isoforms, i.e., HSF2-α and HSF2-β. The two isoforms are products of alternative splicing of HSF2 pre-mRNA. HSF2-α is predominantly found in the testis (352), whereas HSF2-β is synthesized in heart and brain. The HSF2-α form is transcriptionally more active than the HSF2-β form (128). Interestingly, HSF2 operates additionally to HSF1. When both heat shock factors are activated by hemin treatment and heat shock, respectively, transcription of the hsp70-gene is more potent than after activation of HSF1 alone (363).

Evidence is accumulating that the latest discovered HSF, i.e., HSF4, is structurally related to the other three HSFs (302). This particular protein, however, is functionally distinct from the three others since it does not respond to stresses like heat. The precise role of HSF4 is still unknown, aside from its repressing effect of hsp-gene activation. Indeed, the transcription of the hsp70-, hsp90-, and hsp27-genes is reduced when HSF4 is overexpressed. Nakai et al. (302) suggested that HSF4 is not a transcription factor because it lacks the carboxy-terminal repeat shared by HSF1, HSF2, and HSF3 (302).

C. Activation and Deactivation of HSF1

HSF1 is the major stress responsive HSF mediating the heat shock response in mammalian cells (330). The mechanism of action and its regulation and activation have been extensively reviewed by Wu (439). HSF1 is a 75-kDa protein that is constitutively synthesized and localized in the cellular cytosol and nucleus and often associated as a group of inactive monomers in a complex of ~200 kDa (21). In addition, in the resting state, the HSF1 monomers seem to be bound by Hsp70 and Hsp90, the latter being only liberated upon activation by kinases (6, 455).

Upon activation, HSF1 shifts from the cytosolic to the nuclear compartment and becomes organized in an active, DNA-binding homotrimer of ~700 kDa (129, 439). Trimerization is established by arrays of α-helical residues (leucine zippers) in the amino-terminal domain of the protein and is negatively regulated by another leucine zipper domain at the carboxy-terminal site (331, 439). Crystal structure analysis has revealed that the DNA-binding domain of HSF1 contains a bundle of three helices capped by a four-strand antiparallel β-sheet, forming a globular domain of ~90 residues (136). HSF1-DNA binding also depends on the presence of the so-called heat shock elements (HSEs) in the upstream promoter site of the hsp-genes. These HSEs are multiple and have variable numbers of alternating oriented arrays of 5′-nGAAn-3′ (n stands for less conserved nucleotides) (33, 166, 211, 233, 236). The binding of the HSF homotrimers to HSE requires at least two nGAAn units, arranged head-to-head or tail-to-tail (441). Upon binding, the synthesis of new mRNA molecules encoded by related hsp-gene can be initiated.

The HSF1 activity is controlled by multiple regulatory mechanisms, including suppression by Hsp70 and Hsp90 and activation by different cellular signaling cascades (199). Interestingly, HSF1 can be activated into an intermediate state in which it binds to HSE sequences without stimulating gene transcription. For this ultimate process, HSF1 hyperphosphorylation seems to be indispensable (351, 440). Furthermore, trimerization and phosphorylation seem to be far more important for activation of hsp-gene transcription than the absolute cellular content of HSF1 (96).

Many stresses aside from heat activate HSF1. In cultured cells it was found that hypoxia, ethanol, and sodium arsenite increase HSF1-DNA binding and Hsp70 levels (33, 287). The prostaglandins A_1 and A_2 (PGA_1 and PGA_2,

respectively) also enhance HSF1-mediated transcription of hsp70- and hsp90-genes (8, 9). PGA_1 inhibits nuclear factor-κB (NF-κB) by blocking phosphorylation and subsequent degradation of the NF-κB inhibitor protein IκB-α. This inhibition of NF-κB is associated with HSF1 activation (342). The hsp70-gene transcription through PGA_2 only occurs in proliferating but not in confluent cell layers in culture (156, 350) and seems to be cell type dependent. Our group found that hsp70-gene transcription is also stimulated by PGA_1 and PGA_2 in cultured myogenic cells and adult cardiomyocytes, although this expression does not lead to protection against a severe stress (unpublished data). Hyposmotic stress also elicits HSF1-DNA binding, which, however, does not result in hsp70-gene transcription (163). Because salicylates and arachidonic acid also activate HSF1 (178, 186–188), it is tempting to speculate that inflammation and degradation of cellular membranes, resulting in the release of arachidonic acid from membrane phospholipids, lead to enhanced Hsp concentrations in the cell. However, to the best of our knowledge, no enhanced Hsp levels have been found upon treatment with these compounds.

The role of the precise intracellular concentration of individual Hsps on the stress-mediated activation of HSF1 has been investigated by artificially reducing the levels of such Hsps as Hsp70 and Hsp90. Only Hsp90 reduction resulted in HSF1 activation pointing to its negative regulation by this particular Hsp. Heating leads to the dissociation of the constitutive heterocomplexes between HSF1 and Hsp90, because stress-denatured proteins compete for Hsp90 (6, 455). Inversely, by artificially reducing concentrations of nascent polypeptides, the HSF1-elicited hsp70-gene transcription was inhibited, indicating that cells are capable of registration of concentrations of not yet folded nascent proteins or stress-denatured proteins (22).

More recently, investigations have been performed to elucidate the mechanism of HSF1 deactivation, which seems to be regulated by multiple factors. First of all, increasing levels of inducible Hsps repress HSF1 activation in an inverse way, pointing to an autoregulatory mechanism for HSF1 activity (138, 236, 295). Furthermore, phosphorylation has both positive and negative effects on the activity of HSF1 (64, 199, 295). Administration of okadaic acid, a specific and potent inhibitor of serine/threonine phosphatases 2A and 1 to cultured cells, leads to inhibition of HSF1 dissociation and to prolonged activation of hsp70-gene transcription (57, 440). Inversely, blocking the serine/threonine kinase activity inhibits HSF1 phosphorylation and hsp70-gene transcription (440). Second, it was found that HSF1 activity can also be inhibited through phosphorylation of HSF1 serine residues (155), evoked by MAPKs (64). Third, it has been shown that the nuclear heat shock factor binding protein 1 (HSBP1) interacts with the active homotrimeric state of HSF1 during heat shock. More specifically, HSBP1 negatively affects the HSF1-DNA coupling by binding to the HSF1 heptad repeat. Overexpression of HSBP1 blocks the HSF1 activation (354).

Only recently, the HSE in the promoter of hsp-genes was found to be part of a so-called "composite response element" (CRE), consisting of various elements and integrating multiple regulatory signals. For instance, HSEs form CREs with promoter binding elements for two transcription factors, i.e., nuclear factor IL-6 (NF-IL-6) and the signal transducer and activator of transcription-3 (STAT-3). Activation of the STAT-3 transcription factor through the IL-6 receptor attenuates HSF1 activity, while NF-IL-6 consolidates its activity (375). Recently it has been found that interferon-γ (IFN-γ)-mediated activation of another transcription factor, i.e., STAT-1, also synergistically activates HSF1 (376).

IV. FUNCTIONS OF HEAT SHOCK PROTEINS

A. Function of Chaperones and Chaperonins Under Nonstressed Conditions

Most likely, the primary physiological function of Hsps is to fulfill chaperoning activity (28). Molecular chaperones have been defined as a nonrelated class of proteins that mediate the correct folding of other proteins, but do not take part in the final assembly of new structures (109). Although every newly synthesized protein contains within its amino acid sequence the necessary information for ultimate correct folding, this process can be hampered by several factors. For instance, during their synthesis, incomplete amino acid sequences may already associate with other unfinished parts of peptide chains or with totally completed peptides. For correct association and/or folding the amino acid chains need to be entirely synthesized and therefore have to be kept in the unfolded monomer state (348). Second, proteins that need to be translocated to other cellular compartments should also be kept in an unfolded or semi-folded state to pass intracellular membranes. Such physical state can be achieved through binding to chaperone proteins.

Excellent reviews have been written on the chaperone function of Hsps in general (109, 123–125, 137, 145, 343, 379) and on Hsp70 in particular (194). The chaperone role of Hsps in the cardiovascular system has recently been reviewed by Benjamin and McMillan (34).

Chaperonins consist of a class of Hsps that assist in correct protein assembly at a later stage than the chaperones. This process occurs when completed protein chains are released from the ribosomes or are transported to such cell organelles as mitochondria. In eukaryotic cells, a distinction is made between two groups of chaperonins. Group I consists of Hsp60 and Hsp10, both residing in the

mitochondria. Group II comprises the TCP1 T-complex polypeptide (TCP1) subfamily, the members of which can be found in the cytosol (107, 108). TCP1 is, among others, involved in the folding of actin and tubulin and thus indispensible for proper functioning of the cytoskeleton (341).

1. In the cytosol

Hsp70 and Hsc70 act as single entities to exert their chaperone function. A peptide-binding site and enzymatic catalytic site characterize both protein chains. The peptide-binding domain resides in the vicinity of the evolutionary less-conserved carboxy-terminal domain (231), while the catalytic site is situated near the highly conserved amino-terminal domain of the protein (size 44 kDa). The latter site carries the ATPase activity (58, 227, 229), necessary for binding to and release from other protein chains (346). Furthermore, both proteins carry nuclear localization signals (NLS). In the human Hsp70 gene sequence, originally described by Hunt and Morimoto (168), a NLS sequence was presumed to be located in the 246–262 amino acid (AA) region. Coupling of protein kinase to this region indeed led to protein kinase transfer into nucleus and nucleolus (87). However, various sites in the Hsp70 protein can probably affect its nuclear targeting. Milarski and Morimoto (284) showed that an extensive deletion of another region (AA 351–414) inhibits nuclear Hsp70 localization. This result is difficult to interpret because the synthesized protein also lost other physiological properties, like binding of proteins and ATP. In contrast, site-directed mutations in the 246–251 AA sequence confirmed that this region is necessary for nuclear import, but not for viability of the cell after severe stress (201). Furthermore, by transfection of cells with mutants for the tyrosine-524 location, it was found that phosphorylation of this particular amino acid is necessary for both nuclear import and increased resistance against severe stress (205).

Hsc70 also carries a prototype basic NLS in the 246–262 AA region. Partial deletion of this region, however, does not affect Hsc70-mediated nuclear protein import, suggesting that another nonclassical NLS is present in the protein sequence. Lamian et al. (217) found that such an alternative signal is located in the amino terminus of Hsc70.

Under normal, nonstressed conditions Hsp70 chaperones a variety of processes in the cytosol. The protein stabilizes unfolded nascent precursor peptides (30, 137) and keeps them unfolded until they reach the final cellular compartment (18). More specifically, Hsp70 has been found to associate with cytoskeletal proteins (208, 348, 402), cell surface glycoproteins (165), calmodulin (66, 377), and saturated long-chain fatty acids, like palmitate, stearate, and myristate. Several functional explanations have been proposed to explain the interaction with fatty acids. Although Hsp70 could act as intracellular fatty acid transport carrier, it cannot be excluded that fatty acids are required for the proper protein folding by Hsp70 (131). The chaperone also assists in the translocation process across the membranes of the endoplasmic reticulum and mitochondrion (18, 75, 127, 158, 189, 407). Furthermore, Hsp70 guides misfolded proteins to lysosomes (3, 61) or the outer membrane of peroxisomes (424).

Figure 2 presents a model for the cellular chaperone function of Hsc70. Aside from Hsc70, Hsp40 can bind unfolded proteins and couple them to Hsc70 or binds directly to the already formed Hsc70-unfolded protein complex (119). In an ATP hydrolysis-dependent process, Hsc70 folds and releases the newly formed protein. According to the type of the folding process, the complex can have a different fate. Hsp40 is replaced by other proteins such as Hsc70-interacting protein (Hip), the Hsc70-organizing protein (Hop), or the Hsc70-accessory protein (Hap), leading to different interactions of the Hsc70-unfolded protein complex. Coupling to Hip conducts transport of the complex throughout the cell, to Hop the linkage to Hsp90, while binding to Hap stimulates the dissociation of Hsc70 and the unfolded protein (59, 154, 326). The dissociation of Hsc70 from newly folded or refolded proteins occurs after the conversion of ADP to ATP at the surface of the Hsc70 protein. This implies that the Hsc70 protein binding becomes more stable in ischemic/hypoxic periods when cellular ATP concentrations are relatively low (see Fig. 1) (29, 30).

Hsp90, one of the most abundant cytosolic Hsps, binds steroid receptors, protein kinases, intermediate filaments, microtubules, and actin microfilaments in a very specific manner (208). Hsp90 is an essential component of the glucocorticoid receptor, assembled in a complex of several proteins (14, 327). Without the association to Hsp90, intracellular hormone receptors were found to be inactive. Via its interaction with filamentous actin in the cytosol, Hsp90 is thought to target steroid receptors to the nucleus and various kinases to their site of action (86, 327).

It is generally accepted that Hsp90 possesses ATPase activity and can be autophosphorylated on serine and threonine residues (299). It has been found that immobilized Hsp90 molecules can be dissociated from actin by ATP (193).

The 60-kDa chaperonin TCP1 can be seen as the cytosolic counterpart of the mitochondrial Hsp60 chaperonin. It forms a large (970 kDa) hetero-oligomeric protein complex called TriC (TCP1 ring complex), containing TCP1 and several other proteins, among which Hsp70 (122, 145, 228).

Hsp27 is very active in assisting the assembly of macroglobular protein complexes, such as F-actin polymerization. This function, however, is highly dependent on the phosphorylation state and monomeric or multimeric state of Hsp27. In the nonphosphorylated, monomeric state, Hsp27 inhibits F-actin polymerization via spe-

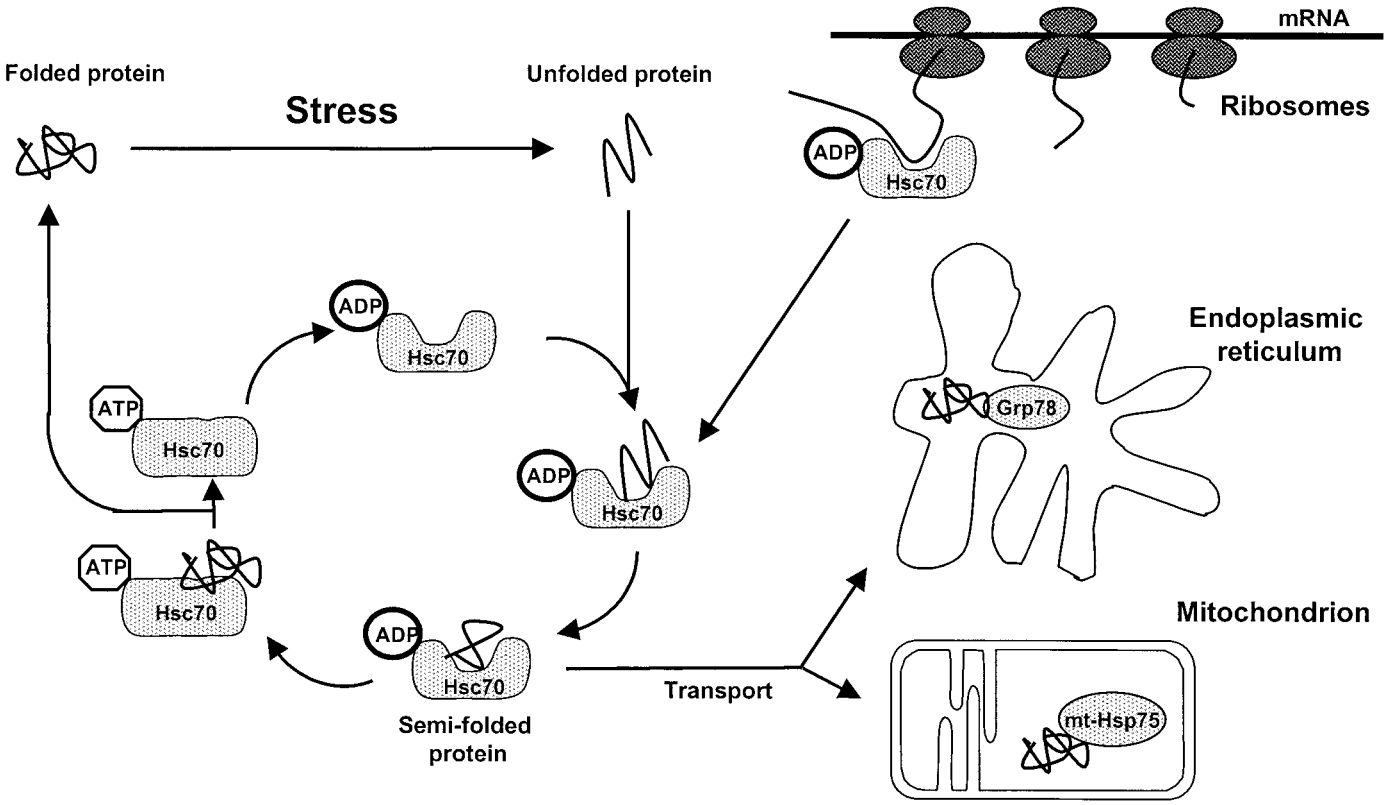

FIG. 2. Scheme of interactions between the chaperones Hsc70 and native peptide chains or unfolding proteins. Binding to the chaperone protein and other associated proteins (not shown, see text) determines the fate of the folded protein. Grp78, glucose-regulated protein 78; mt-Hsp75, mitochondrial 75-kDa heat shock protein.

cific binding to the plus-end of the filaments. In the phosphorylated, multimeric state it does not and thus promotes the polymerization process (37). The other sHsp, αB-crystallin, is more involved in protecting actin and desmin filaments under acidic conditions (36). By binding to the Z-line in the sarcomeres of cardiomyocytes, the protein prevents aggregation of the actin filaments (36, 242, 243).

2. In cellular organelles

Protein chaperoning also occurs within the intracellular compartments. To reach or to remain in these compartments, Hsps are equipped with organelle-targeting sequences. For instance, both Grp78 and Hsp75 possess an amino-terminal extension to reach the endoplasmic reticulum and mitochondrion, respectively (379). In addition, Grp78 contains a carboxy-terminal sequence for retention in the endoplasmic reticulum (132).

In mitochondria, mt-Hsp75 keeps mitochondrially encoded proteins in an assembly competent state (146). Cytosolic Hsc70 ensures efficient presentation of proteins to be transported across the mitochondrial membranes (396). Furthermore, in this organelle the chaperonin Hsp60 prevents aggregation of misfolded proteins and together with Hsp10 aids in the refolding of mitochondrial proteins (252). This process occurs in the so-called Anfinsen or folding cage. This cage consists of two stacked rings, each of them containing seven Hsp60 molecules. A protein chain can be bound inside these stacks, which is then closed on the upper side by one stack of Hsp60 molecules. When ATP is bound to the cage, the protein chain is released within the cage and allowed to fold. The folded chain is released after unbinding of the Hsp10 complex from the cage. The whole process is finished in the seconds time order (107).

In lysosomes, Hsc70 assists in protein degradation by transferring them into this organelle (3, 397). Furthermore, Hsc70 is associated with the cellular centrosome and is required for repair of this organelle after damage (48), probably in collaboration with accessory proteins like p16, a member of the Nm23/nucleoside diphosphate (NDP) kinase family (224).

B. Function of Chaperones and Chaperonins Upon Stress

Compared with the nonstressed situation, much less is known about the various Hsps that exert their chaper-

one function during and after stress. This lack of insight is in part due to the fact that under these circumstances general protein synthesis is completely disturbed and new Hsps appear in various cellular compartments. In addition, many constitutive members redistribute within the cell as newly synthesized inducible Hsps translocate to other cell compartments immediately upon synthesis at the ribosomal site. The role of stress-mediated translocation of Hsps is poorly understood but seems to be pivotal in the protection by these proteins (201, 284). Probably best studied is the stress-mediated translocation of both Hsc70 and Hsp70 into the cellular nucleus, in particular to the nucleolus (403, 432). Upon heat shock, translocation occurs within 60 min and terminates ~3 h later, the time at which the highest content of these proteins is reached (308).

The cellular stress response, especially as a consequence of heating, has been extensively studied in the past 20 years, and excellently reviewed in 1992 by Welch (429). It is well known that heat shock negatively affects the organization of several functional structures in the cell. The Golgi apparatus is disrupted and fragmented. Mitochondria swell and cristae packing changes. Striking alterations occur in the cytoskeleton, more specifically in intermediate filaments. The microtubular network is not affected, but intermediate filaments aggregate to form a tight perinuclear network. This phenomenon, however, is reversible since normal distribution is gradually reappearing after the stress is relieved (435). Within the nucleus, rod-shaped bodies occur consisting of actin filaments. Nucleoli look less condensed, the number and size of the granular ribonucleoprotein components are changed, and the nuclear fibrillar reticulum is reorganized (433). Upon stress the most prominent Hsps present in the nucleolus are the inducible Hsp70 and Hsp110 (433).

The fact that some Hsps translocate to the nucleolus suggests a specific and unique role in the repair and protection of these cellular structures (67). The immediate translocation of newly synthesized Hsp70 into the cell nucleus and nucleolus occurs in various cell types and tissues, including the heart (49, 65, 313, 365, 383, 415, 432). Nuclear Hsp70 accumulation occurs in close vicinity of the preribosomal-containing granular region (434). Normally soluble proteins such as topoisomerases I and II and DNA polymerases α and β become more tightly bound after heat shock and coisolate with the nuclear matrix (45). In such precipitates large amounts of Hsp70 can be found, suggesting binding to matrix-precipitated proteins (65, 412, 427). Recently, the consequence of inhibition of translocation of Hsp70 to the nucleolus has been investigated in more detail. Knowlton (201) mutated the Hsp70 nuclear targeting sequence and investigated nucleolar translocation and cellular viability after heat shock. A relatively small mutation of only six amino acids mitigated nuclear Hsp70 accumulation, but surprisingly did not affect the protection of cellular viability following a severe heat shock. Only when a second mutation was induced, leading to loss of Hsp70 ATPase activity, protection of viability was completely lost. Unfortunately, no definite conclusions can be drawn concerning interactions of both protein regions, because viability was not tested in cells transfected with only mutation of the ATP-binding region. These results suggest that nuclear Hsp70 translocation is not the sole factor responsible for proper cellular protection. Hsps, like Hsc70 (436) and the sHsps (see below), that redistribute to the nucleus have also been considered to play a role in the protection of nucleolar protein structures (65, 412, 427). Hsc70 was found to associate with topoisomerase I and refolded this nuclear protein upon cessation of heat stress. For proper refolding, however, Hsc70 was likely assisted by other proteins, because in vitro refolding with purified Hsc70 alone were not successful (65, 224).

Upon recovery after heat shock, both Hsp70 and Hsc70 exit the nucleolus to accumulate back in the cytoplasm, more specifically in the perinuclear region, along the perimeter of the cell, and in association with large cytosolic phase-dense structures (432, 434). Perinuclear condensation of Hsp70 seems to coincide with reassembly of the centrosome and microtubuli, and also with the cytoplasmic distribution of ribosomes. This suggests that Hsp70 plays a crucial role in the function of these organelles immediately after heat shock and during the subsequent recovery phase (48, 434).

A particular role in the protection of cells against various stresses is played by the constitutively present sHsps, i.e., Hsp27 and αB-crystallin. Both proteins are phosphorylated upon heat stress and associate with structural proteins in sarcomeres, cytoskeleton, and nucleus (17, 196, 410). In sarcomeres, Hsp27 colocalizes with actin in the I band as shown in isolated hyperthermically perfused rat hearts and cultured cardiomyocytes (153). It has been suggested that association with Hsp27 increases the resistance against oxidative stress-induced actin fragmentation and cell death (171). In rat hearts after ischemia, Barbato et al. (24) found that the other sHsp, i.e., αB-crystallin, bound to the cytosolic proteins troponin T and troponin I. The trigger for this binding seems to be the evolving acidosis in the ischemic heart. This interaction may affect postischemic myocardial contractility (24). It cannot be excluded that αB-crystallin also binds to the actin and desmin filaments and prevents their aggregation during and after an ischemic insult (36). These two sHsps can also govern refolding of other proteins. Under in vitro circumstances heat shock- or urea-mediated denaturation of citrate synthase and α-glucosidase could be completely prevented by adding Hsp27 or αB-crystallin (179).

As pointed out above, proteins that lose their normal three-dimensional conformation provoke Hsp synthesis through the activation of HSF1 (13). During and after heat

shock, cytosolic proteins normally aggregate and have a reduced solubility (101, 416). Transient binding to unfolding proteins is caused by the high affinity of Hsps for hydrophobic sites, which are normally covered inside the protein core (101, 317, 319). For the dissociation of Hsp70 from unfolded proteins, ATP is indispensable, whereas high concentrations of ADP or inorganic phosphate slow down the rate of dissocation and increase the rate of Hsp70-protein complex formation and stability of this complex (318).

Although under nonstressed conditions Hsp90 already comprises 1–2% of the total protein content, its synthesis is further stimulated upon heat shock (215). Normally Hsp90 is diffusely distributed in both cytoplasm and nucleus. After heat shock a transient translocation of Hsp90 to the nucleus occurs, reaching a plateau after 15 h (5). In this case, Hsp90 binds to and stabilizes unfolding proteins (180). There are indications that Hsp90 is also involved in thermotolerance, because artificial reduction of the cellular Hsp90 content is associated with lower survival at high temperatures (23).

In samples of mouse liver and brain and in cultured cells it has been shown that Hsp110 is closely associated with the nucleolus under control circumstances. Interestingly, treatment of the cells with ribonuclease results in the loss of the Hsp110 from the nucleus, indicating binding of Hsp110 to RNA, either directly or indirectly via another protein or protein complex (382).

In conclusion, Hsp chaperoning is a permanent cellular event during both nonstressed and stressed conditions. The role of Hsc70, Hsp90, Hsp40, and the sHsps is relatively well understood during nonstressed conditions. However, upon heat shock or other stresses, upregulation of the synthesis and translocation of the various Hsps to other cellular compartments suggest that during evolution tissues developed intrinsic defense mechanisms for rescuing unfolding proteins in various cellular compartments. Further investigations are needed to elucidate the precise role of each of the involved Hsps during and after stress in the various cell organelles.

V. HEAT SHOCK PROTEINS IN THE CARDIOVASCULAR SYSTEM

To date, acute and chronic ischemic heart disease is one of the major causes of death among people in the Western world, despite numerous exogenous pharmacological protective measures like calcium antagonists, coronary vasodilators, and blocking agents of the angiotensin converting enzyme and β-adrenoreceptors. Especially during ischemic disease, the heart could benefit from protective measures from an endogenous source. Upregulation of the synthesis of Hsps is one such phenomenon leading to improved tolerance to ischemia in experimental models. In humans, however, investigations regarding the protective potential are in their infancy.

A. Constitutive and Inducible Cardiac Synthesis of Hsps

In both the vascular and cardiac compartment, heat shock proteins are present and can be induced by specific stressors. The type of proteins expressed in the vascular compartment is somewhat different from that expressed in the heart. In nonstressed, adult mice such Hsps as Hsp27, Hsc70, Hsp70, and Hsp84 are constitutively expressed in a variety of tissues, including the heart (392). In the heart of this species these four Hsps are clearly present, but at low levels compared with other tissues. In contrast, in the adult rabbit, cardiac Hsp70 levels are similar to those in other tissues (253; see Table 4). In unstressed rats the heart contains relatively high αB-crystallin levels, whereas intermediate levels are found for Hsp27 (Table 5). In both rat and human heart, Hsp27 can be found in endothelial cells, smooth muscle cells, and cardiomyocytes, whereas αB-crystallin is only present in cardiomyocytes (247). Although specific investigations of the various HSFs in the heart are scarce, it has been shown that HSF1, HSF2, and HSF4 are present in cardiac tissue (128, 302).

In rabbits after whole body heat shock, cardiac Hsp70 concentrations are much higher than in neural tissues, but still lower than in other, nonneural tissues as liver and kidney (253). As shown in Table 4, in heat-

TABLE 4. *Analysis of Hsc70 and Hsp70 levels in various tissues of rabbit and rat*

	Constitutive Content (Rabbit)		Enhanced Content	
Animal Tissue	Hsc70	Hsp70	Rabbit (Hsp70 5 h after HS)	Rat (Hsp70 24 h after HS)
Retina	281	27	32	ND
Cerebral hemisphere	325	5	6	ND
Brain core	345	10	1	0.6–1
Cerebellum	322	20	32	ND
Brain stem	313	10	6	ND
Kidney	119	17	140	31–39
Liver	100	1	100	100
Lung	ND	ND	ND	11–35
Heart	58	7	90	3–5
Skeletal muscle	103	3	55	37–49
Colon	ND	ND	ND	81–132
Spleen	123	2	74	46–53

Values found in control and heat-shocked tissues were recalculated to the reference value in the liver. For calculation of the rabbit basal Hsp70 levels, the liver values were set at 1. Data of the rabbit are presented as mean values, whereas those of the rat are shown as percentage range. Data were recalculated from densitometric analysis of Western blots for the rabbit (253) and for the rat (27). ND, not determined.

TABLE 5. *Hsp27 and αB-crystallin concentrations in various rat organs at various time points after heat stress*

Tissue	Hours After Heating	Hsp27, ng/mg protein	αB-Crystallin, ng/mg protein
Cerebral cortex	Control	86 ± 2	14 ± 1
	0	75 ± 7	12 ± 2
	8	186 ± 19	21 ± 2
	16	187 ± 12	14 ± 2
Liver	Control	146 ± 3	0.8 ± 0.4
	0	95 ± 6	0.5 ± 0.1
	8	969 ± 240	3.8 ± 1.3
	16	3,090 ± 180	15 ± 1.6
Kidney	Control	123 ± 8	190 ± 16
	0	119 ± 4	253 ± 59
	8	274 ± 39	240 ± 24
	16	190 ± 22	137 ± 24
Lung	Control	2,140 ± 160	70 ± 10
	0	1,250 ± 220	35 ± 10
	8	2,590 ± 360	66 ± 10
	16	3,140 ± 430	101 ± 21
Heart	Control	693 ± 42	3,610 ± 240
	0	614 ± 71	2,820 ± 670
	8	1,160 ± 86	3,600 ± 630
	16	1,710 ± 90	4,390 ± 120
Adrenal	Control	1,310 ± 40	2.5 ± 0.4
	0	1,180 ± 140	2.4 ± 0.6
	8	3,980 ± 380	40.7 ± 12.6
	16	6,000 ± 410	109 ± 18

Values are means ± SE.

shocked rats the Hsp70 content is significantly lower in heart and brain than in colon, liver, kidney, and spleen (27). In these tissues, stress-induced synthesis of new Hsps occurs very rapidly. Within minutes Hsp70 mRNA transcripts are present (84), whereas protein accumulation reaches its maximum at ~12 h after stress induction (70, 329). In the later time domain, the cardiac Hsp70 content slowly decreases but remains detectable up to 192 h after the initial stimulus (190). Immunohistologically, Hsp70 was found to be present in the nucleus of cardiomyocytes, in fibroblasts, and in endothelial cells in the coronary vessel wall within 3 h after stress induction (365). HSF1 was found to be activated upon an ischemic insult, leading to prominent mRNA signals coding for Hsp70 and Hsp90. Under these circumstances, however, HSF2 is not activated (309).

In the adult nonstressed heart, αB-crystallin is constitutively abundant (198, 278) and comprises 1–3% of total soluble protein (192). The protein can be found in high concentrations in the conduction system (222). Inaguma et al. (174) compared the levels of αB-crystallin and Hsp27 in various rat tissues, including the heart during the first 16 h after heat shock (Table 5). The striking finding was the great variability of the tissue Hsp concentrations and the differences between the αB-crystallin and Hsp27 concentrations within the same tissue. In the heart, Hsp27 concentrations double during the first 16 h after heat shock, whereas αB-crystallin increases by 20%.

Although constitutive expression of the various Hsps has been well documented in the adult heart, only limited information is available on the regulation of the constitutive expression and concentrations of Hsps in the embryonic, newborn, and developing heart. Only recently have expression patterns of Hsp70 and Hsc70 in the immature ovine myocardium during the perinatal transition phase and the juvenile phase been described. In the heart, the Hsp70 synthesis seems to be developmentally regulated in both left and right ventricles. In the fetal heart very low Hsp70 levels are found. These levels, however, increase upon development and peak after the first 2 wk after birth. In contrast, the Hsc70 protein contents remain unchanged during left ventricular development, whereas they decrease with age in the right ventricle (378).

αB-Crystallin plays an exceptional role in normal cardiac development, activating genetic programs responsible for cardiac morphogenesis. As early as 8.5 days postconception, αB-crystallin can be detected in the mouse heart and is uniformly distributed in atria and ventricles. In the endothelial cushion, pulmonary trunk, aorta, and endothelium, however, the protein seems to be absent (35).

B. Protection in the Vascular Compartment by Hsps

Upon exposure to environmental stress, all cell types in the blood vessel wall respond with the synthesis of Hsps (11, 152, 364). Aside from heat (207), vascular Hsp synthesis is induced by such triggers as circulating hormones (288, 443), reactive oxygen species (ROS) (245), and sodium arsenite (426). Nitric oxide (NO) is probably involved in heat shock-mediated Hsp70 synthesis in blood vessels, because the NO synthase (NOS) inhibitor N^{ω}-nitro-L-arginine (L-NNA) also inhibits Hsp70-gene transcription. The signal transduction pathway for the activation of Hsp70-gene expression through NO is still unclear, because either increased calcium influx or heat shock-mediated production of ROS could be involved, respectively activating the constitutive NOS and the inducible NOS pathway (251).

In the aorta, as in cardiomyocytes, two isozymes of Hsp32, i.e., heme-oxygenase-1 (HO-1) and HO-2 are constitutively synthesized (1, 114, 314). HO-1 is involved in the degradation of heme to biliverdin, iron, and carbon monoxide. In the aorta, aside from hypoxia and heat, HO-1-gene transcription can be activated rapidly by severe physical stress (112), hemin, hydrogen peroxide (H_2O_2), heavy metals (76, 113, 266), and postischemic myocardial reperfusion (263). Interestingly, enhanced HO-1 synthesis is associated with significant elevation of

intracellular cGMP levels so that smooth muscle cell relaxation and concomitant vasodilation occur (63, 114). Postischemic HO-1 synthesis can be blocked by the addition of such scavenging enzymes as superoxide dismutase and catalase to the perfusate. Inversely, when enhanced concentrations of HO-1 are present in the vascular wall, free radical formation upon severe stress is reduced, pointing to its protective role against oxidative stress-mediated vascular cell damage.

In 1993, Amrani et al. (12) discovered that some essential functions in rat coronary artery endothelial cells are protected against ischemia and reperfusion after preceding whole body heat shock, associated with enhanced Hsp70 levels. In these hearts, endothelial cell-mediated vasodilation elicited through 5-hydroxytryptamine infusion is completely preserved during and after a 4-h lasting ischemic insult combined with intracoronary cardioplegia. The same authors found that protection of the endothelial cell-mediated vasodilation is completely abolished by blocking catalase activity through 3-amino-1,2,4-triazole (3-AT). Later these investigators proposed that the improved postischemic recovery of cardiac mechanical function depends exclusively on the protection by Hsp70 of the coronary endothelium because protection of postischemic cardiac function could be abolished by removing endothelial cells by saponin (11). It can be argued as to whether this hypothesis is valid, because saponin may be so damaging to the coronary vessels that this in itself alters the response to ischemia.

In the endothelium, heat shock-mediated Hsp27 phosphorylation is probably also involved in the protection of specific intracellular structures against environmental stresses. In these cells, metabolic inhibition through a combination of glucose depletion and rotenone addition leads to early breakdown of such cytoskeletal structures as F-actin. A preceding heat shock-mediated increased Hsp27 phosphorylation leads to an improved stability of cytoskeletal F-actin and a better preserved ATP concentration. When dephosphorylation of Hsp27 is prevented through addition of such phosphatase inhibitors as okadaic acid, cantharidin, or sodium orthovanadate, the metabolic block-associated degradation of cytoskeletal F-actin is also prevented, pointing to the stabilizing effect of this particular heat shock protein on cytoskeletal structures (241).

C. Protection of the Heart by Hsps

1. Protection of cultured cardiomyocytes and fibroblasts

To demonstrate the unique protective characteristics of Hsps on the cardiac tissue, numerous studies have been performed on its composing cells in culture. Several cell types have been investigated, from freshly isolated neonatal or adult cardiomyocytes to such cell lines as myogenic C_2C12 (33) and H9c2 cells (197) and fibroblasts (432, 434). Studies performed on isolated cells should be interpreted with caution, because factors like the unnatural cell environment, the number of cell passages, the degree of culture confluency, and the chemical composition of the bathing medium may influence the outcome of the studies. It is known for instance that extracellular matrix proteins, like collagen, attenuate both the constitutive and heat-induced expression of several Hsps in cultured cardiomyocytes (372).

With these shortcomings taken into account, cell culture models have been very helpful in unraveling the expression patterns and mechanism(s) of protection of Hsps. Several reports describe the existence of protection in cultured adult or neonatal cardiomyocytes against a severe, lethal stress challenge after previous activation of hsp-gene transcription. Activation of hsp70- and hsp90-gene transcription can be achieved by heat or metabolic stress (307) and of the hsp70- and HO-1-gene after exposure to the antioxidant compound ebselen (159). That hypoxia and heat stress pretreatment can lead to unexpected and opposite effects has been shown in primary cultures of neonatal cardiomyocytes (297). Hypoxia induced the synthesis of such Hsps as Hsp70, associated with a subsequent improved tolerance to lethal heat stress. In contrast, hyperthermic pretreatment leading to comparable increased Hsp70 levels is not protective against lethal heat stress. From such experiments it could be concluded that enhanced Hsp70 contents are not the sole factor explaining improved stress tolerance (315). This finding could be associated with the cell type used, because Benjamin et al. (33) showed that hypoxia and heat shock offer equipotential protection in myogenic C_2C12 cells. These investigators also showed that ATP depletion alone is sufficient to induce HSF1-DNA binding when oxidative metabolism is impaired by hypoxia (32).

Hsp-mediated protection against the bacteria-derived lipopolysaccharide (LPS) endotoxin has been investigated, among others, in the H9c2 cell. This cell line was isolated from embryonic cardiac tissue, and certain features of cardiac specificity were retained (147, 197). Experimental H9c2 results have been criticized, because of the strong H9c2 homology to skeletal muscle. However, because the potential to upregulate Hsp synthesis is retained (141, 280), they have been explored intensively for characterizing Hsp-mediated protection. Survival of H9c2 cells transfected with the Hsp70-gene is significantly better upon exposure to endotoxin. Endotoxin toxicity is mediated through NO synthesis and cytokines like TNF-α, IL-1β, and IL-6. Therefore, it is very well possible that Hsp70 blocks, in part, the deleterious effects of one of these compounds or their production (60). However, at present no conclusive evidence has been presented that this is indeed the case. Su et al. (380) used H9c2 cells to

investigate the resistance against H_2O_2 after heat pretreatment. Resistance to mild H_2O_2 toxicity already appeared in an early phase, i.e., between 10 and 14 h after heating. Full protection against moderate H_2O_2 concentrations, however, occurred only after 20–24 h (380).

The protective role of Hsps has also been investigated in cultured cells of other origin. In cultured fibroblasts, heat-induced redistribution of Hsp70 and Hsc70 from the cytosol to the cell nucleus has been described in detail by Welch and co-workers and, more recently, by Knowlton and colleagues (201, 205, 432, 434). The above-described increased tolerance toward H_2O_2 challenge depends on the heat shock-elicited Hsp70 and Hsc70 redistribution to the nucleus. Homogeneous distribution of both Hsps throughout nucleus and cytoplasm is required to obtain full protection against moderate H_2O_2 concentrations, occurring only 20–24 h after the challenge (380). This observation could imply that the antioxidant effect of the uniformly distributed Hsps is based on protection of both nuclear and cytoplasmic structures. Otherwise, it could also mean that deleterious effects of the preceding heat shock per se on cellular protein structures mask the improved defense mechanism evoked by Hsps in the early time domain.

2. Protection of the intact heart

a) TRANSIENT CARDIAC ISCHEMIA. In 1988, Currie et al. (82) for the first time reported that whole body heat shock in rats is associated with improvement of cardiac functional recovery after a global ischemic insult, applied 24 h later. A number of remarkable findings were reported: the early postischemic recovery of left ventricular contractility is significantly improved while the postischemic creatine kinase loss is significantly reduced compared with nonpretreated control hearts. Ultrastructural investigation of the heart revealed that mitochondrial morphology is better preserved. In addition, the postischemic activity of the scavenging enzyme catalase was found to be higher than in control hearts. As an indicator of the stress response, the cardiac Hsp70 concentration was significantly increased before the ischemic insult. The authors concluded that the beneficial effects of heat shock pretreatment are likely to be related to both increased radical scavenging activity and the presence of Hsp70 within the cardiac tissue. In the same model, it was shown that physical exercise 3 days before isolated heart perfusion leads to enhanced intracardiac Hsp70 levels and is associated with significantly improved functional recovery after a global ischemic insult. Moreover, the percentage recovery correlated with the degree of training intensity (240). Furthermore, it was found that the transient decay of the cardiac content of Hsp70 during the period following its heat-induced maximal accumulation coincides with the decay of ischemia tolerance (190, 446). This finding is in agreement with the disappearance of inducible Hsps and the loss of thermotolerance in whole animals (381). These and other observations led to the assumption that the degree of postischemic functional recovery correlates with the absolute cardiac Hsp70 tissue contents. Aside from later findings in transgenic animals, in isolated rat hearts it was confirmed that the degree of protection against ischemia is related to the absolute Hsp70 tissue content, that increased stepwise with heat pretreatment at 40, 41, and 42°C (173). Further support was found in isolated papillary muscles, in which the degree of postischemic mechanical recovery correlates with the Hsp70 content in the twin papillary muscle (259).

Hsp-mediated cardioprotection was also established in other mammal species. In isolated rabbit hearts, transient coronary flow reduction (60 min) is better tolerated when the animals are heat-pretreated 24 h earlier (450). The beneficial effect is demonstrated by a better recovery of diastolic and developed left ventricular pressure than in nonpretreated control hearts. Creatine kinase loss is significantly lower, and accumulation of oxidized glutathione, a marker for oxidative stress, is significantly reduced. Cardiac ATP and phosphocreatine levels were found to be better preserved. Furthermore, in intact pigs 1-h coronary artery ligation was significantly better tolerated after the injection of the body temperature-enhancing drug amphetamine 24 h earlier. Postischemic recovery of myocardial segment shortening, developed pressure, and global contractility in the left ventricle occurred significantly faster than in nonpretreated hearts. These phenomena were associated with a less prominent loss of creatine kinase and a higher activity of catalase and superoxide dismutase (262, 265).

Other stress stimuli resulting in enhanced Hsp synthesis without an increase in body temperature, but which enhance Hsp synthesis, are also associated with subsequent transient cardioprotection. An ischemic insult was better tolerated 24 h after the intraperitoneal injection of norepinephrine than in control hearts, as evidenced by a significantly improved postischemic functional recovery of left ventricular developed pressure. Both the hsp70-gene transcription and improvement of postischemic functional recovery could be blocked by the α_1-adrenoceptor blocker prazosin, but not by the β-receptor blocker propranolol. Because α_1-adrenoceptor stimulation activates PKC, it is conceivable that this protein kinase cascade may regulate the transcription of the hsp-genes, as pointed out earlier (276). This notion is further supported by the finding that the PKC inhibitor chelerythrine also blocks the beneficial effect of the delayed norepinephrine-mediated cardioprotection (286).

The report of Knowlton et al. (204) on the induction of Hsp70 synthesis by a short, nonpathological ischemic stress prompted other investigators to perform detailed

studies on ischemia tolerance after a preceding ischemic episode. In an elaborated study on isolated hearts Myrmel et al. (298) showed that the Hsp70 mRNA synthesis increased when coronary flow is gradually reduced from normal to zero flow levels. Hsp70 synthesis could be correlated with the onset of anaerobic metabolism, but not with enzyme leakage from the tissue. The authors hypothesized that anaerobic metabolism is a strong stimulus for hsp70-gene transcription, since the cessation of anaerobic metabolism is associated with complete shutdown of Hsp70 mRNA synthesis. As pointed out by other investigators, a likely candidate for stimulating HSF1-DNA binding is ATP depletion during anaerobic metabolism, which results in enhanced hsp-gene transcription (32).

Investigations of the relationship between expression patterns of Hsp70 and ischemia tolerance within the first 24 h after heat shock led to the conclusion that no significant correlation exists between both parameters in the early phase after hyperthermia. Although Hsp70 was found to accumulate to maximal tissue levels within 6 h after heat shock, improvement of postischemic functional recovery (267) as well as worsening (70) or no effect at all (329) have been reported. It should be emphasized that heat shock itself has transient but profound negative effects on such cellular structures as intermediate filaments (435) and, hence, could mask the early beneficial effects of hsp-gene expression. Furthermore, it cannot be excluded that yet unknown circulating factors produced in the vascular compartment negatively affect cardiac function during the first hours after heat pretreatment (422).

In conclusion, studies on the protective potential of Hsps on the tolerance to transient ischemia reveal that postischemic function can be significantly improved in various mammal species in the time domain beyond 24 h after the induction of Hsp synthesis. Protection has been documented in both isolated hearts and intact animals. It is possible that the presence or absence of protection in the early time domain depends on a number of unknown factors, related to the trigger applied to elicit Hsp synthesis.

B) MYOCARDIAL INFARCTION. Aside from transgenic mice (see below), rats as well as rabbits have been used to investigate the potential of Hsps to limit myocardial infarct size (99, 255). Protection against myocardial infarction as mediated by enhanced Hsp synthesis following whole body heat shock or a short ischemic episode was found to be present but transient (449). For instance, in rabbits, myocardial infarct size significantly declines when infarction is induced 24 h, but not 40 h after heat pretreatment. In addition, infarct size is only reduced when coronary occlusion is kept relatively short. Whereas a 30-min period is well tolerated, a 45-min occlusion in heat-pretreated animals results in infarct sizes comparable to those in control animals. By using a brief ischemic episode as trigger to elicit Hsp70 and Hsp60 synthesis, increased resistance against myocardial infarction was found 24 h later (255). These experiments also shed some light on the mechanism of induction of Hsp synthesis during and/or after the brief ischemic episode. It is known that infusing such ROS-generating complexes as xanthine/xanthine oxidase, irradiated rose bengal, or H_2O_2 can mimic reperfusion-associated events. Such infusions lead to enhanced Hsp70 synthesis that can be blocked only by concomitant administration of superoxide dismutase (212). It is therefore conceivable that Hsp synthesis is evoked by reperfusion rather than the brief ischemic episode itself.

It has also been questioned whether or not Hsps are synthesized in the myocardium during and after myocardial infarction. In situ hibridization on left ventricular tissue revealed accumulation of hsp70 and hsc70 mRNA signals within the ischemic zone. Upon reperfusion, both messenger signals further increased in the ischemic zone but disappeared in the central necrotic region (322). Histological investigation of rat hearts within the first week after myocardial infarction revealed normal Hsp70 but increased Hsp75 and Grp78 levels in the borderzone around the infarcted area. In the infarct center no changes were found in the contents of either of these Hsps. In contrast, in the noninfarcted septum, concentrations of all three Hsps were significantly increased, suggesting that the increased load in this region served as a stress trigger. After 2–3 wk, Hsp concentrations completely normalized in all zones (195). The consequences of these findings are that Hsps may confer some protection to nonlethally injured zones in the heart, such as the zone immediately adjacent to the area completely devoid of perfusion, but also to remote areas performing more compensatory work.

Heat shock pretreatment also has beneficial effects on myocardial stunning occurring after a short ischemic insult. Because stunning is characterized by mild lysis of contractile proteins in combination with a transient decrease of calcium sensitivity of the sarcomeres, stunned hearts show reduced contractility despite a completely normal coronary perfusion (254). In nonpretreated dogs, 3 h of reperfusion following 15 min of coronary circumflex artery occlusion is associated with a significantly reduced preload-recruitable work area and myocardial segment length change in the former ischemic region. Under identical experimental conditions, heat pretreatment 24 h earlier completely prevents these phenomena (339). In mice hearts with genetically induced high Hsp70 levels, the recovery of regional epicardial strain after a short global ischemic insult is faster and better than in Hsp70-negative mice hearts. The maximal strain recovery measured amounted to 88% of the preischemic values and to 58% in wild-type control hearts (401). Because stunning has been described to result, among others, from intra-

cellular ROS, it is conceivable that Hsps attenuate specifically these hazardous molecules through activation of scavenging enzymes (see below) (82, 289).

c) CARDIOPLEGIA. In a number of animal species, using different experimental approaches, the effects of stress pretreatment on the functional recovery following cardioplegic arrest have been investigated. In isolated rat hearts, arrested at 4°C with a cardioplegic solution during 4 h, a preceding whole body heat stress improves function upon return to normal temperatures. This beneficial effect is characterized by improved recovery of cardiac output and peak aortic pressure as well as by a better preserved response of the coronary endothelium to the vasodilatory agent 5-hydroxytryptamine (10). Pig hearts perfused with a warm (42°C) cardioplegic solution during 15 min before a 2-h hypothermic cardioplegic arrest showed significantly better recovery of developed left ventricular pressure, contractility, and segmental shortening upon reperfusion than nonpreheated control hearts. In addition, the pretreated hearts lost less creatine kinase, while the activity of superoxide dismutase (SOD) was significantly higher than in the control hearts (238). This study is one of the rare examples in which the beneficial effects of heat pretreatment can already be observed in a very short time frame after pretreatment.

d) ENDOTOXIN TOLERANCE. Hsp-mediated increased resistance toward unfavorable conditions also extends to the deleterious effects of endotoxins. Doses of intravenously administered endotoxin normally leading to death of 75% of the animals are well tolerated by heat-pretreated animals (345). Inversely, pretreatment with sublethal doses of endotoxin also improves resistance to lethal endotoxin doses, probably through enhanced Hsp synthesis (50, 60, 264, 277). The similarity between heating and endotoxemia in eliciting the heat shock response is also reflected in the inflammatory agents they liberate in the body. Both heat stroke and endotoxin shock are characterized by elevated plasma TNF-α and IL-1 concentrations (39, 46, 312). Furthermore, endotoxin also increases the activity of the scavenging enzymes copper-zinc-SOD (CuZn-SOD), manganese-SOD (Mn-SOD), glutathione peroxidase, and catalase (264). Probably Hsps mediate an increased resistance against the deleterious effects of inflammatory agents and ROS. In endotoxin-pretreated hearts, postischemic levels of malondialdehyde, a final cellular membrane degradation product, are lower than in nonpretreated hearts (264).

D. Effects on Intracellular Processes

1. Energy metabolism

One of the striking observations in heat-pretreated hearts is the better preservation of postischemic mitochondrial ultrastructure (82), suggesting that the mitochondrial oxidative phosphorylation capacity and thus its potential to synthesize high-energy phosphates is better preserved. Heat pretreatment itself has no significant effects on mitochondrial function, since tissue levels of ATP, ADP, AMP, and their degradation products are not affected after whole body heat shock (82). During postischemic reperfusion, however, significantly higher ATP and creatine phosphate tissue levels can be detected in heat-pretreated than control rabbit hearts (450). Comparably, in heat-pretreated rat neonatal cardiomyocytes during metabolic inhibition, the rate of ATP depletion and lactate accumulation occurs significantly slower than in nonpretreated cells (425). These findings, however, could not be confirmed in the intact postischemic heat-pretreated rat hearts (81). The reason for this discrepancy remains to be elucidated.

Protection of oxidative metabolism by Hsps has also been demonstrated by the finding that depolarization of the mitochondrial membrane, as elicited by H_2O_2 challenge, was completely inhibited after heat pretreatment, while the mitochondrial ultrastructure remained preserved. Interestingly, these beneficial effects correlated better with the cellular Hsp70 than the mitochondrial Hsp60 content (324). From the fact that perfusion with pyruvate and not glucose leads to a better postischemic functional outcome of heat-pretreated, isolated hearts, cannot be concluded that mitochondrial oxidation is better protected than glycolysis (259), because pyruvate itself has beneficial effects on the postischemic recovery (409).

2. Calcium homeostasis

Several investigators have addressed the question as to whether Hsp upregulation has beneficial effects on calcium homeostasis during an ischemic challenge. Already in 1988 Currie et al. (82) proposed that the postischemic mitochondrial calcium accumulation is lower in heat-pretreated hearts, which was confirmed in a later study (450). Challenging heat-pretreated rabbit hearts with a submaximal calcium paradox resulted in a better functional recovery compared with nonpretreated hearts (257). Also in papillary muscles isolated from rabbits 24 h after heat pretreatment, calcium handling is completely normal during reoxygenation after a period of hypoxia (259). Further detailed investigation of cardiomyocytes, isolated from rat hearts 24 h after heat shock, revealed that the intracellular calcium homeostasis is not disturbed at normal extracellular calcium concentrations. Diastolic intracellular free calcium concentrations were completely comparable in heat-pretreated and control cardiomyocytes. Calcium sensitivity of the myofilaments was normal at physiological extracellular calcium concentrations. Also challenging these cells with high extracellular calcium levels did not affect contractile function. However,

upon recovery to normal calcium levels, diastolic cell length recovered earlier and better in the heat-pretreated cardiomyocytes (73).

3. Electrical stability

In a number of studies, it has been shown that heat stress pretreatment or the selective induction of such Hsps as Hsp70 before an ischemic insult has beneficial effects on the occurrence of postischemic arrhythmias. In intact rats subjected to a temporary coronary occlusion as well as in isolated rat hearts subjected to transient ischemia, the incidence of postischemic arrhythmias and of ventricular fibrillation is significantly reduced after heat pretreatment (72, 373). In a more recent study, in which heat-pretreated rats were subjected to myocardial infarction at various time points after heat pretreatment, a biphasic reduction of ventricular fibrillation was found reaching peak reductions at 30 min and 72 h after pretreatment. In the same time domains, myocardial infarct size was significantly reduced compared with nonpretreated controls (447). The beneficial effect on rhythm disturbances is likely to be a secondary effect, resulting from smaller infarcts, and is associated with a better and more homogeneous perfusion of the remaining healthy tissue.

4. Lipid metabolism

The role of Hsps in reducing the ischemia-associated degradation of membrane lipids has as yet not been investigated in detail, although many observations point to increased membrane stability after stress pretreatment. In the isolated heat-pretreated rat heart during postischemic reperfusion, the release of arachidonic acid, being a sensitive marker of membrane phospholipid degradation, has been found to be significantly attenuated (411). Furthermore, the reduction of arachidonic acid accumulation in the heat-pretreated heart is a phenomenon occurring only during postischemic reperfusion and not during the ischemic episode, suggesting that membrane stabilization during reperfusion is a key process in heat shock-mediated cardioprotection (72a).

A major part of evidence for an Hsp-associated improved membrane stability comes from experiments performed on noncardiac cell types. For instance, in Hsp70-overexpressing murine fibrosarcoma cells, it has been reported that phospholipase A_2 (PLA_2) is less activated upon exposure to TNF-α, resulting in a decreased accumulation of arachidonic acid, the major product of cellular membrane degradation (178). Interestingly, the general activating inflammatory responses to tissue injury through the action of arachidonic acid and its metabolites also leads to accumulation of Hsp70 mRNA molecules (188). Because local temperatures increase in inflammation, it is difficult to determine the precise role of the degradation products of membrane breakdown. Therefore, attempts have been made to treat cells with arachidonic acid only. Exposure to arachidonic acid induces hsp-gene transcription in a dose-dependent manner (188). Even more interestingly, the combination of inactive low doses of arachidonic acid and slightly increased temperatures, which do not activate the heat shock response themselves, synergistically activate HSF1-DNA binding. This suggests that combining a temperature rise in the physiological range with a component involved in local inflammatory processes can provoke the heat shock response. However, to date, no experimental evidence has been presented that supports the existence of such a mechanism in the heart.

5. Apoptosis

Apoptosis is a consequence of a cascade of biochemical events, is encountered in all tissues and, characterized by blebbing of the plasmalemma, volume loss, condensation of the nucleus and lytic DNA degradation, ultimately leads to the programmed death of the cell. A key role in this process is played by the caspases, enzymes that cleave substrates once activated.

Enhanced tissue levels of Hsps have been reported to inhibit intentionally induced apoptosis. For instance, in Hsp70-overexpressing pulmonary endothelial cells, endotoxin-induced apoptosis is attenuated (437). In cardiomyocytes, isolated from Hsp70-overexpressing hearts, less apoptosis was found after hypoxia-reoxygenation than in control cells (384). When high cellular Hsp70 levels have been induced beforehand, the deleterious effects of Fas-mediated activation of the apoptotic signaling pathway can be inhibited (355). A preceding heat shock-elicited synthesis of Hsp70 and Hsp27 protects against apoptosis (274). This is probably due to an interaction of Hsp70 at the level of the SAPK/JNK signaling pathway (120) and to the blocking of the conversion of procaspase-3 to active caspase 3 (294).

Obviously, competition between signaling pathways occurs when both the apoptotic signaling pathway and the hsp-gene transcription pathway are activated. Pro-apoptotic signals, like activation of the membrane-bound protein Fas, reduce HSF1-DNA binding upon heat shock as a result of absent HSF1 hyperphosphorylation. Furthermore, caspase-1 inhibitors block the inhibitory effect of activated Fas on the activation of hsp-gene transcription. This indicates that Hsp synthesis and induction of apoptosis are mutually inhibitory events within the same cell. More evidence for this hypothesis is provided by the observation that prevention of hsp-gene activation through antioxidant drugs is associated with increased thermal sensitivity and caspase activity, indicating that these cells are apoptotic (130).

6. Scavenging activity

Many studies point to increased scavenging enzyme activity after activation of hsp-gene expression, involving such intracellular enzymes as catalase, SOD, and glutathione peroxidase (31, 82, 264). Enhanced Hsp synthesis modifies the activity of scavenging enzymes more likely via posttranslational modifications rather than by stimulating the synthesis of those scavenging enzymes (80, 373).

Slight differences in the identity of the enzymes activated upon stimulation of hsp-gene transcription have been reported. In hearts isolated from rats pretreated with endotoxin 24 h earlier, a significantly improved postischemic functional recovery has been established, which is associated with increased activity of catalase, but not SOD, glutathione peroxidase, and glutathione reductase (50). In contrast, SOD activity increases in hearts of anesthetized pigs, pretreated during 2 h of warm cardioplegic perfusion. This increase is associated with enhanced Hsp70 tissue levels (238).

In a number of studies investigators attempted to block selectively the activity of the scavenging enzymes to elucidate their specific role in heat shock-mediated cardioprotection. Administration of 3-AT before hyperthermic pretreatment blocks catalase activity and inhibits cardioprotection during a subsequent ischemic insult (12). These observations strongly suggest that the increased catalase activity upon heat shock is responsible for cardioprotection (82, 423). This hypothesis, however, has been contested, because in several studies 3-AT administration to heat-pretreated hearts did not nullify improvement of postischemic functional recovery (289, 373) or the reduction of postischemic infarct size (20). One has to realize, however, that 3-AT seems not to be a specific inhibitor of catalase activity. Administration of this drug to nonheated hearts reduces lipid radical release (289) and suppresses ventricular fibrillation during postischemic reperfusion of the isolated rat heart (373). Moreover, not in all cases, enhanced catalase activity after heat shock is associated with cardioprotection. Isolated heat-pretreated rat hearts challenged with an infusion of H_2O_2 do not recover better than control hearts, despite a significantly increased catalase activity (374). These controversial findings could be explained by different compartmentalization of radicals and scavenging enzymes in the tissue. Within the cardiomyocyte catalase is probably compartmentalized in the mitochondrion (398) and therefore unable to prevent damage to the sarcolemma caused by exogenously administered H_2O_2.

In a recent study, heat-pretreated rats subjected to myocardial infarction showed a significantly reduced infarct size in conjunction with increased Mn-SOD activity. In this case the enhanced activity was paralleled by an elevated Mn-SOD content. Administration of the antioxidant *N*-2-mercaptopropionyl glycine before heat pretreatment completely abolished both the cardioprotective effect and the rise in Mn-SOD activity (447). This observation again points to the difficulties of interpreting results of exogenous antioxidant treatment. It is feasible that administered antioxidants are restricted to the interstitial compartment and thus do not reach the intracellular space to protect intracellular proteins against ROS.

From all these studies it may be concluded that the results obtained regarding the pivotal role of catalase and SOD in cardioprotection after heat shock are conflicting and that the precise role of these and other scavenging enzymes in heat shock-mediated cardioprotection remains to be elucidated.

E. Hsps and Ischemic Preconditioning

A completely different route for activating endogenous cardiac protective mechanisms not related to Hsps is the so-called ischemic ("classic") preconditioning. Several authors have reviewed this phenomenon and its putative mechanisms (26, 296, 320, 335). Essentially different from the protection observed in hearts with enhanced Hsps synthesis is the early time domain in which protection occurs. Whereas in general Hsp-mediated cardioprotection does not occur before 24 h after pretreatment, the ischemic preconditioning-associated protection occurs within the first hours after the ischemic trigger. Although it has been reported that ischemic preconditioning rapidly increases the synthesis of new Hsps and leads to nuclear redistribution of already present Hsps (203, 359), involvement of Hsps in early protection by ischemic preconditioning is as yet unclear. Experiments in which nuclear localization of Hsps is influenced by mutating their NLS could shed more light on the role of Hsps in early protection after ischemic preconditioning. However, recently it has been shown that the sHsps αB-crystallin and Hsp27 translocate to the myofilament/cytoskeletal compartment during ischemic preconditioning. Additional phosphorylation of αB-crystallin on Ser-59 seems to be indispensible for the early protection after ischemic preconditioning (103).

Another key observation linking both endogenous protective mechanisms to each other is the appearance of a so-called "second window of protection," occurring at a typical time lag of 24 h after the ischemic stimuli (391). Typical for this event is the lack of cardioprotection in the period between the first protective period and the appearance of the second one. The molecular changes during the first 24 h after classical ischemic preconditioning are complex and involve changes in protein activity and transcriptional activity, among which the enhanced transcription of such genes as the Hsp70-gene (89, 142, 255). Hsp70 is synthesized within 3 h after the ischemic stimulus,

predominantly in cardiac muscle cells (390). Using gel-mobility shift assay, it was shown that repetitive ischemic stimuli lead to concomitant HSF1 activation. ROS probably play a key role in this activation, because exogenous allopurinol or catalase block HSF1 activation (310). This hypothesis is further supported by the finding that the activity of Mn-SOD changes in a biphasic pattern completely parallel to the cardioprotection profile (160). In addition to Mn-SOD increased activities of endogenous catalase, glutathione peroxidase and glutathione reductase have been documented upon ischemic preconditioning (89).

The prolonged duration of protection makes the second window of protection particularly relevant for its application under clinical conditions. Delayed cardioprotection after ischemic preconditioning was first reported in 1993 (214, 255). In both open-chest rabbit and dog models 24 h after ischemic preconditioning a severe ischemic insult was better tolerated than in control hearts. These findings were confirmed in closed-chest models (448). The improved ischemia tolerance is characterized by increased Hsp70 tissue levels and a decrease in the occurrence of arrhythmias (414) and ventricular fibrillation (448). Furthermore, the accelerated recovery of contractile force is indicative of less stunning (383). Tanaka et al. (389) could not confirm these results in an open-chest rabbit heart 24 h after ischemic preconditioning and ascribed the conflicting outcome of both studies to differences in the experimental protocols and the type of anesthesia applied. Another, more recent study on rat hearts confirmed the negative results (328). It was concluded that ischemic preconditioning does not induce late protection in the rat heart despite enhanced Hsp70 levels and, therefore, should be considered a mechanism distinct from heat shock-mediated cardioprotection.

F. Hsps and Cardiac Aging

An important factor in considering the potential of Hsps to improve cardiac function during and after ischemia is the age of the tissues under investigation. Especially in the Western world, the average life span of the human population is advancing due to major efforts to improve health, extending life expectation. It cannot be denied that tissue aging changes the vulnerability to external stresses. In aged animals and aged humans, a higher vulnerability to cardiac ischemia has been documented (368, 393). It is known that overall gene transcription, mRNA translation, and protein degradation are decreased in senescent animals, whereas the number of malfunctioning proteins is increased (216). Because Hsp-mediated stress protection could be of special interest in aged individuals, several studies have been initiated to evaluate their potential in this population group. Overviews have been written by Heydari et al. (148) and Richardson and Holbrook (337) about the constitutive and stress-mediated expression of hsp-genes.

In almost all aged tissues investigated, the potential for stimulating the synthesis of Hsps upon stress is decreased. The major source of information is not cardiac tissue, but liver (149, 362), brain (41), lymphoid (316), vascular (62, 404, 405) lung, and skin tissue (115) of aged but healthy animals. Lower Hsp synthesis is also found in cultured hepatocytes, isolated from livers of aged animals (438) or in long-lasting cultures of human fibroblasts, reaching the end of their replicative life span (the so-called "cell senescence") (53, 246). Detailed investigation of the reduced heat shock-mediated Hsp70 synthesis in aged human fibroblasts and rat spleen lymphocytes shows a significant decline in HSF1 binding to the HSEs in the Hsp70-gene promoter, associated with markedly decreased Hsp70 synthesis (106, 316). The reduced HSF1-DNA binding is probably not caused by a lower HSF1 content (116) but to a lesser trigger sensitivity, because at higher temperatures or longer periods of heating HSF1 is equally activated and optimal HSF1-HSE binding is achieved (148, 235). Interestingly in the liver, the age-related decline in the induction of hsp70-gene transcription can completely be reversed by caloric restriction (149). Such data suggest that regulation of stress-mediated Hsp synthesis in aged animals is complex and does not depend exclusively on the attenuation of the HSF1-HSE binding in the promoter region of hsp-genes.

Similar to other tissues, the stress-induced synthesis of Hsps is also reduced in the aging cardiovascular system. In the blood vessel wall of aged animals, significantly less Hsp70 is synthesized upon acute hypertension than in young animals (404). Cardiac Hsp70 synthesis upon heat shock is substantially lower in healthy 18-mo-old Wistar-Kyoto rats than in young adult animals of the same strain (43, 121), a finding also observed in other rat strains (239). In our laboratory, we compared the Hsp70 synthesis in 3- and 17-mo-old Lewis rat hearts 24 h after whole body heat stress. Whereas the basal cardiac Hsp70 levels were comparable in nonstressed young and aged rats, after heat stress they were doubled in aged rats, but a fivefold increase was found in young rats. These findings are comparable to those found in heat-stressed male Fisher 344 rats of 12 and 24 mo of age (210). In a comparative study on 2- and 18-mo-old rats, a short transient ischemia-induced stimulation of hsp70-gene transcription resulted in 30% higher Hsp70 levels in the young rat hearts. By extending the ischemic period to 20 min, however, Hsp70 mRNA and protein rose to the same levels in young and aged hearts. This finding confirms that the ability to synthesize Hsps remains intact in aged hearts, although the ischemia-sensing mechanism is less sensitive (311).

In summary, it may be concluded that the heat shock response is reduced in aged animals but that full activa-

tion of the hsp-genes remains possible under certain circumstances. Probably the various HSF1 activation pathways are differentially operational in adult and aged tissues. Due to the limited Hsp70 synthesis upon mild stresses in aged hearts, the protective effects of this particular Hsp on ischemia tolerance are also reduced.

G. Hsps and Cardiac Hypertrophy

Pathological myocardial hypertrophy is acknowledged to be a major risk factor for sudden death, myocardial infarction, and heart failure (226). The pathologically hypertrophied heart is more vulnerable to ischemic damage. Sustained poor cardiac output, permanent arrhythmias, and increased loss of intracellular enzymes are commonly observed during postischemic reperfusion of hypertrophied hearts. This poor function is often associated with myocardial contracture and a substantial underperfusion of subendocardial layers of the left ventricular wall (51, 69, 366, 367). In experimental models of pathological hypertrophy, these phenomena have been observed in hearts of spontaneously hypertensive rats (SHR) as well as of thoracically or abdominally aorta-banded animals. In addition, in adult hearts with fully compensated cardiac hypertrophy, ischemia is better tolerated than in aged hypertrophied hearts, which eventually reach the phase of decompensation (368).

Understanding the potential of hypertrophied cardiac tissue to upregulate Hsp synthesis would be highly valuable, since these proteins could attenuate the poor ischemia tolerance in this type of heart. In animals, cardiac hypertrophy, once established and compensated, has no major effects on the constitutive Hsp tissue levels. Although in cardiomyocytes isolated from rat hearts 2–4 days after thoracic aortic banding Hsps like Hsp70 and Hsp60 are transiently overexpressed (92), completely normal basal Hsp levels are found when cardiac hypertrophy is fully compensated (176). In the adult SHR as well as in the adult aorta-banded rat, basal cardiac Hsp70 concentrations are comparable to those in the age-matched normotensive control (43, 71, 72, 134).

Data on the constitutive Hsp levels in failing hypertrophied hearts are scarce. In pigs with decompensating hypertrophic cardiomyopathy, very low Hsp90 levels have been found in both ventricles, especially in the intraventricular septum. These low Hsp90 levels are not found in compensated cardiomyopathic pig hearts (223). In contrast, right ventricular heart failure elicited by monocrotalin treatment of rats coincides with enhanced right ventricular Hsp70 levels (68).

In hearts of SHR, the stress-mediated hsp-gene transcription seems to be conserved, at least during the compensatory phase of the hypertrophic process (43). Moreover, after whole body heat stress in SHR hearts, the Hsp70 mRNA and protein levels are even higher than in normotensive controls (43, 134). The levels reached, however, appear to relate to the age of the animal. Juvenile heat-pretreated SHRs showed cardiac Hsp70 levels about 3 times higher than age-matched normotensive animals, and more than 10 times higher than nonheated SHRs. Applying the same stress in 18-mo-old animals, however, resulted in comparable increases in Hsp70 levels in SHRs and control hearts (43). This indicates that throughout life the ability to synthesize Hsp70 decreases significantly more in the SHR than in the normotensive control heart.

It is possible that the exaggerated heat shock response of young SHRs is merely a consequence of its disturbed thermoregulation than of an intrinsic higher Hsp synthesis potential (250). SHR breeding experiments show that the gene responsible for thermosensitivity segregates with hypertension in the F_2 generation. Furthermore, one of the loci segregating with hypertension is included in the RT1 complex, the major histocompatibility complex. Because the Hsp70-gene is also located within this complex, it is tempting to believe that the Hsp70-gene is linked to the control of the development of hypertension (133). Also in cells isolated from hypertensive humans, significantly higher Hsp70 mRNA amounts have been found upon heat stress than in cells from normotensive controls (213). This confirms that alterations in the expression of a major enviromentally controlled gene, i.e., Hsp70, may be involved in an increased stress response in hypertensive human individuals.

Cardiac expression of hsp-genes in abdominally aorta-banded animals seems to be less exaggerated than in SHRs. In hypertrophied hearts of such animals, heat shock resulted in a sevenfold increase of Hsp70 mRNA levels compared with a threefold increase in nonhypertrophied controls. Twenty-four hours after heat shock, however, completely comparable increases in Hsp70 levels were found in hypertrophied and nonhypertrophied hearts. In aged animals with long-standing cardiac hypertrophy due to aortic banding, Isoyama (175) described depressed cardiac Hsp synthesis. In our laboratory we investigated compensated hypertrophied hearts of 17-mo-old Lewis rats, aorta-banded at the age of 2 mo (71). Twenty-four hours after heat shock, cardiac Hsp70 protein levels were not increased, contrasting with a doubling of Hsp70 content in the heart of nonhypertensive controls.

Several studies have addressed the question as to whether enhanced Hsp synthesis could be cardioprotective in the hypertrophied heart. This is indeed the case, as shown in young adult hypertrophied hearts from aorta-banded animals in which postischemic functional recovery 24 h after heat shock was significantly better than in nonpretreated controls (72). In this respect it is worth noting that the reperfusion-associated subendocardial underperfusion as normally found in hypertrophied hearts

was completely normalized after heat pretreatment. Although cardioprotection has also been demonstrated after heat pretreatment in aged hypertrophied hearts, upregulation of Hsp70 synthesis seems not to be essential (71).

In conclusion, it seems that the ability of the hypertrophied heart to synthesize pivotal Hsps like Hsp70 decreases significantly with age and varies with the type of hypertrophy. Nevertheless, heat pretreatment prevents the deleterious effects of ischemia and subsequent reperfusion in this type of heart, probably effectuated through the enhanced synthesis of other Hsps than Hsp70.

H. Hsps and Cardiac Transplantation

Induction of Hsps in tissues to be transplanted has drawn the attention of some investigators, but the information available is scarce. To the best of our knowledge, effects of heat pretreatment on the functional outcome after a long-lasting hypothermic storage of cardiac tissue have been investigated only in one study (452). Heat pretreatment of such hearts resulted in a significantly improved and accelerated recovery of developed pressure and coronary flow, while the residual ATP and total energy-rich phosphates tissue content was significantly higher than in non-retreated control hearts. The loss of intracellular enzymes was also significantly reduced.

Hsp70 tissue content has been used as a marker for the risk of rejection of tissue transplants. In transplanted rat hearts in which rejection was established, high levels of Hsp70 and Hsc70 were observed (90, 275). The Hsp70 levels could be reduced by administration of the immunosuppressive agent cyclosporin, being indicative of a direct correlation between the expression of this particular Hsp and the evolution of transplant rejection (90). From such studies it is difficult to determine the precise role of high Hsp tissue concentrations. In transplanted tissues, high Hsp levels probably reflect a response to stressors like inflammation, apoptosis, and/or necrosis.

I. Genetic Manipulation of Hsps in Cardiac Tissue

Several transfection studies have been performed to address the question regarding the specific role of individual Hsps in cell cultures, cell types, and intact animals and to elucidate their mechanism of protection. To this end, the transcription of several hsp-genes has been genetically modified to achieve selective transcriptional activation or inhibition, allowing the elucidation of the potential of individual Hsps or combinations of these proteins to modify protection. In addition, these experiments allowed the differentiation between the degrees of protection afforded by individual Hsps against a particular stress. Overexpression or knock-out of the HSF1- or hsp-genes in transgenic animals allows dissection of the activation pathways and the potential beneficial role of a particular Hsp in the total body, respectively.

1. Overexpression of Hsps

Due to its putative unique protective role, the hsp70-gene has been one of the most transfected hsp-genes so far. As pointed out earlier, hsp70-overexpression in cultured fibroblasts, cardiomyocytes, or H9c2 cells increases resistance to lethal heat stress (230), hypoxia, or metabolic stress (78, 141, 280, 282, 349). Various indicators of cellular viability were found to be improved, including early recovery of the overall protein synthesis after lethal heat stress (237), which seems to be related to rapid dephosphorylation of the protein synthesis initiation factor 2α (56). Investigators in our laboratory have recently shown that the same phenomenon can be observed in H9c2 cells, infected with an adenoviral vector containing the hsp70-gene (369, 413).

Experiments with transfection of other hsp-genes revealed that Hsp90-gene overexpression in H9c2 cells is also associated with increased survival after lethal heat stress, but not after hypoxia or metabolic stress. Overexpression of the hsp60-gene does not enhance survival after one of these stresses (78, 143). Overexpression of genes, coding for sHsps, also results in improved resistance against specific cellular stresses. Landry and colleagues showed that stable overexpression of the human hsp27-gene in cultured Chinese hamster or murine cells confers permanent resistance toward lethal heat shock (218) and is characterized by improved stability of filamentous actin (220). This finding was confirmed by transfecting the Chinese hamster hsp27-gene into mouse NIH/3T3 cells (220). Overexpression of the human hsp27 and αB-crystallin-genes in cultured fibroblasts enhanced the resistance against TNF-α or H_2O_2-induced stress (273).

With regard to Hsp27, it is important to note that the higher resistance toward TNF-α or H_2O_2 is not only related to a higher Hsp27 concentration but also to its degree of phosphorylation, since overexpression of a non-phosphorylatable Hsp27 failed to exert a positive effect (171, 221). The effects of H_2O_2 are also dependent on the cell type involved. For instance, when H_2O_2 is applied to endothelial cells in comparable concentrations as in fibroblasts, unlike in fibroblasts, in endothelial cells stress fibers accumulate and vinculin is recruited around focal adhesion points. In endothelial cells Hsp27 phosphorylation occurs through activation of the p38 MAPK, leading to the activation of the MAPK-activated protein kinase-2/3. This observation points to the critical role of the phosphorylation of Hsp27 in vascular cells, which are constantly exposed to ROS. The immediate response of this specific defense system reduces the toxic effects of the oxidative stress (170).

It is known that introduction of foreign genes into neonatal as well as adult cardiomyocytes is difficult to achieve with classic transfection techniques. For this purpose, adenoviral infection is the only successful way. Various adenovirus systems have been used to infect primary cultures of neonatal and adult rat cardiomyocytes with hsp-genes. Overexpression of Hsp27 significantly protects adult but not neonatal cardiomyocytes against hypoxia, whereas both neonatal and adult cardiomyocytes transfected with the αB-crystallin gene better tolerate hypoxia than control cells (261). In both neonatal cardiomyocytes and H9c2 cells, overexpression of Hsp70 by adenoviral infection leads to increased tolerance to ischemia (282). Suzuki et al. (385) successfully infected intact rat hearts with the hsp70-gene by infusing the hemagglutinating virus of Japan (HJV) liposomes containing the human hsp70-gene into the coronary arteries. Interestingly, whereas the classical adenovirus systems do not seem to cross the endothelial barrier after infusion into coronary arteries, this construct leads to infection of cardiomyocytes throughout the left ventricular wall. The Hsp70 transfection achieved in this way is associated with a significantly improved tolerance to global ischemia.

Taken together, adenoviral infection experiments have been crucial, as transgene animals, to prove that individual Hsps can confer full protection to cardiac cells and tissue against various stresses, including ischemia and hypoxia. Future developments in viral transfection technology that will enhance transfection efficiency in tissues of intact animals will be of crucial importance for determining mechanisms of Hsp-mediated protection in larger mammals than the mouse.

2. Inhibition of Hsp synthesis

Inhibition of the synthesis of individual Hsps can be achieved by applying antisense technology, consisting of introducing specific oligonucleotides into the cell. For antisense technology, a nucleotide sequence is constructed in such a way that it can bind to the complementary sequence on the mRNA coding for the protein to be inhibited. Otherwise, the antisense cDNA can also be introduced so that upon transcription RNA synthesis moves into an antisense direction. The application of this technique for controlling the expression levels of various Hsps in mammalian tissues has been reviewed by Knowlton recently (202).

In cultured cardiomyocytes, Nakano et al. (305) showed that treatment with Hsp70 antisense molecules leads to a specific and almost complete inhibition of Hsp70 synthesis and decreased tolerance to stress compared with control cells, even when the stress is mild. In a comparable setup, antisense-mediated reduction of Hsp90 in cultured mouse cells led to reduced cellular survival upon a hyperthermic challenge (23). However, it is important to point out that titration of antisense molecules is critical, and different concentrations have to be tested to determine optimal inhibition. Even in that case, it is still possible that antisense therapy is not able to completely inhibit Hsp synthesis upon stress treatment (303).

A decline of the transcription of the genes coding for Hsp27 and αB-crystallin resulted in contrasting effects. With the use of adenoviral vectors to introduce antisense cDNA of both hsp-genes in cardiomyocytes, it was found that the loss of intracellular enzymes upon ischemia is increased. In contrast, reduction of αB-crystallin has no effect on cell viability upon severe stress (261).

A different approach has been applied by injecting specific anti-Hsp70 antibodies into fibroblasts, resulting in enhanced vulnerability to heat shock compared with cells overexpressing Hsp70 (336).

3. Transgenic animals

Several successful attempts have been made to overexpress one of the Hsps in whole animals. In heterozygous mice hearts with hsp70-gene overexpression under the β-actin promotor and human cytomegalovirus enhancer significant reductions in infarct size are found (172, 256). In isolated hearts of these mice after a transient ischemic episode a better functional recovery and less intracellular enzyme loss are observed than in hearts of wild-type animals (323). With the use of ^{31}P-NMR spectroscopy, it was shown that transgenic mice hearts overexpressing Hsp70 have a slower ischemia-associated decline of energy-rich phosphate stores than wild-type hearts. Upon reperfusion, acidosis was restored more rapidly to normal levels. In addition, it has been demonstrated that the basal overall protein translation is not affected by the overexpression of the hsp70-gene (333).

Taken together, in the near future transgene animals, in which individual or groups of hsp-genes are overexpressed or knocked out, need to be investigated in more detail. Although initially such an approach will only improve our insight into the mouse heart, it will allow us to develop new strategies in larger mammals, ultimately leading to the understanding of the protective role of individual Hsps in cardiac pathophysiology in humans.

VI. HEAT SHOCK PROTEINS AND THE HUMAN CARDIOVASCULAR SYSTEM

Investigation of the expression levels of Hsps in the human cardiovascular system, aside from the domain of atherosclerosis, has started only recently and largely has been concentrated on the measurement of Hsp tissue concentrations. The knowledge about the potential of stress-mediated upregulation of Hsp synthesis in the human heart is almost nonexistent.

A. Hsps and Atherosclerosis

In atherosclerotic plaques of human blood vessels several members of the Hsp families have been detected. The (patho)physiological significance of the pertinent presence of Hsps in those plaques is still unclear. The most plausible explanation is that they reflect the stressful condition of the cells within the developing plaques. Cell proliferation, inflammation, and chronic ischemia all are events taking part in the multifactorial process of atherosclerosis. The current knowledge about Hsps in atherosclerotic blood vessels has been reviewed by Xu and Wick (445).

Berberian et al. (38) were the first to describe the presence of high concentrations of the major inducible Hsp70 protein in the center of atherosclerotic plaques in human blood vessels. Interestingly, high concentrations of this protein are colocalized with infiltrating macrophages and are particularly localized at the border of necrotic zones in the vessel wall. Aside from Hsp70, increased concentrations of Hsp60 (157, 340) and Hsp90 and Hsp27 (183) have been found in these pathological tissues. A linear relationship between the concentration of Hsp60 and the number of infiltrating T lymphocytes has been described (7, 442). Xu et al. (444) also found coexpression of Hsp60 and the intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule, and E-selectin in endothelial cells exposed to cytokines and low-density lipoproteins, whereas others found coexpression of Hsp60 and ICAM-1 after exposure to endotoxin (358). In a great number of patients with coronary heart disease, high plasma concentrations of anti-Hsp60 antibodies have been documented (157).

The initial event eliciting enhanced Hsp synthesis in the atherosclerotic region remains a subject of discussion. One of the etiological factors of atherosclerosis, i.e., oxidized low-density lipoproteins (Ox-LDL; a cytotoxic lipoprotein), induces Hsp70 synthesis in cultured human endothelial cells (453). Because smooth muscle cells are among the cell types involved in the formation of atheroma, it is obvious to address the question whether Ox-LDL also induce Hsp70 synthesis in these cells. This is indeed the case, as has been demonstrated in cultured human femoral artery smooth muscle cells (454). However, compared with cultured human endothelial cells from umbilical veins, the degree of Hsp70 synthesis is significantly lower. Interestingly, there are indications that smooth muscle cell necrosis as a consequence of circulating toxins is inhibited by treatment of exogenously administered Hsp70 (182). Protection seems to be related to the binding of Hsp70 to the external leaflet of the sarcolemma and not to its internalization in the cell (184). Smooth muscle cell proliferation following scrape wound injury is also inhibited by a preceding heat shock leading to the expression of Hsps in these cells (364).

Another component of atherogenesis is the shear stress acting on the vascular endothelial cell. Large variations in shear stress, which is in fact the tangential component of the hemodynamic force, activate atherogenesis-related genes in the endothelium at lesion-prone sites. Among others, multiple protein kinases leading to protein phosphorylation are activated. The small Hsp27, which is expressed in endothelial cells of the vascular wall, is phosphorylated, among others, upon changes in shear stress, whereas its expression level remains unchanged (232).

B. Hsps and the Human Heart

To date, the data available on the role of Hsps in human myocardium are still scarce. In human autopsy material, incidental findings on Hsp concentrations have been reported, while occasionally Hsp concentrations have been measured in cardiac biopsies taken before, during, and after cardiac surgery. Therefore, reported findings in human cardiac tissues need to be interpreted with care. The characteristics of the tissue samples obtained during cardiac surgery, the hemodynamic circumstances, and serum hormone levels at the moment of tissue preservation should be documented in great detail, since it has been shown in experimental animals that sudden changes in these parameters can affect the synthesis of Hsps. For instance, increased hemodynamic loading through aorta banding (25, 92, 204, 365, 443) and injection of catecholamines, vasopressin, or angiotensin II (207, 288) were found to stimulate Hsp synthesis. Therefore, it cannot be excluded that the reported findings in human tissues are confounded by unknown hemodynamic, hormonal, or other factors and, therefore, do not reflect the basal levels of the Hsps studied (181, 269).

McGrath et al. (270) determined Hsp70 levels in atrial biopsies of patients undergoing cardiopulmonary bypass surgery. The first biopsy was obtained before any surgical manipulation of the heart, the second one immediately following reperfusion after releasing the aortic cross-clamp, and the last one 15 min after weaning from cardiopulmonary bypass. Invariably, all tissue samples showed relatively high Hsp70 concentrations compared with the average values found in nonstressed nonhuman mammalian hearts (270). The authors suggested that either the levels had been artificially enhanced by such peri-operative circumstances as anesthesia and/or drug therapy, or that basal Hsp70 levels are higher in human than other mammalian hearts. The latter is probably not the case, since in hearts from brain-dead patients the Hsp70 mRNA signal in atrial biopsies is significantly lower than in hypertrophied ventricular tissue of patients with tetralogy of Fallot (181). More recently, Knowlton et al. (206) investigated the expression of a number of Hsps in

both dilated and ischemic cardiomyopathy hearts from transplant recipients. Compared with nonused control donor hearts, Hsp27 levels were increased in dilated cardiomyopathic hearts, while Hsp60 levels were enhanced in both dilated and ischemic cardiomyopathic hearts. In contrast, the levels of Hsp70, Hsc70, and Hsp90 were comparable in all three groups of hearts. Furthermore, in sera of patients with dilated cardiomyopathy, significantly enhanced titers of antibodies against Hsp60, Hsp70, and Hsc70 were found (325).

Incidental findings suggest that Hsps can play a role in the maintenance of cellular integrity. For instance, in a child, a systemic mitochondrial disease leading to heart failure has been characterized by decreased mitochondrial enzyme activities and abnormal ultrastructure of the mitochondria as well as a deficiency of mitochondrial Hsp60 (4). Although the association between the phenomena could be incidental, this fatal disorder most likely results from a defective mitochondrial protein import or enzyme assembly, in which Hsp60 plays a key role (47).

Reports on attempts to induce the expression of Hsps before surgical interventions or in the course of ischemic cardiac disease are very limited. Investigation of potentially beneficial effects of inducible Hsps in the human heart is also hampered by the absence of reliable control tissues and the technical difficulty to induce the expression of Hsps, like Hsp70, in the intact human body, especially in the heart. Under experimental conditions, the most potent ways to enhance the tissue content of Hsps have been heating or the use of such toxic stimuli as endotoxin. These techniques, however, cannot be applied in humans, certainly not under surgical routine interventions in the clinical setting. Nevertheless, a limited number of data suggest that the expression level of Hsp70 in diseased human heart tissue can be modified. For instance, during cardiac surgery the Hsp70 and Hsc70 levels have been found to increase significantly before and after the obligatory brief periods of repetitive ischemia. In contrast, cardiac tissue subjected to one continuous period of ischemia does not show increased Hsp levels (386). Furthermore, studies on various types of cultured human cells reveal that they all retain the potential to enhance stress-mediated Hsp synthesis. As such, heat shock has been found to induce Hsp70 synthesis in cultured adult and aged endothelial cells from the human umbilical vein (185), in human fibroblasts (53, 246), and in erythroleukemia cells (8).

To obtain better insight into the physiological behavior of inducible Hsps during surgery, some investigators have mimicked the conditions of surgical interventions in humans in experimental animals. Twenty-four hours after amphetamine pretreatment, pigs were subjected to cardiopulmonary bypass consisting of 60 min of left coronary artery occlusion and 60 min of global hypothermic cardioplegic arrest followed by 60 of min reperfusion (262). Cardiac biopsies taken 3 h after pretreatment revealed significantly enhanced hsp27-, hsp70-, and hsp90-gene expression. Functional parameters during postischemic reperfusion were all significantly improved. Although this study demonstrated a correlation between enhanced cardiac levels of Hsps and improved cardiac function during surgical interventions, it remains questionable whether amphetamine is a suitable drug for preconditioning of the heart, since body temperatures reached uncontrollable high levels (>42°C).

In conclusion, studies on the beneficial properties of inducible Hsps in the diseased human heart are still descriptive, because of the difficulties to make the technique to express these proteins operational. Furthermore, a major problem is the rather long time lapse between the induction of Hsp synthesis and the occurrence of the beneficial effects on cardiac function during and after an ischemic insult (70, 190).

VII. CONCLUSIONS AND FUTURE PERSPECTIVES

The present knowledge of the physiological role of Hsps in healthy tissues points to a crucial role of these proteins in normal tissue growth and in chaperoning intracellular proteins. Under nonstressed conditions, the major task of chaperones, i.e., both folding of nascent proteins and their intracellular transport, is reasonably well understood. So is the activation of the hsp-gene transcription through changes in concentrations of nascent or unfolded proteins. The ability of a number of hsp-genes to upregulate their transcription immediately following the imposition of different kinds of stresses provides the cell with a unique endogenous mechanism of protecting essential proteins and to better withstand a repeated or continued stress. Specifically for the cardiovascular system, detailed understanding of the mastering of this defense mechanism could be essential to salvage cardiac tissue during repetitive ischemic periods. Several pathophysiological situations exist in which cardiac tissue could profit from this defense system, such as chronic unstable angina or coronary balloon angioplasty. But also the viability of tissues to be transplanted could profit from preconditioning by enhancing the synthesis of Hsps.

At present, four problems hamper full exploitation of the protective potential of Hsps in the human cardiovascular system. First of all, the knowledge of hsp-gene transcription and Hsp translation mechanisms in blood vessels and myocardium of human source is still limited, and Hsp tissue levels after induction are essentially unknown. Second, the signal transduction pathways leading to full activation of the various hsp-genes in the human heart and blood vessels are still incompletely understood. Knowledge of these pathways could lead to the development of well-directed synthetic drugs activating hsp-gene

transcription. Third, using the presently known Hsp synthesis initiators, protection appears at the earliest 6–12 h later, implying that such pathophysiological events as acute myocardial infarction can be excluded as a potential therapeutical target. Eventually, earlier protection could be envisaged by using new synthetic drugs that activate hsp-gene transcription without the negative effects on structural proteins of initiators like heat. For instance, promising results have been obtained with the nontoxic hydroxylamine derivative Bimoclomol that activates Hsp synthesis and is associated with protection against ischemia (419). Fourth, we still lack detailed information on the physiological and time-dependent interactions between cellular proteins and the various newly synthesized Hsps. If, for instance, scavenging enzymes play a pivotal role in the Hsp-mediated protection, measures could be taken to directly stimulate intracellular scavenging activity by site-directed activation of Hsps.

Observations in experimental animals that increased Hsp tissue levels limit the deleterious effects of ischemia and reperfusion in aged and hypertrophied hearts, legitimate optimism for therapeutical applications of Hsps in the diseased human heart. Studies in hearts of aged and/or hypertrophied transgenic animals overexpressing or lacking specific Hsps will be instrumental in understanding the mechanism and potential of protection in these pathophysiological conditions. In the same way, new developments in the technology of viral transfection, allowing stable expression of Hsps in well-specified cardiac cells, could be very helpful to extend the applicability of Hsp-mediated cardioprotection to larger mammals and to extrapolate these findings to pathophysiological conditions in human hearts.

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