# Activation of Heat Shock Protein (hsp)70 and Proto-oncogene Expression by $\alpha_1$ Adrenergic Agonists in Rat Aorta with Age

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## Abstract

**Induction of heat shock proteins (hsp) most likely is a homeostatic mechanism in response to metabolic and environmental insults. We have investigated signal transduction mechanisms involved in $\alpha_1$ adrenergic receptor stimulation of hsp70 gene expression in isolated aortas with age. We found that $\alpha_1$ adrenergic agonists directly induced hsp70 mRNA in rat aorta in vitro; the $\alpha_1$ selective antagonist prazosin blocked this effect whereas chloroethylclonidine, an antagonist which has some selectivity for $\alpha_{1B}$ receptors, was ineffective. This response was insensitive to pertussis toxin and was partially blocked by the protein kinase C inhibitor H7. Removal of extracellular calcium attenuated induction of hsp70 mRNA but not the induction of c-fos or c-myc. The induction of hsp70 mRNA by either norepinephrine or by phorbol dibutyrate was blunted in aortas from old (24–27 mo) rats whereas c-fos responses were not diminished in the older vessels. The hsp70 response to elevated temperature (42°C) was not changed with age. Activation of hsp70 expression most likely involves a pertussis toxin insensitive G protein which activates protein kinase C, and requires extracellular calcium. With age, hsp70 gene expression induced by stimulation of $\alpha_1$ adrenergic receptors is markedly attenuated, which could modify responses to stress or vascular injury with aging. (*J. Clin. Invest.* 1996. 97:2316–2323.) Key words: catecholamines • $\alpha_1$ adrenergic receptors • aging • heat shock proteins • vascular smooth muscle**

## Introduction

Acute stress leads to the specific activation of a family of highly conserved genes which encode proteins designated as "stress" or "heat shock" proteins found in most cells from bacteria, plants, and yeast to mammals. Induction of these proteins results from a variety of stressors such as elevated temperature, transient ischemia with reperfusion, exposure to heavy metals, amino acid analogues or viral infection. Multiple heat shock proteins (hsp)[1] are induced ranging in size from 10 to 110 kD with the 70-kD family (hsp70) being the most prominent in many tissues. The induction of heat shock proteins is considered to be a homeostatic defense mechanism of cells in response to metabolic and environmental insults (1). For example, expression of heat shock proteins after brief exposure to heat appears to protect cells from a subsequent traumatic event. In addition, most of the heat shock proteins are expressed constitutively in normal unstressed cells and function as molecular chaperones in protein biosynthesis to facilitate protein folding and translocation (2).

Surgical stress or the mild stress of physical restraint of the intact rat has been shown to induce hsp70 expression selectively in adrenal cortex (3) and media of blood vessels (4). The hsp70 response in adrenal cortex is dependent upon an intact hypothalamic-pituitary-adrenal axis and is mediated by adrenocorticotropin hormone (3). In adrenals, a heat shock transcription factor that binds specifically to the heat shock element has been shown to be elevated by restraint stress. The stress of restraint-induced expression of both hsp70 and hsp27 mRNA in the vasculature is under adrenergic control (4). Intravenous infusion of the adrenergic agonist phenylephrine into rats for 1 h markedly induced the expression of hsp70 mRNA in aorta which was blocked by the $\alpha_1$ selective antagonist prazosin (5). The presence of hsp70 expression in aorta during chronic stress has been shown to be associated with elevated systolic blood pressure (6). A dopamine agonist has also been shown to induce hsp70 in both adrenal cortex and aorta of the rat similar to that of the stress of restraint (7). However, unlike with stress of restraint, hypophysectomy eliminated the dopamine-induced induction of hsp70 in the aorta as well as adrenal, suggesting an effect of a pituitary hormone on dopamine-induced hsp70 mRNA in aorta. Thus, in the intact animal the expression of the different transcripts of hsp70 is complex and appears to depend upon the treatment and tissue examined.

The expression of heat shock proteins has been shown to be markedly reduced with age in both cultured cells and in vivo (8), suggesting a defective protective mechanism with aging. Induction of hsp mRNAs and protein abundance by heat and the arginine analogue canavanine are attenuated with aging in human diploid fibroblasts (9). In intact animals, hsp70 gene expression induced by the stress of restraint is also reduced with aging in rat aorta, vena cava, and adrenal glands when 6 mo and 24 mo old Fischer rats were compared (4). Heat shock protein 70 mRNA induced by a brief period of ischemia, a common clinical stress, also declined in hearts from old rats (10). The decline in heat-induced hsp70 expression in heat-stressed senescent fibroblasts has been correlated with failure to achieve significant activation of heat shock transcription factors (11). Similar findings of decreased ability of heat shock factors to bind to DNA were observed in hepatocytes from old rats exposed to elevated temperature (12) and in

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1. *Abbreviations used in this paper:* CEC, chloroethylclonidine; HSF, heat shock transcription factor; hsp, heat shock protein.

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adrenals from old Fischer rats exposed to restraint stress even though the abundance of heat shock transcription factor 1 (HSF1) in adrenal was unchanged compared to that from young rats (13).

The present experiments were done to elucidate the signal transduction pathways involved in $\alpha_1$ adrenergic induction of hsp70 gene expression in rat aorta. The isolated aorta was chosen as a model system to eliminate some of the variables present in the intact animal. In addition we evaluated the effect of age on expression of hsp70. We found that the addition of $\alpha_1$ adrenergic agonists to isolated aortas in vitro from young animals induced hsp70 mRNA which was blocked by the $\alpha_1$ antagonist prazosin. Furthermore, the induction of hsp70 mRNA by activation of $\alpha_1$ receptors was attenuated with age, mimicking the responses observed with the stress of restraint in intact animals (4, 5). We found signal transduction mechanisms involved in the activation of expression of the gene for hsp70 to be different from activation of the c-fos gene in terms of $\alpha_1$ adrenergic subtypes mediating the response, the importance of extracellular calcium, and the effect of age on gene induction by $\alpha_1$ adrenergic agonists and protein kinase C activators.

## Methods

*Materials.* [$\alpha$-$^{32}$P]dCTP (3,000 Ci/mmol), Hybond nylon filters, random primer labeling system (RPN1601), and ECL Western blotting detection reagents were obtained from Amersham Corp. (Arlington Heights, IL). Nick columns were from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). v-fos and human $\beta$-actin cDNA and human hsp70 genomic DNA were from American Type Culture Collection (Rockville, MD). v-myc genomic DNA was from Clontech Laboratories (Palo Alto, CA). Agarose was from Gibco Laboratories (Grand Island, NY); salmon sperm DNA was from Stratagene Inc. (La Jolla, CA); guanidine thiocyanate and restriction enzymes were from Boehringer Mannheim Corp. (Indianapolis, IN); formamide was from IBI (New Haven , CT). A mouse mAb that recognizes inducible hsp70 (hsp72, SPA#810) was purchased from StressGen Corp. (Victoria, Canada). A goat anti–mouse Ig conjugated to horseradish peroxidase (AMI3404) was from Biosource International (Camarillo, CA). Phenylephrine HCl, norepinephrine bitartrate, timolol maleate, propranolol HCl, pertussis toxin, H7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine), and phorbol 12,13-dibutyrate were purchased from Sigma Chemical Co. (St. Louis, MO). Chloroethylclonidine (CEC) was from Research Biochemicals Inc. (Natick, MA). Prazosin HCl was a gift from Pfizer, Inc. (New York, NY).

*Animals.* Male Sprague-Dawley rats ranging in age from 2 mo (young) to 27 mo (old) were used. The young rats were purchased directly from Bantin and Kingman (Fremont, CA). Aortas from old rats (24–27 mo) were a generous gift from Dr. Salman Azhar (Palo Alto, CA). The Sprague-Dawley rats were allowed to develop in the aging animal facility isolated from the general quarters at the Veterans Affairs Medical Center as previously described (14).

*Preparation of aortas.* Thoracic aortas were removed from Sprague-Dawley rats after decapitation. Fat, connective tissue, and blood were removed. In some experiments vessels were used for contraction studies as described below. Vessels were equilibrated for 60 min at 37°C in modified Krebs buffer (in mM: NaCl 118, KCl 4.7, $NaHCO_3$ 24.9, glucose 10, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2) aerated with 95%$O_2$/5% $CO_2$. Vessels were then incubated with different drugs as indicated. Propranolol ($10^{-6}$ M) or timolol ($10^{-6}$ M) was routinely given 20–30 min before norepinephrine to block beta adrenergic receptors.

*Measurement of smooth muscle contraction.* Aortic rings (3–4 mm) were mounted in tissue bath chambers (20 ml) containing modified Krebs buffer. The tissue was aerated with 95% $O_2$–5% $CO_2$ at 37°C. Rings were equilibrated for 90 min before exposure to drugs. Isometric force of contraction was measured with force-displacement transducers and recorded on a polygraph (Model 7D; Grass Instrument Co., Quincy, MA). Contraction responses were obtained by adding cumulative doses of norepinephrine to the rings of aorta.

*Use of CEC, pertussis toxin and removal of extracellular calcium.* In some experiments aortas were incubated in vitro with CEC ($10^{-4}$ M) for 30 min and then washed extensively as described previously to remove free drug (15). In other experiments, aortas were incubated with pertussis toxin (100 ng/ml) for 2 h before exposure to other drugs. In experiments in which extracellular calcium was removed, all vessels were initially equilibrated for 90 min with Krebs buffer containing 2.5 mM calcium. Subsequently, all vessels were washed twice with Krebs buffer without calcium but containing 2 mM EGTA. Vessels were then treated with phenylephrine or norepinephrine (in the presence of a beta blocker) in Ca-free or Ca-containing buffers.

*RNA isolation and Northern blotting.* After treatment, vessels were finely cut, homogenized by a polytron into denaturing buffer, and total RNA was extracted as described (15). Total RNA (10 μg) was fractionated by 1% agarose gel electrophoresis, and transferred to a nylon filter by capillary action. $^{32}$P labeled genomic DNA probes of human hsp70 (2.3-kb BamHI-Hind III fragment), v-fos (1.0-kb PstI fragment), v-myc (1.52-kb PstI fragment), and human β-actin (1.1-kb EcoRI fragment) were made by random primer labeling according to the manufacturer's instructions. β-actin was used to control for differences in loading. After baking or ultraviolet cross-linking, the filter was initially prehybridized in 50% formamide, 5× SSPE buffer, 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA at 42°C for 2 h and then hybridized in the same solution containing the $^{32}$P-labeled DNA probe for 16–20 h at 42°C. The filter was washed initially with 2× SSC, 0.1% SDS, and then with 0.1× SSC, 0.1% SDS at 60°C (v -fos, v-myc) or at 65°C (hsp70, β-actin), and exposed to Kodak XAR-5 film at −70°C with intensifying screen or to a phosphorimager. The autoradiograms were scanned and quantified using a laser densitometer.

*Protein extraction and Western immunoblotting.* To obtain aortic extracts, the intact vessel was finely minced and homogenized with polytron in a buffer containing 20 mM Tris HCl, 2 mM EDTA, 2.5 mM EGTA, 0.25 M sucrose, 4 μg/ml leupeptin, 10 μg/ml aprotinin, 4 μg/ml antipain, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged 30,000 *g* for 30 min, and an aliquot of the clear supernatant was diluted with 2× sample buffer (62.5 mM Tris HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.025% bromophenol blue). After boiling for 5 min, the aortic extract (25–50 μg protein) was resolved on slab gels using 4.5% acrylamide stacking gel and 5–15% gradient gel. Proteins were transferred onto Immobilon P membranes (Millipore Corp., Bedford, MA) in 10 mM CAPS, pH 11. The membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween (TBS/Tween, 20 mM Tris, pH 7.4, 137 mM NaCl, 0.2% Tween) for 4 h at 37°C. The blots were incubated overnight at 4°C with a mouse mAb that recognizes inducible hsp70 at 1:1,000 dilution in 5% milk in TBS/Tween. After washing in TBS/Tween, the membranes were incubated with horseradish peroxidase–labeled goat anti-mouse Ig secondary antibody (1:1,000) for 2 h at room temperature, and the protein-antibody complex was detected by chemiluminescence and visualized on film.

*Statistical analyses.* To normalize data for densitometer differences among experiments, data were expressed as a fraction or percentage of a specified reference value (designated as 1 or 100) within the same experiment. The one-sample *t* test (two-tailed) was then used to determine significant differences from the reference value. One-way ANOVA was used to compare multiple groups, and those groups differing from others were isolated by the Student-Newman-Keuls method in parametric tests. When normality tests failed, the nonparametric Kruskal-Wallis one way ANOVA on ranks was used in conjunction with the Dunn's method to determine the groups that differed.

## Results

*$\alpha_1$ adrenergic induction of hsp70 mRNA in aorta.* The time course for induction of hsp70 mRNA in aorta by norepinephrine ($10^{-5}$ M) is shown in the presence of the beta adrenergic antagonist propranolol ($10^{-6}$ M given 30 min earlier) (Fig. 1). The basal expression of hsp70 is very low and was variably detected using Northern blotting in the various experiments. The time of maximal abundance of hsp70 mRNA was about 2 h; this time was routinely chosen to measure hsp70 responses in subsequent experiments (Fig. 1). The $\alpha_1$ adrenergic antagonist prazosin ($10^{-6}$ M) blocked induction of hsp70 by NE (Fig. 2) indicating that the response is mediated by $\alpha_1$ adrenergic receptors. These results with isolated aorta support this system as a simplified model of responses mediated in intact rats with stress or intravenous injections of phenylephrine (4, 5).

*Effect of chloroethylclonidine, pertussis toxin, and extracellular calcium on hsp70 mRNA abundance.* To determine which subtype(s) of $\alpha_1$ adrenergic receptors were involved in NE-induced gene expression of hsp70, we tested the effects of CEC, an antagonist that irreversibly inactivates $\alpha_{1B}$ adrenergic receptors. We have found previously that CEC completely blocks $\alpha_1$ receptor–induced c-fos expression in rat aorta (15). However, we have now found that CEC does not attenuate NE-induced hsp70 mRNA (Fig. 2), suggesting that the hsp70 response was mediated by a CEC-insensitive $\alpha_1$ adrenergic subtype in aorta.

To characterize the G protein involved in hsp70 induction by NE, we incubated aortas for 2–4 h with pertussis toxin, an inhibitor which disrupts coupling between receptors and $G_{i/o}$ proteins. We found that pertussis toxin did not block hsp70 or c-fos mRNA induced by norepinephrine (Fig. 3), suggesting that the signal transduction pathway involved a pertussis toxin–insensitive G protein.

To determine the potential role of extracellular calcium in NE-induced hsp70 mRNA, calcium was removed from the bath, as described in Methods. We found hsp70 mRNA induced by $\alpha_1$ adrenergic agents, NE or phenylephrine was dramatically inhibited in the absence of extracellular calcium (Fig. 4, Table I). In contrast, c-fos responses (Fig. 4, Table I) remained intact in the presence or absence of calcium while c-myc responses (Table I) were greater in the absence of extracellular calcium. Extracellular calcium is apparently differentially required for activation of gene expression by $\alpha_1$ receptors in rat aorta.

*Effect of protein kinase C in activation of hsp70 expression.* Since $\alpha_1$ adrenergic stimulation typically enhances formation of diacylglycerol, which in turn activates protein kinase C, we

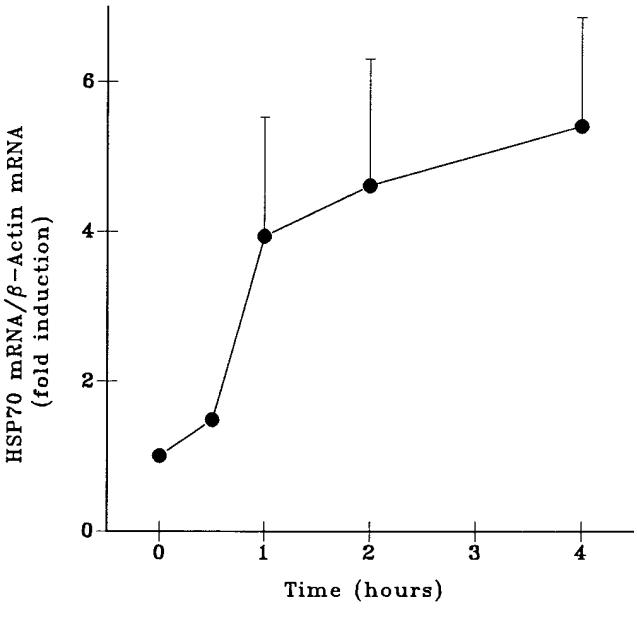


*Figure 1.* Time course of norepinephrine-induced hsp70 mRNA in isolated rat aorta. Rat aortas were incubated at 37°C for the times indicated with norepinephrine ($10^{-5}$ M) in the presence of propranolol ($10^{-6}$ M) added 30 min earlier. Data were quantitated by laser densitometry from autoradiograms of Northern blots. hsp70 mRNA densitometer bands were normalized to those of β-actin mRNA used as an internal control to correct for differences in amount loaded. Data, expressed as fold increase over basal at each time point, are mean±range of two separate experiments.

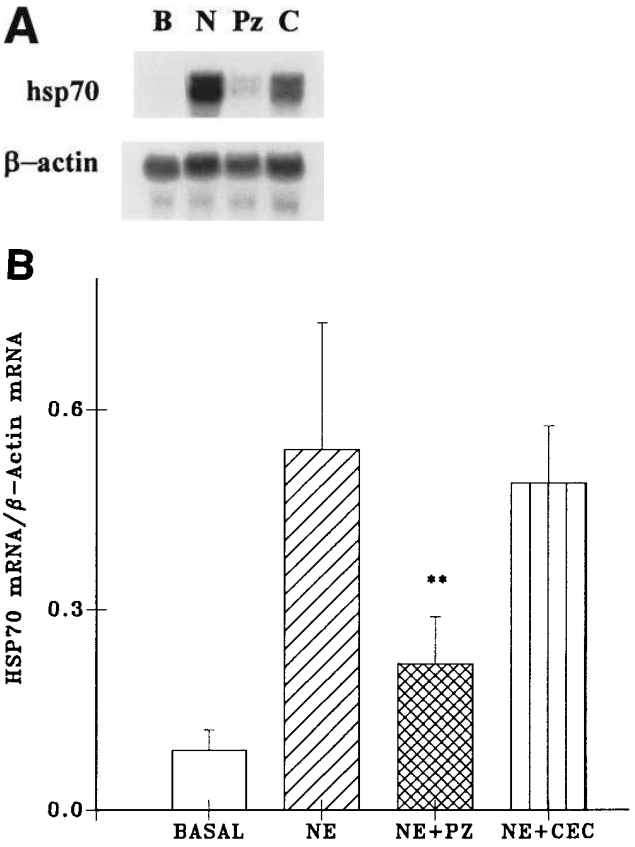


*Figure 2.* Effect of $\alpha_1$ adrenergic antagonists on norepinephrine-induced hsp70 mRNA in rat aorta. Rat aortas were stimulated for 2 h at 37°C with norepinephrine (*NE*, $10^{-5}$ M) in the presence or absence of prazosin (*Pz*) ($10^{-6}$ M). Prazosin was added 15 min before norepinephrine. In other experiments rat aortas were incubated with chloroethylclonidine (*CEC*, $10^{-4}$ M) for 30 min at 37°C. After washing four times with modified Krebs Ringer buffer, the vessels were equilibrated for 30 min and then stimulated with norepinephrine ($10^{-5}$ M) for 2 h. (*A*) Representative Northern blot of hsp70 mRNA and β-actin mRNA. *B*, basal; *N*, norepinephrine; *Pz*, prazosin and norepinephrine; *C*, chloroethylclonidine and norepinephrine. (*B*) Data were quantitated by laser densitometry from autoradiograms of Northern blots. hsp70 mRNA densitometer bands were normalized to those of β-actin mRNA used as an internal control. Data are the means±SEM of 5–8 separate experiments. $^{**}P < 0.01$, compared to NE stimulation.

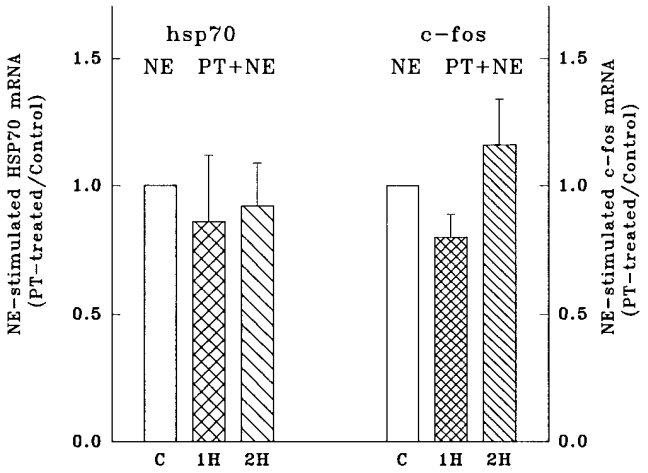


*Figure 3.* Effect of pertussis toxin on norepinephrine-induced hsp70 and c-fos mRNA in rat aorta. Rat aortas were stimulated for 1 or 2 h at 37°C with norepinephrine (*NE*, $10^{-5}$ M) in the presence or absence of pertussis toxin (*PT*) (100 ng/ml). Pertussis toxin was added 2 h before norepinephrine. The beta adrenergic blocker timolol ($10^{-6}$ M) was added 30 min before norepinephrine. Data were quantitated by laser densitometry from autoradiograms of Northern blots. hsp70 (*left*) or c-fos (*right*) mRNA densitometer bands were normalized to those of β-actin mRNA used as an internal control. Data are expressed relative to norepinephrine (*C*) and are the means±SEM of 6–15 separate experiments. *1H*, pertussis toxin pretreatment for 2 h followed by norepinephrine treatment for 1 h at time of maximal c-fos induction; *2H*, pertussis toxin pretreatment for 2 h followed by norepinephrine treatment for 2 h at time of maximal hsp70 induction.

examined the effects of a protein kinase C activator and an enzyme inhibitor on expression of the hsp70 gene. We found phorbol dibutyrate, a potent activator of protein kinase C, increased hsp70 mRNA in rat aorta to a similar extent as norepinephrine. H7, a protein kinase inhibitor, attenuated the hsp70

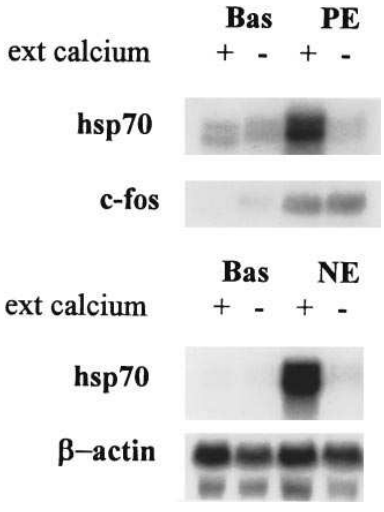


*Figure 4.* Effect of extracellular calcium on phenylephrine- and norepinephrine-induced hsp70 and c-fos mRNA in rat aorta. Rat aortas were stimulated for 1 h at 37°C with phenylephrine (*PE*) ($10^{-5}$ M) or for 2 h at 37°C with norepinephrine (*NE*) ($10^{-5}$ M) in the presence or absence of extracellular calcium as described (Methods). Representative Northern blots of phenylephrine-induced hsp70 and c-fos mRNA and norepinephrine-induced hsp70 mRNA in the presence (+) and absence (−) of extracellular calcium (*ext calcium*) are shown. Basal (*Bas*) in presence (+) or absence (−) of extracellular calcium and phenylephrine (*PE*) or norepinephrine (*NE*) in the presence (+) or absence (−) of extracellular calcium. β-actin mRNA was used as an internal control.

*Table I. Effect of Extracellular Calcium on Induction of hsp70, c-fos, and c-myc mRNA by $\alpha_1$ Adrenergic Agonists*

| | Phenylephrine | Norepinephrine |
|---|---|---|
| hsp70 mRNA | 43.9±17.0% (4)* | 9.0±1.7% (3)‡ |
| c-fos mRNA | 101±26.0% (4) | — |
| c-myc mRNA | — | 159.5±11.1% (3)* |

Phenylephrine- and norepinephrine-induced mRNA abundance in the absence of extracellular $Ca^{2+}$ (expressed as percent response in presence of extracellular $Ca^{2+}$ [100%]). Rat aortas were stimulated for 1 h at 37°C with phenylephrine ($10^{-5}$ M) or for 2 h at 37°C with norepinephrine ($10^{-5}$ M) in the presence or absence of extracellular calcium. Data quantitated by laser densitometry from autoradiograms of Northern blots were normalized to those of β-actin mRNA to correct for any differences in the amount of mRNA loaded. The ratios obtained in the absence of extracellular calcium is expressed as the percentage of that in the presence of extracellular calcium within the same experiment. Data are the means±SEM of the number of separate experiments indicated in parentheses. *$P < 0.05$ and ‡$P < 0.001$, compared to stimulation by α1 adrenergic agonist in the presence of extracellular calcium.

response to norepinephrine (Fig. 5). These results suggest that protein kinase C participates in the signal transduction of $\alpha_1$ adrenergic induction of the hsp70 gene in aorta. In control experiments the inhibitors, H7 as well as prazosin, when added alone, did not change basal hsp70 mRNA levels. Likewise, CEC and pertussis toxin alone were ineffective in inducing hsp70 mRNA.

*Effect of age on norepinephrine induction of hsp70 mRNA.* Udelsman et al. (4) demonstrated that the hsp70 mRNA response to the stress of restraint was markedly attenuated in adrenals and aortas from old rats (24 mo), suggesting decreased adaptive response to stress with aging. In the present experiments we first compared concentration-response curves for NE-induced contraction of aorta from young and old rats. We found the contraction response in older rats equivalent to that in young animals (Fig. 6). We then compared hsp70 mRNA induced by the addition of norepinephrine to aortas from young (2–3 mo) and old (24–27 mo) rats. In preliminary experiments, the effects of norepinephrine on hsp70 mRNA were not significantly different in aortas from 5-mo rats compared to that from 2-mo-old animals. In most experiments we used 2–3-mo old rats as our young group. A marked reduction in NE-stimulated mRNA for both hsp70 and c-myc was observed in aorta from aged rats compared to that from young animals (Fig. 7, Table II). In contrast, norepinephrine-induced c-fos mRNA was not altered with age (Fig. 7, Table II), showing that activation of gene expression by catecholamines was

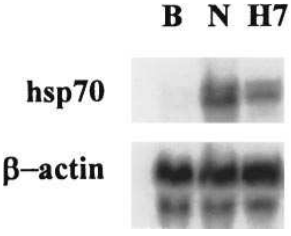


*Figure 5.* Effect of protein kinase C inhibitor on hsp70 mRNA in rat aorta. Rat aortas were stimulated for 2 h at 37°C with norepinephrine (*N*) ($10^{-5}$ M) in the presence or absence of the protein kinase C inhibitor 1-[5-isoquinolinylsulfonyl]-2 methylpiperazine (*H7*, $10^{-5}$ M). Representative Northern blot of hsp70 mRNA and β-actin mRNA used as internal standard is shown. In four experiments H7 inhibited norepinephrine-induced hsp70 about 50% ($P < 0.05$).

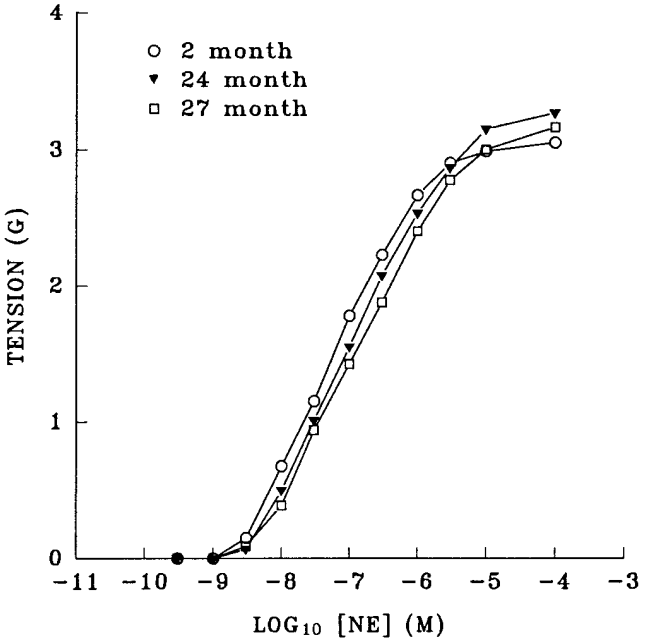


*Figure 6.* Concentration-response curves of norepinephrine-induced contraction of aortic ring segments from young (2 mo) and old (24–27 mo) Sprague-Dawley rats. Each point is the mean of 4, 6, and 4 aortic segments from individual rats 2, 24, and 27 mo old, respectively.

not nonspecifically decreased with age. In addition, the abundance of hsp70 mRNA induced by elevated temperature (42°C) was similar in aortas from young and old animals (Fig. 7, Table II) while that for c-fos tended to increase and that for c-myc decreased in the old rats (Table II). The induction of hsp70 mRNA by phorbol esters was also attenuated with aging whereas the induction of c-fos by phorbol ester was actually enhanced (Fig. 8, Table II). These data suggest that age-related impairment of gene expression depends upon not only the specific gene but also the specific inducer of the same gene.

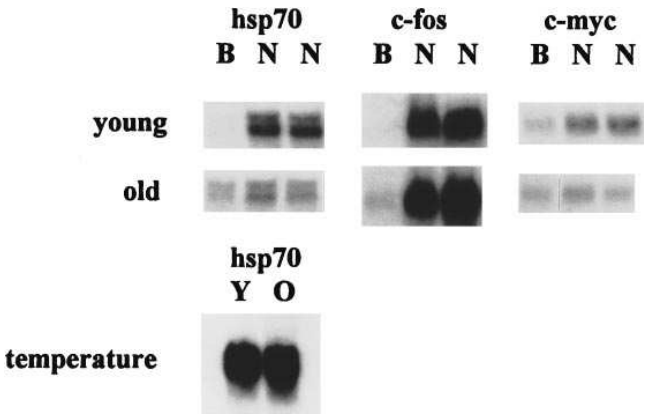


*Figure 7.* Effect of age on norepinephrine-induced hsp70, c-fos, and c-myc mRNA in rat aorta. Aortas from young (2 mo) and old (24–27 mo) Sprague Dawley rats were stimulated for 1 h (for c-fos) or 2 h (for hsp70 and c-myc) at 37°C with norepinephrine ($10^{-5}$ M) after initial block of the beta adrenergic receptor by timolol ($10^{-6}$ M) added 30 min earlier. Some aortas from young (*Y*) and old (*O*) rats were stimulated by heating at 42°C for 1 h (*temperature*). Representative Northern blots of 10 μg of aortic total RNA from individual animals are shown. *B*, basal, *N*, norepinephrine $10^{-5}$ M.

*Table II. Effect of Age on Induction of hsp70, c-fos, and c-myc mRNA by Norepinephrine, Phorbol Dibutyrate, or Heat*

| mRNA | Norepinephrine | PDB | 42°C |
|---|---|---|---|
| hsp70 | 60.8±9.1 (7)* | 48.5±5.7 (4)* | 81.7±19.6 (5) |
| c-fos | 103.3±31.0 (5) | 223.2±16.0 (4)* | 303.6±84.0 (5) |
| c-myc | 63.5±4.9 (8)‡ | 104.1±14.0 (4) | 60.3±10.8 (5)§ |

Stimulation of mRNA expression in aorta from old animals (expressed as percentage of that found in young [100%]). Aortas from young (2 mo) and old (24–27 mo) Sprague-Dawley rats were stimulated at 37°C for 1 h (c-fos) or 2 h (hsp70, c-myc) with norepinephrine ($10^{-5}$ M) or for 1 h at 37°C with phorbol dibutyrate (PDB) ($10^{-6}$ M) or for 1 h at 42°C. Data quantitated by laser densitometry from autoradiograms of Northern blots were normalized to those of β-actin mRNA to correct for differences in the amount loaded. The ratios obtained from aortas from old rats are expressed as the percentage of that from young animals within the same experiment. Data are the means±SEM of the number of separate experiments indicated in parentheses. *$P < 0.01$ compared to young; ‡$P < 0.001$ compared to young; §$P < 0.05$ compared to young.

To determine whether the attenuated hsp70 mRNA expression in aorta from old rats could be due to an autoinhibitory role of elevated hsp70 protein, we measured hsp70 protein levels using an antibody specific for the inducible form (hsp72). In Western immunoblotting experiments we found the abundance of heat shock protein constitutively present in aortas from 24-mo-old rats was ~ 35% that in aortas from 2- and 4-mo-old rats (Fig. 9). The protein abundance of hsp70, however, was not significantly different in aortas from 2- and 4-mo-old rats. In view of the barely detectable expression of basal hsp70 mRNA, it is not possible to compare those values with abundance of hsp70 at the protein level to determine whether or not there might be some correlation between the two values.

## Discussion

The induction of heat shock proteins represents a response to environmental or metabolic stress thought to be a protective or adaptive response to injury. We have shown that hsp70 mRNA can be induced in vitro in the isolated aorta by activating $\alpha_1$ adrenergic receptors by norepinephrine or phenylephrine. The agonist-induced hsp70 response is blocked by the $\alpha_1$ selective adrenergic antagonist prazosin but was insensitive to CEC, an irreversible antagonist with some selectivity for $\alpha_{1B}$ subtype of adrenergic receptors. In contrast, we previously

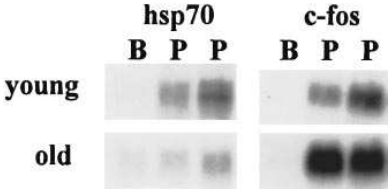


*Figure 8.* Effect of age on phorbol ester-induced hsp70 and c-fos mRNA in rat aorta. Aortas from young (2 mo) and old (24–27 mo) Sprague Dawley rats were stimulated for 1 h at 37°C with phorbol dibutyrate (*P*, $10^{-6}$ M). Representative Northern blots of 10 μg of aortic total RNA from individual animals are shown. *B*, basal, *P*, phorbol dibutyrate $10^{-6}$ M.

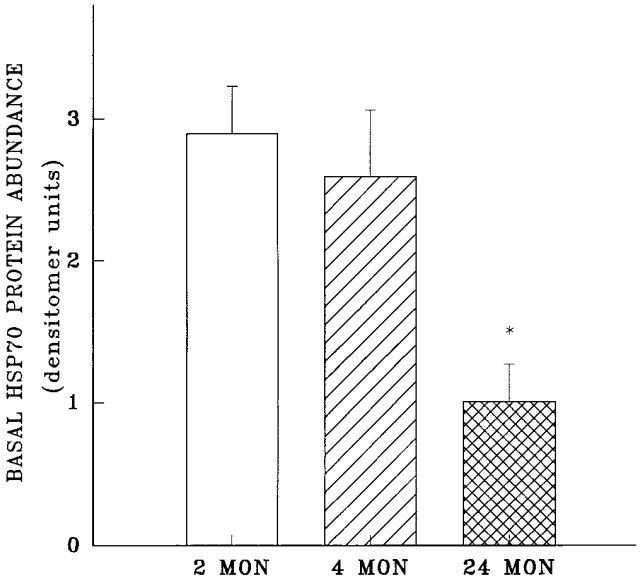


*Figure 9.* Western analysis of hsp70 protein expression in aorta. Aortic extracts from untreated animals 2, 4, and 24 months old were analyzed by Western immunoblotting using an antibody that selectively recognizes the inducible forms of the hsp70 family. Each bar represents the mean±SEM of four rats at each age group. Results were analyzed by one way ANOVA and subsequently by multiple comparison of means (Student-Newman-Keuls method). $*P < 0.05$ compared to either 2 mo or 4 mo group.

found that catecholamime activation of c-fos mRNA expression is fully blocked by both prazosin and CEC (15). Also, $\alpha_1$ agonist induction of hsp70 mRNA in aorta was attenuated by the removal of extracellular calcium while the induction of c-fos was not different in the absence or presence of extracellular calcium. Hsp70 responses to norepinephrine were insensitive to pertussis toxin but were suppressed by a protein kinase C inhibitor H7. In our model system we have found differential age-related defects in $\alpha_1$ receptor activation of gene expression. We found hsp70 mRNA induction by elevated temperature to be similar in aortas from young and old animals. However, induction of hsp70 and c-myc mRNA by norepinephrine was blunted in aortas from old rats but the c-fos response was unchanged with age. Also, the hsp70 response to the protein kinase C activator phorbol dibutyrate was attenuated with age whereas the c-fos response to this stimulus was actually enhanced.

Activation of the heat shock protein gene is mediated by heat shock transcription factor 1 (HSF1), which exists in unstressed cells in a monomeric, non–DNA binding form. Upon stress, HSF1 is converted to a trimer which binds to multiple copies of a consensus nucleotide sequence in the promoter region of heat shock genes termed the heat shock element (16, 17). Increased expression of hsp70 is associated with phosphorylation of HSF1 and is more pronounced in the presence of okadaic acid but diminished in the presence of a tyrosine phosphatase inhibitor (18–20). Thiol reducing agents have been shown to inhibit heat-induced synthesis of heat shock proteins, abundance of hsp70 mRNA, hsp70 gene promoter activity, and HSF DNA binding activity, suggesting involvement of a redox mechanism in the heat shock signal transduction pathway (21, 22).

The pathways involved in transduction of the signal from $\alpha_1$ adrenergic receptors to gene expression of hsp70 in aorta are uncertain. Three subtypes of $\alpha_1$ adrenergic receptor cDNA's have been cloned (23–26) and the International Union of Pharmacology (IUPHAR) recommends that the nomenclature of $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$ be used for the native receptors found in tissues and cells (27). $\alpha_{1A}$ receptors may play a significant role in smooth muscle contraction in rat kidney (27) and prostatic cells (28). $\alpha_{1B}$ receptors play a major role in inducing smooth muscle contraction in spleen and possibly rat aorta. The physiological function of $\alpha_{1D}$ receptors has been unclear although they are extensively expressed in vascular smooth muscle (29), and it has been designated as the receptor predominantly inducing contraction in rat aorta (27). $\alpha_{1B}$ receptors are highly sensitive to complete irreversible inactivation by CEC. CEC also has a strong capacity to inactivate $\alpha_{1D}$ receptors, at least partially, whereas $\alpha_{1A}$ receptors are much less sensitive (27). Rat aorta has been shown to express mRNAs encoding all three subtypes of receptors, as detected by sensitive RNase protection assays (15, 30).

To determine which subtype of $\alpha_1$ adrenergic receptor mediated the induction of hsp70 mRNA in aorta, we used CEC, which blocks irreversibly $\alpha_{1B}$ receptors in pharmacological experiments. CEC did not block norepinephrine induction of hsp70 mRNA suggesting that $\alpha_{1B}$ subtype does not mediate the hsp70 response. On the other hand, activation of c-fos mRNA was completely blocked by CEC (15).

We found norepinephrine induction of hsp70 mRNA in aorta to be pertussis toxin insensitive, compatible with earlier evidence that these receptors are coupled to members of the $G_q$ family (31–33). All three adrenergic receptor subtypes have been found to stimulate phosphoinositide hydrolysis via a pertussis toxin–insensitive G protein (31, 34).

In our experiments in aorta, extracellular calcium appears to be essential for phenylephrine or norepinephrine induction of expression of hsp70 but not the expression of c-fos or c-myc. In cultured rat epithelial cells from kidney proximal tubule, hsp70 mRNA induced by heat was also attenuated when extracellular calcium was reduced (35). The transcription of heat shock genes requires activation of heat shock transcription factor (HSF). In a HeLa cell-free system, treatment with calcium activated the heat shock factor in vitro in a concentration- and time-dependent manner (36). The hsp70 proteins, which associate with a number of cellular proteins, contain a calmodulin binding site postulated to be important in regulation of hsp70 protein-protein interactions as a function of intracellular calcium concentration (37). Relatively little is known about the required extent and duration of elevation of intracellular calcium that is potentially required to contribute to activation of expression of the hsp70 gene compared to that required for c-fos and c-myc. Consequently, it is possible that the requirement for extracellular $[Ca^{2+}]$ in inducing expression of the hsp70 gene could reflect need for a more protracted increase in intracellular $[Ca^{2+}]$ rather than qualitatively different signaling mechanisms.

$\alpha_1$ adrenergic receptors are coupled to activation of phospholipase C, which increases the formation of diacylglycerol and inositol (1, 4, 5) tris-phosphate (IP3). Diacylglycerol can activate protein kinase C which then phosphorylates a wide range of effector proteins. IP3 interacts with receptors which release calcium from intracellular stores. We found hsp70 mRNA induced by norepinephrine in aorta was partially

blocked by the protein kinase C inhibitor H7 when added 1 h before this agonist. Similar results were obtained in prostate and breast carcinoma cells when heat-induced expression of hsp70 and hsp28 gene was suppressed by H7 added 1 h before heating (38). In our experiments, protein kinase C activator phorbol dibutyrate also induced hsp70 mRNA suggesting an involvement of protein kinase C in heat shock gene regulation in vascular smooth muscle.

We found the induction of hsp70 mRNA by norepinephrine was blunted in isolated aorta from old rats compared to responses in aortas from young rats. In older, intact rats, increases in norepinephrine concentrations induced by the stress of immobilization has been shown to be less in paraventricular nucleus of the hypothalamus and in plasma compared to that in young animals, suggesting diminished activation of central and peripheral catecholaminergic systems with aging (39). However, age-related differences in the amount of stimulated catecholamines can not explain our results since aortas from young and old rats were exposed to the same concentration of norepinephrine in vitro. These results are similar to those of Udelsman et al. (4), who found in intact rats diminished hsp70 mRNA in aortas from old rats subjected to the stress of restraint. In adrenals from restrained old Fischer 344 rats the HSF1 was shown to exhibit decreased ability to bind to DNA, although the amount of HSF1 proteins was unchanged (13). The protein hsp70 itself may also play a negative feedback role in regulating the heat shock response (18) by facilitating the dephosphorylation and attenuation of the heat shock element–binding activity of HSF1 (40). In Western experiments we found the abundance of hsp70 in nonstressed aorta from old rats much lower than that from young rats. These results suggest that elevated protein levels of hsp70 constitutively present in aorta does not account for the blunted hsp70 mRNA expression we observed in the old rats. Also, the expression of hsp70 at the protein level may be regulated by posttranscriptional mechanisms as well.

We found no significant age-related decrement in hsp70 mRNA when elevated temperature was used as the inducer in the isolated aorta. Others have observed hyperthermia induced age-related decrement in expression of hsp70 mRNA and/or protein in neutrophils (41), fibroblasts (42), hepatocytes (12), brain, lung, and skin (43). In hepatocytes isolated from old rats (22–28 mo-old) and exposed to elevated temperature of 42.5°C for 30 min, the decrease in induction of hsp70 mRNA was shown to be due to a decline in transcription and decreased binding of the heat shock transcription factor to the heat shock element (12). Unlike the hsp70 gene, norepinephrine induction of c-fos in aorta from older animals was not suppressed in the present experiments. These results suggest that gene induction is not globally impaired in aortas from older animals and that there may be alterations in signaling pathways for activation of some genes but not others in the same vessel. Norepinephrine-induced contraction of aortas from old (24–27 mo) rats was not different from that from young animals, in spite of impaired norepinephrine-stimulated accumulation of hsp70 mRNA in aorta from the old animals. These results suggest the possibility that the signaling mechanisms involved in contraction may diverge from those mediating activation of expression of the hsp70 gene or have different inherent reserves.

In summary, we have shown that $\alpha_1$ adrenergic agonists act directly on $\alpha_1$ adrenergic receptors in aorta to induce gene expression of hsp70, and the induction of hsp70 mRNA was dramatically reduced with age. The hsp70 signaling pathways involve a CEC-insensitive adrenergic receptor subtype (possibly $\alpha_{1A}$) and a pertussis toxin insensitive G protein (such as $G_q$), which activates protein kinase C. In contrast, c-fos signaling pathways involve a CEC-sensitive subtype of adrenergic receptor, likely the $\alpha_{1B}$ receptor. The results suggest the pathways transducing the signal from norepinephrine and phorbol esters to hsp70 gene transcription may be more localized and more susceptible to age than the robust signal of heat shock that may activate hsp70 transcription via multiple pathways. Signal pathways mediating norepinephrine induction of gene expression of hsp70 differed from that of c-fos in terms of $\alpha_1$ adrenergic subtype, extracellular calcium requirement, and vulnerability to age. This in vitro model system mimics the effects of $\alpha_1$ adrenergic induction of hsp70 gene expression in intact animals, thus indicating its potential usefulness in elucidating signal transduction mechanisms with age.

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