Protection Against Necrosis but Not Apoptosis by Heat-Stress Proteins in Vascular Smooth Muscle Cells
Evidence for Distinct Modes of Cell Death

Marie-Josée Champagne, Pierre Dumas, Sergei N. Orlov, Martin R. Bennett, Pavel Hamet, Johanne Tremblay

Abstract—We have reported previously that cultured vascular smooth muscle cells (VSMC) isolated from spontaneously hypertensive rats (SHR) show higher proliferation and cell death than normotensive controls. In addition to protecting cells against death, heat stress proteins (HSPs) appear to play a role in cell proliferation. This investigation examines the involvement of HSP72 and HSP27 in altered SHR VSMC proliferation and death. We have performed detailed discriminatory analysis to characterize which type of VSMC death is induced by heat stress (HS) and serum deprivation. Serum deprivation induced apoptosis (caspase-3 cleavage and DNA laddering) and secondary necrosis, the 2 processes being a continuum of each other. In contrast, acute HS (46°C, 30 minutes), which inhibited BN.Ix and SHR VSMC proliferation by 2-fold, increased necrosis (by 5-fold and 2-fold, respectively) but not apoptosis. HSP72 and HSP27 expression evoked in VSMC by mild HS (44°C, 15 minutes) 6 hours before acute HS prevented the inhibition of proliferation and induction of necrosis with no effect on serum deprivation–induced or staurosporine-induced apoptosis. This induced expression of HSP72 and HSP27 did not eliminate the higher basal proliferation, apoptosis, and necrosis of SHR VSMC compared with BN.Ix VSMC, suggesting that these HSPs are not involved in altered SHR VSMC proliferation and death. Also, although apoptosis and necrosis may be a continuum, in VSMC the 2 processes may be distinguished by HS, in which only necrosis is prevented by prior HSP accumulation. This observation may be of use in designing strategies for cellular protection. (Hypertension. 1999;33:906-913.)

Key Words: muscle, smooth, vascular ■ cell proliferation ■ apoptosis ■ thermotolerance ■ heat-stress proteins

Although cell death has been initially classified into 2 major types, necrosis and apoptosis, a significant amount of debate at present has been devoted to their interrelations. Indeed, the 2 processes (1) may be essentially separate with some overlap, (2) may be a continuum of each other, or (3) may remain as 2 discrete death modes.1,2 DNA fragmentation by specific endonucleases appearing in typical DNA ladders is the most widely used marker of apoptosis,3 although the latter can occur in the absence of DNA fragmentation, and inversely, DNA ladder can also occur early in the course of events leading to necrosis.4 In vascular smooth muscle cells (VSMC), serum deprivation–induced apoptosis is accompanied by DNA laddering.5 Pathways leading to endonuclease activation include stimulation of a family of cysteine proteases (possibly interleukin 1-converting enzyme-like proteases) (see Reference 6 for review). Cardiomyocyte death after acute myocardial infarction has been the classic example of necrosis. However, recent studies performed in rats and humans have shown that apoptosis is observed in the first hours after infarction and that necrosis appears during the later stages of ischemic injury.7,8 The relative importance of apoptosis and necrosis remains to be established. For example, apoptosis is elevated in advanced atherosclerotic lesions, suggesting its involvement in the weakening of plaques,9 but most cells found in ruptured plaques show an ultrastructure characteristic of necrosis.10 Pretreatment with sublethal heat stress (HS) protects against cell death induced in vitro by HS11 or in vivo by ischemia/reperfusion.12 Overexpression of heat stress proteins (HSPs) HSP72 and HSP27 increases the survival of transfected cells during HS.13,14 Furthermore, rat HSP72 expression in transgenic mice has demonstrated the cardioprotective effect of the protein in in vitro and in vivo models of ischemia.15,16 These data suggest that HSP72 and/or HSP27 are involved in the protection conferred by sublethal HS. It is not clear at present whether the cardioprotective function of HSP72 refers to inhibition of necrosis or apoptosis. We therefore performed discriminatory analysis to determine the type of cell death induced by HS and inhibited by HSP72 and HSP27 in cultured VSMC. In addition, the
present experiments were designed to evaluate the relation between the 2 types of cell death in these cells. Some evidence suggests a role of HSPs in hypertension. Their expression is induced during acute hypertension in the rat, and we have demonstrated heightened hsp72 transcription, both in vitro and in vivo, in hypertensive rats, mice, and humans. Also, we have reported an association between hsp72 gene with blood pressure and hsp27 gene with cardiac weight in spontaneously hypertensive rats (SHR) that suggests the involvement of HSPs in the pathogenesis of hypertension and its complications. This work further examines the involvement of HSP72 and HSP27 in hypertension by evaluating their role in the balance between proliferation and cell death that contributes to vascular remodeling in hypertension.

**Methods**

**Cell Cultures**

VSMC, isolated from 10- to 13-week-old male Brown Norway (BN) rats bearing the Ix mutation on chromosome 8 (BN.Ix) and SHR (obtained from Dr Vladimir Kren and Dr Michal Pravenec, Institute of Biology, Charles University, Prague, Czech Republic), which are progenitors of recombinant strains, were used between 9 to 16 passages, as described previously. VSMC-E1A cells were infected with a neomycin-resistant pDORneo retroviral vector containing full-length cDNA coding for the adenovirus protein E1A.

**HS Protocol**

Exponentially growing cells were used for all studies. HS was produced by immersing culture flasks or floating culture plates in a precision water bath (±0.1°C). VSMC were exposed to either mild (44°C, 15 minutes) or acute (46°C, 30 minutes) HS. Thermotolerance was induced by subjecting the cells to mild HS and allowing them to recover for 6 hours at 37°C before acute HS.

**Electrophoresis and Immunodetection**

Cytoplasmic proteins were extracted in hypotonic buffer (10 mmol/L Tris, pH 7.4; 1 mmol/L EDTA; 1 mmol/L dithiothreitol; 1 mmol/L phenylmethylsulfonyl fluoride; 50 μg/mL leupeptin) after 4 cycles of freezing-thawing and centrifugation at 15 000 g for 45 minutes at 4°C. They were electrophoresed on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. HSP72, the inducible form of HSP70, and HSP27 were, respectively, detected with monoclonal antibody SPA-810 and polyclonal antibody SPA-801 (StressGen, Victoria, BC, Canada). Immune complexes were revealed with specific 125 I-labeled antibody (Amersham, Arlington, Ill). Membranes were exposed and analyzed with a PhosphorImager.

**DNA Synthesis, DNA Extraction, and Electrophoresis**

[^H]-thymidine (TdR) incorporation into newly synthesized DNA was performed as outlined previously. DNA extraction and labeling with terminal deoxynucleotidyl transferase (TdT) and [32P]dCTP are described elsewhere. Approximately 0.2 μg of labeled DNA was electrophoresed on 1.5% agarose gel and transferred onto a nylon membrane (Hybond N, Amersham) that was exposed and analyzed with a PhosphorImager.
Quantification of Apoptosis and Necrosis

Apoptosis was induced in VSMC by 24-hour incubation in Dulbecco’s modified Eagle’s medium (DMEM), 0.2% calf serum (CS) (5 hours for VSMC-E1A), or 10% CS staurosporine 0.5 mol/L. To quantify apoptosis in cultured VSMC, we used the chromatin cleavage assay that we have described previously. Briefly, VSMC were labeled with 2 μCi/mL [3H]-TdR in DMEM, 10% CS for 24 hours after inoculation. Labeled DNA was quantified by liquid scintillation spectrometry in culture medium (fraction F1) and in cells (fractions F2 and F3). The cells were incubated for 15 minutes on ice in 10 mmol/L Tris-HCl, pH 8.0/10 mmol/L EDTA/0.5%, Triton X-100. The solution was centrifuged at 15,000 g for 15 minutes at 4°C. The supernatants were transferred to vials (fraction F2), and pellets as well as cells in wells were each solubilized in 0.5 mL 1% SDS/1 mmol/L EDTA and their lysates combined (fraction F3). The levels of necrosis and apoptosis were ascertained by parameters R1 (relative content of extracellular chromatin fragments) and R2 (relative content of intracellular chromatin fragments), respectively, where R1=[A1/(A1+A2+A3)]×100% and R2=[A2/(A1+A2+A3)]×100%, with A1, A2, A3 being the radioactivity of fractions F1, F2, and F3, respectively.

Also, as a measure of necrosis, lactate dehydrogenase (LDH) release was quantified in 100-μL aliquots of culture medium from individual wells of 24-well plates, with the use of a commercially-available kit (Sigma Diagnostics). Percent necrosis corresponds to the percentage of LDH released to total LDH activity.

Statistical Analysis

Data are expressed as mean±SEM. As indicated in the table and figures, the level of significance of differences between the means was evaluated by Student’s t test or by ANOVA. Where appropriate, the data were further analyzed by Bonferroni or Tukey multiple comparison of confidence intervals for all pairwise comparisons within series of data. Correlation coefficients were tested by Pearson’s correlation. The limit of significance was P<0.05.

Results

Effect of HS on VSMC Proliferation

VSMC isolated from the SHR aorta show higher proliferation than VSMC from normotensive Wistar-Kyoto rats. This heightened proliferation corresponds to a 4-hour shortening of the cell cycle. Figure 1 illustrates that under basal conditions, VSMC from SHR also showed 50% higher [3H]-TdR incorporation compared with VSMC from normotensive BN.lx rats (P<0.0012). This was associated with a cell number reached on the seventh day of growth that was almost twice as high in SHR as in BN.lx VSMC (13.1±0.7×104 compared with 7.3±0.5×104 cell/cm2; P<0.001 Student’s t test). Mild HS (44°C, 15 minutes) did not modify the rate of proliferation of BN.lx or SHR VSMC, whereas acute HS (46°C, 30 minutes) inhibited it by 2-fold. Pretreatment of VSMC with mild HS 6 hours before acute HS...
protected against the inhibition of proliferation by acute HS (Figure 1). Thermotolerance induction did not modify the higher proliferation in SHR compared with BN.lx VSMC (P<0.0001).

Effects of HS and Serum Deprivation on VSMC DNA Fragmentation
Agarose gel electrophoresis of TdT-labeled genomic DNA of VSMC is shown in Figure 2. Twenty-four-hour incubation of VSMC with 0.2% CS induced DNA laddering typical of apoptosis (lane 2). In contrast, 24 hours after acute HS, this laddering was absent and only intact and a smear of DNA caused by random cleavage during the necrotic process could be observed (lane 4).

Figure 5. Apoptosis and necrosis of BN.lx and SHR VSMC submitted to mild (44°C, 15 minutes) and acute (46°C, 30 minutes) HS in the presence of 10% CS as described in Methods. Apoptosis corresponds to R₂ levels (%) and necrosis to R₁ levels (%) quantified by chromatin cleavage assay 24 hours after HS as described in Methods. Data are expressed relative to control BN.lx. Mean±SEM of 3 experiments performed in quadruplicate are given. Data were analyzed by ANOVA (P<0.0002, †P<0.0001). Significant differences between control and heat-stressed VSMC were found by ANOVA for necrosis only (P<0.0001 for both strains); Tukey multiple comparison of confidence intervals identified significantly higher levels than in corresponding controls (‡) or thermotolerant VSMC (§).

Kinetics of Serum Deprivation–Induced VSMC Death
We have shown previously that R₁ levels (intracellular DNA fragments) determined by chromatin cleavage assay correlate highly with apoptosis determined on agarose gel after TdT labeling (r=0.98; P<0.0001). The data presented in Figure 3 illustrate that R₁ levels were poorly correlated to LDH release, a recognized marker of necrosis, in control and heat-stressed VSMC (r=0.55; P=0.16 Pearson’s correlation). By contrast, R₂ levels (extracellular DNA fragments) were highly significantly correlated to LDH released in these cells (r=0.92; P<0.0005 Pearson’s correlation). Although the correlation was high between the chromatin cleavage and LDH assays, indicating that chromatin cleavage R₁ fraction

![Figure 6](http://hyper.ahajournals.org/)

**Figure 6.** Cell death of BN.lx and SHR VSMC submitted to acute HS (46°C, 30 minutes) in the presence of 10% CS. R₂ levels (chromatin cleavage assay) and LDH activity released were quantified 8 and 24 hours after acute HS. Data are expressed relative to respective control values determined after 8 or 24 hours of incubation at 37°C in the presence of 10% CS. Mean±SD of 2 experiments performed in quadruplicate (R₂) or 1 experiment performed in duplicate (LDH) are given.
Effect of Mild HS on Serum Deprivation–Induced and Staurosporine-Induced Apoptosis in VSMC from BN.Ix, SHR, and VSMC-E1A

<table>
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<tr>
<th>Strain and Treatment</th>
<th>Serum Deprivation (12)</th>
<th>10% Serum + 0.5 μmol/L Staurosporine (8)</th>
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<tr>
<td>BN.Ix</td>
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<tr>
<td>37°C</td>
<td>148±17</td>
<td>195±20</td>
</tr>
<tr>
<td>44°C</td>
<td>141±13</td>
<td>384±68†‡†</td>
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<td>44°C+6 h 37°C</td>
<td>148±18</td>
<td>225±31</td>
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SHR

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<td>37°C</td>
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<td>253±22*</td>
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<tr>
<td>44°C</td>
<td>168±12*</td>
<td>286±29*</td>
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VSMC-E1A (8)

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<td>44°C</td>
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<td>44°C+6 h 37°C</td>
<td>818±171*</td>
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Effects of Mild HS on Serum Deprivation–Induced or Staurosporine-Induced Apoptosis in VSMC

We have observed maximal cytoplasmic accumulation of HSP72 and HSP27 6 hours after exposure to mild HS (44°C, 15 minutes) (unpublished data, 1998). To discriminate between a protective effect against apoptosis induction caused by immediate cellular modifications associated with HS or HSP synthesis, apoptosis was elicited by 24-hour serum deprivation (0.2% CS) immediately or 6 hours after mild HS (44°C, 15 minutes) (Table). We did not find any significant difference between cells submitted to apoptosis induction immediately or 6 hours after mild HS compared with their controls (10% CS) for both BN.Ix and SHR VSMC. Twenty-four-hour incubation with 0.5 μmol/L staurosporine in DMEM, 10% CS induced apoptosis in BN.Ix and SHR VSMC (Table). Mild HS alone (44°C) or maximal accumulation of HSP72 and HSP27 before apoptosis induction (44°C+6 hours 37°C) did not inhibit staurosporine-induced apoptosis in BN.Ix and SHR VSMC compared with control cells (10% CS) (Table).

Effect of Mild HS on VSMC Death

In control cells, necrosis and apoptosis were significantly higher in SHR than in BN.Ix VSMC (Figure 5) (P<0.0001). Mild HS (44°C, 15 minutes) did not significantly modify either apoptosis or necrosis in both BN.Ix and SHR VSMC. Acute HS (46°C, 30 minutes) had no effect on apoptosis levels but significantly increased necrosis of both BN.Ix and SHR VSMC. Induction was significantly higher for BN.Ix VSMC (P<0.02, Student’s t test), representing ~5-fold and ~2-fold elevations for BN.Ix and SHR VSMC, respectively. Figure 6 shows that although apoptosis (R2 levels) was not significantly induced 8 hours after acute HS, necrosis estimated by released LDH activity was already augmented in both BN.Ix and SHR VSMC. It is therefore evident that HS did not elicit the apoptotic threshold, whereas for necrosis it did so within 8 hours. Thus in these cells, HS-induced necrosis is not secondary to apoptosis. Pretreatment of VSMC with mild HS (44°C, 15 minutes) 6 hours before acute HS (46°C, 30 minutes) protected against HS-induced necrosis (Figure 5). Similar to the control situation, thermotolerant SHR VSMC showed significantly higher apoptosis and necrosis than did BN.Ix VSMC (P<0.0001).

Apoptosis was induced immediately or 6 hours after mild HS (44°C, 15 minutes) by 24-hour incubation in DMEM, 0.2% serum or 10% serum + 0.5 μmol/L staurosporine (BN.Ix and SHR VSMC) or by 5-hour incubation in DMEM, 0% serum (VSMC-E1A). Numbers in parentheses refer to number of samples obtained from experiments performed in quadruplicate. Data (mean±SEM) are expressed relative to their respective basal level (10% serum). Data were analyzed by ANOVA and Bonferroni multiple comparison of confidence intervals (significantly higher than basal level (10% (CS) (*) or induced apoptosis quantified at 37°C (†) or after maximal HSP accumulation (44°C+6 hours 37°C (‡)).

Indeed necrosis is evident from Figure 3 that variability was less with R1 estimation, which we therefore selected for further study. Thus in this study, R1 is a measure of necrosis and R2 of apoptosis.

Figure 4 depicts the kinetics of accumulation of R2 and R1 DNA fragments in BN.Ix and SHR VSMC incubated in the presence of 10% or 0.2% CS. Serum deprivation (0.2% CS) rapidly induced R2 levels. Maximal accumulation of R1 was attained at 24-hour incubation and remained at this level until 48 hours. It was significantly higher for SHR compared with BN.Ix VSMC. R2 levels were also induced by serum deprivation in SHR VSMC but they were delayed so that at 6 hours, they were not significantly different than in the controls. R1 levels were significantly increased in SHR VSMC after 24-hour incubation. This generation of extracellular DNA fragment (R1) appears to be due to secondary necrosis. It is evident here that the apoptotic threshold occurred early (<6 hours), whereas the necrotic threshold appeared later (24 hours). In both cases, the threshold was reached earlier in SHR VSMC.

Effect of HS on VSMC Death

In control cells, necrosis and apoptosis were significantly higher in SHR than in BN.Ix VSMC (Figure 5) (P<0.0001). Mild HS (44°C, 15 minutes) did not significantly modify either apoptosis or necrosis in both BN.Ix and SHR VSMC. Acute HS (46°C, 30 minutes) had no effect on apoptosis...
intensity of full-length CPP32 (Figure 8, lane 3). Mild HS did not prevent the cleavage of CPP32 (lane 4). Acute HS, shown above to induce necrosis, did not lead to CPP32 processing, further confirming its primary character, independent of the apoptotic process (lanes 5 and 6).

**Discussion**

Although the morphological difference between apoptosis and necrosis is well recognized, the fact that they are distinct modes of cell death has been challenged recently.\(^\text{1,31,32}\) This emerging concept implicates mitochondrial proteins, such as cytochrome-c, and their release into the cytosol during mitochondrial permeability transition.\(^\text{33}\) This permeability transition, which is apparently common for all modes of cell death, can lead to cytochrome-c–dependent activation of caspase-3, a prerequisite of apoptosis in certain but not all cell types.\(^\text{33}\) Our results show that in cultured VSMC, serum deprivation induces apoptosis, characterized by caspase-3 activation and typical DNA laddering, followed by secondary necrosis, whereas acute HS (46°C, 30 minutes) evokes only necrosis without caspase activation and DNA laddering. In contrast to what we have observed in serum-deprived VSMC, acute HS-induced necrosis does not appear to be secondary to apoptosis because the latter was absent at any time point earlier than 24 hours, whereas necrosis was already increased 8 hours after acute HS (Figure 6). DNA laddering has been reported during necrosis of cultured canine kidney cells induced by a Ca\(^{2+}\) ionophore and ATP depletion.\(^\text{4}\) In these cells, the activation of DNA fragmentation was dependent on serine rather than cysteine proteases, suggesting that cysteine protease activation could be a more preferable marker of apoptosis than DNA fragmentation itself.\(^\text{4}\) The demonstration of cysteine protease activation in apoptosis but not necrosis in several systems supports this notion.\(^\text{2,34,35}\) The data reported here on VSMC-E1A cells, which express high levels of apoptosis, allowed for the distinction between serum deprivation and HS-induced cell death based on caspase-3 activation (Figure 8).

Our data show that mild HS (44°C, 15 minutes) inhibited acute HS-induced necrosis (Figure 5) but not serum deprivation–induced or staurosporine-induced apoptosis in VSMC (Table). Furthermore, mild HS did not interfere with caspase-3 activation induced by serum deprivation (Figure 8).
The pathway leading to hyperthermic cell death is unknown, but it is generally believed that protein denaturation/aggregation is a key step (review in Reference 36). The chaperone function proposed for many HSPs (review in Reference 37) participates in their protective effect against cell death by their binding to hydrophobic domains of proteins exposed by stress. Considering this, it may be assumed that inhibition of acute HS-induced necrosis in VSMC by prior mild HS could be due to the suppression of protein aggregation by the induced HSP72 and/or HSP27. On the other hand, it has been shown in human cell lines U937 (leukemic cells) and PEER (lymphoid tumor cells) that HS can stimulate the stress kinases p38 and JNK involved in apoptosis.38 Hence, HSP72 overproduction in the PEER cell line inhibits stress kinase activation caused by accumulation of abnormal protein39 and thus prevents HS-induced apoptosis.39 Serum deprivation and inhibition of protein kinase C by staurosporine could evoke apoptosis by a pathway independent of protein denaturation, which would explain the absence of a protective effect of mild HS.

Acute HS (46°C, 30 minutes) inhibited the proliferation of both BN.lx and SHR VSMC by 2-fold (Figure 1). Thermotolerant VSMC that were submitted to mild HS before acute HS were protected against this inhibition. The mechanism explaining the protective effect we observed against acute HS-induced inhibition of proliferation with mild HS requires further investigation.

Previous studies have reported abnormal HSP expression in hypertension18–20 and their involvement in cell proliferation21–23 and death.13–16 Although it remains to be established if increased proliferation is compensatory for increased apoptosis, elevation of both processes suggests higher cell turnover that could be involved in the remodeling of the arterial wall, as we have proposed previously.25 We show here that induction of HSP72 and HSP27 by mild HS did not abolish the higher proliferation, apoptosis, and necrosis of SHR VSMC (Figures 1 and 5) compared with BN.lx VSMC, indicating that these HSPs are not involved in these phenotypes. Also supporting this hypothesis is the apparent dissociation between basal levels of HSP72 and HSP27 and apoptosis or necrosis. Hence, HSP72 and HSP27 levels are the same between BN.lx and SHR VSMC, whereas apoptosis and necrosis are higher in SHR VSMC (Figure 5). In contrast, VSMC-E1A, which showed very low basal levels of HSP72 and HSP27 (Figure 7, A and B), demonstrated a level of apoptosis similar to that of SHR VSMC.

Altogether, our data demonstrate that mild HS protects VSMC against acute HS-elicited inhibition of proliferation and induction of necrosis but not against induction of apoptosis. Furthermore, these data suggest that HSP72 and HSP27 are involved in this protection. Our results do not, however, exclude a role of other HSPs. Because necrosis is elicited by HS without prior apoptosis induction, it appears that apoptosis and necrosis, at least in VSMC, are not always a mere continuum of the same process. Our data also suggest that the cardioprotective effect of HSPs could be due, as shown here for VSMC, to protection against necrosis occurring at later stages of ischemia/reperfusion injury. Finally, HSPs are apparently not involved in the higher proliferation and apoptosis present in SHR VSMC. Accumulation of knowledge on the selective modulation of proliferation, necrosis, and apoptosis could have potential in the development of strategies designed to control vascular remodeling.

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References


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