Increased Transcription of a Major Stress Gene in Spontaneously Hypertensive Mice

Pavel Hamet, Danielle Malo, and Johanne Tremblay

Environmental stress factors, including temperature, modify the severity of hypertension, a genetic disease. Hypertensive animals and humans respond abnormally to heat exposure, and this abnormality is reflected at the cellular level by an increment in a major stress (heat-shock) gene expression. The present studies demonstrate that increased hsp70 gene expression is due to its heightened transcription rate. The genetic basis of environmental susceptibility to hypertension may thus involve an abnormal control of heat-shock genes. (Hypertension 1990; 15:904-908)

Hypertension is a disease with a significant genetic component. Its severity may be modified to a large extent by environmental factors, which have been well documented, both in humans as well as in genetic and experimental hypertensive rodent models. Nutritional modifiers include sodium, calcium, alcohol, excessive caloric intake, and elevated levels of dietary trace elements, such as cadmium. Stressful environmental influences are represented by pain, immobilization stress, and environmental temperature. Hypertensive animals are lethally sensitive to increased environmental temperature, a phenomenon observed in spontaneously hypertensive rats (SHR), as well as in genetically hypertensive mice and hyperthermia-sensitive pigs. The degree of hypertension is also modified by environmental temperature in humans. The increased susceptibility to environmental temperature is not only expressed in the animal as a whole, where it may be a reflection of a thermoregulation abnormality, but is also demonstrable at the cellular level. For instance, we have described increased cell death with temperature elevation in neonatal cardiomyocytes and vascular smooth muscle cells from SHR compared with cells from normotensive Wistar-Kyoto control rats.

To establish a potential genetic linkage of this heightened sensitivity to environmental temperature with hypertension, we recently completed studies demonstrating a higher rate of body temperature increase in hypertensive mice in response to heat. In crossbreeding experiments, this thermosensitivity trait cosegregated with a component of high blood pressure and represented 25% of the difference between normotensive and hypertensive mice. In additional studies, these thermosensitive mice were exposed to repeated brief periods of increased environmental temperature; after several weeks, their blood pressures decreased to levels that were indistinguishable from those of normotensive mice. Thus, it appears that, in this model of genetic hypertension, environmental temperature is a significant modifier of the expression of the disease. It should also be pointed out that similar manipulations of normotensive mice do not have any effect on chronic regulation of their blood pressure; this finding suggests that hypertensive mice are more susceptible to environmental influences.

Since, as mentioned, this increased susceptibility to the environment is observed in the whole body of animals as well as in the cells derived from them, we undertook studies to elucidate its molecular mechanisms. We investigated a set of genes called heat shock genes (hsp), which have been well described in terms of the modulation of their expression by environmental stimuli. The expression of these genes is significantly induced by stimuli such as temperature, calcium ionophore, alcohol, glucose deprivation, cadmium, and many other environmental factors. The major hsp gene belongs to the hsp70 family, which was recently shown to be related to cell thermosensitivity and thermo-resistance. The present study was undertaken to determine whether the elevated hsp70 messenger RNA (mRNA) levels that we described in hypertension are due to an increased transcription rate or to a reflection of the metabolic fate of the mRNA transcripts.

Methods

The genetically hypertensive mouse model was developed by Schlager. The base population was...
derived from an eight-way cross of eight inbred mouse strains; a randomly bred normotensive line was used as the control group. By the thirty-seventh generation, the divergence in blood pressure between hypertensive and normotensive mice was about 50 mm Hg.22

Mice were heat-exposed under anesthesia for 7 minutes in a water bath maintained at 44° C and then allowed to recover for different periods of time.22 Total RNA was extracted from the kidneys by the acid guanidium thiocyanate–phenol–chloroform method for Northern blot analysis.27 RNA samples were denatured by heating (65° C for 10 minutes) in formamide-formaldehyde buffer (0.02 M 3-(N-morpholino)propanesulfonic acid buffer, pH 7.0, 50% formamide, 16% formaldehyde). An equivalent amount (10 μg) of spectrophotometrically determined total cellular RNA, derived from the kidneys of the control and spontaneously hypertensive mice, was electrophoresed through denaturing 1% formaldehyde-agarose gel and was transferred to nylon membranes (Nytran, Schleicher & Schuell, Inc., Keene, New Hampshire).28 The nylon membranes were prehybridized at 42° C for 3 hours in 50% formamide, 750 mM NaCl, 250 mM NaPO4, 5 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), ×5 Denhardt’s solution, and 200 μg/ml sheared and denatured salmon sperm DNA. Probes (hsp70 and β-actin) were prepared according to a random priming technique described previously.29 Hybridizations were performed at 42° C in 50% formamide, ×1 Denhardt’s solution, 750 mM NaCl, 25 mM NaPO4, 5 mM EDTA, 0.5% SDS, 10% dextran, and 200 μg/ml sheared and denatured salmon sperm DNA. After hybridization, the filters were washed with 50% formamide-agarose gel and was transferred to nylon membranes (Ny tran, Schleicher & Schuell, Inc., Keene, New Hampshire).28 The nylon membranes were prehybridized at 42° C for 3 hours in 50% formamide, 750 mM NaCl, 250 mM NaPO4, 5 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), ×5 Denhardt’s solution, and 200 μg/ml sheared and denatured salmon sperm DNA. After hybridization, the filters were washed with 50% formamide, 500 mM NaCl, 25 mM NaPO4, 1 mM EDTA, 0.5% SDS at 42° C for 30 minutes; 75 mM NaCl, 5 mM NaPO4, 1 mM EDTA, 0.1% SDS at 65° C for 30 minutes; and 15 mM NaCl, 2 mM NaPO4, 1 mM EDTA, 0.1% SDS at 65° C for 1 hour. The hsp70 probe, provided by Dr. Larry A. Moran, consisted of a 2.2 kilobase (kb) Stul/Stul gene fragment of cloned mouse hsp68 gene.30 The β-actin probe consisted of a 1.5 kb Psfl/Pstl gene fragment of a cloned rat cytoplasmic β-actin gene.31

Transcription rates of hsp70 gene in normal and spontaneously hypertensive mice after heat shock was carried out in the presence or absence of 1 μg/ml α-amanitin.32 The kidneys were excised and immediately transferred to a solution containing 50 mM ice-cold Tris-HCl (pH 7.5), 25 mM KCl, and 5 mM MgCl₂ (TKM) and 0.25 M sucrose. Tissues were homogenized in a Dounce homogenizer with six to eight strokes of the pestle in 0.25 M sucrose-TKM. After filtration through nylon cloth (75 μ), 2 vol of 2.3 M sucrose-TKM was added. Of this suspension, 9 ml was transferred to a 14-ml polycarbonate tube, underlaid with 2.3 M sucrose-TKM, and centrifuged at 130,000g for 30 minutes at 5° C in an SW40ti rotor (Beckman Instruments, Inc., Palo Alto, California). The pelleted nuclei were resuspended in 100 μl of 50 mM Tris-HCl (pH 8.3), 40% (vol/vol) glycerol, 5 mM MgCl₂, and 0.1 mM EDTA. Nuclei were mixed with 100 μl reaction buffer containing 10 mM Tris-HCl (pH 8.0), 0.5 mM ATP, 0.25 mM CTP, 0.25 mM GTP, 10 mM MnCl₂, 2.5 mM MgCl₂, 300 mM KCl, 20 mM phosphocreatine, 100 μg creatine phosphokinase, and 100 μCi of [α-³²P]UTP (3,000 Ci/mmol, Amersham Canada, Oakville, Ontario, Canada) and incubated at 30° C for 30 minutes. In a given experiment, each preparation contained the same number of nuclei as established by counting in a hemocytometer. After shearing, the lysed nuclei were incubated with 20 units RNase A, 25 units RNAse inhibitor, and 100 μg yeast transfer RNA (tRNA) for 30 minutes at room temperature, and then for 1 hour at 37° C with 75 μg proteinase potassium and 1% SDS. RNA was then extracted with phenol-chloroform and precipitated with ammonium acetate and ethanol. The precipitates were resuspended in 100 μl of 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA (pH 8.0), and the “spun-column” procedure was used to separate free [α-³²P]UTP from labeled RNA.28 RNA was resuspended in 400 μl hybridization buffer containing 50% denionized formamide, 1% SDS, 3 standard saline citrate, ×5 Denhardt’s solution, 1 mM sodium pyrophosphate (pH 7.0), 10 mM sodium phosphate (pH 7.0), and 50 μg/ml yeast tRNA. Using a Bio- Rad (Richmond, California) dot blot apparatus, 500 ng denatured complementary DNA (100° C, 5 minutes) was spotted onto nylon. Filters were prehybridized in hybridization buffer for 4 hours at 42° C. After a 24-hour hybridization at 42° C, the filters were washed in ×2 standard saline citrate for 1 hour at 65° C and in ×1 standard saline citrate for 30 minutes at 65° C and autoradiographed.

Results
Our recent studies have revealed that there is an abnormality in the expression of hsp70 gene in spontaneously hypertensive mice and rats. Elevated mRNA levels parallel to an augmented synthesis of HSP70 protein have been observed in organs from hypertensive mice and rats exposed to heat stress, and similar data have been obtained with cells isolated from them.26

The present investigations were initiated to determine whether these elevated hsp70 mRNA levels are due to an increased transcription rate or are simply a reflection of the metabolic fate of the mRNA transcripts. For this purpose, mRNA levels and transcription rates were measured in 10-week-old anesthetized mice. Total cellular RNA was extracted from their kidneys. Northern blots were hybridized to random phosphorus-32–labeled hsp70 and β-actin probes. An example of such an experiment is illustrated in Figure 1A. In kidneys isolated from mice immediately after heat shock, there was no significant hsp70 expression, while normal β-actin expression was evident (Figure 1B). Thirty minutes after the 7-minute heat exposure, hsp70 mRNA levels were significantly higher in extracts from spontaneously

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hypertensive mice. Figure 1C charts the mean values of densitometric reading of Northern blots obtained in three different experiments involving 18 normotensive and 18 hypertensive mice (three mice per time point). The expression of \( hsp70 \) was significantly higher in hypertensive mice \((p<0.001\) by analysis of variance\) as early as 30 minutes \((p<0.05\) by Bonferroni's contrast analysis\) after the heat shock and persisted for up to 2 hours. A decline in \( hsp70 \) expression followed, and after 4 hours, there was no difference in mRNA levels. The initial velocity appeared more important in hypertensive mice, as rigorously expressed by the analysis of the derived curves (not shown). Furthermore, the maximal levels of mRNA were shifted to the right by 1 hour but decreased more abruptly toward basal values in hypertensive mice.

Subsequent experiments were directed toward the transcription analysis of \( hsp70 \) gene expression. Anesthetized normotensive and hypertensive mice were heat-exposed \((44^\circ\text{C}\) for 7 minutes\) as in previous investigations. A control condition was established: normotensive and hypertensive mice were subjected to water immersion at \(22^\circ\text{C}\) for 7 minutes followed by a 15-minute recovery period before nuclei preparation. Heat-exposed mice were allowed to recover for 0, 15, 30, and 60 minutes after heat shock, and their kidneys were excised and immediately transferred to ice-cold media. After the isolation of nuclei, run-on transcription assays were performed for 30 minutes, before lysis and RNA extraction. Figure 2A provides an example of \( hsp70 \) transcripts in nuclei isolated from mice under control conditions (C in Figure 2), immediately after heat

![Figure 1. Northern blot analysis of hsp70 messenger RNA after whole-body heat exposure of 10-week-old normal and spontaneously hypertensive mice (SHM). After anesthesia, mice were immersed for 7 minutes in a water bath maintained at 44°C and then allowed to recover for different periods of time. Total RNA was extracted from the kidneys. Blots were hybridized to phosphorus-32-labeled hsp70 (panel A) and \( \beta \)-actin (panel B) complementary DNA. Quantitation of hsp70 and \( \beta \)-actin messenger RNA was performed by densitometric scanning, and the levels of hsp70 messenger RNA were corrected for \( \beta \)-actin. Means of three different experiments are charted in panel C. Curves were evaluated by two-way analysis of variance \((p<0.001)\) and by Bonferroni's contrast analysis \((*p<0.05)\).]
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Figure 2. Panel A: Transcription analysis of hsp70 gene expression. Anesthetized normal mice and spontaneously hypertensive mice (SHM) were subjected to whole-body heat exposure (44°C for 7 minutes) and allowed to recover for the time periods indicated (0, 15, 30, and 60 minutes) before kidney removal. After isolation of nuclei, all samples were submitted to an additional period of 30 minutes to allow transcription. Under control conditions (C), mice were anesthetized and immersed for 7 minutes in water kept at 22°C and then allowed to recover for 15 minutes before nuclei preparation. Time 0 represents heat exposure (44°C for 7 minutes) followed immediately by kidney removal. Kidney-radiolabeled RNA was hybridized to filter-bound hsp70 and β-actin complementary DNA. Quantitation of hsp70 transcripts was carried out by densitometric scanning. Panel B: Graph showing transcription levels corrected for β-actin levels. Curves obtained from three different experiments were analyzed by two-way analysis of variance (p<0.001) and subsequently by Bonferroni’s contrast analysis (*p<0.05).

Discussion
As already mentioned, our recent studies have demonstrated a heightened expression of hsp70 gene in tissues from spontaneously hypertensive mice after heat exposure of the whole body. This enhanced expression of hsp70 gene was also observed after heat exposure (0 in Figure 2), and after 15 to 60 minutes of recovery time. Accumulation of nascent RNA, which occurred during the transcription assay, was evident in nuclei isolated immediately (0 in Figure 2) after heat exposure. Since the β-actin transcription rates were not influenced by heat exposure (data not shown), hsp70 levels were corrected for β-actin levels (Figure 2B). To evaluate the specificity of transcription analysis, the effect of α-amanitin was studied. The addition of 1 μg/ml α-amanitin decreased the transcription rates by about 50% in both normotensive and hypertensive mice (Table 1). The three separate experiments involving 18 normotensive and 18 hypertensive mice demonstrated a rapid increase in hsp70 transcription after heat exposure; maximum transcription was achieved after 15 minutes of recovery and declined to control values after 60 minutes. It is evident from Figure 2 that hsp70 transcription, although synchronous, was significantly increased (p<0.0001 by analysis of variance) in spontaneously hypertensive mice as compared with normotensive control mice. These observations confirmed the higher initial velocity of transcription of hsp70 in hypertensive mice.

Table 1. Effect of α-Amanitin on Transcription Rates of hsp70 Gene in Normal and Spontaneously Hypertensive Mice After Heat Shock

<table>
<thead>
<tr>
<th>Animals</th>
<th>α-Amanitin (1 μg/ml)</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td>Normal mice</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Spontaneously hypertensive mice</td>
<td>-</td>
<td>5.3 2.8 48</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>9.4 4.3 55</td>
</tr>
</tbody>
</table>

−: Absence of α-amanitin; +, presence of 1 μg/ml α-amanitin. Anesthetized mice were immersed for 7 minutes in water kept at 44°C and then allowed to recover for 15 minutes. Nuclei were prepared from kidneys and incubated in the presence or absence of 1 μg/ml α-amanitin in 100 ml reaction buffer at 30°C for 30 minutes. Radiolabeled RNA was hybridized to 500 ng filter-bound hsp70 complementary DNA. Quantitation of hsp70 transcripts was carried out by densitometric scanning and expressed as optical density×mm.
exposure of isolated organs from hypertensive mice and rats and was paralleled by an increased synthesis of HSP70 protein. These investigations have clearly shown that the elevated mRNA levels are due to enhanced hsp70 transcription, which may reflect an abnormal regulation of hsp70 gene expression. Although this defect may be due to a genetic anomaly in the regulatory region of hsp70 gene itself, it may also be secondary to the abnormal perception of heat by the cells of hypertensive animals. After heat exposure, heat-stress gene regulatory factors bind to heat-stress elements of the regulatory region of the hsp gene; this binding leads to an increased transcription rate.33-36 At present, the precise site of the abnormality in heat perception in hypertension remains unknown. Nevertheless, the investigations reported here demonstrate, for the first time, an increased transcription rate of a major gene regulated by environmental factors in hypertension. These results suggest the possibility that one of the genetic defects in hypertension is related to enhanced environmental susceptibility reflected by an increment in the expression of stress genes.

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References


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