Influence of Pressure Overload and ACE Inhibitor Therapy on Constitutive Protein mRNA Expression in the Spontaneously Hypertensive Rat

Douglas A. Cipkala, William H. Livingston, and Robert J. Cody

Despite use as constitutive protein standards to quantify mRNA, data are limited regarding alteration of cyclophilin or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) in hypertension or angiotensin converting enzyme (ACE) inhibitor treatment. We assessed these standards in 6 month old Wistar-Kyoto rats (WKY, n = 16), compared to age-matched spontaneously hypertensive rats (SHR, n = 14). Additional SHR (n = 8) had received enalapril for 3 to 4 months at evaluation. Left ventricular (LV) and kidney RNA was extracted for dot blot cyclophilin and G3PDH cDNA hybridization. Cyclophilin and G3PDH mRNA densitometries were expressed as a ratio. Cyclophilin/G3PDH for the WKY, untreated SHR, and enalapril SHR were 1.56 ± 0.33, 1.45 ± 0.42, and 1.49 ± 0.51, respectively, for the LV, and 1.52 ± 0.09, 1.43 ± 0.22, and 1.38 ± 0.22, respectively, for the kidney. Differences were not significant. Relative expression of cyclophilin/G3PDH was unaffected by genetic SHR hypertension, or long term enalapril. Thus, either constitutive mRNA may be confidently used to index structural or functional protein responses, at the transcriptional level, in the SHR.

KEY WORDS: Constitutive proteins, hypertension, mRNA, molecular biology, spontaneously hypertensive rat.

Constitutive proteins such as cyclophilin, a cytosolic cyclosporine binding protein expressed in most organs,1 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a glycolytic enzyme whose structure2 and activity3 have been extensively characterized, are commonly used as standards in the molecular quantification of mRNA, with the targeted protein mRNA expressed as a ratio with one of these constitutive protein mRNAs. It is assumed that the relative expression of these proteins is not altered by an underlying disease state, such as the remodeling that occurs in hypertension, or by a direct pharmacologic effect of therapeutic intervention. This assumption is largely untested in hypertensive animal models, although other diseases have been shown to alter the expression of these proteins. For example, cyclophilin mRNA is increased in the hippocampus of the rat, after hilar lesion-induced seizures.4 Likewise, expression of G3PDH mRNA is altered in hypophysectomized rats treated with thyroid hormone.5 In addi-
tion, target protein mRNA is altered by hypertrophy and angiotensin converting enzyme (ACE) inhibitor therapy, requiring clarification of such methodological issues.

In the current study, we tested the hypothesis that constitutive protein mRNA is differentially expressed in the pressure overload spontaneously hypertensive rat (SHR), compared to normal Wistar-Kyoto (WKY) rats, and that chronic treatment with the ACE inhibitor enalapril would alter constitutive protein mRNA, compared to the untreated SHR. We measured the relative expression of cyclophilin to G3PDH mRNA in both the left ventricle and kidney, to determine if there were significant differences between normal, untreated SHR and enalapril-treated SHR groups.

MATERIALS AND METHODS

Animals All experiments utilized WKY or SHR strains (Harlan Sprague Dawley Suppliers), weighing 250–350 g, following approval by the Institutional Laboratory Animal Care and Use Committee of the Ohio State University. Enalapril (supplied by Merck, West Point, PA) was dissolved in drinking water, with a daily target intake of 10 mg. Water intake was measured daily and the enalapril concentration was adjusted to ensure consistent daily intake. All rodents were followed for 3 to 4 months, following treatment group assignment, and all were approximately 6 months of age at the time of terminal study. Terminal hemodynamic studies and tissue acquisition were performed on individual animals, brought to the laboratory on the day of study.

Reagents and Probes Reagents were purchased from Sigma Chemical, Inc., St. Louis, MO, unless noted. The G3PDH probe was purchased from CLONTECH Laboratories, Inc., Palo Alto, CA. The cyclophilin probe was kindly provided by Stanley Rubin, MD, Long Beach Veteran’s Administration Hospital.

Terminal Hemodynamic Study Hemodynamic study was performed, as previously described.8 Rats were induced and anesthetized with inhaled halothane. Electrocardiographic monitoring was obtained. Tracheal ventilation was a mixture of room air and oxygen. The right carotid artery was cannulated with a 2-French catheter (Millar Instruments, Houston, TX), advanced to the ascending aorta. Thoracotomy was performed, and a volumetric flow probe (Transonic Systems, Ithaca, NY) was placed on the ascending aorta for estimation of cardiac output (milliliters/minute) from normalized aortic flow. All hemodynamic signals were recorded on digital tape (Teac, Montebello, CA; model RD-111TN). Systemic arterial resistance (units) was the ratio of mean arterial pressure to normalized cardiac output. Following hemodynamic assessment, the left kidney was removed and snap frozen in liquid nitrogen. Animals were euthanized with intravenous KCl under general anesthesia. The heart was immediately isolated, the left ventricle was rapidly dissected, and snap frozen in liquid nitrogen. All samples were stored at −80°C until analysis.

RNA quantification Approximately 50 mg of each tissue sample was homogenized for 30 sec in 700 μL 4 mol/L guanidine thiocyanate, and 5% b-mercaptoethanol. We then added 80 μL of 10% sarcosyl, 80 μL of 2.5 mol/L sodium acetate/8.5 mol/L acetic acid, and 700 μL phenol/chloroform/isomyl alcohol (25:24:1). The tubes were vortexed and the homogenate was allowed to settle on ice for several minutes. The homogenates were centrifuged at 10,400 g for 30 min and the upper aqueous phase containing the RNA was transferred to another tube. We added 700 μL isopropanol; this was followed by overnight storage at −20°C to precipitate the RNA.

Tubes were then spun for 45 min at 20,300 g. The supernatant was removed under vacuum, and the RNA pellet was resuspended in 300 μL 4 mol/L guanidine thiocyanate and 5% b-mercaptoethanol. To reprecipitate the RNA pellet, 300 μL of isopropanol was added and tubes were vortexed and allowed to stand for 1 h at −20°C. After centrifugation for 30 min at 12500 rpm, the liquid was vacuumed. The final wash of the RNA pellet was 500 μL of 70% ethanol, followed by gentle shaking, centrifuging, and vacuuming.

The RNA pellets were allowed to dry for approximately 15 min. They were then resuspended in 100 μL of 0.1% diethylpyrocarbonate (DEPC) water. Total RNA was quantified spectrophotometrically by adding 5 μL RNA to 1.0 mL of 0.1% DEPC water and measuring optical density at 260 nm. A unit of optical density at 260 nm is equivalent to 40 μg RNA. RNA purity was confirmed by measuring the absorbance ratio at 260 and 280 nm. The sample was considered acceptably pure if the ratio was between 1.5 and 2.

To prepare the RNA for the nylon membrane, 2 μg RNA was added to 30 μL of 20X sodium chloride/sodium citrate (SSC) and 20 μL 37% formaldehyde. DEPC water was added to bring the final volume to 100 μL and the solution was incubated at 60°C for 15 min. The nylon membrane was presoaked along with two sheets of filter paper in 15X SSC for approximately 15 min. Low vacuum was applied to the assembled manifold, and each well was washed with 0.5 mL of 15X SSC. The RNA was loaded onto the membrane followed by 0.5 mL of 15X SSC to rinse the wells. The RNA was UV cross-linked to the membranes to assure firm attachment of the RNA to the membrane.

The blots were prehybridized at 42°C in a cylinder with 15 mL filtered solution of 50% deionized for-
mamide, 5× Denhardt’s reagent, 0.5% sodium dodecyl sulfate (SDS), 5× sodium chloride/sodium phosphate/EDTA (SSPE), and 200 μg denatured salmon sperm DNA for 3 h. The cDNA probe was radiolabeled with 32P by using a Boehringer-Manheim Nick Translation kit (Boehringer-Mannheim, Inc., Indianapolis, IN). The probe was purified by passage over a NICK-lution kit (Pharmacia Biosystems, Inc., Piscataway, NJ). The probe was denatured by placing it in boiling water for 10 min followed by 2 min on ice. The prehybridization solution was decanted and 15 mL of the hybridization solution, containing radiolabeled probe instead of salmon sperm, was added and allowed to incubate for 20 min and then twice for 20 min in 400 mL of 2× SSPE/0.1% SOS at room temperature. A final wash was performed in 500 mL of 0.1× SSPE/0.1% SDS for 30 min at 42°C. The blots were dried with a paper towel, wrapped in cellophane and taped to an intensifying screen in an autoradiography cassette. Film (Kodak XAR-5; Rochester, NY) was loaded in the cassette and allowed to expose at -80°C until adequate intensity of the samples were observed. Films were developed and analyzed using a CCD-72 camera (Dage-MTI, Inc., Michigan City, IN) and Optimas version 4.0 software (Optimas Corp., Seattle, WA).

Statistics All statistical analyses were one way analysis of variance. Intergroup differences were determined by the Bonferroni-Dunn post hoc adjusted t test. Statistical significance following adjustment for multiple groups was \( P < .05 \). All values are expressed as the mean ± one standard deviation.

RESULTS

The hemodynamic profile of the treatment groups is summarized in Table 1. The untreated SHR group had significantly elevated heart rate (402 ± 44 beats/min), mean arterial pressure (152 ± 27 mm Hg), normalized aortic flow (107 ± 33 mL/min/kg body weight), and arterial resistance (1.51 ± 0.39 units) compared to the WKY group. The SHR group that was treated with enalapril demonstrated significant reduction in mean arterial pressure (94 ± 16 mm Hg) and arterial resistance (0.67 ± 0.19 units). Heart rate was essentially unchanged (410 ± 38 beats/min), and aortic flow increased towards normal (148 ± 30 mL/min).

Analysis of the constitutive protein mRNAs are given in Table 1. The cyclophilin/G3PDH mRNA expression in the left ventricle of the WKY group was 1.56 ± 0.33 (n = 16). The ratio for the untreated SHR, and the enalapril treated SHR were 1.45 ± 0.42 (n = 14), and 1.49 ± 0.51 (n = 8), respectively. The ratio of cyclophilin/G3PDH mRNA for kidney samples was 1.52 ± 0.09 for the WKY, 1.43 ± 0.22 for the untreated SHR, and 1.38 ± 0.22 for the enalapril treated SHR, respectively. There was no significant difference among treatment groups. The results for the kidney closely resembled those observed from the left ventricle, with each treatment group expressing cyclophilin approximately 1.4 times more than G3PDH (Table 1).

### TABLE 1. HEMODYNAMIC PROFILES AND MOLECULAR CHARACTERISTICS OF CYCLOPHILIN AND G3PDH IN MYOCARDIAL AND RENAL TISSUES: NORMOTENSIVE AND HYPERTENSIVE ANIMALS

<table>
<thead>
<tr>
<th></th>
<th>Normal WKY (n = 14)</th>
<th>Untreated SHR (n = 16)</th>
<th>SHR + Enalapril (n = 8)</th>
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<tbody>
<tr>
<td>Heart rate</td>
<td>304 ± 55</td>
<td>402 ± 44*</td>
<td>410 ± 38†</td>
</tr>
<tr>
<td>Mean arterial pressure</td>
<td>81 ± 16</td>
<td>152 ± 27*</td>
<td>94 ± 16*</td>
</tr>
<tr>
<td>Normalized aortic flow</td>
<td>179 ± 43</td>
<td>107 ± 33*</td>
<td>148 ± 30†</td>
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<tr>
<td>Arterial resistance</td>
<td>0.47 ± 0.15</td>
<td>1.51 ± 0.39*</td>
<td>0.67 ± 0.19†</td>
</tr>
<tr>
<td>Left ventricle</td>
<td></td>
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<tr>
<td>Cyclophilin densitometry</td>
<td>46.9 ± 5.4 (34.1, 54.3)</td>
<td>52.1 ± 7.8 (30.3, 63.4)</td>
<td>60.1 ± 22.0 (37.3, 112.0)</td>
</tr>
<tr>
<td>G3PDH densitometry</td>
<td>31.4 ± 7.6 (15.8, 38.8)</td>
<td>38.6 ± 10.8 (15.4, 52.4)</td>
<td>41.0 ± 8.1 (23.0, 49.3)</td>
</tr>
<tr>
<td>Cyclophilin/G3PDH</td>
<td>1.56 ± 0.33 (1.17, 1.90)</td>
<td>1.45 ± 0.42 (1.08, 2.67)</td>
<td>1.49 ± 0.51 (1.17, 2.69)</td>
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<tr>
<td>Kidney</td>
<td></td>
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<tr>
<td>Cyclophilin densitometry</td>
<td>55.2 ± 6.6 (41.9, 65.2)</td>
<td>52.1 ± 9.5 (36.7, 66.3)</td>
<td>51.0 ± 6.9 (42.7, 64.9)</td>
</tr>
<tr>
<td>G3PDH densitometry</td>
<td>36.6 ± 5.4 (28.3, 45.3)</td>
<td>37.5 ± 9.8 (22.6, 50.9)</td>
<td>38.2 ± 9.9 (24.6, 52.1)</td>
</tr>
<tr>
<td>Cyclophilin/G3PDH</td>
<td>1.52 ± 0.09 (1.36, 1.66)</td>
<td>1.43 ± 0.22 (1.10, 2.29)</td>
<td>1.38 ± 0.22 (1.11, 1.74)</td>
</tr>
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</table>

* \( P < .05 \) when compared to normal WKY animals. † \( P < .05 \), compared to untreated SHR. Values in parentheses represent the range for each group.
DISCUSSION

At the time of evaluation, there were significant differences in the hemodynamic profiles of the different groups. In the SHR, significant increase of blood pressure was mediated by increased vascular resistance. Enalapril-treated SHR demonstrated return of blood pressure and resistance to normal levels, following 3 to 4 months of treatment. Despite these differences, relative mRNA expression for the two constitutive proteins cyclophilin and G3PDH was not significantly different. In experimental investigations, either cyclophilin or G3PDH is typically used as the indexing mRNA for a wide variety of target protein mRNA expression. It has been tacitly presumed that these constitutive proteins are expressed in a uniform manner in tissue, and that their expression is not altered by disease states, or the presence of pharmacologic intervention. Accordingly, any change in the ratio of target protein to constitutive protein mRNAs assumed to be due to a change in the target protein mRNA. However, if the constitutive protein mRNA expression is altered by the type of tissue, the presence of disease states such as hypertension, or pharmacologic intervention, then the observed change in ratio may in fact be unrelated to changes in the target protein mRNA. In view of the known changes of myocardial, vascular, and renal remodeling which occur in hypertension, and the physiologic adaptation which occurs following reduction of angiotensin II in response to ACE inhibitor therapy, it is important that these apparent standards be characterized. Based upon the observations in the current study, it appears that these constitutive protein mRNAs are expressed to comparable degrees in the WKY and SHR. Furthermore, the expression is not directly affected by ACE inhibitors, despite a significant reduction of blood pressure, and by inference, reduction of angiotensin II.

Although a peptide with high sequence homology to cyclophilin whose expression in the kidney and liver is regulated by hypertension, its differential expression does not seem to interfere with probing for cyclophilin in the dot blot assay. An argument can be made that both cyclophilin and G3PDH expression were reduced or enhanced by the same relative amount by hypertension and use of enalapril, thus yielding the same overall ratio even though they are not expressed in equal amounts. One would have to postulate that these two unrelated proteins have reduced or enhanced proportional expression under very different physiologic states. There may be a wide range of variability in the raw densitometric data, usually caused by one or two samples per blot that are either over- or underexposed, when compared to the rest of the samples. While it is difficult to attribute such abnormal data to a specific cause, possibilities include difficulties during the terminal tissue harvest, such as tissue ischemia, or inaccurate loading of the RNA sample on the membrane blot. Following radiographic development, over- or underexposed dot samples must be assessed and censored accordingly.

These findings indicate that neither the presence of genetic hypertension in the SHR, nor long term treatment with an ACE inhibitor in this model, alter the relative expression of these two constitutive proteins, at the level of transcription. Therefore, either probe may be confidently used as the index mRNA for the assessment of structural or functional protein responses which occur in the SHR.

REFERENCES