Aortic Connexin43 Is Decreased During Hypertension Induced by Inhibition of Nitric Oxide Synthase

Jacques-Antoine Haefliger, Paolo Meda, Andrea Formenton, Philippe Wiesel, Anne Zanchi, Hans R. Brunner, Pascal Nicod, Daniel Hayoz

Abstract—Connexin43 (Cx43), the predominant gap junction protein in vessels and heart, is involved in the control of cell-to-cell communication and is thought to modulate the contractility of the vascular wall and the electrical coupling of cardiac myocytes. We have investigated the effects of arterial hypertension induced by inhibition of nitric oxide synthase on the expression of Cx43 in aorta and heart as well as on the distensibility of the carotid artery. Administration of 0.4 g/L N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) to rats in their drinking water for 4 weeks increased intra-arterial mean blood pressure, wall thickness of aorta and carotid artery (25%), and heart weight (17%). Analysis of heart mRNA demonstrated increased expression of the fetal skeletal α-actin and of atrial natriuretic peptide but not of Cx43. In contrast, Cx43 mRNA and protein were decreased by 50% in the aortas of L-NAME–treated rats that did not show increased carotid distensibility. Because these data contrasted with those obtained in the 2-kidney, 1 clip model of rat hypertension, which is characterized by increased arterial distensibility and Cx43 expression in aorta, we investigated by Western blot analysis the posttranslational modifications of Cx43. We found that Cx43 was more phosphorylated in the aorta of 2-kidney, 1 clip rats than in that of L-NAME or control rats, which indicated a differential regulation of Cx43 in different models of hypertension. The data suggest that the cell-to-cell communication mediated by Cx43 channels may help regulate the elasticity of the vascular wall. (Arterioscler Thromb Vasc Biol. 1999;19:1615-1622.)

Key Words: connexin • aorta • heart • elasticity • hypertension • nitric oxide

Gap junctions comprise specialized channels that represent 1 pathway by which vertebrate cells communicate and ensure the electrical and mechanical coupling of different types of muscle cells. In vessels, gap junctions provide a pathway to modulate the contractile activity of smooth muscle cells. In the myocardium, junctional coupling ensures the same function and is also implicated in the propagation and synchronization of electrical activity. Characterization of the proteins that form gap junctions is expected to provide insights on the possible involvement of junctional channels during cardiac and arterial hypertrophy associated with hypertension.

We have recently shown that in 2 hypertensive rat models that feature a comparable degree of hypertension, as a result of either the clipping of 1 renal artery or of a DOCA-salt treatment, increased levels of Cx43 were associated with a marked hypertrophy of smooth muscle cells in the aortic wall. Under these conditions, the isobaric distensibility of the carotid has been shown to increase because of a reduced elastic modulus that corresponds to a reduction of wall material stiffness. These biomechanical changes are not observed in another rat model in which a degree of hypertension similar to that observed in the 2-kidney, 1 clip (2K-1C) and the DOCA-salt models can be induced by inhibiting nitric oxide synthase with N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME). In this model, hypertension is associated with limited cardiovascular hypertrophy and with small changes in the isobaric distensibility of the carotid. Therefore, the model is of interest to investigate whether connexin changes are similar in all forms of hypertension or vary as a function of the mechanisms that lead to increased blood pressure and/or of the cellular changes that occur in vessels. Specifically, we have assessed whether the expression of Cx43 is altered in the aorta and heart of rats made hypertensive by oral treatment with L-NAME under conditions associated with normal carotid distensibility.

Methods

Induction of Hypertension

Normotensive male Wistar-Kyoto rats (Ifca Credo, L’Arbesles, France) that weighed 140 to 180 g at the beginning of the experiment were allowed free access to ordinary rat chow (UAR, A04) that contained 100 μmol of sodium per gram. The nitric oxide synthase inhibitor L-NAME (Sigma Chemical Co) was administered in drinking water over a 4-week period at a dose of 0.4 g/L. The L-NAME solution was made fresh every 3 days. Four rats had the left renal artery depressed by a solid U-shaped silver clip of 0.2 mm internal diameter (2K-1C renal hypertension model). Except for the clipping, 4 rats were exposed to the same surgical manipulations.
After the rats were returned to their cages, they were kept on a regular diet with free access to water for 4 weeks. All rats were housed at constant temperature and humidity and under standard light/dark cycles.

Measurement of Blood Pressure

Procedures for rat care, surgery, and euthanasia were approved by our institutional review committee for animal experiments. For surgery, all rats were anesthetized with halothane (Arovet AG). Twenty-four hours before the rats were killed, they were instrumented with 2 catheters: 1 in the right internal iliac artery and the other in the right femoral vein. Both catheters (PE-10, Portex Ltd) were exteriorized between the scapulas and filled with a heparinized 0.9% NaCl solution before rats were placed in individual cages. On the day of the experiment, the rats were placed in a Plexiglas tube to continuously record intra-arterial pressure and heart rate with the use of a data acquisition system. After an initial 1-hour period in which hemodynamic parameters reached baseline values, blood pressure was measured for 10 minutes. The animals were then killed with an overdose of methohexital sodium. Immediately afterward, 50 mL of diethyl pyrocarbonate (Sigma) in PBS (DEPC-PBS) was rapidly infused through the left ventricle to wash out blood cells and to avoid RNA degradation. Heart and aorta were then removed, the hearts were weighted, and all tissues were rapidly frozen in liquid nitrogen.

RNA Isolation and Northern Blot Analysis

Hearts and aortas were homogenized in a 4 mol/L guanidine hydroxycyanate buffer that contained 25 mmol/L sodium citrate and 100 mmol/L β-mercaptoethanol by use of a Kinematic Polytron blender (Kriens). Total RNA was extracted by the acid guanidinium isothiocyanate method, and yields were evaluated by absorbance at 260 nm. Ten to 15 μg of total RNA was size-fractionated on 1% agarose gels that contained 8% formaldehyde (Fluka) and 1× MOPS buffer (Fluka). RNAs were transferred overnight to Gene Screen membranes (Du Pont de Nemours GmbH, NEN Division) by capillary transfer in the presence of 1× SSC. Membranes were UV cross-linked and vacuum-baked for 2 hours at 80°C. After prehybridization, total mRNA levels were determined by hybridization with random primed (Boehringer Mannheim) cDNA probes specific for Cx43, GAPDH, ANF, and skeletal α-actin that were labeled with [32P]dCTP (Amersham). Hybridizations were performed overnight at 42°C in the presence of 5× SSPE, 50% formamide, 5× Denhardt’s solution, 5% SDS, 100 μg/mL purified salmon sperm DNA (Sigma), and 100 μg/mL polyuridylic acid (Boehringer Mannheim). Blots were washed 3 times for 10 minutes at 42°C in 2× SSC, 1% SDS, and 3 times for 20 minutes in 2× SSC that contained 0.1% SDS. Exposure times of all membranes to x-ray film (X-Omat AR, Kodak) were chosen to optimize the signals under conditions to prevent saturation. To normalize signal levels, the same filters were rehybridized with probes for the ubiquitously expressed gene GAPDH.

The cDNA clone coding for rat Cx43 (clone G2, 1.6 kb) and the 1.1-kb (HindIII–EcoRI) fragment of GAPDH cDNA were used. The probe for skeletal α-actin was obtained by polymerase chain reaction (PCR) amplification of rat genomic DNA, which was prepared as follows. Two centimeters of rat tail were cut and digested over night at 55°C in 0.7 mL Tris-HCl (pH 8.0) that contained 100 mmol/L EDTA, 0.5% SDS, and 500 μg/mL proteinase K. DNA was purified by phenol/chloroform extraction and ethanol precipitation. To generate PCR fragments, the sense primer sequence, originated at position 2881, was 5′-GTC CAC CTT CCA GCA GAT GT-3′ and the antisense primer, originated at position 3146, was 5′-GGT TTC CAT TTC CTT CCA CA-3′. Both primers were synthesized by MWG-Biotech. PCR reactions were started with 1 μg of rat genomic DNA, 20 ng of sense and antisense primers, 200 mmol/L dNTP in 10× PCR buffer, and 1,5 mmol/L MgCl2 (Gibco-BRL) under the following conditions: 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. The 265-bp product obtained after 30 cycles was gel-purified and used as a probe.

Measurement of Carotid Distensibility

Rats were anesthetized with halothane through a mouthpiece and placed on a heating pad. The right common carotid artery was cannulated with a PE-50 catheter filled with a heparinized 0.9% NaCl solution. Right carotid pressure and left carotid inner diameter were measured simultaneously with an A-mode ultrasonic echotracking device (NIUS-02, Asulab). Variations in the diameter of the common carotid artery were evaluated with a precision of ~1 μm. For signal transduction, a 10-MHz focalized transducer positioned perpendicularly to the arterial axis was used in the Doppler mode. Arterial wall movements that produced echoes of larger amplitude than those of surrounding tissues were visualized on an oscilloscope and tagged with electronic tracers. Ten successive diameter-pressure recordings per animal were obtained during a 5-minute period and averaged for analysis. The simultaneous measurements of arterial diameter and blood pressure were processed on line to calculate a database of arterial wall-diameter relations, which was subsequently converted into an arterial cross-sectional compliance-pressure curve for the entire range of operating blood pressures. This curve is best fitted by an arc-tangent function as described by Langerwouters et al. In the case of a cylindrical vessel, cross-sectional compliance is given by δS/δP, in which δS is the change in arterial cross section and δP is the change in blood pressure. Arterial cross-sectional distensibility (D) is the inverse of the Peterson elastic modulus, i.e., the compliance value normalized for the cross-section (S). It is defined as D=(1/S)×(δS/δP).

Morphometry

The abdominal artery was pressurized for 15 minutes at 80 mm Hg by a continuous perfusion of 4% paraformaldehyde solution in PBS. The fixed aorta was excised, and fragments were frozen in OCT medium, sectioned at a thickness of 8 μm, and stained with hemalum–eosin. Sections were dehydrated and mounted with glycerol. After projection at a final magnification of ×180, the intima and media thicknesses were measured by semiautomatic planimetry with a Quantimeter 500+ system (Leica AG).

For histological analysis of the left common carotid artery, rats were killed with a lethal dose of pentobarbital. The carotid artery was excised, fixed in 4% paraformaldehyde, and processed for histological examination. Paraffin-embedded tissue blocks were sectioned at a thickness of 5 μm and stained with hematoxylin–eosin. Measurements were performed under a Diaphot microscope (Nikon) that was fitted with a scale in the ocular. The intima-media thickness and lumen diameter measurements were performed at a ×200 magnification by evaluating 2 sections per animal and 6 microscopic fields around the circular profile of the cross-sectioned carotids.

The intima-media cross-sectional area (CSA) of both aorta and carotid arteries was determined according to the following formula

\[ \text{CSA} = \pi \left( \text{Lumen Radius} + \text{Media-Intima Thickness} \right)^2 - \text{Lumen Radius}^2 \]

as reported. Cardiac weight index (CWI) was calculated according to the following formula

\[ \text{CWI} = \text{[Heart Weight (mg)/Body Weight (g)]} \]

For histological analysis of hearts, rats were perfused with PBS and their hearts were rapidly excised and fixed by immersion in Bouin’s solution. The hearts were transversely cut at a level that corresponded to half the length of the left ventricle, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin–eosin.

Immunofluorescence

For indirect immunofluorescence, anesthetized rats (2 per group) were perfused with 20 mL of PBS, and the heart and aorta were rapidly excised and quickly frozen in 2-methylbutane that was cooled in liquid nitrogen. Fragments were frozen in OCT medium (Miles Inc) and sectioned at ~6-μm thickness in cryostat. Sections were rinsed in PBS and incubated for 30 minutes in a buffer that contained 0.5% BSA. Sections were then incubated for 1 hour in the presence of a monoclonal antibody to Cx43 (Zymed Laboratories Inc) that was diluted 1:250 in PBS. Primary antibodies were detected with anti-mouse immunoglobulins labeled with FITC (Biosystem). Sections were then rinsed in PBS, stained with Evans’ blue, viewed with a Axioshot microscope (Zeiss), and photographed on Kodak TMAX 400 films.
Western Blot Analysis
Immediately after the rats were killed, L-NAME, 2K-1C, and control animals (4 rats per group) were infused with 30 mL of PBS and their aortas were excised and rapidly frozen. The organs were homogenized with a Kinematic Polytron blender (Kinematika) in 100 mmol/L Tris-HCl, pH 7.4, supplemented with 20 mmol/L EDTA, 1 mg/mL pepstatin A, 1 mg/mL antipain (Merck), 1 mmol/L benzamidine, 40 kallikrein inactivator units/mL aprotinin, 2 mmol/L PMSF (Sigma), and 1 mmol/L diisopropyl fluorophosphate (Aldrich Chemical Co.). The homogenates were passed through a syringe to break DNA and centrifuged for 10 minutes at 3000 g to pellet intact cells and elastic fibers. Supernatants were collected. Total protein content was determined by the DC protein assay reagent kit (Bio-Rad Laboratories). Samples were fractionated by electrophoresis in a 12.5% acrylamide gel and immunoblotted for 20 hours onto Immobilon polyvinylidene difluoride membranes (Millipore Co.) at a constant voltage of 25 V. The membranes were preincubated for 3 hours at room temperature in PBS that contained 3% BSA (blocking buffer) and then incubated for 4 hours with a monoclonal antibody to Cx43 (Zymed Laboratories Inc) that was diluted 1:10,000 in blocking buffer. After the immunoblots were repeatedly rinsed in PBS and PBS+0.1% Tween 20, they were incubated for 2 hours with an anti-mouse Ig antibody coupled to alkaline phosphatase (Dako Diagnostic AG) that was diluted 1:5000. The bands were developed with the BCIP-NBT method (AP development reagent, Bio-Rad Laboratories).

Statistical Analysis
Densitometric analysis of signals detected on Northern blots, mRNA, and Western blots was performed with a Molecular Dynamics scanner (Sunnyvale), which integrates areas and corrects for background. Signals of specific transcripts were related to the corresponding GAPDH signals and expressed relative to the lowest control ratio, which was assigned the arbitrary value of 1. Data were expressed as mean ± SEM.

Values obtained in the L-NAME-treated rats were compared with those of controls with the use of a 1-way ANOVA (superANOVA and the Bonferroni-Dunn test. Relative mRNA and/or protein levels were calculated from Northern and Western blot analyses and compared with superANOVA and Fisher’s protected least significant difference test. Statistical significance was defined at a value of $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***) . Distensibility-pressure curves were established within operating pressures in which upper and lower limits represented the mean systolic and diastolic pressure curves, respectively. For statistical evaluation, the area beneath the curves of the overlapping pressure ranges was compared by use of the Scheffe test.

Results
Effects of L-NAME on Aorta and Carotid Artery
The characteristics of the rats on the day of the experiment and the morphometric parameters evaluated are shown in the Table. The body weight of L-NAME–treated rats was significantly lower than that of the corresponding controls, although the mean intra-arterial blood pressure of the treated animals was higher ($P<0.01$) than that observed in normotensive controls.

Compared with controls, the hypertensive animals showed a 25% thickening of aortic intima-media (Table) and featured a 25% increase in the CSA of aorta ($P<0.01$) and left carotid artery ($P<0.05$), despite a constant lumen diameter (Table). Histological analysis further revealed that the thickness of the aortic wall was larger in hypertensive than in normotensive animals (Figure 1).

Figure 2 shows the distensibility-pressure curves of the right common carotid artery of L-NAME hypertensive and controls animals. Comparison between the control and the treated group was performed over the range of overlapping pressures (120 to 140 mm Hg). In both groups of animals, the distensibility of the common carotid decreased with increasing blood pressure. A trend toward a reduced distensibility-pressure curve was seen in the L-NAME–treated rats. However, this reduction did not reach statistical significance in the range of blood pressures (120 to 140 mm Hg) that could be compared.

Effects of L-NAME Treatment on Cx43 of Aorta
Quantitative assessment of total aorta mRNA revealed that the transcript for Cx43, which was mostly contributed to by the aortas of 2K-1C rats than in that of both controls and L-NAME animals (Figure 4). The number of immunofluorescence spots that located Cx43 was clearly larger in the aorta of control rats compared with that of L-NAME–treated rats (Figure 5).

- $P<0.05$ vs control.
- $P<0.01$ vs control.
- $P<0.001$ vs control.

Values are mean ± SEM; n indicates the number of animals tested. L-NAME: 0.4 g/L L-NAME in the drinking water. Control: no L-NAME in the drinking water. Cross sectional area was determined according to the following equation: $CSA = \pi (R^2 - \rho^2)$, in which $R$ is the lumen radius ($\rho$) plus the media-intima thickness.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>Mean Blood Pressure, mm Hg</th>
<th>Aortic Lumen Radius, µm</th>
<th>Aortic Cross-Sectional Area, $\times 10^4$ µm$^2$</th>
<th>Carotid Artery Lumen Radius, µm</th>
<th>Carotid Artery Cross Sectional Area, $\times 10^4$ µm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-NAME</td>
<td>334±5†</td>
<td>188±6†</td>
<td>713±7</td>
<td>34.3±2.2†</td>
<td>463±18</td>
<td>88±5*</td>
</tr>
<tr>
<td>n</td>
<td>13</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Control</td>
<td>362±4</td>
<td>124±2</td>
<td>707±9</td>
<td>27.3±1.7†</td>
<td>489±10</td>
<td>70±3</td>
</tr>
<tr>
<td>n</td>
<td>16</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

Table. The body weight of L-NAME–treated rats was significantly lower than that of the corresponding controls, although the mean intra-arterial blood pressure of the treated animals was higher ($P<0.01$) than that observed in normotensive controls.
Effects of L-NAME Treatment on Cx43 of Heart

Hearts of hypertensive rats were hypertrophied compared with those of normotensive controls as indicated by a 17% increase ($P<0.001$) in CWI (Figure 1) and by histology. In agreement with this change, Northern blot analysis of heart showed a 3-fold increase in the expression of ANF mRNA and a 2-fold increase in the expression of skeletal $\alpha$-actin mRNA in the hypertensive rats (Figure 6). Quantitative assessment of Northern blot analysis showed that the expression of Cx43 mRNA was similar in the hypertrophied hearts of hypertensive rats and in those of normotensive controls (Figure 6). Also, the levels and distribution of Cx43 were similar in the 2 groups of rats (Figures 4 and 5).

Discussion

When exposed to chronic hypertension, conduit arteries undergo profound functional and structural changes that are characterized by an outward hypertrophic remodeling with preserved isobaric luminal diameter.$^{15,31}$ This remodeling, which results from the hypertrophy of smooth muscle cells and the alterations of extracellular matrix, may be regarded as an adaptation to normalize wall stress.$^{32-34}$ However, this adaptation may also modify the mechanical properties of arteries, which could be detrimental in the long term.$^{15}$ Previous studies have shown that the distensibility and compliance of various arteries increase under isobaric conditions in both 2K-1C and SHR hypertensive animals,$^{15,20,35}$ which suggests that changes in tissue composition and architecture permit arteries to maintain adequate elastic properties in spite of increased blood pressure. In contrast, as shown here, hypertension caused by the inhibition of nitric oxide is not associated with an increase in the isobaric distensibility of carotid, despite a thickening of the arterial wall that is similar to that observed in other experimental models of hypertension. The different viscoelastic properties of the common carotid arteries of 2K-1C and L-NAME–treated rats implies a differential composition or organization of tissues that comprise the wall of resistance arteries. Although the hypertension achieved after L-NAME treatment was similar to that obtained in the 2K-1C and DOCA-salt models, it also features unique characteristics. Thus, L-NAME–induced hypertension
is associated with impaired endothelium-dependent relaxation, which is important to modulate the viscosity of the vessel wall, and may, in conjunction with an altered balance between reactive oxygen species and endogenous antioxidants, account for the lack of autoregulation observed in the L-NAME model.15 L-NAME hypertensive rats showed a decreased expression of Cx43 in the smooth muscle cells of aorta that contrasted with the previously reported increase in the expression of this protein in the smooth muscle cells of both 2K-1C and DOCA-salt hypertension models.11,12 These findings suggest that the cell-to-cell communications mediated by Cx43 are specifically implicated in the lack of regulation of the elastic properties of arteries.

The significance of the decrease in Cx43 in the L-NAME–treated rats remains to be elucidated. Several electrophysiological studies have suggested that gap junction proteins may be important to coordinate the mechanical contractions of smooth muscle cells, possibly to ensure a proper modulation of the vasomotor tone of the aortic wall.4,38,39 Certainly, Cx43 can provide an intercellular pathway for the syncytial functioning of distant smooth muscle cells that could be recruited for synchronous contraction through propagation of second messengers that are gap-junction permeant.8,9

This study also provides the first evidence for a different posttranslational regulation of Cx43 in the aortic smooth muscle cells of 2 different models of hypertension in rats. Thus, although the degree of Cx43 phosphorylation increased in the 2K-1C animals, it decreased in the L-NAME–treated rats and featured a similar degree of hypertension, which expressed a form of immunoreactive Cx43 that, as judged by its mobility, appeared essentially nonphosphorylated. Because connexin phosphorylation can affect the extent of communication,9–11 this difference could result in selective regulation of the exchange molecules involved in the hypertrophy (2K-1C model) and polyploidy (L-NAME model) of vascular smooth muscle cells. Blockage of nitric oxide production of endothelial cells after treatment with L-NAME is expected to decrease apoptosis and to promote the prolif-
eration of smooth muscle cells,\textsuperscript{42,43} which accounts for their accumulation and polyplody in the wall of aorta. In addition, the reduced expression of Cx43 in the aorta of L-NAME-treated rats may help, such as in atherosclerotic lesions,\textsuperscript{44} upregulate the adhesion of monocytes and macrophages to the aorta; it is known that after inhibition of nitric oxide production by L-NAME treatment, this adhesion increases.\textsuperscript{18}

The L-NAME-treated rats we studied also presented with cardiac hypertrophy that was reflected by an increase in heart index and in the mRNA expression of skeletal $\alpha$-actin\textsuperscript{45–47} and ANF.\textsuperscript{48} However, this hypertrophy was not associated with changes in the levels of Cx43, which confirmed the data observed in 2 other models of rat hypertension that featured a larger thickening of ventricular wall.\textsuperscript{11,12} The data indicate that Cx43 is not involved in the myocardial adaptation that accompanies a hypertension-induced increase in the load of the left ventricle. It remains to be shown whether any of the other connexins that colocalize with Cx43 at the gap junc-

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Expression of Cx43 in aorta and heart. Immunofluorescence labeling with specific mouse monoclonal antibodies located Cx43 as discrete spots dispersed throughout the media of control aortae (A). A marked decrease in the number of immunofluorescent spots was observed in the thickened media of L-NAME hypertensive rats (B). L indicates the lumen. C and D, Immunofluorescence located Cx43 at intercalated disks throughout left ventricular myocardium. No obvious difference in the number and pattern of these bands was noticed between L-NAME (D) and control rats (C). Bar=23 $\mu$m.}
\end{figure}
tions of myocardial cells\textsuperscript{49,50} are differentially regulated under the same conditions.

In summary, our data indicate that Cx43 is differentially regulated in the hypertrophic muscle cells of heart and aorta of L-NAME–treated animals. The altered expression and phosphorylation of Cx43 in the aortas of these rats raises the possibility that this gap junction protein may contribute to the lack of autoregulation in arterial distensibility.

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References


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