Cytosolic Free Calcium of Aorta in Hypertensive Rats

Chronic Inhibition of Angiotensin Converting Enzyme

Toshio Sada, Hiroyuki Koike, Masahiro Ikeda, Koichi Sato, Hiroshi Ozaki, and Hideaki Karaki

Cytosolic free calcium concentration ([Ca\(^{2+}\)]\(_i\)) and muscle tension were simultaneously measured in aortic tissue isolated from spontaneously hypertensive rats (SHR), normotensive Wistar-Kyoto (WKY) rats, and SHR chronically treated with a novel angiotensin converting enzyme inhibitor, CS-622. In the presence of 2.5 mM Ca\(^{2+}\) in the bathing solution, aortic [Ca\(^{2+}\)]\(_i\), measured with fura-2 was higher in SHR than in WKY rats, and it was almost the same in CS-622-treated SHR and untreated WKY rats. Increase of external Ca\(^{2+}\) concentration from zero to 2.5 mM elicited a contraction in SHR aortas but not in aortas from both CS-622-treated SHR and untreated WKY rats. When the aortas were contracted by 60 mM K\(^+\), however, [Ca\(^{2+}\)]\(_i\), as well as developed tension was similar in the three groups. CGP-28392 (10\(^{-4}\) M), a Ca\(^{2+}\) channel activator, induced a rhythmic activity superimposed on a gradual increase of [Ca\(^{2+}\)]\(_i\), and tension in SHR aortas but not in the aortas of CS-622-treated SHR or untreated WKY rats. These results suggest that SHR aortas have a higher myogenic tone due to increased [Ca\(^{2+}\)]\(_i\), than WKY rat aortas and that the increased [Ca\(^{2+}\)]\(_i\), is attributed to alterations of dihydropyridine-sensitive Ca\(^{2+}\) channels in SHR aortas. Further, the decrease of the vascular tone induced by long-term administration of the angiotensin converting enzyme inhibitor may be due to a reduction of increased [Ca\(^{2+}\)]\(_i\), in SHR.

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The cytosolic free calcium concentration ([Ca\(^{2+}\)]\(_i\)) of vascular smooth muscle cells (VSMC) is a major factor that determines the vascular tone.\(^1\) Because high [Ca\(^{2+}\)]\(_i\), in VSMC has been implicated in the pathogenesis of hypertension, it is important to determine whether [Ca\(^{2+}\)]\(_i\), in VSMC is increased in spontaneously hypertensive rats (SHR) or essential hypertensive subjects compared with normotensive controls. A large body of evidence has indicated that [Ca\(^{2+}\)]\(_i\), levels in platelet or other blood cells are increased in SHR and hypertensive patients.\(^2\)-\(^5\) However, whether [Ca\(^{2+}\)]\(_i\), in VSMC is increased in spontaneously hypertensive rats (SHR) or essential hypertensive subjects compared with normotensive controls is controversial.\(^6\)-\(^7\) Sugiyama et al,\(^8\) using primary cultured VSMC from the aorta and cultured VSMC from the sixth passage, demonstrated that [Ca\(^{2+}\)]\(_i\), was markedly elevated in 8-week-old SHR but not in 4-week-old SHR compared with age-matched normotensive Wistar-Kyoto (WKY) rats. Erne and Hermsemeyer,\(^9\) using cultured VSMC from the azygous vein from neonatal rats (3 days old), demonstrated that VSMC from SHR had higher levels of [Ca\(^{2+}\)]\(_i\), in a resting condition than those of WKY rats at the peripheral rim area of the cell but not at the central region of the cell. In these studies, however, it is uncertain whether the nature of dispersed, single VSMC is identical with that of VSMC lying in a tissue. An increase of Ca\(^{2+}\)-dependent myogenic tone has been demonstrated in SHR vessels,\(^10\)-\(^11\) although the evidence is not available that this increased muscle tone is due to the increased [Ca\(^{2+}\)]\(_i\),. To elucidate the relation between [Ca\(^{2+}\)]\(_i\), and vascular tone, it is essential to measure these parameters simultaneously in vascular tissues.

Angiotensin converting enzyme (ACE) inhibitors lower blood pressure not only in hypertensive patients and animals with high plasma renin activity (PRA) but also in those with normal or low PRA. Many reports\(^12\)-\(^14\) support the contention that the lowering of blood pressure correlates better with the inhibition of tissue ACE than with the inhibition of serum ACE. However, the precise mechanism responsible for the antihypertensive action of ACE inhibitors, particularly...
during long-term treatment, has not been established. Recently, we found that chronic administration of an ACE inhibitor, CS-622, lowered blood pressure of SHR and suppressed Ca\(^{2+}\)-dependent myogenic tone of SHR aorta, whereas the suppression was not achieved by either chronic administration of hydralazine or acute inhibition of ACE.15 These observations suggest that the suppression of exaggerated vascular tone contributes to the chronic antihypertensive action of ACE inhibitors.

The purpose of the present study was to examine if SHR aortas have higher [Ca\(^{2+}\)] than WKY rat aortas and if chronic inhibition of ACE decreases [Ca\(^{2+}\)] in SHR aortas. We measured [Ca\(^{2+}\)] and vascular tension simultaneously by the use of a fluorescent Ca\(^{2+}\) indicator, fura-2,16 in the aortas excised from SHR, WKY rats, and SHR chronically treated with CS-622.

**Methods**

**Animals and Regimen of Drug Treatment**

Male SHR and age-matched normotensive WKY rats were obtained from Hoshino Laboratory Animals, Yashio, Saitama, Japan. Twelve-week-old rats were divided into three groups: the control SHR group (n=10) received vehicle for CS-622 (NaHCO\(_3\) (n=7) did not receive any drug.

Systolic blood pressure was determined in conscious, restrained rats by the tail-cuff plethysmographic method (PE-300, Narco Biosystems Inc., Houston, Tex.). The water intake was measured during drug administration, and the daily dose of CS-622 was estimated to be about 10 mg/kg/day.

**Simultaneous Measurement of Cytosolic Free Calcium Level and Muscle Tension in Aortic Tissue**

The thoracic aorta was isolated from rats of each group and cut into spiral strips (2 mm wide and 10 mm long). Endothelium was removed by gently rubbing the intimal surface with a cotton stick in physiological salt solution (PSS). PSS contained (mM): NaCl 136.8, KCl 5.6, CaCl\(_2\) 2.5, MgCl\(_2\) 1.0, glucose 5.5, and HEPES (N-2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid) 10.0 (pH 7.4, 37° C). High K\(^+\) solution was made by substituting NaCl with equimolar KCl. Ca\(^{2+}\)-free solution was made by removing CaCl\(_2\) from PSS. These solutions were bubbled continuously with 100% O\(_2\).

[Ca\(^{2+}\)] was measured simultaneously with muscle tension as reported by Ozaki et al17 and Sato et al.18 The aortic strip was loaded with 7×10\(^{-6}\) M acetoxymethyl ester of fura-2 (fura-2/AM) under protection from light in the presence of 0.2% cremophor EL, a nontoxic detergent, at room temperature (20–25° C). After the loading period of 3–5 hours, the aortic strip was rinsed with PSS and was held horizontally in a bath (7 ml) attached to a fluorimeter (CAF-100, Japan Spectroscopic, Tokyo). The bottom of the bath was made of quartz glass. One end of the strip was connected, with an initial resting tension of 1 g, to a strain gauge transducer (Toyo Boldwin, Tokyo) to monitor the isometric tension. Through the bottom of the muscle bath, excitation light (a spot 2–3 mm in diameter) was given to a part of the muscle that had no hole due to vascular branching. The light was obtained from a xenon high pressure lamp (75 W) equipped with a rotating wheel that had 340 (±5.5) nm and 380 (±5.5) nm interference filters. The muscle strip was excited alternately at a cycle of 48 Hz with two excitation wavelengths (340 and 380 nm), and fluorescence emitted from the tissue was led to a photomultiplier through a 500 (±10) nm filter. The intensity of fluorescence induced by excitation at 340 nm (F\(_{340}\)) was automatically divided by that induced by excitation at 380 nm (F\(_{380}\)) to obtain the ratio (R\(_{340/380}\)). In the muscle strips loaded with fura-2, an increase of [Ca\(^{2+}\)] caused an increase of F\(_{340}\) a decrease of F\(_{380}\) and thus a greater increase of R\(_{340/380}\).17-20 To distinguish between the fura-2 Ca\(^{2+}\) signal and autofluorescence or movement artifact, F\(_{340}\) and F\(_{380}\) were always monitored. Only those preparations in which F\(_{340}\) and F\(_{380}\) changed in mirror image were used in the present experiments. The time constant of the optical system was 0.25 second.

After tension and fluorescence (F\(_{340}\), F\(_{380}\), and R\(_{340/380}\)) stabilized, the tissue was conditioned by two applications of 60 mM K\(^+\). In the first series of experiments, Ca\(^{2+}\) concentration in bathing medium was increased from zero to 2.5 mM in a cumulative manner. In the second series of experiments, a Ca\(^{2+}\) channel activator, CGP-28392 (10\(^{-6}\) M), was added to the bathing solution. In the third series of experiments, nicardipine (10\(^{-7}\) M) was added to the bathing solution. At the end of each experiment, 10\(^{-5}\) M ionomycin (in 60 mM K\(^{+}\) solution) and 4 mM EGTA were sequentially added to determine the maximum (R\(_{\text{max}}\)) and the minimum (R\(_{\text{min}}\)) respectively. In a separate experiment, it was confirmed that further increase in the concentration of ionomycin or EGTA produced little change in R\(_{\text{max}}\) and R\(_{\text{min}}\) respectively. The muscle tension before the application of the test agent (CaCl\(_2\), CGP-28392, or nicardipine) was taken as 0%, and the tension induced by 10\(^{-5}\) M ionomycin (in 60 mM K\(^{+}\)) was taken as 100%.

The following equation has been widely used to calculate [Ca\(^{2+}\)];

\[
[Ca^{2+}] = \alpha \times K_d
\]

where "\(\alpha\)" represents \((R - R_{\text{min}})/(R_{\text{max}} - R)\)×"\(\beta\)\), R represents R\(_{340/380}\), "\(\beta\)" represents the ratio of F\(_{340}\) measured in EGTA plus ionomycin to that measured in Ca\(^{2+}\) plus ionomycin, and K\(_d\) represents the dissociation constant of fura-2 for Ca\(^{2+}\), which is calculated to be 224 nM in PSS.16,18,20,21 Using this method (internal calibration method), absolute [Ca\(^{2+}\)] is cal-
Soda et al
Intracellular Calcium in SHR Aorta

TABLE 1. Systolic Blood Pressure of Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Systolic blood pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control SHR</td>
<td>10</td>
<td>211±4</td>
</tr>
<tr>
<td>CS-622-treated SHR</td>
<td>7</td>
<td>130±6*</td>
</tr>
<tr>
<td>Age-matched WKY rats</td>
<td>7</td>
<td>137±3*</td>
</tr>
</tbody>
</table>

Twelve-week-old spontaneously hypertensive rats (SHR) received vehicle (NaHCO3 and KHCO3 each 1 g/l) or CS-622 (100 mg/l in the vehicle, about 10 mg/kg/day) in their drinking water for 8 weeks. Systolic blood pressure was measured by tail plethysmography. Wistar-Kyoto (WKY) rats (20 weeks old) did not receive drug or vehicle. Values are expressed as mean±SEM. n, number of experiments.

*p<0.01 significantly different from control SHR by Duncan’s test.

calculated even in the presence of background fluorescence. However, because the dissociation constant in smooth muscle cytoplasm may be greater than 224 nM, we used the “a” instead of [Ca2+] as an indicator of [Ca2+] in the present study.

Data were expressed as mean±SEM. Statistical difference was evaluated by Duncan’s multiple range test when variances were uniform and by Cochran-Cox test when variances were not uniform.

Drugs

The following drugs were used: CS-622 [α-{(2S,6R)-6-[(1S)-1-ethoxycarbonyl-3-phenylpropyl] amino-5-oxo-2-(2-thienyl) perhydro-1,4-thiazepin-4-yl} acetic acid hydrochloride] (Research Laboratories, Sankyo Co., Ltd., Tokyo), fura-2/AM (DMSO solution, Dojindo Laboratories, Kumamoto, Japan), CGP-28392 (Ciba-Geigy Ltd., Basel, Switzerland), nicardipine hydrochloride (Sigma Chemical Co., St Louis, Mo.), ionomycin (Calbiochem Corp., La Jolla, Calif.), cremophor EL (Nakarai Chemicals, Ltd., Kyoto, Japan), EGTA (Tokyo Kasei Kogyo Co., Ltd., Tokyo), N,N-dimethylacetamide (Wako Pure Chemical Industries Ltd., Osaka, Japan), and HEPES (Dojindo Laboratories, Kumamoto, Japan). To make the stock solution, ionomycin and CGP-28392 were dissolved in ethanol and N,N-dimethylacetamide, respectively.

Results

Blood Pressure of Rats

Administration of CS-622 (100 mg/l in drinking water, about 10 mg/kg/day) for 8 weeks lowered systolic blood pressure of SHR from 203±5 to 130±6 mm Hg (n=7), whereas vehicle alone did not. Systolic blood pressure of CS-622-treated SHR was similar to that of untreated WKY rats (Table 1). At the end of the dosing period, the aorta was excised and used in experiments described below.

Effects of Alteration in Calcium Concentration of Bathing Medium on Fura-2 Calcium Signal and Muscle Tension in Rat Aortas

Figure 1 shows typical recordings of cytosolic free calcium concentration ([Ca2+]i) in terms of “a” (see Methods) and muscle tension simultaneously measured in fura-2-loaded aortas isolated from control SHR, CS-622-treated SHR, and untreated WKY rats. When Ca2+ concentration of bathing medium was increased from zero to 0.5 and to 2.5 mM, [Ca2+], was increased in aortas from all the groups. However, the increase was much greater in the control SHR group than in the other two groups. Addition of Ca2+ elicited a contraction in aortas from control SHR, but not in aortas from CS-622–treated SHR or untreated WKY rats.

Figure 2 (A and B) summarizes [Ca2+], and muscle tension in aortas isolated from the three groups in the presence of different external Ca2+ and K+ concentrations. [Ca2+]i and muscle tension in 60 mM K+ solution were almost identical in the three groups.

![Figure 1](image-url)  
**Figure 1.** Typical recordings of cytosolic free calcium concentration ([Ca2+]i) (upper trace) and muscle tension (lower trace) simultaneously measured in aortic strips loaded with fura-2. Aortas isolated from control spontaneously hypertensive rats (SHR) (panel A), from SHR treated with CS-622 (about 10 mg/kg/day) for 8 weeks (panel B), and from untreated age-matched Wistar-Kyoto (WKY) rats (panel C) are shown. Ca2+ concentration of bathing medium was increased from zero (0 Ca) to 0.5 mM (0.5 Ca) and to 2.5 mM (2.5 Ca), followed by application of 60 mM K+ (60 K). Ionomycin (IM) (10^-3 M) and EGTA (4 mM) were further added to obtain the maximum and minimum levels of [Ca2+]i, respectively. Dotted lines indicate the level of [Ca2+]i in 2.5 mM Ca2+ medium. [Ca2+]i is shown by “a” calculated as follows: “a”=([R−Rmin]/(Rmax−R)×“B” (see Methods).
normal solution (2.5 mM Ca\(^{2+}\), 5.9 mM K\(^+\)), however, aortic [Ca\(^{2+}\)] and muscle tension of control SHR (0.83±0.10 and 18.4±4.3%, respectively) were higher than those in CS-622-treated SHR (0.40±0.04 and 1.2±0.5%) or untreated WKY rats (0.45±0.07 and 1.2±0.5%). These data indicate that SHR aortas have higher [Ca\(^{2+}\)], and tension than WKY rat aortas in a resting condition and that long-term treatment with CS-622 reduces [Ca\(^{2+}\)] and tension of SHR aortas to levels similar to those of WKY rats.

Effects of a Calcium Channel Activator on Fura-2 Calcium Signal and Muscle Tension in Rat Aortas

Figure 3 shows typical recordings of [Ca\(^{2+}\)], and muscle tension simultaneously measured in aortas from the three groups of rats. In the aortas from the control SHR, addition of CGP-28392 at 10\(^{-6}\) M produced a rhythmic increase of [Ca\(^{2+}\)], and a contraction with rhythmic activity. On the other hand, CGP-28392 produced only a slight increase in [Ca\(^{2+}\)], and no change in muscle tension in the aortas isolated from both CS-622-treated SHR and untreated WKY rats.

Figure 4 (A and B) summarizes the effect of CGP-28392 on aortic [Ca\(^{2+}\)] and muscle tension, respectively. The basal [Ca\(^{2+}\)], of the control SHR group was higher than that of the CS-622-treated SHR or untreated WKY rat group (Base in Figure 4A). These basal [Ca\(^{2+}\)], levels were lower than those in Figure 2A (calcium 2.5, potassium 5.9). In the experiments where external Ca\(^{2+}\) concentration was changed (Figures 1 and 2), aortas had been exposed to Ca\(^{2+}\)-free medium (without EGTA) before Ca\(^{2+}\) was added to the bathing solution. This may account for the slightly greater...

Figure 2. Bar graphs showing changes in cytosolic free calcium concentration (panel A) and muscle tension (panel B) in aortas from control spontaneously hypertensive rats (SHR), CS-622-treated SHR, and untreated Wistar-Kyoto (WKY) rats. This figure summarizes data obtained from experiments where Ca\(^{2+}\) concentration of bathing medium was increased from zero to 0.5 mM and to 2.5 mM, followed by application of 60 mM K\(^+\). Cytosolic free calcium level was shown by "a." Tension was expressed as a percentage of contraction induced by 10\(^{-5}\) M ionomycin (see Methods). Each column represents mean±SEM of 7–10 experiments. *p<0.05; **p<0.01 compared with control SHR group.

Figure 3. Simultaneous recordings of cytosolic free calcium concentration ([Ca\(^{2+}\)]) (upper trace) and muscle tension (lower trace) in fura-2–loaded aortic strips when 10\(^{-6}\) M CGP-28392 (CGP) was added. Aortas isolated from control spontaneously hypertensive rats (SHR) (panel A), from SHR treated with CS-622 (about 10 mg/kg/day) for 8 weeks (panel B), and from untreated age-matched Wistar-Kyoto (WKY) rats (panel C) are shown. After CGP application, K\(^+\) was increased to 60 mM (60 K). Finally, ionomycin (IM) (10\(^{-3}\) M) and EGTA (4 mM) were added to obtain the maximum and minimum levels of [Ca\(^{2+}\)], respectively. [Ca\(^{2+}\)], is shown by "a" calculated as follows: "a"=[(R–R\(_{\text{min}}\))/(R\(_{\text{max}}\)-R)]× "B" (see Methods).
Intracellular Calcium in SHR Aorta

Sada et al

Figure 4. Bar graphs showing changes in cytosolic free calcium concentration (panel A) and developed tension (panel B) induced by CGP-28392 (10^-6 M) in aortas from control spontaneously hypertensive rats (SHR), CS-622-treated SHR, and untreated Wistar-Kyoto (WKY) rats. This figure summarizes data obtained from experiments where CGP was added, followed by application of high K+ (60 mM). Cytosolic free calcium level was shown by "a." Developed tension was expressed as a percentage of the maximum contraction induced by 10^-5 M ionomycin (see Methods). Each column represents mean±SEM of 7-10 experiments. *p<0.05; **p<0.01 compared with control SHR group.

Effects of Nicardipine on Basal Calcium Level and Basal Tension in Rat Aortas

Nicardipine (10^-7 M) was applied to the aortic muscle in a resting condition. As shown in an example (Figure 5), nicardipine decreased basal [Ca^{2+}] from 0.74±0.08 to 0.39±0.06 and resting tension (-22±8%) in SHR aorta (n=4), but the agent induced only a slight decrease in these parameters (from 0.42±0.08 to 0.32±0.02 in [Ca^{2+}], and -4±1% in tension) in WKY rat aortas (n=3). These results suggest that dihydropyridine-sensitive Ca^{2+} channels are activated to increase resting [Ca^{2+}] in SHR aorta.

Discussion

Changes in membrane properties of VSMC have been implicated in the alteration of vascular function in hypertension. Several studies, including ours, have indicated that the voltage-dependent Ca^{2+} channels are altered in arteries isolated from SHR: 1) Ca^{2+} channel activators such as Bay K-8644 and CGP-28392 elicit greater contractions in SHR arteries than normotensive WKY rat arteries; 2) the resting tension is higher in SHR arteries than in WKY rat arteries, and it is decreased by Ca^{2+} channel antagonists like nicardipine; 3) SHR arteries are more sensitive to K+ depolarization than WKY rat arteries. None of these studies,
however, measured the [Ca$^{2+}$], of arteries simultaneously with muscle tension.

The present study demonstrated: 1) the increase of [Ca$^{2+}$], and tension in response to an increase of Ca$^{2+}$ concentration in the bathing solution was greater in SHR aortas than in WKY rat aortas, 2) the increase of [Ca$^{2+}$], and tension in response to CGP-28392 was greater in SHR aortas than in WKY rat aortas, 3) nicardipine decreased resting [Ca$^{2+}$], and tension to a greater extent in SHR aortas than in WKY rat aortas. These findings suggest that SHR aortas have a higher myogenic tone due to increased [Ca$^{2+}$], than WKY rat aortas and that the increased [Ca$^{2+}$], is attributed to alterations of dihydropyridine-sensitive Ca$^{2+}$ channels in SHR aortas. This may explain more clearly the role of [Ca$^{2+}$] of VSMC in the pathogenesis of hypertension in this particular animal model of hypertension, the SHR.

Although the increased vascular tone could be due to the elevated [Ca$^{2+}$], in VSMC, it could also be due to an increased Ca$^{2+}$ sensitivity of the regulatory or contractile proteins. However, the later possibility seems to be remote because of the observation that Ca$^{2+}$ sensitivity of contractile apparatus in SHR arteries does not differ from that in WKY rat arteries in chemically skinned muscles.\cite{38,39} The [Ca$^{2+}$], tension curves constructed from data of three different groups of rats (Figure 6) support this notion: It appears that values from the three different groups lie on a single curve, although there are not enough data in the middle range of [Ca$^{2+}$], for WKY rats and CS-622-treated SHR.

CS-622 is a selective ACE inhibitor that does not have any other pharmacological actions.\cite{40} Our previous studies showed that long-term administration of CS-622 suppressed the increased Ca$^{2+}$-dependent resting tone and CGP-28392-induced contraction in SHR aorta and that these suppressions were never achieved by either long-term treatment with hydralazine or acute inhibition of aortic ACE in vitro.\cite{5,25} In the present study, long-term treatment with CS-622 (10 mg/kg/day) lowered blood pressure of SHR to the same level as that of WKY rats (Table 1) and lowered both [Ca$^{2+}$], and resting tone in SHR aortas to levels in WKY rat aortas (Figure 2). Exaggerated response of SHR aortas in [Ca$^{2+}$], and tension development in response to CGP-28392 was also normalized by chronic inhibition of ACE. These observations suggest that [Ca$^{2+}$], of VSMC is under long-term control by the renin-angiotensin system and that a decrease of [Ca$^{2+}$], in VSMC is an important factor underlying antihypertensive action of chronic ACE inhibition. But we do not know how the chronic inhibition of ACE lowers [Ca$^{2+}$], in SHR aortas. One possible explanation is that chronic inhibition of ACE alters the voltage-related functions of dihydropyridine-sensitive Ca$^{2+}$ channels in SHR arteries, or decreases the number of Ca$^{2+}$ channels, thereby decreasing Ca$^{2+}$ influx through these channels. To elucidate precise mechanisms, further studies are needed.

In summary, the present study demonstrated that both resting tension and [Ca$^{2+}$], were increased in SHR aorta and that chronic inhibition of ACE normalized these abnormal features of SHR aorta. These observations suggest that the activity or expression of dihydropyridine-sensitive Ca$^{2+}$ channels of VSMC is under long-term control by the renin-angiotensin system and is increased in SHR aorta.

Acknowledgment

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References


![Figure 6. Line graph showing relation between cytosolic free calcium concentration ([Ca$^{2+}$]) and muscle tension when external Ca$^{2+}$ concentration was altered in aortas isolated from control spontaneously hypertensive rats (SHR), CS-622-treated SHR, and untreated Wistar-Kyoto (WKY) rats. Averaged [Ca$^{2+}$], and tensions (panels A and B, respectively, in Figure 2) were plotted. Curved line indicates relation for control SHR group.](image-url)


**Key Words**  • calcium  • vascular smooth muscle  • fluorescent indicators  • converting enzyme inhibition  • spontaneously hypertensive rats
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