Caveolae Dysfunction Contributes to Impaired Relaxation Induced by Nitric Oxide Donor in Aorta from Renal Hypertensive Rats


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ABSTRACT

Relaxation induced by nitric oxide (NO) donors is impaired in renal hypertensive two kidney-one clip (2K-1C) rat aortas. It has been proposed that caveolae are important in signal transduction and Ca\(^{2+}\) homeostasis. Therefore, in the present study we investigate the integrity of caveolae in vascular smooth muscle cells (VSMCs), as well as their influence on the effects produced by NO released from both the new NO donor [Ru(NH\(_2\)\(_2\))\(_2\) (terpy)NO] (TERPY) and sodium nitroprusside (SNP) on 2K-1C rat aorta. The potency of both TERPY and SNP was lower in the 2K-1C aorta than in the normotensive aorta (two kidney (2K)), whereas the maximal relaxant effect (ME) was similar in both 2K-1C and 2K aortas. In the 2K aorta, methyl-\(\beta\)-cyclodextrin (CD) reduced both the potency of TERPY and SNP, and their ME compared with the control, but it had no effect on the potency and ME of these NO donors in 2K-1C aortas. The decrease in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_c\)]) induced by TERPY was larger in 2K than in 2K-1C, and this effect was inhibited by CD in 2K cells only. Aortic VSMCs from 2K rats presented a larger number of caveolae than those from 2K-1C rats. Treatment with CD reduced the number of caveolae in both 2K and 2K-1C aortic VSMCs. Our results support the idea that caveolae play a critical role in the relaxant effect and in the decrease in [Ca\(^{2+}\)\(_c\)] induced by NO, and they could be responsible for impaired aorta relaxation by NO in renal hypertensive rats.

Caveolae are flask-shaped invaginations of the plasma membrane that are abundant in endothelial cells and vascular smooth muscle cells (VSMCs) (Voldstedlund et al., 2001). They are rich in cholesterol, glycosphingolipids, and the structural protein caveolin. Cholesterol is a very important component of caveolae, and it appears to be crucial to the maintenance of the structural integrity of this vesicular complex, because caveolae disappear in cells that are depleted of cholesterol. Previous studies have shown that cell exposure to sterol-binding agents such as methyl-\(\beta\)-cyclodextrin removes cholesterol from the plasmalemma. This causes caveolae disassembly (Linder et al., 2005), whereas the general morphology of the tissue is preserved.

It has been proposed that caveolae are important in signal transduction. Diverse functional roles have been ascribed to them, including regulation of macromolecular transport, cell volume regulation, and Ca\(^{2+}\) homeostasis (Taggart, 2001). Voltage-gated Na\(^+\) channels have been reported to be localized in cardiac myocyte caveolae (Yarbrough et al., 2002), and \(\alpha\)-subunits of L-type Ca\(^{2+}\) channels can be found in caveolin-enriched membranes in the smooth muscle (Darby et al., 2000). In addition, caveolin expression seems to enhance or to be required for the swelling-induced Cl\(^-\) channel function (Okada, 1999). Na\(^+\)/K\(^+\)-ATPase pump and Na\(^+\)/Ca\(^{2+}\) exchange (Moore et al., 1993) are also found in caveolae. Furthermore, in many smooth muscles, caveolae are often in close proximity to the underlying network of the peripheral sarcoplasmic reticulum, commonly separated by distances as little as 10 to 40 nm (Nixon et al., 1994). Therefore, the fact that caveolae are located adjacent to the sarcoplasmic reticulum, close to channels, pumps, and exchangers important for Ca\(^{2+}\) mobilization and transport, supports the notion that caveolae may influence Ca\(^{2+}\) signaling.

ABBREVIATIONS: VSMC, vascular smooth muscle cell; NO, nitric oxide; TERPY, [Ru(NH\(_2\)\(_2\))\(_2\) (terpy)NO]\(^{3+}\); SNP, sodium nitroprusside; 2K-1C, two kidney-one clip or renal hypertensive; 2K, two kidney or normotensive; PSS, physiological saline solution; CD, methyl-\(\beta\)-cyclodextrin; ME, maximal relaxant effect; YC-1, 3-(5-hydroxymethyl-2-furyl)-1-benzyl indazole.
Drab et al. (2001) have demonstrated extensive changes in the cardiovascular system of knockout mice to caveolin-1, which is a structural protein essential for caveola formation. Various diseases lead to changes in caveolin levels and/or to the appearance of caveola (Taggart, 2001). However, in the case of renovascular hypertension, there are no studies describing caveola in the VSMCs from thoracic aortas.

Hypertension has been associated with vascular reactivity impairment to relaxant agents. In several models of experimental hypertension the NO-dependent relaxation is reduced. Impaired relaxation to NO, both endothelium-dependent (Callera et al., 2000, 2004) and endothelium-independent (Heitzer et al., 1999; Callera et al., 2000, 2004; Bonaventura et al., 2005), has been observed in arteries from renovascular hypertensive rats. This impairment has been attributed to several factors such as endothelial dysfunction by decreased NO release and/or endothelium-derived hyperpolarizing factor (Manenti et al., 1995), increased production of superoxide anions (Heitzer et al., 1999), and alterations in the membrane potential of VSMCs (Callera et al., 2000). On the basis of this information, it seems that impaired relaxation to NO in arteries from hypertensive rats occurs due to endothelial dysfunction and to some dysfunction in the vascular smooth muscle. Thus, the aim of this study was to investigate dysfunction in the vascular smooth muscle. We hypothesized that the caveola in VSMCs are disassembled, thus contributing to the exogenous NO effect in reducing the cytosolic Ca\(^{2+}\) concentration. Therefore, in the present study we investigated the integrity of the caveola in VSMCs from the thoracic aorta and their influence on the relaxant effect of NO released from both the new NO donor ([Ru(NH\(_3\)H\(_2\))(terpy)NO\(^{-}\)]\(^{1+}\) (TERPY) (de Lima et al., 2006) and the classic NO donor sodium nitroprusside (SNP) on 2K-1C rat aorta.

Materials and Methods

Experimental Animals

Renovascular hypertension was induced in rats following the 2K-1C Goldblatt model. In brief, male Wistar rats (180–200 g) were anesthetized and submitted to a midline laparotomy. Then, a silver clip with an internal diameter of 0.20 mm was placed around the left renal artery. Normotensive 2K rats were submitted to laparotomy only. Animals were maintained on standard rat chow with a 12-h light/dark cycle and given free access to food and water. The systolic blood pressure was measured weekly in nonanesthetized animals by an indirect tail-cuff method (MLT125R pulse transducer/pressure cuff coupled to the PowerLab 4/8 analog-to-digital converter; AD Instruments Pty Ltd., Castle Hill, Australia), and rats were considered hypertensive when the systolic blood pressure was higher than 160 mm Hg. Experimental protocols followed standards and policies of the University of São Paulo’s Animal Care and Use Committee.

Vascular Reactivity Studies

Six weeks after surgery, rats were killed by decapitation, and the thoracic aortas were isolated. Aortic rings, 4 mm in length, were placed in bath chambers (10 ml) for isolated organs containing physiological salt solution (PSS) at 37°C, continuously bubbled with 95% O\(_2\) and 5% CO\(_2\), pH 7.4. Two fine stainless steel holders were placed through the lumen of the aortic rings; one of the holders was fixed to the tissue chamber and the other was connected to an F-60 force-displacement transducer, and the contractile/relaxant responses were recorded on a polygraph (Narco Biosystems Inc., Houston, TX). The aortic rings were submitted to a tension of 1.5 g, which was readjusted every 15 min throughout a 60-min equilibration period before addition of the given drug. An optimal basal tension of 1.5 g in aortic rings from both 2K and 2K-1C rats was previously standardized by exposing the vessels to 90 mM KCl under various resting tensions (0.25–2.5 g). The endothelium was mechanically removed by gently rubbing the intimal surface with stainless steel holders. Endothelial integrity was qualitatively assessed by the degree of relaxation caused by 1 \(\mu\)M acetylcholine in the presence of contractile tone induced by phenylephrine (0.1 \(\mu\)M). As our studies required endothelium denuded aortas, the rings were discarded if there was any degree of relaxation to avoid the possible influence of endothelial factors.

After the endothelial integrity was assessed, aortic rings were precontracted with phenylephrine (100 nM). When the plateau was reached, concentration-effect curves to the NO donors TERPY (1 mM–300 \(\mu\)M) (de Lima et al., 2006) and SNP (0.1 mM–10 \(\mu\)M) were constructed in the 2K and 2K-1C aortic rings. Methyl-\(\beta\)-cyclodextrin (CD) (10 mM) (Linder et al., 2005), a caveola disassembler, was added 60 min before phenylephrine to examine the effect of CD on the relaxation induced by the NO donors. Concentration-effect curves to both TERPY and SNP were constructed in aortic rings from 2K and 2K-1C rats, precontracted with phenylephrine in the presence or in the absence of CD. Control responses were obtained in experiments where CD was replaced by vehicle (PSS).

Vascular Smooth Muscle Cell Isolation

To confirm the results obtained with TERPY in the aorta tissue by vascular reactivity studies, all the experiments with TERPY were performed using smooth muscle cells isolated from the aorta using confocal microscopy. Six weeks after the surgery, rats were killed by decapitation, and the thoracic aortas were isolated. VSMCs were isolated from the aorta by enzymatic digestion. In brief, the aortas were dissected and longitudinally opened. The endothelium and the adventitia were removed, and the tissue was minced into small pieces, which were incubated in Ca\(^{2+}\)-free Hanks’ solution containing collagenase type II-S (0.03 mg/ml). The tissue was gently shaken in this solution for 25 min at 37°C and bubbled with a carbogen mixture. After that, bovine serum albumin (type 1, 10 mg/ml) was added to the vessel fragments present in Ca\(^{2+}\)-free Hanks’ solution, and the cells were released by mechanical dispersion with a Pasteur pipette. The resultant cell suspension was centrifuged at 1000 rpm for 3 min and suspended in Dulbecco’s modified Eagle’s medium containing glutamine (2 mM), HEPES (pH 7.4, 20 mM), penicillin (10,000 U/ml), and streptomycin (10,000 \(\mu\)g/ml) (Palmberg and Thyberg, 1986). The cells were plated on glass coverslips, kept in a humidified 37°C incubator gassed with 5% CO\(_2\), and used 3 h after plating, in a serum-free medium. A similar procedure was carried out for 2K and 2K-1C rats.

Ca\(^{2+}\) Imaging with Fluorescent Probe and Confocal Microscopy

Dye Loading and Treatments. To assess cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), VSMCs were loaded with Fluo-3-AM (10 \(\mu\)M) for 30 min at room temperature. Excess dye was removed by washing out the dye with bath solution and allowing 30 min for intracellular deesterification of Fluo-3-AM. CD (10 mM) was added 60 min before cell loading with Fluo-3-AM in a humidified 37°C incubator gassed with 5% CO\(_2\), and used 3 h after plating, in a serum-free medium. A similar procedure was carried out for 2K and 2K-1C rats.

Microscopy and Image Analysis. The cells were imaged in Hanks’ buffer (pH 7.4). [Ca\(^{2+}\)]\(_i\), was assessed by a confocal scanning laser microscope (Leica TCS SP5). Fluo-3-AM fluorescence was excited with the 488 nm line of an argon ion laser, and the emitted fluorescence was measured at 510 nm. A time course software was used to capture images of the cells at 0.830-s intervals (cyt), in the Live Data Mode acquisition. By using the laser scanning confocal microscope computer software, the intensity of the intracellular maximal or minimal fluorescence was measured. The initial fluorescence intensity value obtained at \(t = 0\) was designated \(F_0\), and the
final fluorescence intensity value obtained after stimulation with TERPY (10 µM) was designated \( F \). In this way, the percentage of the difference in fluorescence intensity (%ΔFI), which reflects the decrease in \([\text{Ca}^{2+}]_c\) was obtained for each protocol in relation to the control (100%): %ΔFI = (\( F_0 \) - \( F/F_0 \)) × 100.

**Electron Microscopy**

Procedures were performed as described by Moreira et al. (1996). In brief, rat aorta treated or not treated with CD (10 mM) for 60 min was dissected out and fixed by immersion in a solution containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1 mM sodium cacodylate buffer for 24 h. Preparations were kept in sodium cacodylate (0.1 mM) in a freezer until the next step.

Postfixation was carried out in 1% osmium tetroxide for 1 h. The muscle was washed twice in the cacodylate buffer and twice in a sodium acetate buffer (0.1 mM, pH 5.0, adjusted with acetic acid) for 10 min each time, stained in block with 2% uranyl acetate in a sodium acetate buffer (0.1 mol/liter, pH 5.0) overnight, and washed in the acetate buffer. Osmium and uranyl acetate steps were performed on ice. The samples were then dehydrated using a graded series of ethanol (50, 75, 85, and 95%, 10 min each), ending in two changes of 100% ethanol for 5 min, followed by 100% propylene oxide to complete dehydration. The infiltration was done using Araldite resin (CY 212; Agar Scientific, Essex, England) and propylene oxide in a 1:1 ratio overnight, 2:1 ratio for 4 h, 3:1 ratio for 4 h; and 100% resin for 24 h. The infiltrated samples were placed in Beam capsules containing fresh Araldite and placed in an oven at 60°C for 36 h to polymerize. The plastic blocks were trimmed and sectioned in a Leica Ultracut UCT ultramicrotome. Semithin sections of 0.5 µm were stained with 1% toluidine blue to choose the appropriate areas for ultrathin sectioning (60–70 nm) under light microscopy. Ultrathin sections were collected on Pyoloform and carbon-coated single slot grids, and they were contrasted with uranyl acetate and lead citrate. Electron micrographs were taken at an initial magnification of 8000×, and they were photographically enlarged on the computer screen to a magnification of 50,000×.

Morphometry and quantitative analysis were performed with the NIH ImageJ software from National Institutes of Health, Research Services Branch (Bethesda, MD) over scanned negatives on a Macintosh G4 computer. Caveolae were counted in the cytosolic space next...
to the membrane. Results are expressed as the number of the caveolae per cell area (square micrometer).

**Statistical Analysis**

Data are expressed as means ± S.E.M. In each set of experiments, n indicates the number of rats studied. The values for vascular reactivity responses to TERPY and SNP are expressed as the percentage of the preceding contraction induced by phenylephrine. The concentration of the agonist producing a half-maximal response (EC50) was determined after logit transformation of the normalized concentration-response curves, and it is reported as the negative logarithm (−log EC50 = pD2 values) of the mean of individual values for each tissue, using GraphPad Prism (version 3.0; GraphPad Software Inc., San Diego, CA). The maximal relaxant effect (ME) was considered to be the maximal amplitude response reached in concentration-effect curves for relaxant agents. The decrease in [Ca2+]c in cells stimulated with TERPY was obtained from %ΔFI. The values for electron microscopy are expressed as the number of the caveolae per square micrometer. Statistical analysis of the results was performed using GraphPad Prism. Statistical significance was tested by one-way analysis of variance (post hoc test: Newman-Keuls) and Student’s t test. Values of *, p < 0.05, **, p < 0.01, and ***, p < 0.001 were considered to be significant.

**Materials**

Glutaraldehyde, paraformaldehyde, sodium cacodylate, osmium tetroxide, sodium acetate, propylene oxide, araldite plastic, uranyl acetate, and lead citrate were purchased from electron microscopy sciences (EMS, Fort Washington, PA). The composition of the PSS was the following: 130.0 mM NaCl, 1.6 mM CaCl2, 4.7 mM KCl, 1.17 mM MgSO4, 1.18 mM KH2PO4, 14.9 mM NaHCO3, 0.026 mM EDTA, and 5.5 mM dextrose.

**Drugs.** Acetylcholine, phenylephrine, sodium nitroprusside, methyl-β-cyclodextrin, and Fluo-3AM were obtained from Sigma-Aldrich (St. Louis, MO). [Ru(NH.NHq)(terpy)NO]3 was synthesized at the Laboratory of Analytical Chemistry by Dr. Roberto Santana da Silva.

**Results**

**Vascular Reactivity.** As shown in Fig. 1A, the new NO donor TERPY induced a concentration-dependent relaxation in endothelium-denuded aortas contracted with...
phenylephrine. The potency of TERPY in inducing relaxation was greater in the 2K rat aortas (pD2: 7.05 ± 0.07, n = 6) than in the 2K-1C rat aortas (pD2: 6.51 ± 0.06, p < 0.01, n = 6), whereas the maximal relaxant effect was similar in both cases (2K: 108.6 ± 2.0%; 2K-1C: 110.7 ± 0.7%). Similar results were obtained in the case of relaxation by the classic NO donor SNP. The potency of SNP in inducing relaxation was greater in the 2K rat aortas (pD2: 8.90 ± 0.08, n = 7) than in the 2K-1C rat aortas (pD2: 8.44 ± 0.15, p < 0.01, n = 6), and the maximal relaxant effect was similar in both aortas (2K: 114.3 ± 3.4%, n = 7; 2K-1C, 110.3 ± 1.2%, n = 6) (Fig. 2A).

To verify the influence of caveolae on the relaxation induced by NO donors, we treated aortas with CD (10 mM), a caveolae disassembler. Our results showed that the relaxation induced by TERPY was inhibited by treating of 2K aortic rings with CD (pD2: 6.55 ± 0.09; ME: 97.9 ± 1.7%, n = 6), compared with the nontreated aortic rings (pD2: 7.05 ± 0.07, p < 0.01; ME: 108.6 ± 2.0%, p < 0.05; n = 6) (Fig. 1B). On the other hand, as shown in Fig. 1C, CD did not change the relaxation induced by TERPY in 2K-1C aortic rings (pD2: 6.33 ± 0.11; ME: 104.5 ± 2.6%; n = 6) versus control (pD2: 6.51 ± 0.06; ME: 110.7 ± 0.7%; n = 6). In the same way, the relaxation induced by SNP was inhibited by treating 2K aortic rings with CD (pD2: 8.47 ± 0.07; ME: 101.7 ± 1.4%; n = 7), compared with the nontreated aortic rings (pD2: 8.90 ± 0.08, p < 0.01; ME: 114.3 ± 3.4%, p < 0.01; n = 7) (Fig. 2B). However, CD did not change the relaxation induced by SNP in 2K-1C aortic rings (pD2: 8.46 ± 0.12; ME: 103.6 ± 2.6%; n = 6) versus control (pD2: 8.44 ± 0.15; ME: 110.3 ± 1.2%; n = 6) (Fig. 2C).

Confocal Microscopy. To confirm the results obtained with TERPY in the aorta tissue by vascular reactivity studies, we used confocal microscopy. Our results show that the reduction of [Ca2+]c in response to TERPY was more significant in the case of cells from 2K rats (%ΔFI: 55.71 ± 4.81%, n = 5) compared with 2K-1C aortic cells (%ΔFI: 36.29 ± 2.92%, p < 0.001, n = 4), as shown in Fig. 3. These results corroborate the vascular reactivity to TERPY in 2K and 2K-1C aortic rings, as shown in Fig. 1A.

We also verified that the reduction in [Ca2+]c, induced by TERPY was lower in 2K cells treated with CD (%ΔFI: 35.49 ± 5.46%, n = 4) compared with those from 2K rats (%ΔFI: 55.71 ± 4.81%, p < 0.001, n = 5) (Fig. 3). These results corroborate the vascular reactivity to TERPY in 2K aorta treated with CD as shown in Fig. 1B.

CD did not change the decrease in [Ca2+]c induced by TERPY in 2K-1C aortic cells (%ΔFI: 37.20 ± 1.14%, n = 4) versus control (%ΔFI: 36.29 ± 2.92%, p < 0.05) (Fig. 3), corroborating the vascular reactivity to TERPY in 2K-1C aorta treated with CD as shown in Fig. 1C.

Electron Microscopy. To investigate the integrity of caveolae in VSMCs and to verify whether treatment with CD (10 mM) would reduce the number of caveolae in VSMCs from the aorta, we performed out experiments with electron microscopy. Our results gave evidence of a larger amount of caveolae in 2K (73.20 ± 10.74 caveolae/μm², n = 3) than in 2K-1C VSMCs (30.33 ± 8.68 caveolae/μm², n = 5) (Fig. 4). Moreover, treatment with CD (10 mM) reduced the number of the caveolae per square micrometer in both 2K (38.80 ± 7.55 caveolae/μm², n = 5) and 2K-1C aortic cells (14.00 ± 0.80 caveolae/μm², n = 3) (Fig. 4).

These results are structurally visualized in the representative electron micrographs from 2K and 2K-1C aortas that were not treated with CD (Fig. 5A) and of those treated with the cholesterol-binding agent CD (Fig. 5B). The photographs show that the caveolae are mainly aligned under the sarcolemma, forming periodical clusters, but treatment with CD disassembles the linear distribution of the caveolae and the uniformity of the cell.

Discussion

To investigate the influence of caveolae in aortas from 2K-1C rats on the relaxation induced by NO donors, we used the new NO donor TERPY and the classic NO donor SNP. TERPY is a thermally stable nitrosyl ruthenium complex that is also stable in physiological pH (de Lima et al., 2006). In the present work, we found decreased sensitivity of 2K-1C rat aortic rings to the NO donors TERPY and SNP. The effect

![Fig. 3. Effect of CD on the reduction of ([Ca2+]c) to TERPY in aortic smooth muscle cells from 2K and 2K-1C rats. %ΔFI shows average of percent reduction in FI that indicates a reduction [Ca2+]c. Differences in %ΔFI values to TERPY are indicated as ***, p < 0.001, 2K versus 2K + CD, 2K-1C, 2K-1C + CD.](image)

![Fig. 4. Number of caveolae square micrometer in aortic smooth muscle cells from 2K and 2K-1C rats. The tissues were treated to design the electron microscopy protocol, as described under Materials and Methods. Bars represent the means ± S.E.M. of three to five electron micrographs counted in areas of similar magnitudes. The values are expressed as the number of caveolae per area (square micrometers). *, significant difference (p < 0.05) in relation to the caveolae number in 2K-1C and 2K + CD aorta. **, significant difference (p < 0.01) in relation to the number of caveolae in 2K aorta cells.](image)
of TERPY in reducing $[\text{Ca}^{2+}]_c$ is also impaired in 2K-1C rats. In other studies using different NO donors, a reduction in the sensitivity to NO in the 2K-1C rat aortas was also reported (Heitzer et al., 1999; Callera et al., 2000, 2004; Bonaventura et al., 2005).

In the present study, when aortic rings and aortic cells were treated with CD, the effect of TERPY was impaired in 2K rats only, and the effect of SNP was impaired in 2K aortic rings only. On the other hand, in renal arteries and mesenteric arteries from normotensive rats treated with 1 mM CD, relaxation by SNP was not impaired (Xu et al., 2007). In the same way, in rabbit aorta treated with 2% 2-hydroxypropyl-β-cyclodextrin relaxation by SNP was not impaired either (Darblade et al., 2001). In the aorta of normotensive rats, Linder et al. (2005) demonstrated that 1 mM CD did not change the endothelium-independent relaxation to YC-1. However, this change was found to occur with 10 mM CD. Probably these contrasting results are due to different concentrations of caveolae disassemblers and/or different experimental animals. These results indicate an impaired relaxation by TERPY and SNP, as well as a failure of the new NO donor in decreasing $[\text{Ca}^{2+}]_c$, which could be related with the reduced number of caveolae in 2K-1C aortas.

Some functions have been attributed to caveolae, including $\text{Ca}^{2+}$ homeostasis. Several studies have revealed that the caveolae from smooth muscle cells contain a $\text{Ca}^{2+}$-ATPase pump (Fujimoto et al., 1992), an ATP-sensitive K$^+$ channel (Sampson et al., 2004), a voltage-activated K$^+$ channel (Martens et al., 2004), a large conductance $\text{Ca}^{2+}$-activated K$^+$ channel (Lu et al., 2006), a Na$^+$/K$^+$-ATPase pump, and a Na$^+$/Ca$^{2+}$ exchange (Moore et al., 1993). Moreover, soluble guanylyl cyclase, which is the natural NO receptor, is translocated to caveolar domains to be activated by NO (Zabel et al., 1997). These results indicate that caveolae have some roles in $\text{Ca}^{2+}$ homeostasis and in the NO relaxant mechanisms as pumps, channels, exchangers, and receptors that have been found in caveolae are either activated or inhibited by NO.

The NO-induced vasorelaxation involves the activation of soluble guanylyl cyclase mainly, which catalyzes the conversion of GTP into cGMP (Arnold et al., 1977). Acting as a second messenger, cGMP activates a family of serine/threonine protein kinases called cGMP-dependent protein kinases, which induce relaxation by decreasing $[\text{Ca}^{2+}]_c$ and Ca$^{2+}$ desensitization of the actin-myosin contractile system (Lee et al., 1997). Experimental studies have identified various molecular targets that can be regulated by cGMP-dependent protein kinases, including the $\text{Ca}^{2+}$-ATPase pump (Yoshida et al., 1991), $\text{Ca}^{2+}$-activated K$^+$ channel (Robertson et al., 1993), and Na$^+$/Ca$^{2+}$ exchange (Furukawa et al., 1991). Various NO cGMP-independent mechanisms have been reported in a number of studies, including the direct activation of K$^+$ channels (Bolotina et al., 1994; Homer and Wanstall, 2000), as well as the activation of the Na$^+$/K$^+$/ATPase pump (Homer and Wanstall, 2000).

In the present study, we demonstrated that the number of caveolae is lower in the cells from aortas treated with CD in both groups (2K and 2K-1C). The reduced number of caveolae in VSMCs upon treatment with CD has been reported in other studies (Ushio-Fukai et al., 2001; Dreja et al., 2002). Because caveolae are enriched in cholesterol (4–8-fold compared with the rest of the membrane), these organelles disappear in cells that are depleted of cholesterol (Darblade et al., 2001). Exposure of cells to sterol-binding agents such as CD and filipin removes cholesterol from the plasmalemma, which causes the caveolae to disassemble (Rothberg et al., 1990, 1992). CD is a membrane-impermeable molecule that depletes cellular cholesterol content through solubilization of the plasmalemmal cholesterol (Kilsdonk et al., 1995). This cholesterol-binding agent has been efficiently used as a pharmacological tool to study the role of caveolae (Dreja et al., 2002; Je et al., 2004).
In summary, a decreased number of caveolae play a critical role in the reduced sensitivity to NO in aortas from 2K-1C rats, and it could be the reason for the impaired relaxation by NO donors. To our knowledge, this is the first work showing that there is a lower number of caveolae in aortic smooth muscle cells from renal hypertensive rats (2K-1C). Our results support the idea that caveolae play a key role in the vascular effects of nitric oxide. The evaluation of this question may provide insights for the design of tools to restore normal blood pressure levels in hypertensive states.

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