Carter, Rebecca W., and Nancy L. Kanagy. Mechanism of enhanced calcium sensitivity and α₂-AR vasoreactivity in chronic NOS inhibition hypertension. Am J Physiol Heart Circ Physiol 284: H309–H316, 2003. First published September 19, 2002; 10.1152/ajpheart.00453.2002.—PKC augments calcium sensitivity in spontaneously hypertensive rats and contributes to α₂-adrenergic receptor (AR) contraction in rabbit saphenous vein. We showed previously that denuded aortic rings from Nω-nitro-l-arginine-treated hypertensive rats (LHR) contract more to CaCl$_2$ and to the α₂-AR agonist UK-14304 than do rings from normotensive rats (NR). We hypothesized that enhanced PKC activity or a change in PKC isoform contributes to augmented calcium sensitivity and enhanced α₂-AR contraction in LHR aorta. Current studies demonstrate that non-isofrom-specific PKC inhibitors reduced UK-14304 contraction in both NR and LHR aorta. However, the calcium-dependent PKC inhibitor Gö-6976 only attenuated contraction in LHR aorta. Additionally, UK-14304 translocated PKC-δ to the membrane in NR aorta, whereas PKC-α was translocated to the membrane in LHR aorta. Finally, in ionomycin-permeabilized aorta Gö-6976 eliminated enhanced basal and augmented α₂-AR-stimulated calcium sensitivity in LHR aorta but did not affect NR contraction. Together, these data suggest that PKC-α contributes to augmented calcium sensitivity and α₂-AR reactivity after chronic nitric oxide synthase inhibition hyperten-

sion.

nitric oxide; α₂-adrenergic receptors; protein kinase C; vascular smooth muscle; calcium sensitivity

α₂-ADRENERGIC RECEPTORS (AR) are G$\text{f}$ protein-coupled receptors present within the vasculature on both the endothelium and the vascular smooth muscle (2). On stimulation, endothelial receptors activate nitric oxide (NO) synthase (NOS) and trigger NO release whereas vascular smooth muscle receptors promote vasoconstriction (1, 2, 13, 14). In vivo, the endothelial and vascular receptors work in concert to provide an appropriate response to catecholamines. However, damage to the endothelium results in enhanced vasoreactivity to α₂-AR stimulation. We showed previously (14, 23) that vascular reactivity to CaCl$_2$ and to the α₂-AR agonist UK-14304 is augmented in denuded aorta and mesenteric arteries from chronically NOS-inhibited hypertensive rats [Nω-nitro-l-arginine (L-NNA) hypertensive rats; LHR]. These data suggest an altered vascular smooth muscle response to these stimuli in this model of hypertension. However, we have not identified the mechanism(s) for this increased vaso-

activity.

Although we have determined that L-type calcium channels, tyrosine kinases, extracellular signal-regulated kinase, and Rho-associated kinase contribute to α₂-AR contraction, none of these is responsible for increased calcium sensitivity or augmented α₂-AR contraction in LHR (7, 13, 23). Therefore, other kinases linked to both α₂-AR and calcium sensitivity may be affected in NOS inhibition hypertension.

PKC is a family of signaling molecules with 11 isoforms encoded by 10 genes and divided into 3 subfamilies based on differences in regulatory domains (21). The conventional or calcium-dependent isoforms, α, β, and γ, have both calcium-binding and lipid-binding domains. The novel or calcium-independent isoforms, δ, ε, η, and θ, contain a lipid-binding domain but no calcium-binding domain. The atypical isoforms, λ, τ, and ζ, contain neither a calcium-binding nor a lipid-binding domain (21). Many PKC isoforms, including α, δ, ε, η, and ζ, are present in vascular smooth muscle (21) and participate in contraction by augmenting calcium sensitivity (5, 8, 9, 11, 24) or by increasing the open probability of L-type calcium channels (4, 24). In α₂-AR contraction, Aburto et al. (1) showed that the PKC inhibitors calphostin C and staurosporine inhibit α₂-AR contraction in rabbit saphenous vein, suggesting that PKC contributes to the contraction. Additionally, Kanashiro et al. (15) demonstrated that PKC-α is activated by phenylephrine in the aorta from pregnant rats after NOS inhibition. Together, these data indicate that PKC may play a role in augmented vasoactivity to α₂-AR and CaCl$_2$ in LHR aorta.

The goal of this study was to evaluate the contribution of PKC to α₂-AR contraction as a potential mechanism of augmented calcium sensitivity and enhanced α₂-AR contraction after NOS inhibition hypertension. We hypothesized that enhanced PKC activity or re-

Address for reprint requests and other correspondence: R. W. Carter, 915 Camino de Salud, Vascular Physiology Research Division, Dept. of Cell Biology and Physiology, Univ. of New Mexico Health Sciences Center, Albuquerque, NM 87131 (E-mail: bcarter@salud.unm.edu).

http://www.ajpheart.org 0363-6135/03 $5.00 Copyright © 2003 the American Physiological Society H309
cruietment of additional PKC isoforms augments α2-AR contraction and calcium sensitivity after chronic in vivo NOS inhibition.

METHODS AND MATERIALS

Animals. Male Sprague-Dawley rats (250–300 g) drank tap water containing 0.5 g/l 1-γ-NNA (1-γ-NNA hypertensive rats; LHR) or vehicle (normotensive rats; NR) for 14 days. Blood pressures (tail cuff; IITC, Woodland Hills, CA) and animal weight were measured on days 0, 7, and 14. Mean systolic blood pressures on day 14 were 136 ± 3 and 201 ± 2 mmHg for NR and LHR, respectively. After the 14-day treatment period, animals were anesthetized with pentobarbital sodium (60 mg/kg ip). Thoracic aorta were removed and placed in ice-cold physiological saline solution (PSS; in mM: 130 NaCl, 4.7 KCl, 1.18 KH2PO4, 1.17 MgSO4·7H2O, 14.9 NaHCO3, 5.5 dextrose, 0.026 CaNa2-EDTA, and 1.6 CaCl2, pH 7.3). Vessels were cleaned of visible fat, cut into 4-mm rings, and denuded of endothelium with the tip of closed sharp forceps.

Calcium-tension measurements. Endothelium-denuded aortic rings were cut into helical strips and incubated overnight at 4°C in PSS containing fura 2-AM (2 µM), cromophor-EL (0.2 µmol/ml), and pluronic acid (0.02%). After incubation, strip ends were clamped with metal clips (Kent Scientific) and the clips were affixed to stainless steel hooks attached to a myograph (Kent Scientific) and placed in a heated water bath mounted in the base of a Nikon Diaphot 300 microscope fitted with a ×10 Nikon fluor objective. Strips were perfused with PSS maintained at 37°C and bubbled with 95% O2-5% CO2 for 60 min. During the equilibration period, strips were stretched to 2,000-g passive tension to achieve maximal active tension generation (appropriate tension determined by length-tension curves; data not shown). Indomethacin (1 µM) was added to the perfusate in the final 30 min of equilibration. Strips were exposed to a single concentration of phenylephrine (0.1 µM) to determine viability. Lack of relaxation to acetylcholine (1 µM) in phenylephrine-contracted rings was used to check for endothelium removal. Rings were washed until no active tension remained. Rings were then exposed to cumulative concentrations of UK-14304 (10^{-8}–10^{-5} M) in the presence or absence of increasing concentrations of PKC inhibitors. Time controls were performed with each experiment, and data were not reported if differences were apparent in time controls.

Three separate PKC inhibitors were used. Calphostin C and chelerythrine chloride are unique non-isofrom-specific PKC inhibitors. Calphostin is an inhibitor of the PKC regulatory subunit, whereas chelerythrine is an inhibitor of the ATP binding site (9, 16). Therefore, these non-isofrom-specific PKC inhibitors should attenuate the activity of all PKC isoforms. The third PKC inhibitor used, Gö-6976, inhibits the ATP binding site of conventional PKC molecule and therefore should only attenuate Ca2+-dependent PKC activity (37). In separate experiments, viable rings were incubated for 30 min with one of the PKC inhibitors or vehicle (DMSO). After incubation, calcium-containing PSS was replaced with calcium-free PSS in the continued presence of the inhibitor or vehicle. Ionomycin (1.5 µM) was added to the bath to permeabilize vascular smooth muscle cells. Tension was allowed to stabilize, and then 0.4 or 0.8 mM CaCl2 was added. Contraction in permeabilized segments was allowed to reach a steady state, after which UK-14304 (10^{-8}–10^{-5} M) was added to the bath to evaluate basal calcium sensitivity and α2-AR contraction independent of increases in intracellular calcium.

There is some evidence that high concentrations of intracellular calcium can activate PKC-α (19), and we found that in 0.8 mM CaCl2 Gö-6976 attenuated CaCl2 contraction in both NR and LHR aorta (data not shown). To avoid direct CaCl2 stimulation of PKC-α in experiments testing PKC-α effect on calcium sensitivity, we used the lowest concentration of CaCl2 that consistently contracted both NR and LHR aorta, 0.4 mM CaCl2.

PKC activity. Rings were hung in water-jacketed tissue baths as for contractile experiments and equilibrated for 60 min. After equilibration, tissues were exposed to the α2-AR agonist UK-14304 (10 µM). Exactly 10 min after agonist stimulation (appropriate activation time determined by time course study; data not shown), rings were removed from baths and placed in ice-cold homogenization buffer [in mM: 50 Tris·HCl, 1 DTT, 10 benzamidine, and 10 mM EGTA with complete protease inhibitor (Roche Mannheim), pH 7.5]. Homogenate was centrifuged at 13,000 g for 3 min at 4°C. The supernatant was removed and centrifuged at 100,000 g for 20 min. The supernatant (cytosolic fraction) was removed, and the pellet (membrane fraction) was resuspended in homogenization buffer plus 1% Triton X-100. Protein concentration of each fraction was determined with a modified Lowry method (Pierce). Inactive PKC is found in the cytosol and translocates to the membrane on activation; therefore, PKC activity can be estimated by the amount of PKC present in the membrane fraction versus the amount present in the cytosolic fraction.

For Western blot analysis, protein concentrations were normalized and samples were loaded into a 7.5% acrylamide gel for resolution with electrophoresis. Separated proteins were transferred onto polyvinylidene difluorid membranes and blocked 1 h with Tris-buffered saline containing 0.1% Tween 20, 5% milk, and 3% bovine serum albumin. Blots were incubated overnight at 4°C with anti-PKC-α or anti-PKC-β (1:500), followed by secondary antibody for 1 h at room temperature (1:10,000) and developed with enhanced chemiluminescence reagents. Positive controls were also loaded. After analyses, blots were stained with Coomassie blue to ensure equivalent protein loading. Protein levels were com-
PKC-α ENHANCES HYPERTENSIVE VASOREACTIVITY

Fig. 1. N°-nitro-L-arginine hypertensive rat (LHR) aorta contracts more to the α2-adrenergic receptor (AR) agonist UK-14304 and to CaCl2 with the same or reduced increase in intracellular calcium concentration ([Ca2+]i). Fura 2-AM-loaded, endothelium-denuded normotensive rate (NR) and LHR thoracic aortic strips were used to generate consecutive cumulative concentration response curves to UK-14304 (10⁻⁸–10⁻⁵ M) while fluorescence (340- to 380-nm ratio) was measured (A). Additionally, ionomycin-permeabilized strips were exposed to 2 different concentrations of CaCl2 (0.8 and 1.6 mM; B). Each data point represents an increasing concentration of UK-14304 or CaCl2. *Statistically significant difference between NR and LHR; n = 7 (NR) and 5 (LHR).

Enhanced calcium sensitivity in LHR aorta. To evaluate calcium sensitivity, concurrent calcium-tension measurements were generated with fura 2-AM-loaded aortic strips. Basal fluorescence was not different between NR and LHR aorta (mean 340-to-380 ratio: NR 0.69 ± 0.02, LHR 0.69 ± 0.03). Strips were then exposed to cumulative concentrations of UK-14304 (10⁻⁸–10⁻⁵ M). LHR strips were more sensitive to UK-14304 than NR strips; however, change in fluorescence was less in LHR than in NR, suggesting an increase in α2-AR-induced calcium sensitivity (Fig. 1A). Similarly, CaCl2 (0.8 and 1.6 mM) produced a greater contraction with similar calcium increases in ionomycin-permeabilized LHR strips than in NR strips, suggesting augmented basal calcium sensitivity (Fig. 1B). To determine whether UK-14304 was capable of increasing intracellular calcium above that generated by CaCl2 in ionomycin-permeabilized strips, UK-14304 was added to the bath after contraction to CaCl2 had plateaued. Although UK-14304 increased tension, it did not change the 340-to-380 ratio (Table 1). These data suggest that in ionomycin-permeabilized vessels, UK-14304 does not change intracellular calcium concentrations, and they validate the use of this method to study basal and α2-AR-stimulated calcium sensitivity.

Both NR and LHR aortic segments were adversely affected by overnight incubation with fura 2-AM, and overall fewer LHR strips were viable on testing the following day. To avoid the confounding problem of decreased viability of LHR strips, freshly isolated ionomycin-permeabilized aortic rings were also exposed to CaCl2, followed by cumulative concentrations of UK-14304. CaCl2 (0.8 mM) and UK-14304 (10⁻⁸–10⁻⁵ M) contracted LHR more than NR rings (Fig. 2, A and B).

Table 1. Effect of UK-14304 on NR and LHR aorta

<table>
<thead>
<tr>
<th></th>
<th>0.8 mM CaCl2</th>
<th>1.6 mM CaCl2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CaCl2</td>
<td>CaCl2 + UK-14304</td>
</tr>
<tr>
<td><strong>NR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tension, mg</td>
<td>625 ± 57</td>
<td>825 ± 50</td>
</tr>
<tr>
<td>Δ340/380</td>
<td>0.09 ± 0.01</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td><strong>LHR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tension, mg</td>
<td>1,083 ± 96a</td>
<td>1,864 ± 170a</td>
</tr>
<tr>
<td>Δ340/380</td>
<td>0.09 ± 0.02</td>
<td>0.09 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE. NR, normotensive rat; LHR, N°-nitro-L-arginine-treated hypertensive rat; Δ340/380, change in 340- to 380-nm ratio. *Significant difference between NR and LHR.

AJP-Heart Circ Physiol • VOL 284 • JANUARY 2003 • www.ajpheart.org
supporting the conclusion that there is enhanced basal and UK-14304-stimulated calcium sensitivity in LHR aorta.

Contribution of PKC to \(\alpha_2\)-AR contraction. Aortic rings were exposed to cumulative concentrations of UK-14304 (10\(^{-9}\)–10\(^{-5}\) M) in the presence of increasing concentrations of the PKC inhibitors chelerythrine chloride (5 and 10 \(\mu\)M) or calphostin C (0.1 and 0.5 \(\mu\)M). Both inhibitors significantly attenuated the contraction to UK-14304 in both NR and LHR aorta (Fig. 3), suggesting that PKC contributes to the \(\alpha_2\)-AR contraction. PKC activity assays performed on cytosolic and membrane fractions of aortic homogenates confirmed that PKC is translocated from the cytosol to the membrane after UK-14304 stimulation. However, UK-14304 stimulation of PKC activity was not greater in LHR homogenates than in NR homogenates (Fig. 4).

Both chelerythrine and calphostin are non-isoform-specific inhibitors of PKC. To evaluate whether a calcium-independent PKC isoform is activated after \(\alpha_2\)-AR stimulation, Western blot analysis of PKC-\(\beta\) in membrane and cytosolic aortic homogenate fractions was performed. PKC-\(\beta\) expression was similar in LHR and NR aorta (ratio of membrane to cytosolic PKC-\(\beta\): NR, 2.19 \(\pm\) 0.55; LHR, 1.93 \(\pm\) 0.34); however, UK-14304 (10 \(\mu\)M) translocated PKC-\(\beta\) from the cytosolic fraction to the membrane fraction in NR aorta only (Fig. 4). This suggests that PKC-\(\beta\) is normally activated by \(\alpha_2\)-AR, but not in chronic NOS inhibition hypertension. Together, these data suggest that PKC plays a large role in \(\alpha_2\)-AR contraction in both NR and LHR aorta. Furthermore, although total PKC activity after UK-14304 stimulation is not different between NR and LHR, the isoform of PKC contributing to \(\alpha_2\)-AR contraction may differ between NR and LHR aorta.

To evaluate the role of PKC in \(\alpha_2\)-AR-stimulated and basal calcium sensitivity, ionomycin-permeabilized aortic rings in the presence of chelerythrine (10 \(\mu\)M) or vehicle (DMSO) were exposed to CaCl\(_2\) (0.8 mM). After the contraction plateaued, cumulative concentrations of UK-14304 (10\(^{-9}\)–10\(^{-5}\) M) were added to the bath. LHR aorta contracted more to CaCl\(_2\) than did NR aorta. However, chelerythrine attenuated only LHR

![Fig. 2. Ionomycin-permeabilized denuded aortic rings from LHR contract more to CaCl\(_2\) and to the \(\alpha_2\)-AR agonist UK-14304 than NR rings. Aortic rings in calcium-free buffer were permeabilized with the calcium ionophore ionomycin (1.5 \(\mu\)M) and exposed to 0.8 mM CaCl\(_2\). The contraction was allowed to plateau (A), and the rings were then exposed to cumulative concentrations of UK-14304 (10\(^{-8}\)–10\(^{-6}\) M; B). *Statistically significant difference between NR and LHR; \(n = 5\).](http://ajpheart.physiology.org/)

![Fig. 3. The PKC inhibitors chelerythrine chloride and calphostin C attenuate UK-14304 contraction. Treatment of NR (A and C) and LHR (B and D) endothelium-denuded aortic rings with chelerythrine chloride (1 and 10 \(\mu\)M; A and B) and calphostin C (0.1 and 0.5 \(\mu\)M; C and D) significantly and concentration-dependently attenuated contraction to the \(\alpha_2\)-AR agonist UK-14304 (10\(^{-9}\)–10\(^{-5}\) M). *Statistically significant difference between vehicle and treatment; \(n = 7\).](http://ajpheart.physiology.org/)
PKC-α ENHANCES HYPERTENSIVE VASOREACTIVITY

H313

To evaluate the role that calcium-dependent PKC plays in basal and α2-AR-stimulated calcium sensitivity, ionomycin-permeabilized aortic rings in the presence of Gő-6976 (1 μM) or vehicle were exposed to 0.4 mM CaCl2. After contraction plateaued, rings were exposed to cumulative concentrations of UK-14304 (10<sup>-8</sup>–10<sup>-5</sup> M). Gő-6976 attenuated the contraction to CaCl2 and the cumulative contraction to UK-14304 in LHR aorta and not in NR aorta (Fig. 8, A and B), so that contractions to CaCl2 and UK-14304 in NR and LHR aorta were not different in the presence of Gő-6976. These data suggest that calcium-dependent PKC augments basal and α2-AR-stimulated calcium sensitivity in LHR aorta.

DISCUSSION

The goal of this study was to evaluate enhanced PKC activity as a potential cause of augmented α2-AR reactivity in aorta from chronic NOS-inhibited hypertensive rats. We observed that, in addition to enhanced α2-AR contractile sensitivity, LHR aorta have increased basal and α2-AR-stimulated calcium sensitivity. This increase in calcium sensitivity may in fact be an underlying mechanism for augmented α2-AR contractility, although decreased sarcoplasmic reticulum buffering capacity in LHR arteries cannot be excluded as a mechanism. PKC has been shown to affect both calcium entry (4, 24) and calcium sensitivity (5, 8, 9, 11, 25). Therefore, we sought to evaluate the contribu-

CaCl2 contraction, so that in the presence of chelerythrine, the CaCl2 contractions were no longer different (Fig. 5A). Chelerythrine attenuated contraction to UK-14304 similarly in NR and LHR ionomycin-permeabilized aortic rings (Fig. 5B). These results suggest that PKC contributes to α2-AR-stimulated calcium sensitivity in both NR and LHR aorta but basal augmentation of calcium sensitivity in LHR aorta may be due to enhanced PKC activity.

**Contribution of calcium-dependent PKC to α2-AR contraction.** To determine whether an additional PKC isoform was recruited during α2-AR contraction in LHR aorta, rings were exposed to cumulative concentrations of UK-14304 in the presence of the calcium-dependent PKC inhibitor Gő-6976 (0.1 or 1 μM) or vehicle. Gő-6976 attenuated contraction to UK-14304 only in LHR aorta and not in NR aorta (Fig. 6). Similarly, Western blot analysis showed that PKC-α is translocated to the membrane after UK-14304 (10 μM) stimulation in LHR aortic homogenates only (Fig. 7), suggesting that PKC-α is only activated by α2-AR in LHR aorta. However, PKC-α expression was similar in NR and LHR aorta (ratio of membrane to cytosolic PKC-α: NR, 1.91 ± 0.11; LHR, 2.38 ± 0.26).

**Fig. 4.** UK-14304 translocates PKC activity to the membrane fraction. More [32P]ATP was incorporated in a PKC-specific substrate by the membrane fraction of aortic homogenates treated with the α2-AR agonist UK-14304 (10 μM) than in basal homogenates (A; n = 5). Similarly, Western blots show more PKC-α in the membrane fraction of NR aortic homogenates after UK-14304 exposure (B; n = 3). Representative blots are shown in C. cyt, Cytosolic; mem, membrane. *Statistically significant difference between vehicle and treatment.

**Fig. 5.** The PKC inhibitor chelerythrine chloride attenuates contraction to CaCl2 and the cumulative contraction to UK-14304 in both LHR and NR ionomycin-permeabilized aortic rings. Endothelium-de-
tion of PKC to α2-AR contraction. We found that two separate inhibitors of PKC, calphostin C and chelerythrine chloride, significantly attenuated α2-AR contraction in NR and LHR aorta and noted that PKC-δ is translocated to the membrane fraction of aortic homogenates after α2-AR stimulation in NR aorta. Together, these data extend previous suggestions that PKC participates in α2-AR contraction in normotensive rat aorta and suggest that a Ca2+-independent isoform of PKC, PKC-δ, is activated after α2-AR stimulation and may mediate contraction in normotensive rat aorta. However, although PKC contributes to α2-AR contraction in LHR aorta, these data also suggest that PKC-δ does not mediate this contraction and that a change in PKC isoform occurs with NOS inhibition hypertensive males.

Interestingly, chelerythrine significantly attenuated α2-AR-stimulated calcium sensitivity in both NR and LHR, but only reduced basal calcium sensitivity in LHR aorta. This suggests that α2-AR contraction augments calcium sensitivity via receptor-stimulated PKC activity. Several other studies showed that calcium sensitivity is augmented in hypertension (28, 29) and suggested that PKC may play a role in this (3, 23, 32). Data presented here confirm these studies. Chelerythrine attenuated contraction to CaCl2 in LHR aorta only, suggesting that PKC, at least in part, is responsible for augmented basal calcium sensitivity in LHR in the absence of receptor stimulation.

Kanashiro et al. (15) reported that phorbol 12,13-dibutyrate and phenylephrine stimulate translocation of PKC-α to the membrane fraction in aortic homogenates from virgin female rats, but this translocation is abolished in pregnant rats. Treatment with NG-nitro-L-arginine (L-NAME) prevented PKC translocation. Therefore, it is possible that PKC-α translocation is mediated by NOS activity in these studies.

Fig. 6. The calcium-dependent PKC inhibitor Gö-6976 attenuates UK-14304 contraction in LHR aortic rings only. Treatment of NR (A) and LHR (B) endothelium-denuded aortic rings with Gö-6976 (0.1 and 1 μM) significantly attenuated contraction to the α2-AR agonist UK-14304 (10−9–10−5 M) in LHR rings only. *Statistically significant difference between vehicle and treatment; n = 7.

Fig. 7. The α2-AR agonist UK-1430 translocates PKC-α to the membrane only in LHR aorta. A: membrane and cytosolic fractions of aortic homogenates in the presence and absence of UK-14304 (10 μM) were subjected to Western blot analysis and probed for PKC-α. More PKC-α is present in the membrane fraction of LHR aortic homogenates after UK-14304 stimulation, indicating translocation to the membrane. Data are shown as the ratio of membrane to cytosolic PKC-α. Representative blots are shown in B. *Statistically significant difference between basal and UK-14304 treated; n = 5.

Fig. 8. The calcium-dependent PKC inhibitor Gö-6976 attenuates contraction to CaCl2 and the α2-AR agonist UK-14304 in ionomycin-permeabilized LHR aortic rings only. Endothelium-denuded aortic rings in calcium-free buffer were permeabilized with the calcium ionophore ionomycin (1.5 μM) in the presence of Gö-6976 (1 μM) or vehicle. CaCl2 (0.4 mM) was added to the bath, and the contraction was allowed to plateau (A). Rings were then exposed to cumulative concentrations of UK-14304 (10−8–10−5 M; B). The presence of Gö-6976 abolished augmented contraction to CaCl2 and UK-14304 in LHR aorta. *Statistically significant difference between vehicle and treatment; n = 5.
PKC-α ENHANCES HYPERTENSIVE VASOREACTIVITY

H315

L-arginine methyl ester restored PDBu and phenylephrine translocation of PKC-α. Similarly, we show that chronic L-NNA treatment recruits PKC-α.

The calcium-dependent PKC inhibitor Gö-6976 attenuated α2-AR contraction in LHR aorta, but not in NR aorta, suggesting that NOS inhibition hypertension results in the recruitment of a Ca^{2+}-dependent PKC isoform to the α2-AR contractile pathway. Additionally, Western blot analysis demonstrated that PKC-α was translocated to the membrane fraction of LHR but not NR aorta after α2-AR stimulation, suggesting that PKC-α mediates α2-AR contraction in LHR aorta. This PKC isoform may also contribute to basal and α2-AR-stimulated calcium sensitivity in LHR aorta but not in NR aorta. Therefore, the translocation of PKC-α to the membrane fraction after α2-AR stimulation in LHR aorta and not in NR aorta further suggests that PKC-α is recruited to the α2-AR signal transduction cascade after chronic NOS inhibition hypertension to augment vasoreactivity by increasing calcium sensitivity.

The PKC activity data suggest a tendency for increased basal PKC activity in LHR, but this did not reach significance. It is possible that the assay was not sensitive enough to detect small changes in activity without isoform-specific assays. Therefore, without a more sensitive, isoform-specific PKC assay it cannot be concluded that PKC activity is elevated in LHR aorta at baseline.

Together, these data suggest that in NOS inhibition hypertension there is a switching of the PKC isoform participating in α2-AR contraction from Ca^{2+} independent to Ca^{2+} dependent. Increased pressure or hypertrophy could precipitate the isoform change (9, 16). This change in isoform appears to mediate the increase in α2-AR-stimulated calcium sensitivity as well as α2-AR vasoreactivity. However, we did not examine the role of other PKC isoforms, and one or more of these isoforms may also be involved. Future studies should investigate these other PKC isoforms to more fully understand the α2-AR contractile pathway and changes that occur with hypertension. Additionally, the contribution of PKC-α to vasoconstriction in hypertension and to other contractile pathways is unknown and will be tested in future experiments.

Upstream activation of PKC also remains an open question. Previous studies suggest that PLC activation is an unlikely mediator because inositol 1,4,5-trisphosphate production is not increased after α2-AR stimulation (20) and intracellular calcium release contributes minimally to α2-AR contraction (20, 23). It is possible that PLD releases diacylglycerol to activate PKC, as suggested by Deth and co-workers (1). However, these earlier studies relied on wortmannin as a specific PLD inhibitor. More recently, wortmannin has also been found to be an effective inhibitor of myosin light chain kinase and phosphatidlyinositol 3-kinase (PI3K) (24, 35). This is especially troublesome because several studies have demonstrated that PI3K can activate PKC either through the production of 3,4,5-inositol trisphosphate or through phosphorylation of the PKC molecule (6, 18, 31, 34). Preliminary studies in our lab have shown that the PI3K inhibitor LY-294002 significantly attenuates α2-AR contraction in both NR and LHR aorta (unpublished observations). Future experiments will review the ability of PI3K to activate PKC in this pathway.

In conclusion, this study provides novel evidence that the mechanism of augmented α2-AR contractile reactivity after chronic NOS inhibition hypertension is due, at least in part, to a PKC-dependent increase in basal and receptor-stimulated calcium sensitivity. Moreover, NOS inhibition hypertension recruits a calcium-dependent PKC isoform, most likely PKC-α, to the α2-AR contractile pathway. It will be important for future studies to determine whether PKC contributes to induction or maintenance of NOS-deficient hypertension.

The authors express special thanks to Pam Allgood for expert technical assistance. This work was supported by an Arovastatin Research Award and National Heart, Lung, and Blood Institute Grant HL-63832.

REFERENCES


