Role of protein kinase C in electrical-stimulation-induced neuronal nitric oxide release in mesenteric arteries from hypertensive rats

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ABSTRACT

This study examines the influence of hypertension on neuronal nitric oxide (NO) release and its modulation by protein kinase C (PKC). For this purpose, mesenteric segments without endothelium were obtained from Wistar–Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs), and neurogenic NO release induced by electrical field stimulation (EFS) was examined in these segments. EFS induced frequency-dependent contractions. The NO synthase inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) and the sensorial neurotoxin capsaicin increased EFS-induced contractions in SHR segments, but did not affect these contractions in segments from WKY rats. In segments from SHRs, the increase in EFS-induced response by capsaicin was further increased by the combination of capsaicin and L-NAME. EFS-induced contractions in SHR arteries were unaltered by the protein synthesis inhibitor cycloheximide or by 2-amine-5,6-dihydro-6-methyl-4H-1,3-tiazine (AMT), an inhibitor of inducible NO synthase, and increased by the guanylate cyclase inhibitor Methylene Blue. In these arteries, capsaicin plus the PKC inhibitor calphostin C increased the contractions elicited by EFS; the addition of L-NAME did not affect this increase. Phorbol 12,13-dibutyrate (PDBu) did not modify the response to EFS in these arteries pretreated with capsaicin, although a combination of PDBu and L-NAME was effective. These results indicate that, in mesenteric arteries, EFS induces the release of NO from perivascular nitrergic nerves and of neuropeptides from sensory nerves, but only in hypertensive rats. The NO released is synthesized by constitutive neuronal NO synthase in a manner that is positively modulated by PKC, an enzyme that seems to be activated in hypertension.

INTRODUCTION

Nitric oxide (NO), which is essentially synthesized in endothelial cells, plays an important role in the regulation of vasomotor tone [1,2]. Certain pathological processes, such as diabetes and hypertension, may alter NO formation [2]. Some arteries, such as the cerebral and mesenteric arteries, have perivascular nitrergic innervation; when electrically stimulated, they release NO, which in turn induces vasodilation, thus participating in the regulation of vascular tone [3–6]. Studies with various tissue preparations indicate that the phosphorylation of NO synthases (NOSs) by various agents, such as activators of protein kinase C (PKC), is associated with

Key words: electrical field stimulation, nitrergic innervation, protein kinase C, rat mesenteric artery, spontaneously hypertensive rat.

Abbreviations: AMT, 2-amine-5,6-dihydro-6-methyl-4H-1,3-tiazine; EFS, electrical field stimulation; KHS, Krebs–Henseleit solution; NA, noradrenaline; L-NAME, N\textsuperscript{G}-nitro-L-arginine methyl ester; NOS, nitric oxide synthase; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; SHR, spontaneously hypertensive rat; SNP, sodium nitroprusside; WKY, Wistar–Kyoto.

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changes that usually lower NOS activity [2,7–10], although increased NOS activity has also been described [11,12].

Hypertension has been found to increase PKC activity in various tissues [13–15], and this finding suggests the involvement of PKC in the development and maintenance of this disease. Our previous results indicate that PKC is involved in the regulation of neuronal NOS activity [16], which is assumed to be altered in diabetes, probably due to enhanced PKC activity.

Hypertension has also been associated with alterations in endothelial NO synthesis [13,17], as well as the activation of inducible NOS [18], but nothing is yet known about possible changes to neuronal NO release in hypertension. The objective of the present work was to assess the functional role of nitrergic innervation, and the possible participation of PKC, in neuronal NO synthesis, using mesenteric arteries from hypertensive rats.

METHODS

Tissue preparation

The present study used 6-month-old male normotensive Wistar–Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs) (body weight 200–250 g). Systolic blood pressure was determined by tail cuff plethysmography. The average systolic blood pressure of the animals (means ± S. E. M.) was 105 ± 7 mmHg in WKY rats (n = 20) and 170 ± 9 mmHg in SHRs (n = 25; P < 0.05). Animals were killed by CO₂ inhalation; the first branch of the mesenteric artery was then carefully dissected out, cleaned of connective tissue and placed in Krebs–Henseleit solution (KHS) at 4 °C. The investigation conformed to guidelines in the Guide for the Care and Use of Laboratory Animals, published by the U.S.A. National Institutes of Health (NIH publication no. 85.23; revised 1985).

Contractile responses

For isometric tension recording, each artery was divided into segments of 2 mm in length. Each segment was set up in an organ bath, and changes in segment tone were recorded using the method of Nielsen and Owman [19]. The organ bath contained 5 ml of KHS at 37 °C, bubbled continuously with a 95% O₂/5% CO₂ mixture (pH 7.4). Two horizontally arranged stainless steel pins, 75 μm in diameter, were passed through the lumen of the vascular cylinder. One pin was fixed to the wall of the organ bath, and the other was connected vertically to a strain gauge for isometric tension recording. The isometric contraction was recorded through a force–displacement transducer (Grass FTO3C; Quincy, MA, U.S.A.) connected to a polygraph (Grass model 7D). For electrical field stimulation (EFS) experiments, the segments were mounted between two platinum electrodes 0.5 cm apart, connected to a stimulator (Cibertec model CS9) modified to supply adequate current strength. The segments were subjected to a tension of 0.5 g, which was re-adjusted every 15 min during a 90 min equilibration period before drug administration. Then the vessels were exposed to 75 mmol/l K⁺ to check their functional integrity.

As the object of the study was to analyse the vasomotor role of nitrergic innervation in these arteries, all experiments were performed with endothelium-denuded segments, to eliminate this source of vascular NO. This avoided possible actions of the various drugs used on the endothelial cells, which might lead to misinterpretation of results. Endothelium was removed by gently rubbing the luminal surface of segments with a wooden stick of small diameter. The absence of endothelium was tested by the inability of 10 μmol/l acetylcholine to relax segments precontracted with 1 μmol/l noradrenaline (NA). Endothelium removal did not alter the contractions elicited by 75 mmol/l K⁺.

Frequency–response curves to EFS or concentration–response curves to NA (10 nmol/l–10 μmol/l) were performed. The parameters used for EFS were 200 mA/0.3 ms/1–16 Hz for 30 s, with an interval of 1 min between stimulii; this time was required to recover basal tone. A washout period of at least 1 h between consecutive curves was necessary to avoid desensitization. Four successive frequency–response or NA concentration–response curves, separated by 1 h intervals, indicated similar contractile responses. When the effects of 0.1 μmol/l tetrodotoxin or 1 μmol/l phentolamine on the contraction elicited by EFS were assessed, these were added to the bath 20 min before EFS.

To determine the participation of nitrergic and sensory innervation in EFS- and NA-induced responses, 10 μmol/l N⁵-nitro-L-arginine methyl ester (L-NAME; a NOS inhibitor) or 0.5 μmol/l capsaicin (a sensory neurotoxin) respectively was added to the bath at 40 or 60 min, before recording a second frequency–response curve or concentration–response curve to NA.

We studied the effects of 10 μmol/l Methylene Blue (a guanylate cyclase inhibitor), 5 nmol/l 2-amino-5,6-dihydro-6-methyl-4H-1,3-tiazine (AMT) and 10 μmol/l cycloheximide (a protein synthesis inhibitor) on EFS- and NA-induced responses in mesenteric arteries from SHRs. The first two substances were added to the bath 40 min before the second frequency–response curve or concentration–response curve to NA. Cycloheximide was added to the original KHS at the time of artery removal from the animal, and incubation was for 6 h. In some cases, a third curve was performed in the presence of two drugs. When two or more drugs were administered, they were added to the bath simultaneously.

The possible role of PKC in the modulation of NOS activity was investigated in segments from SHRs, by analysing the effects of 0.1 μmol/l calphostin C (an inhibitor of PKC) or 1 nmol/l phorbol 12,13-dibutyrate.
Hypertension, protein kinase C and neurogenic NO release

Figure 1 Frequency-dependent and NA-concentration-dependent contractions in mesenteric artery segments from WKY rats and SHRs

Results (means ± S.E.M.) are expressed in mg. The numbers of segments are indicated in parentheses.

(PDBu) (an activator of PKC) on EFS- and NA-induced responses. Since PKC has been described as also being able to increase the activity of sensory innervation [20], these experiments were performed in the presence of capsaicin. For this purpose, a first curve was performed in the presence of this sensorial neurotoxin; then calphostin C or PDBu was added 40 min before a second curve. A third curve was obtained in the presence of calphostin C plus l-NAME or PDBu plus l-NAME and capsaicin.

The ability of sodium nitroprusside (SNP; 0.1 nmol/l–10 μmol/l) to induce relaxation in arteries from WKY rats and SHRs was assessed in some segments precontracted with NA.

Drugs and chemicals

The composition of KHS was as follows (mmol/l): NaCl 115, CaCl₂ 2.5, KCl 4.6, KH₂PO₄ 1.2, MgSO₄ · 7H₂O 1.2, NaHCO₃ 25, glucose 11.1 and Na₂ EDTA 0.03 (to prevent the oxidation of unstable substances). Drugs used were: l-NA hydrochloride, acetylcholine chloride, tetrodotoxin, calphostin C, PDBu acetate, SNP, l-NAME hydrochloride, capsaicin, Methylene Blue, cycloheximide (all from Sigma, St. Louis, MO, U.S.A.) and AMT hydrochloride (Research Biochemical International, Natick, MA, U.S.A.). Stock solutions (10 mmol/l) of drugs were prepared in distilled water, except for NA, which was dissolved in NaCl (0.9%)/ascorbic acid (0.01% w/v), and calphostin C and PDBu, which were dissolved in DMSO. It was observed that the vehicle DMSO did not alter basal tone or the EFS-induced response. These solutions were kept at ~20 °C, and appropriate dilutions were made in KHS on the day of the experiment.

Data analysis

The responses elicited by EFS or NA were expressed in mg in order to compare them between WKY rats and SHRs, and as a percentage of the contraction induced by 75 mmol/l K⁺ when examining the effects of different drugs on these responses. The relaxation caused by SNP was expressed as a percentage of the initial contraction induced by 1 μmol/l NA. Results are given as means ± S.E.M. Statistical analysis was carried out by means of two-way ANOVA for comparison between groups, or repeated-measures ANOVA to compare treatments. A P value of < 0.05 was considered significant.

RESULTS

Frequency (1, 2, 4, 8 and 16 Hz)–contraction curves performed in segments lacking endothelium obtained from WKY rats and SHRs were similar (Figure 1). However, when the response was expressed as a percentage of the contraction elicited by 75 mmol/l K⁺ [WKY rats, 916 ± 46 mg (n = 35); SHRs, 672 ± 33 mg (n = 55); P < 0.0001], the results obtained at each frequency were higher for segments from SHRs than for those from WKY rats (Table 1). Likewise, it was observed that three successive curves performed in the same segment and separated by 1 h intervals were similar. EFS-induced contractions were practically abolished by 0.1 μmol/l tetrodotoxin and markedly reduced by 1 μmol/l phentolamine in segments from both WKY rats and SHRs (Table 1). Two successive concentration–response curves to NA (10 nmol/l–10 μmol/l), separated by a 1 h interval, were similar in arterial segments from WKY rats and SHRs. However, the contractions elicited by NA were greater in WKY than in SHR arteries (Figure 1).

The contraction induced by EFS was significantly increased in SHR segments by preincubation of the segments with 10 μmol/l l-NAME or 0.5 μmol/l capsaicin; contractions in WKY rat segments were not
Table 1  Effects of tetrodotoxin (0.1 μmol/l) and phentolamine (1 μmol/l) on the frequency–contraction curve in mesenteric artery segments from WKY rats and SHRs

Results (means ± S.E.M.; n = 5 paired experiments) are expressed as a percentage of the response elicited by 75 mmol/l K⁺ (100%). Significance of differences: *P < 0.001 compared with the respective control; †P < 0.01 compared with WKY rats.

<table>
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<th>4</th>
<th>8</th>
<th>16</th>
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<tr>
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Figure 2  Influence of L-NAME,capsaicin, Methylene Blue,AMT,cycloheximide or the combination of capsaicin + L-NAME on the frequency–contraction curves performed in mesenteric artery segments from WKY rats and SHRs

Results (means ± S.E.M.) are expressed as a percentage of the contraction induced by 75 mmol/l K⁺. The numbers of unpaired experiments are indicated in parentheses; n = number of paired experiments.

In WKY arteries, capsaicin did not affect the frequency–contraction curve; however, the addition of L-NAME further increased the response elicited by EFS in SHR segments (Figure 2). In these segments, incubation with 5 nmol/l AMT or 10 μmol/l Methylene Blue did not affect or increased respectively the EFS-induced contraction; incubating the segments with 10 μmol/l cycloheximide for 6 h, after their removal from the animal, did not affect this contraction (Figure 2).

In SHR arteries preincubated with 0.5 μmol/l capsaicin, the addition of 0.1 μmol/l calphostin C increased the contractile response to EFS; this increase was not altered by the combination of calphostin C and L-NAME.
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Figure 3 Effects of calphostin C and PDBu, with or without L-NAME, on the frequency–contraction curves performed in SHR mesenteric artery segments preincubated with capsaicin

Results (means ± S.E.M.) are expressed as a percentage of the contraction induced by 75 mmol/l K⁺; n = number of paired experiments.

Figure 4 Concentration–response curves to SNP in mesenteric artery segments from WKY rats and SHRs

Results (means ± S.E.M.) are expressed as percentage inhibition of contraction induced by NA. Numbers of segments are indicated in parentheses.

capsaicin-pretreated segments, PDBu (1 nmol/l) did not modify the response to EFS. The combination of both drugs with L-NAME increased the vasoconstrictor response in arteries from SHRs (Figure 3).

Both an increased and a decreased vasoconstrictor response to NA have been described in hypertension [13,21,22]. NA elicited higher contractile responses in mesenteric arteries from WKY rats than in those from SHRs, and the same occurred for the responses induced by K⁺ [23,24]. This decrease could be due, at least partially, to alterations of vessel structure associated with vascular remodelling and vascular smooth muscle cell geometric disorganization caused by hypertension [25]. However, the contractile response to EFS was lower in segments from WKY rats than in those from SHRs when expressed as a percentage of the response induced by 75 mmol/l K⁺, which agrees with previous findings in hypertensive rats [13,26].

The presence of the NOS inhibitor L-NAME increased the vasoconstrictor responses induced by EFS in segments from SHRs, but did not affect the response in [WKY rats, 865 ± 118 mg (n = 6); SHRs, 912 ± 55 mg (n = 5); P > 0.05], SNP (0.1 nmol/l–10 μmol/l) induced a concentration-dependent relaxation, which was greater in segments from SHRs than in those from WKY rats (Figure 4).

DISCUSSION

The present results show that EFS induces contractile responses in endothelium-denuded mesenteric artery segments from WKY rats and SHRs, and that these contractions appear to be mediated by the release of NA from adrenergic nerve terminals, with a subsequent activation of α-adrenoceptors. Indeed, these contractions were virtually abolished by tetrodotoxin and markedly reduced by phentolamine, which respectively block nerve impulse propagation and α-adrenoceptors, as reported for mesenteric arteries from Wistar and Sprague–Dawley rats [6,16].
segments from WKY rats. This suggests increased neurogenic NO formation in segments from SHRs, which could compensate for the increased vascular tone in these rats. It is important to note that neurogenic NO release in response to EFS depends on the rat strain, i.e. in the present paper we show the absence of neurogenic NO release in WKY rats, while the participation of neuronal NO was noted in Wistar and Sprague–Dawley rats under the same experimental conditions [6,16]. Further data supporting the increased release of neurogenic NO from SHR segments are the results obtained with Methylene Blue, a guanylate cyclase inhibitor, which also increased the response to EFS.

In order to study the possibility that hypertension might increase the sensitivity of smooth muscle cells to released NO, the response induced by the NO donor, SNP, was assessed. SNP induced a greater vasodilator response in NA-precontracted segments from SHRs than in those from WKY rats. However, this difference would not explain the inability of l-NAME to increase the contractile response induced by EFS. These data suggest that the increase in the vasomotor response to EFS observed in SHRs in the presence of l-NAME is limited to nitrergic nerve terminals that produce an excess of NO, probably through enhanced activation of these nerves. The increased NO release seems to parallel the enhanced adrenergic nerve activity that has been suggested as a possible compensatory mechanism in this illness [16].

Several authors have shown that various protein kinases, including PKC, can phosphorylate neuronal NOS [2,8,9,12], which normally results in inhibition of this enzyme, although stimulation has also been reported [7,9,27]. Furthermore, the activity of PKC in various tissues is enhanced in hypertension, and this effect is probably involved in the pathogenesis of the illness [13–15]. Our next objective was to analyse the possible participation of PKC in the regulation of NOS activity in hypertension. It has been reported that activating PKC with PDBu increases the release of peptides (substance P and calcitonin-gene-related peptide) from rat sensory neurons [20]. In the present study, acute in vitro denervation of sensory nerves with capsaicin increased the contraction to EFS in arteries from SHRs, but did not affect EFS-induced contractions in WKY rat arteries. This finding indicates that EFS only releases these peptidergic substances from perivascular sensory nerves in SHRs. However, sensory innervation does not seem to participate in the response induced by EFS in segments from WKY rats, in contrast with earlier results [28,29]. This might be explained by different experimental conditions, since we have observed, using the methods described in the present paper, a lack of participation by this innervation in the EFS-induced response in mesenteric arteries from Sprague–Dawley and Wistar rats [6,16].

The effects of PKC on neuronal NOS activity in hypertension were assessed in experiments performed in the presence of capsaicin, in order to denervate the perivascular sensory nerves and thus avoid possible interference between sensory nerves and the nitrergic nerves.

Incubating segments with capsaicin plus calphostin C, a PKC inhibitor [30,31], increased the contraction elicited by EFS. This increase was unaltered by the further addition of l-NAME. These results suggest that the PKC of nitrergic nerves is activated in hypertension, and that this activation stimulates neuronal NOS, since l-NAME did not modify the contractile response. To confirm this assumption, PKC was activated with the phorbol ester PDBu. In SHR arteries pretreated with capsaicin, PDBu did not modify the contraction elicited by EFS, while the combination of PDB plus l-NAME increased the EFS-induced contraction, which could indicate the absence of PDBu activation of PKC, since a low concentration of PDBu was used. However, this is unlikely, because the low concentration of PDBu used did not produce contraction, although it was enough to modulate NOS activation [16]. Higher concentrations were not used, since they might have induced contractions [32,33], which would affect the results. All these results suggest that the degree of PKC activation alone is enough to induce maximal NOS activity in hypertension.

The capacity of vascular smooth muscle to synthesize and release NO by activating inducible NOS, which can be modulated by PKC, has been reported previously [34,35]. Preincubation with the protein synthesis inhibitor cycloheximide or with AMT, an inhibitor of inducible NOS [36,37], did not modify the vasoconstrictor responses induced by EFS in arteries from SHRs, indicating that this NOS isoform is not involved in these responses.

The concentration–response curves to NA were unaltered in SHR arteries by l-NAME, AMT, Methylene Blue, capsaicin, calphostin C and PDBu; likewise, l-NAME and capsaicin had no effect in WKY rat arteries. These results suggest that the effects exerted by some of these drugs on the contraction elicited by EFS occur only at the level of sensory and nitrergic nerves.

In conclusion, the present results indicate that EFS induced neuronal NO release by mesenteric arteries from SHRs, but not by those from WKY rats, and that this release was mediated by a constitutive NOS isoform. This enzyme is positively modulated by PKC, which seems to be activated in hypertension.

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