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Endothelial dysfunction in aortic rings and mesenteric beds isolated from deoxycorticosterone acetate hypertensive rats: possible involvement of protein kinase C

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Abstract

The main objectives of this study were to investigate the effects of deoxycorticosterone acetate (DOCA)-induced hypertension on the aortic and mesenteric vascular responses to vasodilator and vasoconstrictor agents and also to elucidate whether protein kinase C (PKC) was involved in these responses, by using chelerythrine and calphostin C, the inhibitors of protein kinase C. Hypertension was induced in male Sprague–Dawley rats (200–250 g) by DOCA-salt injection [20 mg/kg, twice weekly for 5 weeks, subcutaneously (s.c.)] and NaCl (1%) was added to their drinking water. Control rats received a saline injection (0.5 ml/kg, twice weekly for 5 weeks, s.c.), then the animals were anaesthetised [thiopental, 30 mg/kg, intraperitoneally (i.p.)] and the arterial blood pressure was measured. Mean arterial blood pressure in control and hypertensive rats were 98 ± 7.5 and 163 ± 3.5 mmHg, respectively ($P < 0.0001$). In the in vitro studies, rings of descending aorta and mesenteric beds were precontracted with phenylephrine and then concentration–response curves to acetylcholine and sodium nitroprusside were constructed. In the tissue removed from hypertensive rats, the responses to acetylcholine, but not to sodium nitroprusside, were significantly reduced. However, addition of chelerythrine (10 μM) or calphostin C (100 nM) to the organ bath significantly restored these impaired responses. Our data suggest that protein kinase C plays a crucial role in the endothelial dysfunction induced by hypertension.

Keywords: Hypertension; Aorta; Mesenteric vascular bed; Chelerythrine; Calphostin C

1. Introduction

Endothelial dysfunction is a consequence of imbalance between the synthesis, release or effect of endothelium-derived relaxing factors such as nitric oxide and prostacyclin or of endothelium-derived hyperpolarizing factor, and the vasoconstrictor substances mainly, cyclooxygenase products, endothelin and angiotensin II (Yanagisawa et al., 1988; Vanhoutte, 1992). The imbalance manifests itself as a reduction in endothelium-dependent vasodilation or an increase in vasoconstriction. Studies in both human (Panza et al., 1990; Paniagua et al., 2001) and experimental animals (Luscher et al., 1988) have shown that endothelium-dependent vasodilation has been impaired in hypertensive subjects compared with that in healthy subjects, resulting in an overall increase in vascular tone. On the other hand, implication of altered activity of protein kinase C (PKC) has been established in the pathophysiology of many diseases such as diabetes (Akita, 2002) and hypertension (Fareh et al., 2000). Roles of protein kinases in many metabolic processes of phosphate transferring and their importance in cell communication and function in physiological and pathological states have been characterized (Kiley and Jaken, 1994; Nishizuka, 1995). Considering the absence of any reports on the effects of these enzymes in the mediating responses to vasodilators during hypertension induced by deoxycorticosterone acetate (DOCA) salt, this study became of interest.

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2. Materials and methods

2.1. Induction of experimental hypertension

The experiments were performed in accordance with the Animals (scientific procedures) Act of 1986 (Britain) and conformance to the National Institutes of Health guidelines for the use of experimental animals in Britain. This study was carried out with male Sprague–Dawley rats (Razi Institutes; Mashhad, Iran), weighing between 250 and 300 g. The rats were housed in temperature- and humidity-controlled, light-cycled quarters. The animals were randomly divided into two groups including control and hypertensive. Control rats received saline injection [0.5 ml/kg, twice weekly for 5 weeks, subcutaneously (s.c.); $n=20$] whereas hypertension was induced by DOCA-salt injection (20 mg/kg, twice weekly for 5 weeks, s.c.; $n=20$), and NaCl (1%) was added to their drinking water (Bockman et al., 1992).

2.2. Studies in anaesthetised rats

Five weeks after saline or DOCA injection, the animals were anaesthetised with sodium thiopental [30 mg kg^{-1} by intraperitoneal (i.p.) injection]. The right common carotid artery was catheterised for the measurement of blood pressure; right and left jugular veins were cannulated for the administration, throughout the experiment, of anaesthetic (sodium thiopental, 10 mg kg^{-1}) and of different agents such as acetylcholine, sodium nitroprusside and phenylephrine, respectively. The trachea was cannulated and the animals were allowed to breathe spontaneously. Body temperature was recorded using a rectal thermistor probe and was maintained at 37 ± 0.5 °C using an incandescent lamp placed over the abdomen. After 20 min (for stabilization), arterial blood pressure (systolic, diastolic and mean) and heart rate were measured.

2.3. Studies on the isolated aortic rings

The descending thoracic aorta was excised and trimmed free of adhering fat and connective tissue (Cordellini, 1999). The aorta was cut into rings (width, 2 mm) and was vertically mounted in a 10-ml organ bath containing normal Krebs solution (mentioned below) for 1 h. The Krebs solution was maintained at 37 °C and was bubbled with a mixture of 95% O_2 and 5% CO_2. The preparations were allowed to equilibrate for at least 1 h under a resting tension of 1 g that was maintained throughout. Changes in tension were recorded with an isometric transducer and were displayed on a Washington recorder. Cumulative concentration–response curves for phenylephrine (10^{-9}–10^{-5} M), acetylcholine (10^{-6}–10^{-3} M) and sodium nitroprusside (10^{-8}–10^{-6} M) were constructed in tissues removed from either control or hypertensive rats.

2.4. Studies on the isolated perfused mesenteric bed

The abdominal cavity was opened by a midline incision through the linea alba and the mesenteric bed was excised using the procedure described by McGregor, 1965. The animals were given heparin [1000 u kg^{-1} intravenously (i.v.)] just before cannulation of the mesenteric artery and removal of the preparation. The mesenteric bed was perfused through a cannula inserted into the superior mesenteric artery using Krebs–Henseleit solution of the following composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4$, H_2O 1.2, KH_2PO_4, $2H_2O$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and glucose 11.1 in distilled water. This solution was maintained at 37 °C, bubbled with 5% CO_2 and 95% O_2 and was perfused at a constant rate (5 ml min^{-1}; Gilson Minipuls 2). The isolated, perfused preparations were placed on a Petri dish which was supported in a heated water bath (37 °C). The perfusate, which flowed from the cut ends of the vessels at the intestinal margin of the mesentery, was removed at a rate of 5 ml min^{-1} to prevent accumulation in the bath. The tissue was allowed to equilibrate for 30 min before the start of the experiments. The cumulative concentration–response curves for phenylephrine (10^{-8}–10^{-6} M), acetylcholine (10^{-8}–10^{-6} M) and for sodium nitroprusside (10^{-8}–10^{-6} M) were constructed in tissue removed from either control or hypertensive rats.

To determine the possible roles of protein kinase C in the responses to acetylcholine, the aortic rings and mesenteric beds were perfused with the Krebs solution plus chelerythrine (10^{-6} M) for 20 min.

2.5. Drugs

The following drugs were used: deoxycorticosterone acetate (Iran–Hormone), chelerythrine chloride, calphostin C, phenylephrine hydrochloride, acetylcholine chloride, sodium nitroprusside, heparin sodium and dimethylsulphoxide (DMSO) which were obtained from Sigma. Sodium chloride, potassium chloride, magnesium sulfate, sodium hydrogen carbonate, potassium dihydrogen orthophosphate, D-glucose and calcium chloride were obtained from Merck Laboratories. Sodium thiopental was obtained from Biochemie, Vienna, Austria. Phenylephrine and acetylcholine solutions were freshly prepared in normal saline and were diluted with 0.9-w/v sodium chloride solution. All drugs except chelerythrine and calphostin C were dissolved in distilled water and were then diluted with normal saline. Chelerythrine and calphostin C were dissolved in DMSO (final concentration of DMSO in the bathing solution was 0.01% or less). All stock solutions were kept at −20 °C.

2.6. Statistical analysis of data

Results were expressed throughout as means ± S.E.M. and were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey–Kramer multiple compar-

ison test (for comparison of responses to phenylephrine and ACh in aortic and mesenteric preparations). A P value of <0.05 was considered to be significant.

3. Results

3.1. Arterial blood pressure, body and heart weight

Systolic, diastolic and mean arterial blood pressure were significantly increased in DOCA-salt-treated rats (20 mg/kg, twice weekly for 5 weeks, s.c., plus NaCl (1%) added to the animals' drinking water for 5 weeks) as compared to controls (0.5 ml/kg, twice weekly for 5 weeks, s.c.; Table 1). DOCA treatment reduced the body weight [DOCA-treated (g): 244 ± 2 vs. control 379 ± 7, $P<0.001$] and heart weight was significantly increased [DOCA-treated (mg): 1384 ± 22 vs. control 1130 ± 37, $P<0.01$], resulting in a higher heart to body weight index.

3.2. Effects of DOCA treatment on responses to different agents in anaesthetised rats

Administration of acetylcholine (1 and 10 μg/100 g) to the anaesthetised rats (either control, $n=4$; or hypertensive, $n=5$) resulted in a dose-dependent reduction in the arterial blood pressure (Fig. 1). DOCA-treated rats showed a response to the intravenous administration of sodium nitroprusside and phenylephrine similar to that of the control (data not shown).

3.3. Effects of chelerythrine and calphostin C on aortic responses to acetylcholine, phenylephrine and sodium nitroprusside

Addition of phenylephrine (10^{-9}–10^{-5} M) to the rat aortic rings incubated in normal Krebs solution produced a concentration-dependent contraction (Fig. 2). Contractile responses to phenylephrine were significantly increased in aortic rings isolated from DOCA-treated rats. Therefore, to obtain responses comparable to those of preparations removed from controls, all aortic rings removed from hypertensive rats were precontracted with 5×10^{-8} M of phenylephrine. Addition of chelerythrine (10 μM for 20 min) or calphostin C (100 nM for 20 min) to the Krebs solution did not modify the basal tension and responses to phenylephrine in this tissue (data not shown). In the precontracted rings (with phenylephrine, 50 nM) removed from DOCA-treated rats, the relaxant effects of acetylcholine (10^{-6}–10^{-3} M) and also the maximal response to acetylcholine were significantly decreased in comparison to those of controls (Fig. 3). Although chelerythrine or cal-

Table 1
Hemodynamic effects of deoxycorticosterone acetate salt administration (hypertensive, 20 mg kg^{-1}, twice weekly for 5 weeks, s.c.) plus NaCl (1%, added to the rats' drinking water) in male rats

Groups	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Mean arterial blood pressure (mmHg)	Heart rate (beats/min)
Control	111 ± 8.5	87 ± 5.9	98 ± 7.5	402 ± 22
Hypertensive	185 ± 3.4[a]	154 ± 3.8[a]	163 ± 3.5[a]	506 ± 12[b]

Control rats received saline injection (0.5 ml/kg, twice weekly, for 5 weeks, s.c.). The values are given in mean ± S.E.M. of 15 experiments.
[a] $P<0.001$ vs. control.
[b] $P<0.05$ vs. control.

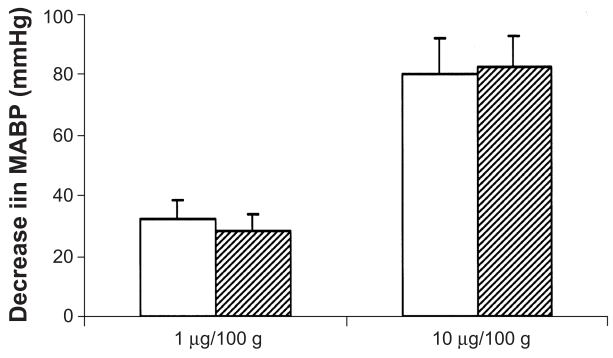

Fig 1. Decrease in mean arterial blood pressure in response to different doses of acetylcholine (1 and 10 μg/100 g body weight) in control (open bars, $n=4$) and hypertensive rats (hatched bars, $n=5$). DOCA treatment did not modify the vasodilator responses to acetylcholine.

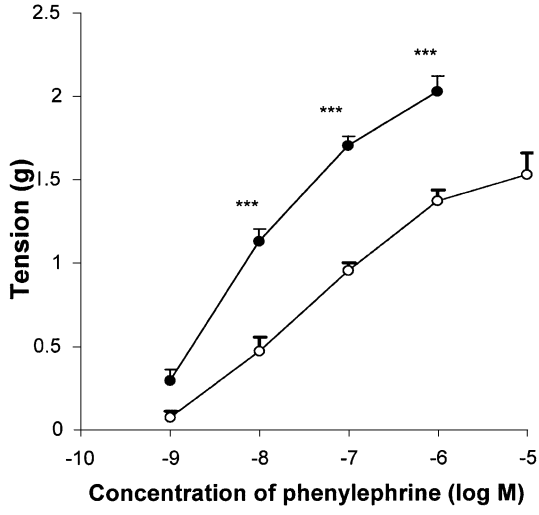

Fig 2. The contractile effects of different concentration of phenylephrine in isolated aortic rings removed from saline-treated [0.5 ml/kg, twice weekly for 5 weeks, s.c. (○)] and DOCA-salt treated rats [20 mg/kg, twice weekly for 5 weeks, s.c., plus NaCl (1%) added to the rats' drinking water for 5 weeks (●)]. The responses to phenylephrine were significantly increased in tissue removed from DOCA-treated rats. One-way ANOVA performed on aortic responses to phenylephrine at all concentrations showed a significant difference among treatment groups ($P<0.001$). Results of Tukey–Kramer multiple comparison test at each concentration showed a significant difference between groups receiving saline injection and those received DOCA (***$P<0.001$). Each value is the mean ± S.E.M. of four to five observations.

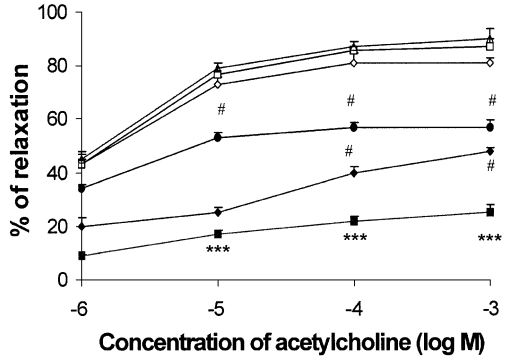

Fig 3. Percentage (%) of relaxation in response to different concentration of acetylcholine (10^{-6}–10^{-3} M) in precontracted aortic rings removed from saline-treated rats (0.5 ml/kg, twice weekly for 5 weeks, s.c.) in the absence (□) or in the presence of either chelerythrine [10 μM, incubated for 20 min before addition of phenylephrine (△)] or calphostin C [100 nM, incubated for 20 min before addition of phenylephrine (◇)]; and in tissue removed from DOCA-treated rats (20 mg/kg, twice weekly for 5 weeks, s.c., plus NaCl (1%) added to the rats' drinking water for 5 weeks) in the absence (■) or presence of either chelerythrine [10 μM, incubated for 20 min before addition of phenylephrine (●)] or calphostin C [100 nM, incubated for 20 min before addition of phenylephrine (◆)]. The responses to acetylcholine were significantly reduced in the tissue removed from DOCA-treated rats. One-way ANOVA performed on aortic responses to acetylcholine at all concentrations showed a significant difference among treatment groups ($P<0.001$). Results of Tukey–Kramer multiple comparison test at each time showed a significant difference between groups receiving saline injection and those received DOCA (***$P<0.001$ vs. control). Incubation of tissue with chelerythrine or calphostin C did not modify responses to acetylcholine in rings isolated from saline-treated but significantly restored the responses to acetylcholine in those removed from DOCA-treated rats ($^{\#}P<0.01$ vs. hypertensive). Each value is the mean ± S.E.M. of four to five observations.

phostin C pretreatment did not modify the responses to acetylcholine in the rings isolated from saline-treated rats, they significantly restored the responses to acetylcholine in the rings removed from DOCA-treated animals (Fig. 3). Responses to sodium nitroprusside in precontracted rings were not modified by DOCA treatment (data not shown).

3.4. Effects of chelerythrine and calphostin C on mesenteric responses to acetylcholine, phenylephrine and sodium nitroprusside

The contractile responses to different concentrations of phenylephrine in mesenteric bed removed from DOCA-treated rats were similar to those of the controls (Fig. 4). In precontracted mesenteric beds (with phenylephrine, 10^{-6} M) isolated from hypertensive rats, the responses to acetylcholine, but not to sodium nitroprusside, were significantly reduced. For instance, the changes in basal perfusion pressure in response to acetylcholine (10^{-6} M) in tissue removed from saline and DOCA-treated rats were: -41.6 ± 4.9, $+17.2 \pm 3.6$ mmHg ($P<0.05$), respectively (Fig. 5). Preincubation of tissue with chelerythrine (1 μM for 20 min) did not change the basal perfusion pressure but significantly increased the vasodilator responses to acetylcholine (Fig. 5). Incubation of mesenteric bed removed

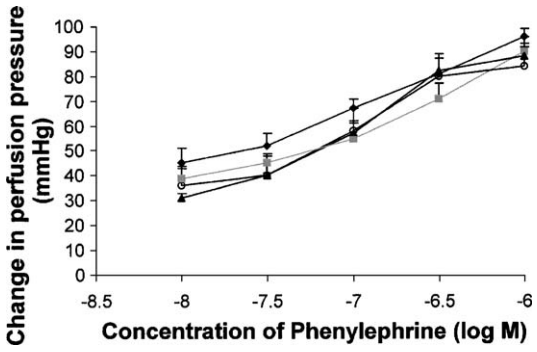

Fig 4. Change in mesenteric perfusion pressure (mmHg) in response to different concentration of phenylephrine (10^{-8}–10^{-6} M) in tissue removed from saline-treated rats either in the absence [0.5 ml/kg, twice weekly for 5 weeks, s.c. (◆)] or in the presence of chelerythrine [10 μM, incubated for 20 min before addition of phenylephrine (○)]; and in tissue removed from DOCA-salt treated rats either in the absence [20 mg/kg, twice weekly for 5 weeks, s.c., plus NaCl (1%) added to the rats' drinking water for 5 weeks (■)] or in the presence of chelerythrine [10 μM, incubated for 20 min before addition of phenylephrine (▲)]. The responses to phenylephrine in the isolated mesenteric beds were not modified by DOCA treatment. Each value is the mean ± S.E.M. of four to five observations.

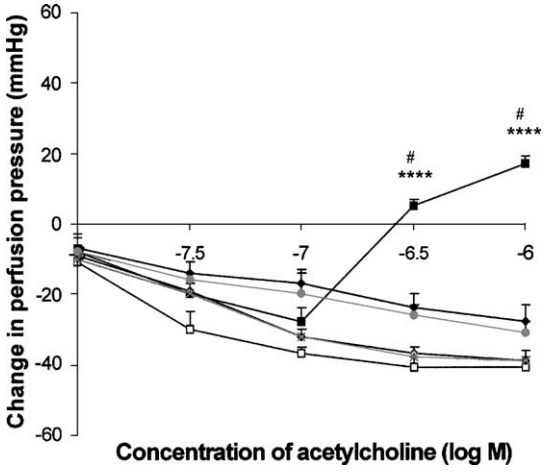

Fig 5. Change in mesenteric perfusion pressure (mmHg) in response to different concentration of acetylcholine (10^{-8}–10^{-6} M): in precontracted mesenteric beds removed from saline-treated rats (0.5 ml/kg, twice weekly for 5 weeks, s.c.) in the absence (□) or presence of either chelerythrine [10 μM, incubated for 20 min before addition of phenylephrine (◇)] or of calphostin C [100 nM, incubated for 20 min before addition of phenylephrine (×)]; and in tissue removed from DOCA-treated rats (20 mg/kg, twice weekly for 5 weeks, s.c.) plus NaCl (1%) added to the rats' drinking water for 5 weeks, in the absence (■) or presence of either chelerythrine [10 μM, incubated for 20 min before addition of phenylephrine (●)] or of calphostin C (◆). The responses to acetylcholine were significantly reduced in tissue removed from DOCA-treated rats. One-way ANOVA performed on mesenteric responses to acetylcholine at all concentrations showed a significant difference among treatment groups ($P<0.001$). Results of Tukey–Kramer multiple comparison test at each time showed a significant difference between groups receiving saline injection and those received DOCA (****$P<0.0001$ vs. control). Incubation of tissue with chelerythrine or calphostin C did not modify responses to acetylcholine in rings isolated from saline-treated but significantly restored the responses to acetylcholine in those removed from DOCA-treated rats ($^{\#}P<0.01$ vs. hypertensive). Each value is the mean ± S.E.M. of four to five observations.

from control or from DOCA-treated rats caused a nonsignificant reduction in the mesenteric basal perfusion pressure. However, calphostin C pretreatment did cause a significant increase in responses to acetylcholine in tissues removed from DOCA-treated rats (Fig. 5). Tissue removed from DOCA-treated rats showed responses to sodium nitroprusside similar to those of saline-treated rats (data not shown).

4. Discussion

In the present study, administration of DOCA-salt and replacement of tap water by NaCl (1%) for 5 weeks increased the arterial blood pressure which confirms previous work (Bockman et al., 1992). The increase in heart to body weight index represents a cardiac hypertrophy in DOCA-salt hypertensive rats which is similar to previous reports (Matsumura et al., 1999). Numerous in vitro studies indicate that endothelium-mediated relaxation is reduced in DOCA-salt hypertension (Luscher et al., 1988; White et al., 1996; Somers et al., 2000). It has also been reported that responses to acetylcholine are reduced in patients with essential hypertension (Linder et al., 1990). However, our in vivo studies showed that responses to acetylcholine are not affected by DOCA treatment. Although the reasons for these disparate results are not yet clear, species-specific variations and the model of hypertension are important to note. In aortic rings removed from DOCA-treated rats, pathological studies did not show any structural changes (data not shown), but contractile responses to phenylephrine were significantly augmented. Several explanations may exist. This could be due to the increase in Ca^{2+} concentration in aortic rings isolated from DOCA-treated rats. Another possibility is that DOCA treatment increases or sensitizes the á-adrenoreceptors in this tissue. In addition, it has been shown that nitric oxide synthase activity is altered in different experimental models of hypertension including DOCA-induced hypertension (Sullivan et al., 2002). These various explanations should be verified by further studies.

Different mediators such as nitric oxide (NO) and prostaglandins mediate the basal perfusion pressure in the rat mesenteric bed (Fatehi-Hassanabad et al., 1995). In the present study, the basal perfusion pressure was similar in mesenteric bed removed from either saline- or DOCA-treated rats, which shows that basal nitric oxide/prostaglandin release is not affected by DOCA injection. Responses to different concentrations of phenylephrine were not modified by DOCA treatment in isolated mesenteric beds, which is similar to the previous report (Ayangade-Johnson and Joshua, 2001). However, small mesenteric arteries removed from spontaneously hypertensive rats showed structural changes such as medial hypertrophy and increased arterial wall and width wall/lumen ratio (Dickhout and Lee, 1997). In contrast to the absence of any change in the basal release of NO, it seems that stimulated release of NO by endothelial cells of aorta and by mesenteric beds removed from DOCA-treated rats is affected, as indicated by responses to acetylcholine, an agent reported to stimulate the release of endogenous NO. The absence of a change in responses to sodium nitroprusside in the aortic rings and mesenteric beds removed from DOCA-salt hypertensive rats would also support the hypothesis that at least the guanylate cyclase system in vascular smooth muscle of these animals is not impaired. Mesenteric preparations removed from DOCA-hypertensive rats showed a bimodal effect in response to acetylcholine. Considering the present data, it is not easy to explain this effect of acetylcholine. However, in the tissues removed from hypertensive rats (probably with endothelial damage), the high concentrations of acetylcholine may have induced the release of some contracting factors, such as calcium, from the intracellular stores via activation of muscarinic receptors on vascular smooth muscles.

Protein kinase C (PKC) is a key element in signal transduction and cell regulation, eliciting a variety of cellular responses by phosphorylating target proteins on serine and threonine residues. The PKC molecule contains two domains: a regulatory domain that interacts with calcium, phosphatidylserine and diacylglycerol; and a catalytic domain with binding sites for ATP and protein substrates. In the present study, we used potent, specific and dissimilar inhibitors of PKC, chelerythrine and calphostin C, which interact with the catalytic domain and the diacylglycerol binding to the regulatory domain of PKC, respectively (Kobayashi et al., 1989; Herbert et al., 1990). Addition of chelerythrine and calphostin C to the Krebs failed to modify the responses to phenylephrine in aortic rings removed from DOCA-treated rats but significantly improved the vasodilator responses to acetylcholine in both aortic and mesenteric preparations removed from DOCA-treated rats. The impaired vascular responses to acetylcholine could be due to either the receptor or the G protein alteration induced by DOCA treatment. In hypertension, it has been shown that the defect in receptor-mediated vasorelaxation (e.g., reduced β-badrenergic responsiveness) is associated with an increase in serine–threonine receptor phosphorylation (Feldman et al., 1995). On the other hand, G protein-coupled-receptor phosphorylation is mediated by at least two classes of serine–threonine kinases including protein kinase C and members of the G protein-coupled receptor kinase family; therefore, it seems that the beneficial effects of chelerythrine and calphostin C on endothelial-dependent relaxation to acetylcholine are exerted through the change in receptor phosphorylation in aortic and mesenteric preparations removed from DOCA-treated rats. In summary, this study showed that hypertension induced by injection of DOCA-salt impaired the aortic and mesenteric vascular responses to acetylcholine, an endothelium-dependent vasodilator, in rats. The novel finding of this study was that inhibition of protein kinase C by chelerythrine or calphostin C significantly restored these impaired responses to acetylcholine.

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