Calcium, Phosphoinositide, and 1,2-Diacylglycerol Responses of Blood Vessels of Deoxycorticosterone Acetate–Salt Hypertensive Rats to Endothelin-1

Jean-Pierre Flückiger, Paul V. Nguyen, Guo Li, Xiao-Ping Yang, and Ernesto L. Schiffrin

In previous studies a decreased responsiveness to endothelin-1 (ET-1) of conduit arteries and resistance vessels of deoxycorticosterone acetate (DOCA)–salt hypertensive rats was found in comparison with uninephrectomized controls. Decreased isometric force, number of receptors, and inositol phosphate accumulation were reported in the DOCA-salt animals. In the present study effects of ET-1 on cytosolic free calcium, inositol phosphates, and 1,2-diacylglycerol were investigated in blood vessels of DOCA-salt hypertensive rats. Basal cytosolic free calcium, measured with the fluorescent dye fura-2, was 201 ± 41 nmol/l in mesenteric arteries of DOCA-salt rats and 45 ± 9 nmol/l in uninephrectomized controls (p < 0.01). The maximal response of cytosolic free calcium (to 30 nmol/l ET-1) was 176 ± 22% of the basal value for DOCA-salt and 242 ± 6% for uninephrectomized rats (p < 0.05). The concentration giving 50% of the maximum response was 9.0 and 6.5 nmol/l for DOCA-salt rats and controls, respectively. Inositol phosphate production after stimulation with 100 nmol/l ET-1 in the presence of LiCl was lower by at least 30% (p < 0.01) in both aorta and mesenteric arteries of DOCA-salt hypertensive versus control rats. Basal levels of diacylglycerol in aorta were similar in DOCA-salt rats and in controls and did not respond to a 100 nmol/l ET-1 stimulation in the DOCA-salt rats, in contrast to the increase found in the control uninephrectomized rats (p < 0.05). Thus, the diminished response to ET-1 of DOCA-salt rat arteries may be due to a lower density of ET-1 receptors, resulting in a blunted signal transduction, as reflected by decreased responses of inositol phosphate, cytosolic free calcium, and diacylglycerol.

Key Words • endothelins • phospholipids • diglycerides • signal transduction • arteries

Deoxycorticosterone acetate (DOCA)–salt hypertensive rats have been shown to have an altered response to norepinephrine, angiotensin II, arginine vasopressin, and endothelin-1 (ET-1).1–5 These observations may be related to changes at the receptor level1–3 as well as to modifications of the intracellular signal transduction mechanisms3–5 of vascular smooth muscle cells. ET-1 is a potent 21-amino acid peptide vasoconstrictor, secreted by endothelial cells, first described by Yanagisawa et al in 1988.6 Although endothelin infusion may raise blood pressure, its role in hypertension is unproven.7 The vascular effects of this peptide have been extensively investigated: endothelins most likely act as paracrine hormones and exert their cellular effects through the phosphoinositide pathway, through calcium mobilization and influx, and by phospholipase A2 activation.8–9 In a previous study we reported that the density of mesenteric artery ET-1 receptors and vascular responses to ET-1 are decreased in DOCA-salt hypertensive rats.3 Moreover, we demonstrated that vascular accumulation of inositol phosphates (InsPs) in response to ET-1 was significantly lower in DOCA-salt hypertensive rats when compared with uninephrectomized controls. These observations led to the question of whether diacylglycerol (DG) production and intracellular calcium metabolism are also altered in DOCA-salt hypertensive rats. It is indeed well established that DG can be produced by phosphatidylcholine breakdown as well as through the phosphatidylinositol pathway.10 Therefore, DG levels might not change in parallel with endothelin-stimulated InsP production in vascular smooth muscle cells. ET-1 has also been shown to exert a wide range of effects on calcium metabolism: low ET-1 concentrations (<1 nmol/l) provoke calcium influx through calcium channels,8,11–12 whereas higher concentrations stimulate inositol 1,4,5-trisphosphate–dependent calcium release from intracellular stores in the first step of contraction, followed by calcium entry during the sustained phase.8,11–14 Taken together, these data suggest multiple steps in the regulation of smooth muscle contraction by endothelin.
In the present study, we have therefore investigated the responses of intracellular calcium, InsP, and DG to ET-1 in thoracic aorta and mesenteric arteries of DOCA-salt rats with hypertension of a 3-week duration in comparison to uninephrectomized control rats.

Methods

Materials

Chemicals were of the highest reagent grade available. Ionomycin, cardioliopin, octyl β-glucoside, dimethyl sulfoxide, and β-arachidonyl-γ-stearoyl L-α-phosphatidic acid (sodium salt) were from Sigma Chemical Co., (St. Louis, Mo.), ET-1 (human, porcine) was purchased from Peninsula Laboratories, Belmont, Calif. Myo-2-[3H]inositol were from Amersham, Oakville, Canada. Fura-2 AM and Pluronic F127 were from Molecular Probes, Inc., Eugene, Ore., and DG kinase was from Lipidex, Inc., Westfield, N.J.

Animal Experiments

The study was approved by the Animal Care Committee of the Clinical Research Institute. Male Sprague-Dawley rats (n=95; Charles River Laboratories, St. Constant, Canada) weighing 150–200 g were uninephrectomized under ether anesthesia. Silicone rubber, impregnated with DOCA (130 mg per rat), was implanted subcutaneously.1-3,15 Animals received 1% saline to drink. Control rats (n=100) were uninephrectomized and implanted with silicone rubber without DOCA and were offered tap water to drink. Rats were studied 3 weeks after hypertension was apparent in the DOCA-treated group (systolic blood pressure >150 mm Hg). Blood pressure was measured with the tail-cuff method, with a model 1010 crystal microphone as a pulse detector, fitted with a 7PR preamplifier, coupled to a model 7 polygraph (Grass Instrument Co., Quincy, Mass.). The thoracic aorta and mesenteric arteries were removed from rats after decapitation and immersed in a Krebs solution maintained at 4°C while the fat of blood vessels was mechanically removed.

Intracellular Free Calcium Measurement

Mesenteric arteries were incubated at 37°C in 20 ml Krebs solution with the following composition in millimoles per liter: NaCl 118, KCl 4.65, MgSO4 1.18, KH2PO4 1.18, CaCl2 2.5, glucose 10, NaHCO3 25, adjusted to pH 7.4. Oxygenation and pH were maintained by bubbling the solution with a 95% O2-5% CO2 mixture. After a 120-minute stabilization period, the mesenteric arteries were incubated for 60 minutes with the calcium-sensitive fluorescent dye fura-2 AM. Fura-2 AM was prepared from frozen aliquots of a stock solution made up with dimethyl sulfoxide containing 0.02% Pluronic F127. The final dye concentration was 4 μmol/l, and dimethyl sulfoxide did not exceed 0.4%. Tissues and solutions were then carefully transferred to a Petri dish. Small second- and third-order arteries were removed from the mesenteric arterial bed and transferred to a silicone-coated aluminum well adjusted to a thermostated tissue bath. The 3-mm-diameter well faced the excitation beam (340 and 380 nm) of a CAF-100 spectrofluorometer (Japan Spectroscopic Co., Ltd., Tokyo). The experimental solutions were perfused at a rate of 1.1 ml/min from the bottom of the well and flowed away through an upper nylon grill that maintained the resistance arteries in place. The emissions at 510 nm for both incident wavelengths as well as their fluorescence ratio at 340 and 380 nm were recorded on a Grass model 7 polygraph. Tissues were first challenged with 124 nmol/l K+, and after washout, cumulative concentration-response curves were obtained for ET-1. The dose was increased as soon as a plateau was reached (after 5 minutes at ET-1 concentrations <10 nmol/l and after 1 minute at 10 nmol/l or higher concentrations) to reduce duration of experiments in order to diminish the photobleaching of fura-2.16 Maximum intracellular calcium concentrations were achieved with a perfusion of 10 μmol/l ionomycin and minimum values were obtained with 2.5 mmol/l EGTA instead of calcium in a Krebs solution. Calcium concentrations ([Ca2+]i) were calculated according to the formula of Grynkiewicz et al.17: [Ca2+]i=[Kd(R−Rmin/Rmax−R)]β, with a Kd (dissociation constant) of fura-2 for calcium assumed to be 224 nmol/l at 37°C. R is the ratio of fluorescence of the sample at 340 and 380 nm; Rmax and Rmin are the ratios for fura-2 free acid at these wavelengths in the presence of saturating calcium (with ionomycin) and EGTA, respectively; β is the ratio of fluorescence at 380 nm in the presence of EGTA over fluorescence at 380 nm in the presence of ionomycin. Rmax obtained with this preparation was approximately 2 versus 6 obtained with suspensions of A-10 cells (rat aortic smooth muscle cell line from American Tissue Culture Collection, Rockville, Md.) and 12 with acellular media with the same instrument. Rmax, Rmin, and β were similar when measurements were made in blood vessels of DOCA-salt and control rats. Rmax of control mesenteric arteries was 0.70±0.06 and that of DOCA-salt rats was 0.79±0.05 (n=5 and 6, respectively). Rmax of control blood vessels was 1.90±0.23 and that of DOCA-salt rats was 1.90±0.10. In control rats, β was 1.39±0.19; in DOCA-salt rats, β was 1.60±0.13.

Insitol Phosphate Measurement

Mesenteric arteries and thoracic aortas were incubated in a Krebs-Ringer solution containing 2% bovine serum albumin. Tissues were incubated with 100 μCi/ml [3H]myoinositol for 5 hours at 37°C with constant stirring in a 95% O2-5% CO2 atmosphere. Excess radioactivity was washed away in a Krebs-bicarbonate solution. LiCl (20 mmol/l) was added to inhibit inositol monophosphatases. After a 30-minute stabilization period, tissues were challenged with a solution containing 100 mmol/l ET-1. The reaction was stopped by placing tissues in liquid nitrogen. Tissues were then weighed. The extraction and separation of InsP was performed as described elsewhere.3 In brief, tissues were extracted overnight in chloroform/methanol/HCl (1:2:0.05 [vol/ vol]). After separation with chloroform and H2O, the aqueous phase was partially evaporated and neutralized. Filtered samples were then injected into a high-performance liquid chromatography system (Beckman Instruments, Inc., Fullerton, Calif.) equipped with a Partisol 10 SAX precolumn and a Partisil 5 SAX analytical column. The different [3H]myoinositol phosphates were separated with a three-step gradient of ammonium formate. Radioactivity was monitored on-line with a Beckman 171 radioisotope detector. Because.

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the mass of organs was higher in DOCA-salt rats, data were normalized according to wet weight. Each result was obtained from one aorta or mesenteric vascular bed.

**Diacylglycerol Measurement**

Thoracic aortas of DOCA and control rats were dissected, cut into six rings (one for each time point), and then preincubated as described for mesenteric arteries for calcium determination. Arteries were stimulated with 100 nmol/l ET-1, and the time course of the response was studied. The reaction was stopped by placing the tissues in liquid nitrogen. Frozen tissues were weighed and subsequently ground with a Vari-Mix III grinder (Densply Inc., Toronto, Canada). DG was determined according to the technique of Preiss et al. 18 Briefly, the powder obtained after grinding was extracted with 3 ml chloroform/methanol (1:2 [vol/vol]) mixture. Phases were separated by adding 1 ml chloroform and 1 ml NaCl (1 mol/l). DG residues were then phosphorylated with a DG kinase (Lipidex) in an imidazole buffer in the presence of [γ-32P]ATP (1–2x 10^5 cpm). The reaction was stopped after 30 minutes. Lipids were extracted by splitting the chloroform/methanol phase with perchloric acid. The organic phase was dried and resuspended in 100 μl methanol/chloroform (2:1 [vol/vol]). Twenty microliters was spotted on silica gel thin layer chromatography plates and separated with a chloroform/methanol/acetic acid (65:15:5 [vol/vol/vol]) mixture. After autoradiography, the spots corresponding to phosphatidic acid (identified with β-arachidonoyl-γ-stearoyl L α-phosphatidic acid used as standard) were scraped (0.5 cm above and 1 cm below the spot corresponding to the standard, total 3.5-cm length to include DG with different fatty acid composition) and counted in an LKB beta counter.

**Biochemical Measurements**

From the blood collected after decapitation, plasma renin activity was measured in tubes containing 5 mmol/l potassium edetate (final concentration). Tubes were placed on ice and were subsequently centrifuged at 3,000 rpm at 4°C. Plasma renin activity was measured by radioimmunoassay of angiotensin I after a 2-hour incubation at pH 6.5, as previously reported. 5

**Data Analysis**

The ALLFIT program 19 based on the four-parameter logistic equation was used to fit the data from dose–response curves for the determination of the maximum response and the concentration giving 50% of the maximum response (EC50). All results are presented as mean±SEM. Statistical differences between groups were analyzed with Student’s t test and by one- or two-way analysis of variance followed by a t test, with Bonferroni’s correction for multiple comparisons. 20

**Results**

**Weight, Systolic Blood Pressure, and Plasma Renin Activity**

Table 1 shows typical data obtained for a representative group of rats. Systolic blood pressure was significantly higher and plasma renin activity was lower in DOCA-salt rats (p < 0.001).

**Intracellular Free Calcium Concentration in Mesenteric Arteries**

Basal cytosolic free calcium concentration in mesenteric arteries of DOCA-salt hypertensive rats was higher than in control animals (201±41 versus 45±9 nmol/l, p <0.01, n=6 and 5, respectively; Figure 1A). The EC50 for ET-1 stimulation of calcium levels was 9.0±2.0 and 6.5±1.4 nmol/l for DOCA and control rats, respectively.

**TABLE 1. Weight, Systolic Blood Pressure, and Plasma Renin Activity of Deoxycorticosterone Acetate–Salt Hypertensive Rats and Their Uninephrectomized Controls**

<table>
<thead>
<tr>
<th>Measurements</th>
<th>DOCA-salt hypertensive rats (n=31)</th>
<th>Control rats (n=33)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>326±7</td>
<td>396±6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>184±4</td>
<td>103±1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma renin activity (ng Angiotensin I • ml^-1 • hr^-1)</td>
<td>0.24±0.04</td>
<td>1.37±17</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean±SEM. DOCA, deoxycorticosterone acetate.

![Figure 1](http://hyper.ahajournals.org/Downloadedfrom.jpg)

**Figure 1.** Line plots show effect of cumulative doses of endothelin-1 (ET-1) on intracellular free calcium concentration in mesenteric arteries. Panel A: Absolute values. Panel B: Percent of basal values. Solid circles, deoxycorticosterone acetate (DOCA)-salt hypertensive rats; open squares, controls. *p<0.05, two-way analysis of variance between groups. Basal and stimulated cytosolic free calcium concentrations in response to 30 and 100 nmol/l ET-1 were higher in DOCA-salt rats than in controls ( p<0.05, Student’s t test with Bonferroni’s correction).
FIGURE 2. Line plots show responses of inositol monophosphate (InsP₁) and inositol bisphosphate (InsP₂) to 100 nmol/l endothelin-1 in blood vessels. Panel A: Aortas. Panel B: Mesenteric arteries. Solid circles, deoxycorticosterone acetate (DOCA)-salt hypertensive rats; open squares, controls. *p<0.01, analysis of variance between groups. Basal levels (dpm/mg wet wt) in aorta were 506±96 in control and 765±220 in DOCA-salt rats for InsP₁ and 158±29 and 159±28 for InsP₂, respectively. Basal levels in mesenteric arteries (same units) were 774±211 and 655±277 for InsP₁ and 229±46 and 150±27 for InsP₂ in control and DOCA-salt rats, respectively (no significant difference between both groups).

Production of Inositol Phosphates in Blood Vessels

Thoracic aortas of control rats weighed 82.0±1.3 mg and those of DOCA-salt rats 100.7±1.4 mg (p<0.01). The mesenteric vasculature of control rats weighed 65.4±2.3 mg and that of DOCA-salt rats 125.6±5.9 mg (p<0.01). Figure 2 shows the time course of accumulation of InsPs in aorta and mesenteric arteries of DOCA and control rats stimulated with 100 nmol/l ET-1. Basal levels (disintegrations per minute per milligram wet weight) in aorta were 506±96 in control and 765±220 in DOCA-salt rats for inositol monophosphate (InsP₁) and 158±29 and 159±28 for inositol bisphosphate (InsP₂), respectively. In mesenteric arteries basal levels of InsPs (disintegrations per minute per milligram wet weight) were 774±211 and 655±277 for InsP₁ and 229±46 and 150±27 for InsP₂ in control and DOCA-salt rats, respectively (no significant difference between both groups). The pattern of response was the same for both tissues. Between 5 and 30 minutes of stimulation with ET-1, DOCA-salt rats exhibited overall significantly lower levels of InsP₁ and InsP₂ in aorta and of InsP₂ in mesenteric arteries (p<0.01).

Production of Diacylglycerol in Aorta

The mass of thoracic aorta of DOCA-salt rats (64.2±1.9 mg) was greater than that of control rats (49.2±2.3 mg, p<0.01). The time course of DG production in aorta, stimulated with 100 nmol/l ET-1, is shown in Figure 3. Basal values were 66±8 pmol/mg wet wt for DOCA-salt rats and 110±12 pmol/mg wet wt for control rats (not significantly different by t test with Bonferroni's correction). The control group (n=12) showed a biphasic response to stimulation. No significant response was observed in the DOCA-salt hypertensive group (n=10). DG production was lower (p<0.05) in aortas from DOCA-salt hypertensive rats than in controls at 0.5 and 30 minutes after stimulation.
Discussion

We previously demonstrated that, within 2 weeks of developing hypertension in DOCA-salt hypertensive rats, changes in responses of blood vessels to ET-1 were localized at the receptor level (decreased number of binding sites) as well as at the level of InsP metabolism. The aim of the present study was to further investigate the levels of InsP, calcium, and DG in response to ET-1 in blood vessels of DOCA-salt rats after 3 weeks of established hypertension. InsPs in DOCA-salt hypertensive rats responded significantly less than in controls when tissues were challenged during a long period (30 minutes) with 100 nmol/l ET-1. The responses of thoracic aorta and mesenteric arteries had the same pattern, showing that alterations were similar in large conduit arteries and in resistance arteries, in agreement with our previous results demonstrating blunted vascular responses to ET-1 in conduit arteries such as aortic and mesenteric artery rings and in mesenteric resistance arteries of DOCA-salt hypertensive rats. The data of InsP accumulation on long-term stimulation agree with previous findings we have reported but is in contrast to increased responsiveness of InsP to ET-1 shown in another study, which may relate to duration of hypertension. In another study, aortic rings of DOCA-salt hypertensive rats had a contractile response that tended to be smaller than that of controls, in agreement with our current and previous data, although the difference did not reach statistical significance. Microvessels (15-μm diameter) of DOCA rats responded more to ET-1 but showed no structural alterations. In our previous report, the mass of arteries from hypertensive animals was increased. In the study of small mesenteric resistance arteries (200–250 μm i.d.) from DOCA-salt hypertensive rats, we have found that these present extensive morphological alterations and exhibit decreased responses to ET-1 at 4–5 weeks after surgery. It is therefore possible that in severe DOCA-salt hypertension of several weeks in duration, in association with structural alterations, endothelin receptors are decreased in number and biochemical responses are downregulated (Reference 3 and this study), which results in blunting of vascular responses to ET-1.22,23

The basal intracellular calcium concentration in the hypertensive rats was fourfold that of controls. This finding may be an expression of increased calcium turnover in part caused by altered activity of plasma membrane Ca-ATPase22 and in response to exaggerated activity of different hormones in the DOCA-salt hypertensive rat. Aortas of spontaneously hypertensive rats show increased calcium levels, whereas no difference was observed in the mesenteric arterial bed. No differences were found between free calcium concentrations in platelets of DOCA-salt rats and spontaneously hypertensive rats and their controls. ET-1 is known to provoke calcium release and entry into the cell.24-27 In the present study, no attempt was made to differentiate between the calcium from both sources. The change in intracellular calcium concentration in response to ET-1 tended to be greater in mesenteric arteries of DOCA-salt rats, but this difference was not significant, which may depend in part on the reduced InsP production blunting the effect of increased calcium available for release from intracellular stores. When expressed as a percentage to eliminate the effect of the higher basal concentration, the calcium response was proportionately lower in the hypertensive group. Thus, this observation could be related in part to the decreased production of InsP, secondary to a reduced number of endothelin receptors. It may also be in part the result of the negative feedback of calcium on endothelin-induced calcium entry, which has been recently reported.

DG production is stimulated by ET-1 in cultured smooth muscle and other cells.26,27 In normotensive controls, DG appeared to follow a biphasic increase after stimulation with ET-1. DG levels did not change significantly after stimulation by ET-1 in DOCA-salt rats in contrast to controls. After stimulation with norepinephrine, no difference in DG levels was found in spontaneously hypertensive rats relative to Wistar-Kyoto rats at 20 weeks. It should be pointed out that DG accumulation in response to different vasoconstricting agents in vessels of normotensive rats may be difficult to demonstrate because of rapid metabolism of DG to phosphatidic acid. Because absolute amounts of DG produced in response to ET-1 were lower in vessels from DOCA-salt hypertensive rats, it is likely that protein kinase C stimulation by DG will be lower in DOCA-salt rats than in controls, even if the actual cytosolic free calcium concentration achieved in the former is higher, with greater translocation of protein kinase C to the membrane, but this remains to be demonstrated.

In conclusion, responses of InsP, cytosolic free calcium, and DG to ET-1 appear to be decreased in DOCA-salt hypertensive rats. Decreased density of ET-1 receptors in blood vessels of DOCA-salt rats, as described elsewhere, may result in decreased phosphoinositide, calcium, and DG responses, which in turn underlie the blunted vascular reactivity to endothelin.

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