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Cocaine toxic effect on endothelium-dependent vasorelaxation: an in vitro study on rabbit aorta

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Abstract

Effects of cocaine on vascular endothelium relaxing properties and the related mechanism were investigated in vitro in rabbit aorta. Several vasorelaxing agents with different mechanisms, i.e. acetylcholine, substance P, calcium ionophore A23187, 2,5-di-*tert*-butylhydroquinone, or sodium nitroprusside, were employed. Cocaine effects on the vascular response to relaxing agents in cumulative (acetylcholine, substance P, or A23187) or single dose (2,5-di-*tert*-butyl-hydroquinone) were performed in endothelium-intact aortic rings precontracted with phenylephrine. Relaxing activity of cumulative doses of sodium nitroprusside was evaluated in endothelium-denuded aortic rings, in the presence of cocaine. Cocaine significantly reduced endothelium-dependent relaxations induced by acetylcholine, or substance P. By contrast A23187 endothelium-mediated relaxation as well as endothelium-independent relaxation by sodium nitroprusside were unaffected by cocaine. Furthermore, cocaine significantly increased endothelium-dependent relaxation response to 2,5-di-*tert*-butylhydroquinone, a sarcoplasmic Ca^{2+}-ATPase pump inhibitor, in the aortic rings. These findings indicate that cocaine reduces nitric oxide release from vascular endothelium apparently through the inhibiting action of Ca^{2+}-ATPase pump.

Keywords: Cocaine; Endothelium; Nitric oxide; Rabbit aorta

1. Introduction

Interest in the cardiovascular toxicity of cocaine has greatly increased due to its more diffused use as a recreational drug. Illicit use of cocaine is frequently associated with acute cardiovascular events, including stroke, myocardial infarction, coronary thrombosis, arrhythmia and sudden death (Rezkalla et al., 1990; Isner and Chokshi, 1991; Minor et al., 1991; Om, 1992; Mouhaffel et al., 1995; Kaufman et al., 1998). Premature atherosclerosis has also been reported after chronic cocaine abuse (Billman, 1990; Kolodgie et al., 1991). Although it has been established that inhibitory action on catecholamine re-uptake is the main basis for cocaine-induced cardiovascular toxic effects (Billman, 1990; Isner and Chokshi, 1991; Om, 1992) alternative explanations, including alterations in platelet and vascular endothelium functions induced by cocaine, have also been suggested.

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Cocaine has, in fact, been shown to reduce the agonist concentrations required to induce aggregating response in rabbit (Togna et al., 1985) and human platelets (Rezkalla et al., 1993; Heesch et al., 1996), presumably by activating the prostaglandin system (Eichhorn et al., 1992; Togna et al., 1996), with the consequent increase in thromboxane production. Cocaine activation, i.e. significant expression of P-selectin on circulating platelet surface, has also been reported in conscious dogs (Kugelmass et al., 1995).

An abnormal endothelial function could explain the focal vasospasm and thrombosis (Flores et al., 1990; Egashira et al., 1991; Havranek et al., 1996) as well as initiation of the atherosclerosis process (Heng and Haberfeld, 1987; Mouhaffel et al., 1995) induced by cocaine; however, the role of cocaine in altering endothelium-mediated vascular tone remains discordant (Egashira et al., 1991; Kuhn et al., 1992). In an in vivo study (Havranek et al., 1996) it was shown that, during acetylcholine infusion, forearm blood flow was significantly lower in long-term cocaine users than in control subjects, thus indicating that cocaine impairs endothelium-dependent vasorelaxation. Among possible mechanisms, the authors (Havranek et al., 1996) include enhancement of endothelial-derived contracting factor activity, an increased sensitivity to muscarinic stimulation of vascular smooth muscle, as well as a direct toxic effect by cocaine on vascular endothelial cells. More recently, Mo et al. (1998), investigating the role of vascular endothelial relaxing mediator in cocaine-induced hypertension in rats, observed that vasoconstriction induced by cocaine is blocked by pretreatment with L-NMMA and the authors hypothesised a cocaine-induced inhibiting activity on local vasodilator endothelium-derived nitric oxide (NO).

In this study the possible mechanisms involved in the toxic effects of cocaine on vascular endothelium relaxing properties were investigated in rabbit aorta. We therefore examined and compared the in vitro cocaine effect on endothelium-mediated relaxing response induced in phenylephrine (PE)-precontracted rabbit aortic rings by: (1) acetylcholine (ACh); (2) substance P (SP); (3) calcium ionophore A23187; or (4) 2,5-di-*tert*-butylhydroquinone (DBHQ). The effect of cocaine on endothelium-independent relaxation by sodium nitroprusside (SNP) was also tested.

2. Materials and methods

2.1. Isolated rabbit aortic rings

Adult male New Zealand rabbits (2.5–3.0 kg) were sacrificed under sodium pentobarbital anaesthesia (45 mg/kg i.v.) and the thoracic aorta rapidly excised and transferred to Krebs buffer solution. The aorta was then carefully freed of connective tissue, and cut into rings of 2–3 mm width. In alternate rings, the endothelium was removed by gently rubbing the intima surface with a steel stick. The aortic rings were then suspended in organ baths containing 4 ml Krebs solution (in mM: KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, Na_2 CaEDTA 0.026, NaCl 118, $NaHCO_3$ 25, $CaCl_2$ 1.2 and glucose 11.1), continuously aerated with a mixture of 95% O_2–5% CO_2 and maintained at 37 °C. Rings were connected to a force transducer (Ugo Basile isometric transducer mod. 7003) for continuous recording of changes in isometric tension (Ugo Basile 'Gemini' mod. 7070). Preparations were mounted under 4 g resting tension and allowed to equilibrate for approximately 1 h. During this period, solutions in the organ baths were replaced with freshly gassed, warmed Krebs solution every 15 min. After 30 min of equilibration, indomethacin (10 μM) was added to the Krebs medium to prevent endothelial vasoactive prostanoid formation.

2.2. Experimental protocol

After the equilibration period, rings were repeatedly exposed to PE (1 μM) until reproducible contraction responses were obtained. ACh (1 μM) was then added to the organ bath to assess aortic ring endothelial integrity. Endothelium was considered intact when a relaxation $> 50\%$ of maximum PE contraction was recorded. The rings were then rinsed twice with Krebs solution before starting the experiments. Relaxation studies in PE-precontracted aortic rings were conducted as follows.

Endothelium-intact or endothelium-denuded rabbit aortic rings were contracted with 1 μM PE. Cocaine hydrochloride (0.01–0.5 mM), or distilled water in controls, was added to the organ bath when contraction induced by PE reached plateau level and 2 min before the addition of vasorelaxing agents. ACh (0.01–1 μM), SP (0.1–10 nM), or A23185 (0.001–10 μM) were added in a cumulative manner; DBHQ was added as a single dose (30 μM). In parallel experiments, the relaxing activity of cumulative doses of SNP (0.01–10 μM) in the presence of cocaine (0.01–0.5 mM) was performed in endothelium-denuded aortic rings.

Contractile response of aortic rings to PE was measured as increase above rest tension. Relaxation of aortic rings was expressed as percentage of PE-induced contraction.

2.3. Drugs

PE hydrochloride, SNP, DBHQ were provided by Fluka Chemie AG (Buchs, CH); ACh chloride, SP acetate salt, calcium ionophore A23187 and indomethacin by Sigma Chemicals Co. (St. Louis, MO); cocaine hydrochloride was supplied by SALARS (Como, Italy). Cocaine hydrochloride, PE, ACh, SP and SNP were dissolved in distilled water and added to the organ bath medium in a volume range of 20–200 μl; drug concentrations are reported as final molar concentrations in the organ bath. Indomethacin was dissolved in 150 mM $NaHCO_3$; A23187 and DBHQ in dimethyl sulphoxide (DMSO). Final organ bath concentration of DMSO did not exceed 0.025%. Preliminary experiments ascertained the lack of response of aortic tissue to DMSO in the concentrations employed.

2.4. Data analysis

Data were expressed as the mean ± SE. One-way analysis of variance (ANOVA) was used for the comparisons between the effects of cocaine doses on DBHQ relaxation. While two-way ANOVAs with repeated measures were used for the comparisons between the effects of cocaine doses on ACh, A23187, SNP and SP relaxation, respectively. Post-hoc comparisons were performed by Duncan test. Statistical significance was set at $P < 0.01$.

3. Results and discussion

Endothelium-intact PE (1 μM) precontracted aortic rings relaxed in a concentration-dependent manner in response to the cumulative addition of ACh (Fig. 1), SP (Fig. 2), A23187 (Fig. 3, Panel A). The relaxing response to ACh, SP and A23187, but not to SNP (Fig. 3, Panel B) did not occur in rings mechanically denuded of endothelium.

Evidence is accumulating that ACh and SP induce an NO-mediated endothelium-dependent relaxant effect in rabbit aorta (Regoli et al., 1984; Rees et al., 1989; Fujimoto and Itoh, 1997). [Under our experimental conditions the incubation of rabbit thoracic aortic rings with 100 μM L-NAME, a competitive inhibitor of nitric oxide synthase, completely abolished the endothelium-dependent relaxation induced by these drugs (data not shown)].

Cocaine (0.1–0.5 mM) added to the organ bath when aortic rings reached a stable contractile tension by PE, caused a significant concentration dependent inhibition of the vascular relaxing responses to 1 μM ACh [$F(20, 150) = 2.6$; $P < 0.001$] (Fig. 1). The inhibitory effect was fully reversible after repeated washings.

In blood vessel, the release of NO evoked by ACh is mediated via the activation of muscarinic receptors M_3 subtype (Jaiswal et al., 1991). It has been reported that high concentrations of cocaine interfere with muscarinic receptors in cardiac tissues (M_2 subtype) and in the brain (M_1 subtype) (Sharkey et al. 1988; Billman and Lappi, 1993).

To verify whether the cocaine inhibitory effect on ACh induced relaxation could be ascribed to a muscarinic receptor antagonism at vascular endothelium level (M_3 subtype), we evaluated the cocaine effect on endothelium-dependent relaxation by SP. It is well known that SP-induced relaxation in rabbit aorta is mediated by endothelial NK_1 receptors (Rubino et al., 1992) which share with M_3 receptors the ability to stimulate

phosphoinositide turnover and to increase cellular IP_3 levels (Guard and Watson, 1991). Our data showed that the vascular relaxing response to SP (10 nM) was also inhibited in a concentration-dependent manner by cocaine [$F(6, 32) = 11.7$; $P < 0.0001$] (Fig. 2) suggesting that cocaine-induced inhibition of endothelium-mediated relaxation is caused by its interactions beyond the binding site of muscarinic endothelial receptors.

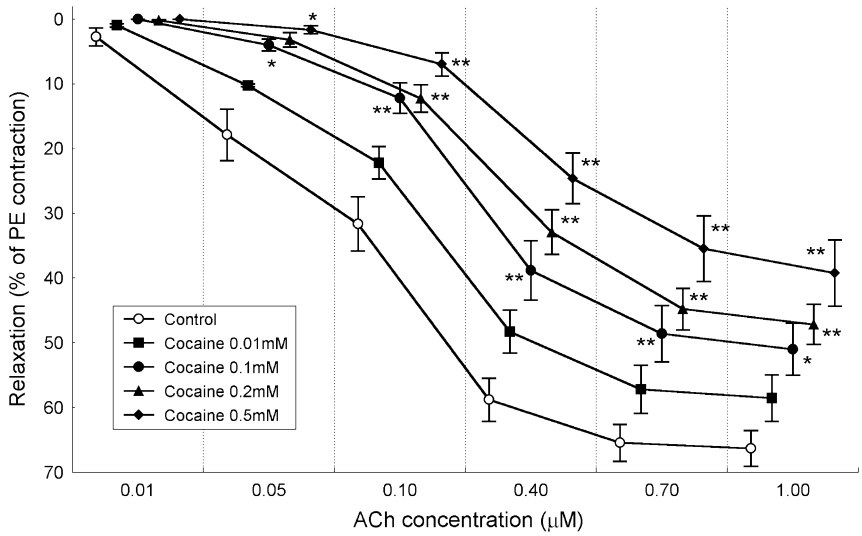

Fig. 1. Effect of cocaine on the endothelium-dependent relaxation evoked by acetylcholine (ACh) in rabbit aortic rings precontracted with phenylephrine (PE, 1 μM). Experiments were performed in the presence of 10 μM indomethacin. Cocaine was added to the organ bath when contraction induced by PE reached plateau level and 2 min before ACh addition. Results (mean ± SE of seven independent experiments) are expressed as percentage of PE-induced contraction. *$P < 0.01$; **$P < 0.001$.

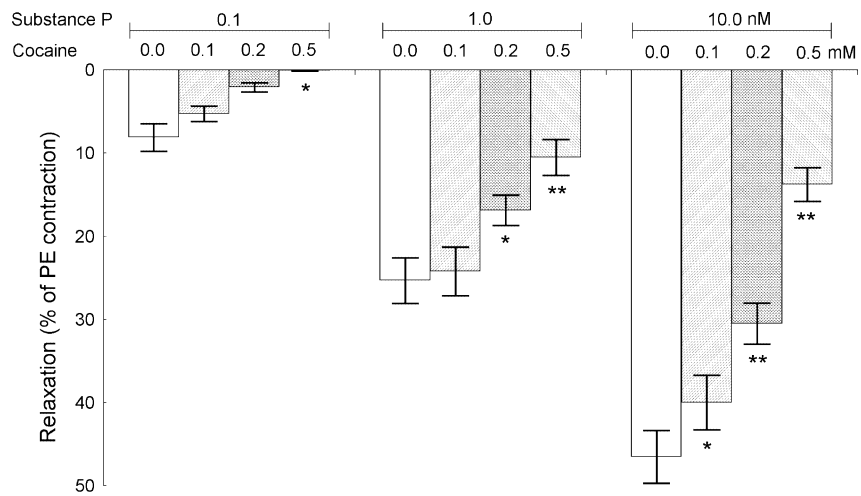

Fig. 2. Bar graph shows the effect of cocaine on the endothelium-dependent relaxation elicited by substance P (SP) in rabbit aortic rings precontracted with phenylephrine (PE, 1 μM). Experiments were performed in the presence of 10 μM indomethacin. Cocaine was added to the organ bath when contraction induced by PE reached plateau level and 2 min before SP addition. Results (mean ± SE of five independent experiments) are expressed as percentage of PE-induced contraction. *$P < 0.01$;**$P < 0.001$.

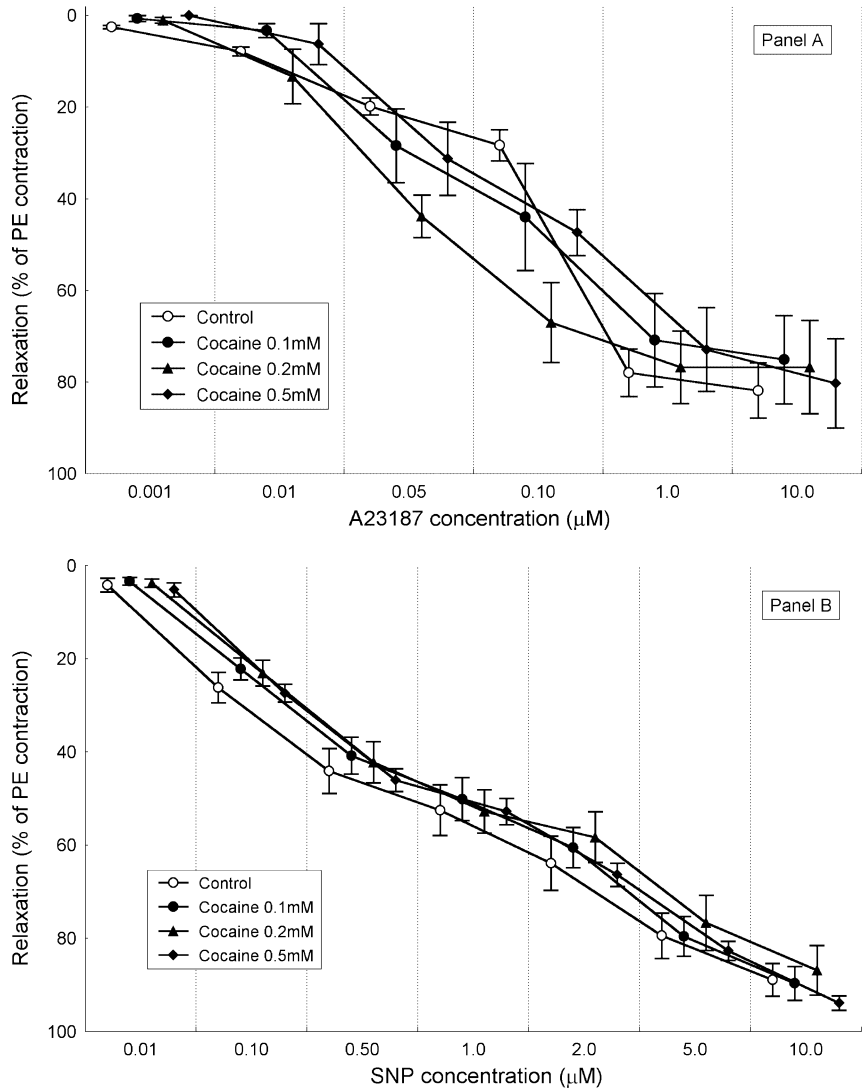

Fig. 3. Concentration-response curve for A23187 in endothelium-intact (Panel A) and for sodium nitroprusside (SNP) in endothelium-denuded (Panel B) rabbit aortic rings precontracted with phenylephrine (PE, 1 μM) (five and six independent experiments, respectively). Experiments were performed in the presence of 10 μM indomethacin. Cocaine was added to the organ bath when contraction induced by PE reached plateau level and 2 min before the addition of the relaxing agent. Results (mean ± SE) are expressed as percentage of PE-induced contraction.

Using NO-donor SNP in rings mechanically denuded of endothelium, we assayed the possibility that cocaine could interfere with vascular smooth muscle reactivity to NO. Results showed that cocaine did not affect the vascular relaxing response to 50 μM SNP [$F(21, 140)$; $P < 1.0$] (Fig. 3, Panel B). The fact that cocaine reduces endothelium-dependent relaxation induced in PE-precontracted rabbit aortic rings by ACh or SP, leaving the vasorelaxing response of endothelium-denuded rings to SNP unchanged, indicates that cocaine interferes with NO synthesis at endothelium level, without affecting vascular smooth muscle reactivity to NO.

As shown in Fig. 3 (Panel A), the relaxing response to 10 μM A23187, an agent that causes

NO-synthase activation by elevating cytosolic Ca^{2+} levels in endothelial cells (Itoh et al., 1985), was also unaffected [$F(15, 80)$; $P < 1.0$] by cocaine (0.1–0.5 mM). The lack of effect on A23187 induced vasorelaxation indicates that cocaine does not directly inhibit constitutive NO synthase. It also suggests that inhibition of ACh- or SP-induced vasorelaxation could be ascribed to cocaine interference at prior steps of cytosolic Ca^{2+} increase. It is well known that elevation of [Ca^{2+}] by ACh and other agonists, including SP, results from inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]-induced Ca^{2+} mobilisation from internal stores and consequent Ca^{2+} influx from extracellular space (Dolor et al., 1992; Schilling et al., 1992; Sharma and Davis, 1995; Hutcheson and Griffith, 1997).

With use of DBHQ, the cocaine effect on calcium internal stores was examined. DBHQ, by inhibiting endoplasmic reticulum Ca^{2+}-ATPase (Luo et al., 1993), induces an NO-mediated vasorelaxation (Fusi et al., 1999). This effect is the consequence of increased Ca^{2+} influx in endothelial cells (via receptor-operated channels) triggered by impaired refilling of intracellular calcium stores and subsequent store depletion. In endothelium-intact PE precontracted aortic rings cocaine induced a significant increase in the relaxing response to 30 μM DBHQ [$F(3, 44) = 6.4$; $P < 0.01$] (Fig. 4). The potentiating effect of cocaine on vasorelaxation by DBHQ suggests the ability of cocaine to induce stored Ca^{2+} depletion, presumably by an impairment of the Ca^{2+} refilling of intracellular stores. It is known that calcium influx in endothelial cells is closely coupled to, and regulated by, the status of the intracellular calcium pool and thus, to the store depletion rate (Schilling et al., 1992; Moritoki et al., 1996).

Prevention of IP_3-sensitive intracellular calcium store refilling could also account for the inhibitory effect of cocaine on endothelial-dependent relaxation by ACh and SP. Amerini et al. (1996) demonstrated that, in the presence of a Ca^{2+}-ATPase inhibitor, the relaxing response elicited in rabbit aortic rings by ACh or SP was significantly reduced. Further indirect support is provided by Schilling et al. (1992) who, in a culture of vascular endothelial cells, observed an attenuation of response to subsequent additions of agonists, such as bradykinin, as a consequence of calcium depletion from Ins(1,4,5)P_3-sensitive internal stores, induced by a Ca^{2+}-ATPase inhibitor.

It may therefore be concluded that cocaine alters NO release from vascular endothelial cells by interfering with its synthesis, apparently

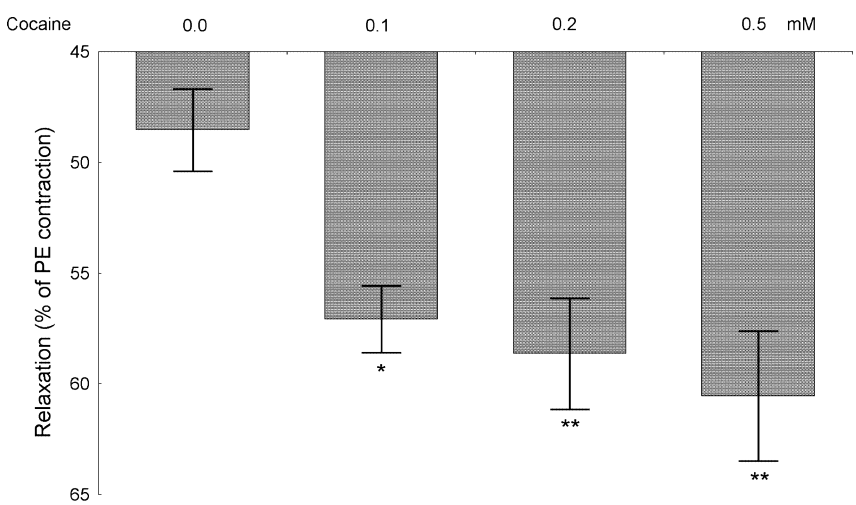

Fig. 4. Bar graph shows the effect of cocaine on relaxation elicited by 2,5-di-*tert*-butylhydroquinone (DBHQ, 30 μM) in endothelium-intact rabbit aortic rings precontracted with phenylephrine (PE, 1 μM). Cocaine was added to the organ bath when contraction induced by PE reached plateau level and 2 min before DBHQ addition. Results (mean ± SE of 12 independent experiments) are expressed as percentage of PE-induced contraction. *$P < 0.01$; **$P < 0.001$.

through an inhibiting effect on the sarcoplasmic Ca^{2+}-ATPase pump. These findings contribute to clarifying the toxicological profile of cocaine on the vascular target, providing a further explanation for cocaine's capacity to induce vascular disorders.

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