Effects of Cadmium *in Vitro* on Contractile and Relaxant Responses of Isolated Rat Aortas

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Abstract

Objective: Cadmium is known to affect the vascular tone of isolated blood vessels *in vitro* and the arterial pressure of rats *in vivo*. However, the mechanisms of cadmium actions on the vascular system have not been clarified. To elucidate the actions of cadmium on vascular tonus, effects of cadmium on vasocontractile and vasorelaxant responses *in vitro* were investigated using aortic strips isolated from rats.

Methods: Aortic strips isolated from male Wistar rats were incubated with CdCl₂ (10 µM) for 24 hr, washed with fresh CdCl₂-free medium, and then used for measurement of isometric tension and Western blot analysis of eNOS (endothelial nitric oxide synthase) and iNOS (inducible nitric oxide synthase).

Results: In the aortas pretreated with cadmium *in vitro*, the contractile response to phenylephrine was significantly higher than that in the control aortic strips pretreated with a vehicle. The sodium nitroprusside-induced relaxing response was significantly higher in the aortic strips pretreated with cadmium for 24 hr, compared with that in the control pretreated with a vehicle. The isoproterenol-induced relaxing response was also significantly higher in the cadmium-accumulated aortic strips. *In vitro* cadmium treatment slightly but not significantly increased the acetylcholine-induced relaxation of the aortic strips. Cadmium treatment induced expression of iNOS and significantly increased expression of eNOS in the aortic strips, while it did not affect expression of β-actin.

Conclusions: Cadmium treatment *in vitro* augmented the α1 adrenoceptor-mediated contractile response, even though eNOS and iNOS were upregulated by cadmium treatment. NO-induced and β-adrenoceptor-mediated relaxing responses were also augmented by cadmium treatment. These results suggest that both vasocontractile and vasorelaxing responses are augmented in cadmium-accumulated aortas.

Key words: cadmium intoxication, vasoconstriction, vasodilation, vascular smooth muscle, vascular endothelium

Introduction

Cadmium is known to affect blood pressure, but the direction of the effects by cadmium is controversial. In previous epidemiological studies, cadmium levels in blood, urine and hair have been reported to be higher in hypertensive subjects than in normotensive subjects (1–3). In contrast, the blood

pressure of the inhabitants of areas with heavy cadmium pollution was lower than that of those without pollution (4). Similar findings have been reported in previous experimental studies: rats receiving oral administration of low doses of cadmium showed hypertension, while rats receiving high doses of cadmium showed hypotension (5–8). Although changes in vasoreactivity, blood noradrenaline levels and sodium excretion have been suggested to be involved in the *in vivo* effects of cadmium on blood pressure as mentioned above (9–11), the exact mechanisms for cadmium action on blood pressure have not yet been determined. Cadmium is known to directly influence the contractility of isolated vascular smooth muscle *in vitro* (12–16). These *in vitro* actions of cadmium are also concentration-dependent: low concentrations of cadmium potentiate vasoconstriction (12–14),

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whereas high concentrations inhibit it (15, 16). However, only a few studies have investigated the vasocontractility of blood vessels isolated from cadmium-intoxicated animals, and the results are controversial: chronic cadmium intoxication resulted in an increase (17, 18) or a decrease (19) in norepinephrine-induced contraction of isolated rat arteries. The vasoactive substances released from the vascular endothelium, such as nitric oxide (NO), are important regulators of vascular tonus (20). Although a previous study demonstrated that endothelium-dependent NO-mediated relaxation is inhibited by cadmium *in vitro* (21), there have been no studies on endothelium-dependent relaxation of vessels isolated from cadmium-intoxicated animals. Moreover, vasocontractile function is known to be modulated by vasorelaxing substances such as NO. Especially, vasocontraction mediated by α -adrenergic agonists such as norepinephrine and phenylephrine is augmented by removal of the endothelium of isolated blood vessels (22). This implies that vasocontraction is suppressed in the presence of the endothelium by vasorelaxing substances released from endothelial cells. Therefore, it was speculated that cadmium affected vascular tone through its actions on production and/or release of relaxing substances from the endothelium. The purpose of the present study was thus to investigate the *in vitro* effects of cadmium accumulation in blood vessels on their contractile and relaxant responses.

Materials and Methods

Tissue preparation

Thoracic aortas isolated from rats were used for isometric tension experiments to evaluate vasocontractile and vasorelaxant functions. The study protocols regarding treatment of animals were in accordance with the "Guidelines for Experiments Using Laboratory Animals in Yamagata University School of Medicine". The rats were anesthetized with sodium pentobarbital (50 mg/kg) and killed by exsanguination. Rat aortic strips were prepared by the following procedure under sterile conditions. The thoracic aortas were removed and immediately placed in Dulbecco's Modified Eagle's Medium (DMEM) with 0.1% bovine serum albumin, 4 mM glutamate, 100 U/ml penicillin and 100 μ g/ml streptomycin. After removal of excess fat and connective tissue, ring-shaped strips (4 mm long) were prepared. Each aortic strip was then put in a well of a 24-well plate containing 0.5 ml of DMEM. The plates were incubated in a humidified atmosphere at 37°C under 5% CO₂-95% air. First, the strips were stabilized in DMEM for 3 hrs, and then cadmium (10 μ M, final concentration of CdCl₂) or a vehicle (distilled water) was added to the medium. After 24 hr of incubation with cadmium or the vehicle, the aortic strips were washed three times with cadmium-free DMEM and used for the experiments.

Measurement of isometric tension

Each aortic strip was cut longitudinally and suspended vertically in a 10-ml organ bath filled with Krebs-Ringer bicarbonate solution (mM: NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, glucose 10 and NaHCO₃ 25, pH 7.4 at 37°C). A force-displacement transducer was attached to each strip. Care was taken not to injure the intimal layer of the aortas during preparation of the aortic strips. After 1 hr of equilibrium

with a resting tension of 1 g, changes in isometric force were recorded. The aortic strips were first contracted with 60 mM KCl. After washing out the bath solution, the KCl-induced contraction protocol was repeated once more. The preliminary experiments showed that a constant contraction by KCl of the aortic strips was obtained after these two protocols of KCl contraction. Then, each experimental protocol was started. To obtain a concentration-response relationship of contraction induced by phenylephrine, an α 1-adrenergic agonist, phenylephrine was cumulatively added to the organ bath. The contractile response obtained was expressed in terms of mg force (mg tissue)⁻¹. Relaxation of the aortic strips was detected as a decrease in the isometric tension of the aortic strips precontracted with phenylephrine. Relaxants were also cumulatively added when the phenylephrine-induced contraction reached a plateau. Since the degree of relaxing response is known to be dependent on the precontractile forces, the precontractile forces were adjusted using different concentrations of phenylephrine for the control and cadmium-pretreated aortic strips. The control aortic strips were precontracted with phenylephrine at an EC₇₀₋₈₀ for phenylephrine contraction, which was determined in each experiment prior to the relaxation experiment. The aortic strips pretreated with cadmium were precontracted with phenylephrine at a concentration which could induce a degree of contractile force similar to that induced by an EC₇₀₋₈₀ of phenylephrine contraction in each control aortic strip. The relaxant response was expressed in terms of percentage decrease of tension/phenylephrine-induced precontractile force. After measurement of isometric tension, each aortic strip was blotted and weighed.

Western blot analysis

Protein expression of iNOS and eNOS was determined by standard Western blot analysis. Aortic rings of 4 mm in length after incubation with cadmium or a vehicle were homogenized on ice in a lysate buffer (50 mM Tris, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 μ g/l leupeptin, 1 mM Na₃VO₄ and 1 mM NaF) using a common glass-glass type homogenizer. The homogenate was sonicated briefly and then centrifuged for 10 min at 15,000 $\times$ g. Protein content in the supernatant was determined by the Bradford method and aliquots were added to electrophoresis sample buffer [65 mM Tris/HCl (pH 6.8), 10% glycerol, 2.5% β -mercaptoethanol and 2% sodium dodecylsulfate with 0.01% bromophenol-blue] and heated at 100°C for 5 min before SDS-polyacrylamide gel electrophoresis (PAGE). Aliquots containing the same amounts of protein (5–10 μ l) were subjected to SDS-PAGE on 7.5% polyacrylamide slab gels and blotted onto a polyvinylidene difluoride membrane. The blots were blocked for 1 hr in Tris-buffered saline (TBS: 150 mM NaCl, 20 mM Tris, pH 7.5) containing 5% nonfat milk and incubated overnight with polyclonal rabbit anti-(mouse) iNOS IgG (diluted 1:1000; Transduction Laboratory) or polyclonal rabbit anti-(mouse) eNOS IgG (diluted 1:1000; Transduction Laboratory) at 4°C. After washing with TBS, the membrane was incubated with goat anti-rabbit alkaline phosphatase-conjugated antibody (diluted 1:1000) for 1 hr. After removal of antibodies by washing, proteins were detected by enhanced chemilumines-

cence method using an immunoblot assay kit (Immun-Blot Assay Kit, BioRad). In all of the electrophoresis experiments, a protein standard ladder (Kaleidoscope prestained standard, BioRad) was used for estimation of protein molecular size. Sample protein content per lane was checked by determination of expression of β -actin, a house-keeping gene protein, using anti-(mouse) β -actin antibody. Western blots were digitized using a high-resolution image scanner and evaluated densitometrically by gel-analysis software. The arbitrary units are expressed as % of the level with LPS (1 μ g/ml) stimulation for iNOS and % of the control (without cadmium) for eNOS.

Chemicals

Phenylephrine hydrochloride (Sigma), acetylcholine chloride, sodium nitroprusside and *dl*-isoproterenol hydrochloride (Wako) were dissolved in distilled water to make stock solutions of 10 mM, 10 mM, 1 mM and 10 mM, respectively, and were kept at 4°C.

Statistical analysis

The data are shown as means $\pm$ SE. Statistical analysis was done using Student's *t* test. *P* values less than 0.05 were regarded as significant. The data of protein expression evaluated by Western blot analysis were analyzed using Mann-Whitney U test.

Results

Effects of cadmium on phenylephrine-induced contraction of rat aortic strips

Phenylephrine-induced contraction was significantly augmented by *in vitro* pretreatment with cadmium (10 μ M) for 24 hrs, compared with that in the control pretreated with a vehicle (Fig. 1).

Effects of cadmium on sodium nitroprusside-induced relaxation of rat aortic strips

Sodium nitroprusside-induced relaxation was significantly potentiated in the aortic strips pretreated with cadmium *in vitro* compared with that in the control aortic strips pretreated with a vehicle (Fig. 2).

Fig. 1 Effects of pretreatment with cadmium (10 μ M) *in vitro* on phenylephrine-induced contraction of aortic strips isolated from rats. Asterisks denote significant differences between the cadmium-pretreated and control groups. *n*=8.

Effects of cadmium on acetylcholine-induced relaxation of rat aortic strips

Acetylcholine-induced relaxing response in the aortic strips pretreated with cadmium *in vitro* was slightly increased compared with that in the control, although the difference was not significant (Fig. 3).

Effects of cadmium on isoproterenol-induced relaxation of rat aortic strips

Isoproterenol-induced relaxation was significantly potentiated in the aortic strips pretreated with cadmium *in vitro* compared with that in the control pretreated with a vehicle (Fig. 4).

Fig. 2 Effects of pretreatment with cadmium (10 μ M) *in vitro* on sodium nitroprusside-induced relaxation of rat aortic strips. The control aortic strips were precontracted with phenylephrine at an EC₇₀₋₈₀ for phenylephrine contraction. The aortic strips pretreated with cadmium were precontracted by phenylephrine at a concentration which could induce a degree of contractile tension similar to that induced by the EC₇₀₋₈₀ of phenylephrine contraction in the control aortic strips. After the precontractile tension by phenylephrine reached a plateau, the relaxing response was induced by addition of sodium nitroprusside in a cumulative manner. Asterisks denote significant differences between the cadmium-pretreated and control groups. *n*=8.

Fig. 3 Effects of pretreatment with cadmium (10 μ M) *in vitro* on acetylcholine-induced relaxation of rat aortic strips. The control aortic strips were precontracted with phenylephrine at an EC₇₀₋₈₀ for phenylephrine contraction. The aortic strips pretreated with cadmium were precontracted by phenylephrine at a concentration which could induce a degree of contractile tension similar to that induced by the EC₇₀₋₈₀ of phenylephrine contraction in the control aortic strips. After the precontractile tension by phenylephrine reached a plateau, the relaxing response was induced by addition of acetylcholine in a cumulative manner. *n*=7.

Fig. 4 Effects of cadmium on isoproterenol-induced relaxation of rat aortic strips. Effects of pretreatment with cadmium (10 μ M) *in vitro* on isoproterenol-induced relaxation of rat aortic strips. The control aortic strips were precontracted with phenylephrine at an EC_{70-80} for phenylephrine contraction. The aortic strips pretreated with cadmium were precontracted by phenylephrine at a concentration which could induce a degree of contractile tension similar to that induced by the EC_{70-80} of phenylephrine contraction in the control aortic strips. After the precontractile tension by phenylephrine reached a plateau, the relaxing response was induced by addition of isoproterenol in a cumulative manner. Asterisks denote significant differences between the cadmium-pretreated and control groups. $n=4$.

Effects of cadmium on expression of eNOS and iNOS in rat aortic strips

Fig. 5 displays protein expression of iNOS and eNOS evaluated by Western blot analysis. eNOS was constitutively expressed in aortic strips, while iNOS expression was only slightly detected at the basal condition without stimulation. In cadmium-treated aortas, expression of eNOS and iNOS was significantly higher than that in the control treated with a vehicle. The level of iNOS expression was about 30% of the maximum level induced by LPS at 1 μ M. On the other hand,

expression of β -actin in the cadmium-treated aortas was comparable with that in the control.

Discussion

Contractility of isolated blood vessels is known to be inhibited in the presence of cadmium in the medium, due to depression of transplasmalemmal Ca^{2+} entry through voltage-dependent Ca^{2+} channels (23). Thus, cadmium is used as an inorganic antagonist of Ca^{2+} channels. In addition, cadmium also depresses an extracellular Ca^{2+} -independent component of agonist-induced contraction by depressing an intracellular contractile mechanism (24). On the other hand, vasoconstriction by noradrenaline has been reported to be increased when the blood vessels were incubated with lower concentrations of cadmium (15, 16). However, there have been few studies showing the effects of cadmium accumulation in the vascular smooth muscle on its contractility. A recent study showed that the average cadmium concentration in the medial layer of human abdominal aortas obtained by biopsies was 7 μ M (25). Thus, we used aortas that had been incubated with 10 μ M of cadmium and washed with a fresh medium not containing cadmium, in the present tension experiments. The contractile response to phenylephrine, an α 1-adrenoceptor agonist, in which phosphoinositide (PI) hydrolysis is known to be involved (26), was augmented by cadmium accumulation in the rat aortic strips. In our previous study (27), the aortic strips that had been preincubated with cadmium at the same concentration as that used in the present study also showed an increase in 5-hydroxytryptamine (5-HT)-induced contraction, but not in KCl-induced contraction, which is not mediated by receptor stimulation but by plasmalemmal depolarization. Moreover, we demonstrated that 5-HT-induced PI hydrolysis was also augmented in cadmium-pretreated aortic strips (27). Therefore, the augmenta-

Fig. 5 Effects of cadmium on protein expression of iNOS (left panel) and eNOS (right panel). Aortic strips were incubated with cadmium (10 μ M), LPS (1 μ M) or a vehicle (ctrl) for 24 hr. Upper: Densitometric analysis of gel photographs. Lower: Representative gel photographs of iNOS and eNOS expression. The protein levels of iNOS and eNOS on gel photographs were quantified by densitometry. The arbitrary units are expressed as a % of the level with LPS stimulation for iNOS and a % of the control (without cadmium) for eNOS. *, significantly different ($P<0.05$) from the control treated with a vehicle (ctrl). $n=3$.

tion of phenylephrine contraction in cadmium-accumulated aortic strips may be also due to increase in PI hydrolysis. Thus, the augmenting action of cadmium on the contractility of vascular smooth muscle possibly induces hypertension in cadmium intoxication.

Vascular endothelium plays obligatory roles in the regulation of vascular tonus (20). NO is a major endogenous vasodilator from the vascular endothelium (28). Since human primary hypertension is associated with increased peripheral vascular resistance (28), there are theoretical reasons why reduced NO production or bioavailability could lead to vasoconstriction, and hence, increased peripheral vascular resistance, resulting in hypertension. Incubation of blood vessels with cadmium was reported to inhibit endothelium-dependent vasorelaxation (21), which is mainly mediated by NO from the vascular endothelium (29). However, it has not been determined whether vasorelaxation is altered in cadmium-accumulated blood vessels. The preservation of endothelium-dependent vasorelaxing response is often used to demonstrate that the vascular endothelium remains intact. In the cadmium-accumulated aortic strips, the endothelium-dependent vasorelaxing response due to endothelial NO as well as expression of eNOS was rather higher than that in the control. These findings indicate that the endothelial cells are not injured by cadmium treatment in our system, although the metal can exhibit cytotoxicity in the cells (30, 31).

In the present study, nitroprusside-induced relaxation, which is not dependent on the endothelium but is due to exogenous NO, was significantly augmented in aortic strips with cadmium accumulation *in vitro*. Similarly, the isoproterenol-induced relaxing response of the rat aortic strips, which is not dependent on the endothelium but mediated via β -adrenoceptors of vascular smooth muscle cells, was also significantly augmented in aortic strips with cadmium accumulation *in vitro*. Moreover, acetylcholine-induced relaxation, which is mainly mediated by NO from the endothe-

lium in rat aortas (32), was also slightly but not significantly augmented in the cadmium-accumulated aortic strips. Thus, relaxing responses to NO and β -adrenoceptor stimulation were increased in cadmium-accumulated vascular smooth muscle, suggesting that vasorelaxation in cadmium-accumulated aortic strips is enhanced in a nonspecific manner. A recent study has shown that sensitivity of noradrenaline-induced contraction to nitro-L-arginine, an inhibitor of NO synthesis, was higher in arteries isolated from cadmium-intoxicated rats than in those from control rats (18). This finding indirectly implies that vasorelaxation by NO released from the endothelium was increased in blood vessels in cadmium intoxication, and agrees with the above present findings. Although the level of NO production was not measured in the present study, the expression of both eNOS and iNOS was significantly increased in the cadmium-treated aortic strips. Therefore, a slight increase in acetylcholine-induced relaxation, which is mediated by NO through eNOS activation, is associated with increased eNOS expression in cadmium-treated aortic strips. To the best of our knowledge, this is the first study demonstrating that eNOS and iNOS expression is increased in cadmium-accumulated arteries. In addition, NO spontaneously released through eNOS is known to suppress vasoconstriction induced by α -adrenoceptor agonists such as phenylephrine (22). Even under the condition of the expression of both eNOS and iNOS being augmented by cadmium, possibly resulting in increased suppression of vasoconstriction, contraction by α -adrenoceptor activation was augmented in cadmium-accumulated aortas. These results suggest that both α -adrenoceptor-mediated contraction and NO-mediated relaxation of smooth muscle cells are increased in cadmium-accumulated aortic strips.

In conclusion, both phenylephrine-induced contraction and NO-mediated and β -adrenoceptor-mediated relaxation are augmented by acute accumulation of cadmium in blood vessels.

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