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Role of nitric oxide synthase inhibition in the acute hypertensive response to intracerebroventricular cadmium

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1 In the rat, intracerebroventricular (i.c.v.) injection of cadmium, a pollutant with long biological half-life, causes a sustained increase in blood pressure at doses that are ineffective by peripheral route. Since cadmium inhibits calcium-calmodulin constitutive nitric oxide (NO) synthase in cytosolic preparations of rat brain, this mechanism may be responsible for the acute pressor action of this heavy metal.

2 To test this possibility, we evaluated the effect of i.c.v. injection of 88 nmol cadmium in normotensive unanaesthetized Wistar rats, which were i.c.v. pre-treated with: (1) saline (control), (2) L-arginine (L-Arg), to increase the availability of substrate for NO biosynthesis, (3) D-arginine (D-Arg), (4) 3-[4-morpholinyl]-sydnonimine-hydrochloride (SIN-1), an NO donor, or (5) $CaCl_2$, a cofactor of brain calcium-calmodulin-dependent $cNOS_I$. In additional experiments, the levels of L-citrulline (the stable equimolar product derived from enzymatic cleavage of L-Arg by NO synthase) were determined in the brain of vehicle- or cadmium-treated rats.

3 The pressor response to cadmium reached its nadir at 5 min (43 ± 4 mmHg) and lasted over 20 min in controls. L-Citrulline/protein content was reduced from 35 up to 50% in the cerebral cortex, pons, hippocampus, striatus, hypothalamus ($P<0.01$) of cadmium-treated rats compared with controls. Central injection of N^G nitro-L-arginine-methylester (L-NAME) also reduced the levels of L-citrulline in the brain.

4 Both the magnitude and duration of the response were attenuated by 1.21 and 2.42 μmol SIN-1 (32 ± 3 and 15 ± 4 mmHg, $P<0.05$), or 1 μmol $CaCl_2$ (6 ± 4 mmHg, $P<0.05$). Selectivity of action exerted by SIN-1 was confirmed by the use of another NO donor, S-nitroso-N-acetyl-penicillamine (SNAP). Both L-Arg and D-Arg caused a mild but significant attenuation in the main phase of the pressor response evoked by cadmium. However, only L-Arg reduced the magnitude of the delayed, pressor response. Despite their similarity in ability to attenuate the cadmium-induced pressure effect, L-Arg and its isomer exerted differential biochemical changes in brain L-citrulline, as L-Arg normalized cadmium-induced reduction in L-citrulline levels, whereas i.c.v. D-Arg did not.

5 We conclude that the pressor effect of i.c.v. cadmium is due, at least in part, to reduced NO formation, consequent to inhibition of brain NO synthase. Accumulation of cadmium in the central nervous system could interfere with central mechanisms (including NO synthase) implicated in the regulation of cardiovascular function.

Keywords: Heavy metals; calcium; blood pressure; nitric oxide synthase; central nervous system

Introduction

Cadmium is a toxic environmental pollutant, derived from industrial refining operations and cigarette smoking. Its very long biological half-life is responsible for nearly irreversible accumulation in the human organism (Nordberg, 1984). Blood and tissue cadmium concentrations reportedly correlate with blood pressure (BP) levels in man (Glauser *et al*., 1976; Templeton & Cherian, 1983; Fontana & Boulos, 1988; Whittemore *et al*., 1991), though this relationship was not confirmed by recent epidemiological studies (Staessen & Lauwerys, 1993).

Cadmium administration, by the oral or intraperitoneal route, is able to increase blood pressure levels in rats (Perry *et al*., 1977; Revis, 1977; Balaraman *et al*., 1989). Recently, we found that cadmium, at doses not sufficient to induce pressor effects by the peripheral route, is able to induce sustained BP increases when injected by the intracerebroventricular (i.c.v.) route (Madeddu *et al*., 1993). Yet, the mechanism responsible for this central cardiovascular response has not been elucidated. Heavy metals, including cadmium, have been found to interact with transmembrane calcium influx in the central nervous system (SNC) (Suszkiw *et al*., 1984). Consistent with this possibility is our finding that central administration of calcium antagonists protects against the pressor effect of cadmium in rats (Madeddu *et al*., 1993). On entering neuronal cells, cadmium could act as an 'analogue-antagonist' of calcium on intracellular receptors. In this regard, there is evidence that cadmium can substitute for calcium in binding neuronal calmodulin, thus affecting the activity of enzymes that are calcium-calmodulin regulated (Haberman *et al*., 1983; Cheung, 1984). One of such enzymes, nitric oxide synthase, isoform I ($cNOS_I$), is constitutively expressed in the brain, spinal cord, sympathetic ganglia, adrenal glands and peripheral nitrergic nerves (Bredt *et al*., 1990). Following agonistic-induced elevation of intracellular calcium and in the presence of specific cofactors, neural $cNOS_I$ catalyzes the conversion of L-Arginine (L-Arg) to nitric oxide (NO) plus L-citrulline (Knowles *et al*., 1989). A vasodilator action of NO generated in the CNS is suggested by the finding that central injection of NOS inhibitors increases systemic BP and enhances sympathetic nerve activity (El Karib *et al*., 1993;

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Toda *et al*., 1993; Harada *et al*., 1993; Dominiczak & Bohr, 1995; Cabrera & Bhor, 1995).

Based on these observations, we hypothesized that the pressor response to central administration of cadmium is due to inhibition of brain calcium-calmodulin-dependent $cNOS_I$, leading to reduction in the generation of NO and unbalanced prevalence of tonic pressor mechanisms. To test this possibility, we determined if the pressor response to cadmium is prevented by i.c.v. pretreatment with L-Arg (the natural substrate for NO biosynthesis), 3-[4-morpholinyl]-sydnonimine-hydrochloride (SIN-1; an NO donor) and $CaCl_2$ (a cofactor of brain calcium-calmodulin-dependent $cNOS_I$). In addition, the levels of L-citrulline, the stable equimolar product derived from the enzymatic cleavage of L-Arg by NOS, were determined in the brain of vehicle- or cadmium-treated rats.

Methods

Animals

Male Wistar rats (Morini, Milan, Italy) weighing 280 to 300 g were housed at constant temperature ($24 \pm 1^{\circ}C$) and humidity ($60 \pm 3\%$) with a 12 h light/dark cycle and had free access to water and rat chow. All procedures complied with the standards for care and use of animal subjects as stated in Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md, U.S.A.). In addition, all protocols were approved by the Institutional Animal Care and Research Advisory Committee at the University of Sassari.

Surgical procedures

Rats were anaesthetized with ketamine chloridrate (45 mg kg^{-1} body weight, Parke-Davis, Milan, Italy) and diazepam (5 mg kg^{-1} body weight, Roche, Milan, Italy). A 22 gauge stainless steel cannula fitted into a 3×4 mm membrane-valve plastic block (Umberto Danuso, Milan, Italy) was placed stereotaxically into the left lateral cerebral ventricle (1.5 mm lateral and 1.0 mm posterior to the bregma, and 4.5 mm deep from the skull surface). The plastic block was anchored to the skull with screws embedded in dental acrylic cement. At the end of the surgical procedure ampicillin (1.25 μg in 5 μl saline) was injected by the i.c.v. route to prevent infection.

Four days later, a polyethylene catheter (PE-10 connected to a PE-50, Clay Adams, Parsippany, NJ, U.S.A.) was filled with heparin-treated saline and passed into the abdominal aorta via the left femoral artery; the catheter was then tunnelled under the skin and brought out of the back of the neck.

Blood pressure measurement and drug delivery

Experiments were performed in unanaesthetized rats, placed in cylindrical plastic restrainers, 5 days after cerebroventricular cannula implantation. Mean BP (MBP) and heart rate (HR) were measured via the femoral catheter by the use of a Statham transducer (Gould, Oxford, Calif., U.S.A.) connected to a four channel Quartet Recorder (Ugo Basile, Biological Research Apparatus, Comerio, Italy). Injections via the cerebroventricular cannula were performed with a 25 μl syringe (Hamilton, Reno, NV, U.S.A.); injection volume for drug delivery was 5 μl, followed by additional 5 μl saline to flush the cannula. At the end of the experiment, the correct placement of the cerebroventricular cannula was tested by determining the dipsogenic and pressor response to i.c.v. angiotensin II.

Experiment 1: MBP effects of i.c.v. L-Arg, SIN-1 or $CaCl_2$

After 15 min stabilization, rats ($n = 4$, each group) received an i.c.v. injection of saline (vehicle), L-Arg (1 μM), SIN-1 (2.42 μM) or $CaCl_2$ (1 μM). MBP was measured during the stabilization period and for an additional 25 min after i.c.v. administration of the above compounds.

Experiment 2: effects of L-Arg, D-Arg, SIN-1 or $CaCl_2$ on the pressor response to i.c.v. cadmium acetate

After 15 min of stabilization, rats received an i.c.v. bolus injection of saline (vehicle, $n = 11$), D-Arg (1 μM, $n = 5$), L-Arg (1 μM, $n = 11$), SIN-1 (0.48, 1.21 and 2.42 μM, $n = 8$, 8 and 12, respectively), or $CaCl_2$ (0.1, 0.3 and 1 μM, $n = 8$, 8 and 11, respectively). Five min later, cadmium acetate (88 nM) was injected by the i.c.v. route. MBP and HR were measured during the stabilization period before the cadmium acetate injection and then for an additional 20 min.

Additional experiments were performed to determine if the pressor effects of other agents were attenuated by SIN-1, $CaCl_2$ or L-Arg. Rats received vehicle, 2.42 μM SIN-1, 1 μM $CaCl_2$ or 1 μM L-Arg by the i.c.v. route ($n = 8$ each group). Five min later, they were injected with 25 nM 5-hydroxytryptamine (5-HT), intracerebroventricularly. In a separate set of experiments, rats received vehicle or 2.42 μM SIN-1 by the i.c.v. route ($n = 6$ each group). Five min later they were injected with 2 nM angiotensin II, intracerebroventricularly. MBP and HR were measured before and after the injection of 5-HT or angiotensin II.

Finally, the effect of i.c.v. S-nitroso-N-acetyl-penicillamine (SNAP, 1.21 μM, $n = 5$) on the pressor response to cadmium acetate was tested. MBP was measured in basal conditions, before the cadmium acetate injection and then for an additional 5 min.

Experiment 3: effects of i.c.v. cadmium acetate on L-citrulline levels in the brain

Rats ($n = 6$ per group) were instrumented with an i.c.v. cannula as described above. Five days later, they were given an i.c.v. bolus injection of vehicle (saline), cadmium acetate (88 nM), L-Arg (1 μM), D-Arg (1 μM), N^G-nitro-L-arginine-methyl-ester (L-NAME, 1 μM), L-Arg + cadmium acetate, or D-Arg + cadmium acetate. Ten minutes later, the rats were killed by cervical dislocation and brains were quickly removed and placed on ice. Selected regions (pons, hippocampus, striatus, hypothalamus, cerebral cortex) were homogenized at 4°C in a solution of methanol/water (80/20, vol/vol) with an Elvehim Glass Teflon Homogeniser; 100 μl of the homogenate was used to measure protein concentration. The remaining was sonicated with a microtip Brenson Sonifier (at 75% of maximum power) and then centrifuged at 12,500 *g* for 10 min at 4°C. The supernatants were desiccated in a vacuum speed centrifuge and stored at −20°C until L-citrulline assay.

Analytical procedures

L-Citrulline was measured by *o*-phthaldialdehyde precolumn derivatization, reversed-phase high-performance liquid chromatography (h.p.l.c.) and fluorimetric detection (Curró *et al*., 1996). The L-citrulline derivative was analysed with a Li-Chrosphere 100 RP-18 column (Merck, Darmstadt, Ger-

many). The chromatography was performed with a linear gradient (flow rate of 1.3 ml min^{-1}) starting with 0.05 M aqueous potassium dihydrogen phosphate, pH 6.3, and ending after 15 min with acetonitrile:H_2O:methanol (4:4:2). The latter solvent was continued for 5 min and the column was then re-equilibrated for 5 min with the initial buffer before the infection of the next sample. The h.p.l.c. system (Jasco, Tokyo, Japan) consisted of a 880-PU pump, a 880-02 controller for gradient programming, a Rheodyne injection valve 7125i with a 100 μl filling loop and 821-FP spectrofluorometer. Excitation/emission wave lengths were maintained at 330/450 nm. The detector was coupled to a CR6A Chromatopak (Shimadzu, Kyoto, Japan). The chemicals utilized were of analytical grade and the solvents were of chromatographic grade.

Statistical analysis

All data are expressed as mean $\pm$ s.e.mean. Multivariate repeated-measures analysis of variance was performed to test interaction between time and grouping factor. Then univariate analysis of variance was used to test for differences between groups and over time. Changes caused by the pretreatments were included in the analysis. Differences within or between groups were determined by Student's paired or unpaired t tests with the Bonferroni multiple comparison adjustment. A probability (P) value less than 0.05 was considered significant.

Substances

Cadmium acetate and calcium chloride were purchased from Aldrich Chemical Company (Milan, Italy); SIN-1 was kindly donated by Cassella AG (Frankfurt/Main, Germany), angiotensin II, 5-HT, L-Arg, D-Arg, SNAP, and L-NAME were purchased from Sigma Chemical Company (Milan, Italy). All these compounds were dissolved in sterile saline immediately before the experiment.

Results

Experiment 1: MBP effects of i.c.v. L-Arg, SIN-1 or $CaCl_2$

Basal MBP did not significantly differ between the groups at the end of stabilization period (Vehicle: 123 ± 2, L-Arg: 120 ± 4, SIN-1: 117 ± 2, $CaCl_2$: 126 ± 7 mmHg). Injection of saline or L-Arg by the i.c.v. route did not affect MBP (Figure 1). Central administration of $CaCl_2$ decreased MBP levels by 12 mmHg at 25 min (from 126 ± 7 to 114 ± 6 mmHg, $P<0.05$), though this change did not differ from that observed in controls given i.c.v. saline (from 123 ± 2 to 120 ± 2 mmHg). MBP increased transiently following i.c.v. SIN-1 (from 117 ± 2 to 135 ± 2 mmHg at 1 min, $P<0.05$). It returned to basal levels within 5 min and then remained similar to that of vehicle-treated rats until 21 min, when a mild but significant fall in MBP was detected ($P<0.05$). No significant change in HR was observed in any group over the duration of the experiment.

Experiment 2: effects of L-Arg, SIN-1 or $CaCl_2$ on the pressor response to i.c.v. cadmium acetate

During the period that preceded the administration of cadmium acetate, no significant difference in MBP and HR was detected between the groups given vehicle, L-Arg or D-Arg. Central injection of cadmium induced a prompt and sustained increase in MBP that peaked at 5 min (43 ± 4 mmHg) and lasted over 20 min (Figure 2). The main phase of the pressor response to cadmium was significantly attenuated in rats pretreated with L-Arg or D-Arg (32 ± 3 mmHg and 27 ± 5 at 5 min, respectively, $P<0.05$). In addition, L-Arg was able to attenuate the delayed pressor response to cadmium, while D-Arg was ineffective. The bradycardia evoked by cadmium was not altered by pretreatment with L-Arg or D-Arg.

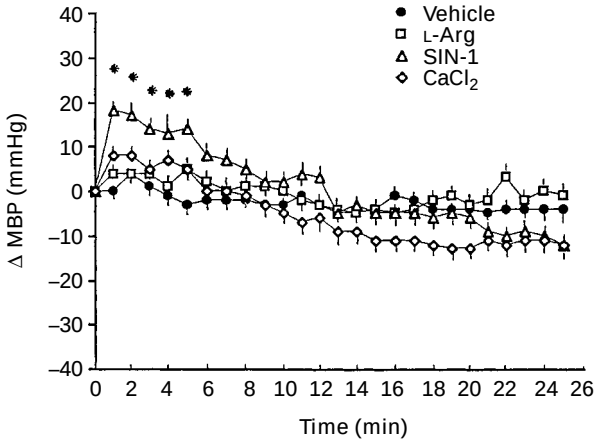

Figure 1 Effects of intracerebroventricular injection of vehicle, L-arginine (L-Arg, 1.0 μM), SIN-1 (2.42 μM), $CaCl_2$ (1.0 μM) on the mean blood pressure of unanaesthetized Wistar rats. Values are expressed as mean and represent absolute changes from baseline values recorded at the end of stabilization period (time 0); vertical lines show s.e.mean. Each group consisted of 4 rats. *$P<0.05$ versus vehicle group.

MBP was transiently increased by i.c.v. SIN-1 (Figure 3). It returned to levels similar to baseline at time 0, when cadmium acetate was injected. The pressor response to cadmium was attenuated by SIN-1 and the magnitude of this effect was dose-related (43 ± 4, 43 ± 3, 32 ± 3 and 15 ± 4 mmHg at 5 min with 0, 0.48, 1.21, and 2.42 μM, respectively, $P<0.05$). The bradycardia induced by cadmium was nullified by i.c.v. pretreatment with 2.42 μM SIN-1.

Pretreatment with SNAP, another NO donor, significantly attenuated the pressor effect of cadmium (21 ± 2 versus 43 ± 4 mmHg in controls at 5 min, $P<0.05$).

The pressor response to cadmium was attenuated by i.c.v. $CaCl_2$ in a dose-related manner, as shown in Figure 4. The cadmium-induced bradycardia was nullified by pretreatment with $CaCl_2$.

Immediately before the administration of 25 nM 5-HT, MBP and HR levels did not differ significantly between rats given vehicle, L-Arg (1 μM), $CaCl_2$ (1 μM) or SIN-1 (2.42 μM). The pressor response and the bradycardia evoked by the i.c.v. administration of 5-HT were significantly reduced by i.c.v. pretreatment with $CaCl_2$, while they were not affected by L-Arg, or SIN-1 (Figure 5).

Pretreatment with 2.42 μM SIN-1 did not alter the pressor response and the bradycardia induced by the i.c.v. administration of 2 nM angiotensin II (Figure 6).

Experiment 3: effects of i.c.v. cadmium acetate on L-citrulline levels in the brain

L-Citrulline levels in the brain were reduced in rats given i.c.v. cadmium acetate compared with controls (Table 1).

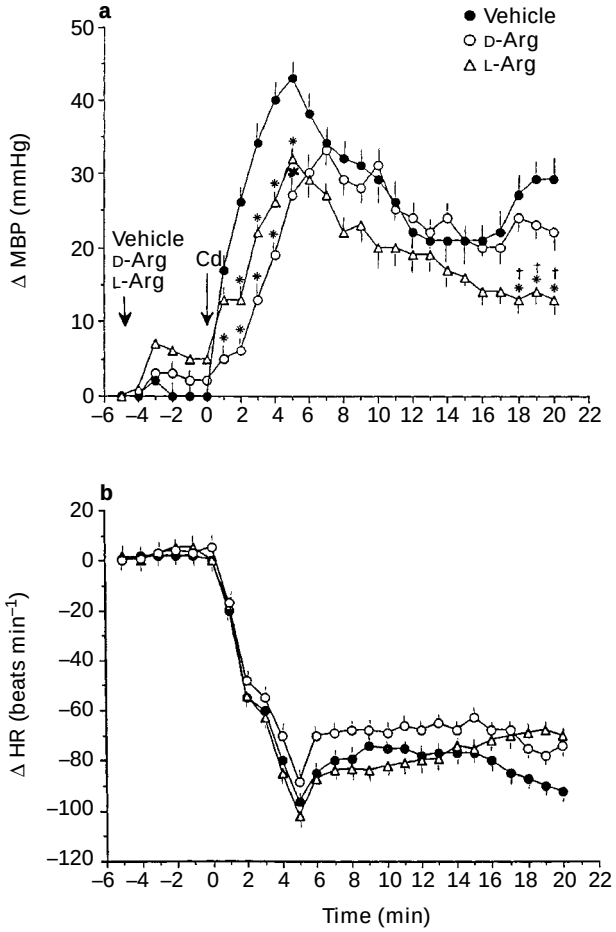

Figure 2 Effects of intracerebroventricular cadmium on (a) the mean blood pressure (MBP) and (b) heart rate (HR) of unanaesthetized Wistar rats pretreated with intracerebroventricular vehicle ($n=11$), L-arginine (L-Arg, 1.0 μM; $n=11$), or D-arginine (D-Arg, 1.0 μM; $n=5$). Cadmium acetate (Cd, 88 nM) was injected 5 min after vehicle, L-Arg or D-Arg. Values are expressed as mean and represent absolute changes from baseline values recorded at the end of stabilization period (time, -5 min); vertical lines show s.e.mean. $^*P<0.05$ versus vehicle group, $\dagger P<0.05$ versus D-Arg group.

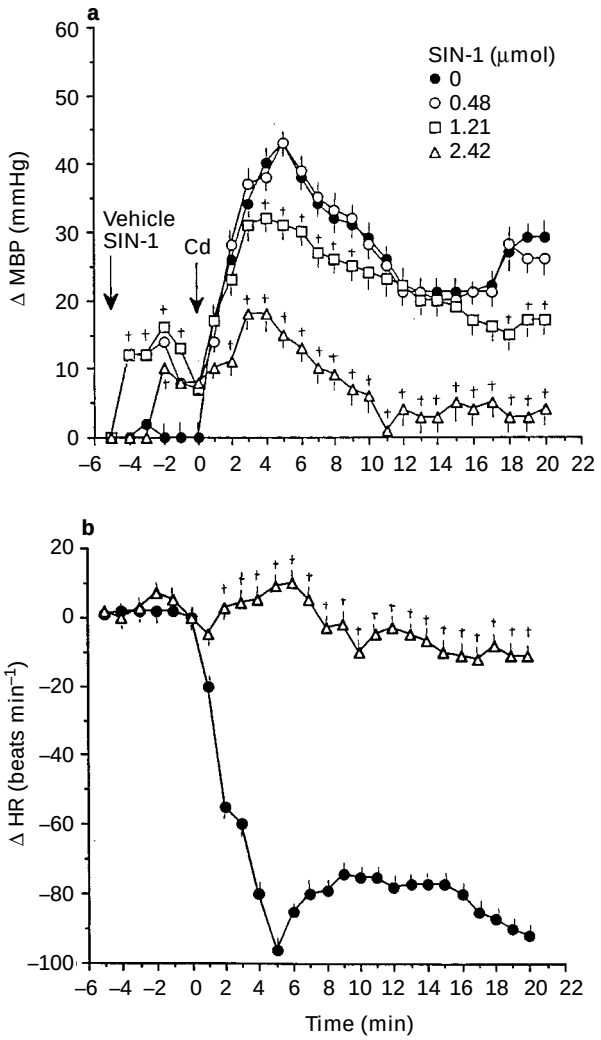

Figure 3 Effects of intracerebroventricular cadmium on (a) the mean blood pressure (MBP) and (b) heart rate (HR) of unanaesthetized Wistar rats pretreated with intracerebroventricular SIN-1 ($n=$ at least 8 for each dose) or vehicle ($n=11$). Cadmium acetate (Cd, 88 nM) was injected 5 min after SIN-1 or vehicle. HR values were not recorded in rats given 0.48 or 1.21 μM SIN-1. Values are expressed as mean and represent absolute changes from baseline values recorded at the end of the stabilization period (time, -5 min); vertical lines show s.e.mean. $\dagger P<0.05$ versus vehicle.

The between group-difference was highest in pons, followed by striatus, hypothalamus, hippocampus and cortex. The brain L-citrulline levels of rats given L-Arg were higher compared with controls, whereas they were unaltered in rats given D-Arg and reduced in rats treated with L-NAME. L-Arg significantly attenuated the reduction in brain L-citrulline levels induced by i.c.v cadmium, whereas D-Arg did not.

Discussion

The present study confirms our previous results that central injection of cadmium increases the BP of rats. The finding that the same dose is ineffective by the peripheral route indicates that central mechanisms are responsible for the pressor effect induced by this heavy metal (Madeddu *et al*., 1993).

The presence of $cNOS_1$ has been recognized in the rat and bovine brain (Knowles *et al*., 1989). This enzyme was subsequently purified, cloned, sequenced and found to be expressed (Bredt *et al*., 1990). Levels of brain $cNOS_1$ expression are altered in the two-kidney, one clip model of hypertension (Krukoff *et al*., 1995). Most important is the finding that the central administration of NOS inhibitors, functioning as false substrates, causes an elevation in rat BP at doses that are ineffective by the intravenous route (El Karib *et al*., 1993; Toda *et al*., 1993; Harada *et al*., 1993). These studies suggest that NO generated in the brain causes a centrally mediated vasodilatation. In fact, the i.c.v. administration of NO donors decreases the BP of normotensive rats, as does i.c.v. $CaCl_2$, which stimulates the $cNOS_1$ to release NO in a cardiovascular regulatory centre (Cabrera & Bhor, 1993). Consistent with the above findings are our results showing modest, but significant decreases in BP following the central injection of $CaCl_2$ or SIN-1. The vasodepressor response to SIN-1 was preceded by a brief increase in BP. It appears unlikely that this brief increase in pressure is related to elevated NO levels in the brain, as it was not observed with SNAP, another NO-donor (P. Madeddu, unpublished observations). Our finding that the central administration of L-Arg does not affect the basal BP levels of conscious rats confirms previous experiments performed in anaesthetized, baroreceptor denervated rats (Togashi *et al*., 1992). It is conceivable that, under basal conditions, endogenous L-Arg is not a rate-limiting step

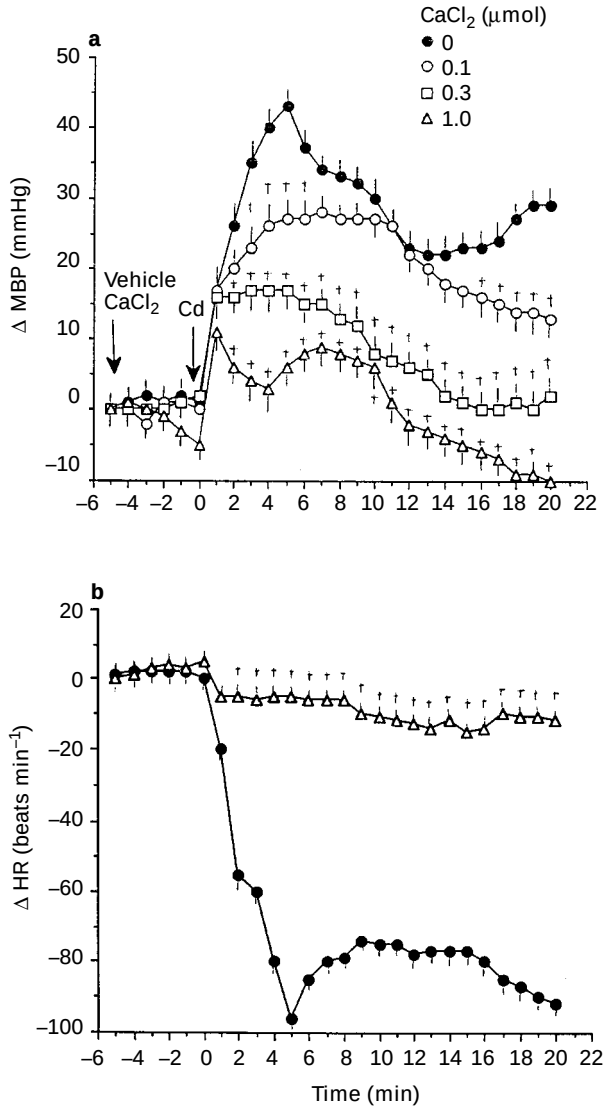

Figure 4 Effects of intracerebroventricular cadmium on (a) the mean blood pressure (MBP) and (b) heart rate (HR) of unanaesthetized Wistar rats pretreated with intracerebroventricular $CaCl_2$ (n=at least 8 for each dose) or vehicle (n=11). Cadmium acetate (Cd, 88 nM) was injected 5 min after $CaCl_2$ or vehicle. HR values were not recorded in rats given 0.1 or 0.3 μM $CaCl_2$. Values are expressed as mean and represent absolute changes from baseline values recorded at the end of stabilization period (time, −5 min); vertical lines show s.e.mean. †$P<0.05$ versus vehicle.

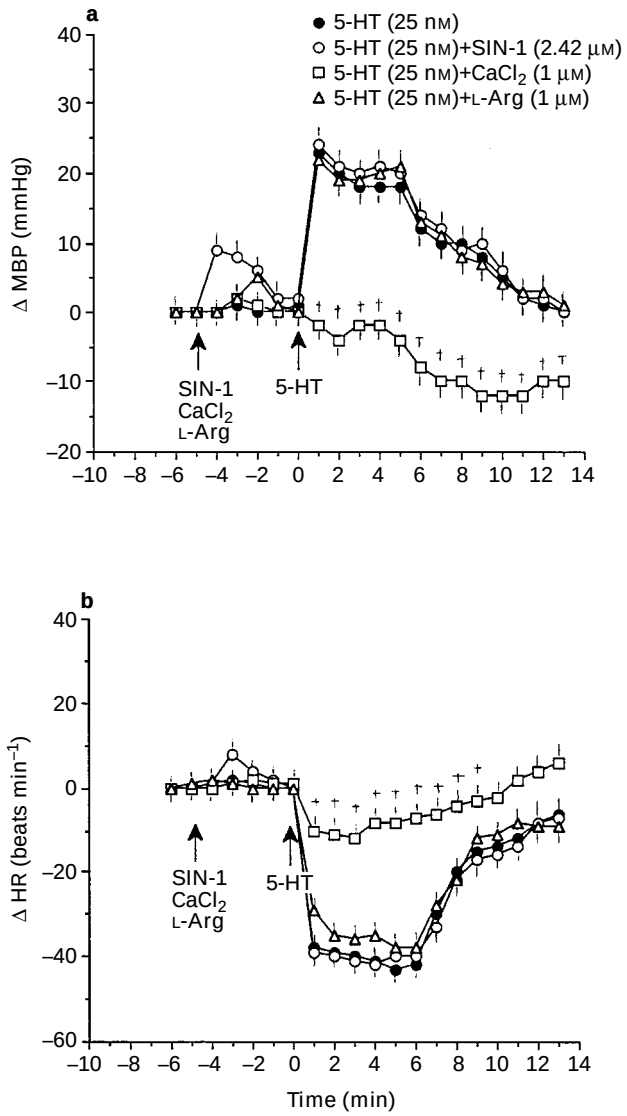

Figure 5 Effects of intracerebroventricular 5-HT (25 nM) on (a) the mean blood pressure (MBP) and (b) heart rate (HR) of unanaesthetized Wistar rats pretreated with vehicle, SIN-1 (2.42 μM), $CaCl_2$ (1 μM) or L-Arg (1 μM). Rats received vehicle, SIN-1, $CaCl_2$ or L-Arg by the i.c.v. route (n=8 for each group). Five minutes later, they were injected with 5-HT. Values are expressed as mean and represent absolute changes from baseline values recorded at the end of stabilization period (time, −5 min); vertical lines show s.e.mean. †$P<0.05$ versus vehicle.

in NO production by $cNOS_1$. However, exogenously supplied L-Arg attenuates the pressor effect induced by NOS inhibitors injected into the lateral cerebral ventricle or into the brain stem nuclei of rats and rabbits (Togashi *et al*., 1992; Tseng *et al*., 1996).

We speculated that the brain $cNOS_1$ might play a role in the acute pressor response to cadmium. The reason for testing this hypothesis was that cadmium, as well as other heavy metal cations, inhibits $cNOS_1$ activity in the cytosolic preparation from rat brain (Joshi & Desaiah, 1994; Mittal *et al*., 1995). Possible mechanisms for this action include: (1) direct binding to the catalytic site(s), (2) interference with the electron transfer during catalysis, or (3) induction of unfavourable conformational changes in the enzyme structure. In addition, cadmium may compete with calcium, that is required for the activation of $cNOS_1$, either by antagonizing transmembrane calcium influx or by substituting for it in calmodulin-dependent enzymatic reactions (Haberman *et al*., 1983; Suszkiw *et al*., 1984; Cheung, 1984). Our results indicate that cadmium-induced pressor effect is mediated, at least in part, by inhibition of brain NOS. Consistent with this possibility is the recognition of decreased levels of L-citrulline in the brain of cadmium-treated rats. L-Citrulline is the stable equimolar derivative of $cNOS_1$ enzymatic reaction on L-Arg and its reduction indirectly reflects the rate of NO production (Curró *et al*., 1996). Indeed, we found that i.c.v. administration of L-NAME, an NOS inhibitor, actually lowers the levels of L-citrulline in the brain, while administration of L-Arg increases them. It is still possible that the lower levels of L-citrulline are, at least in part, the consequence of an accelerated metabolism of L-citrulline and conversion to L-Arg. Yet, this metabolic pathway has been demonstrated only in endothelial cells, where it could help maintain sufficient levels of L-Arg during periods of prolonged NO release (Hecker *et al*., 1990). However, enzymes responsible for L-citrulline conversion to L-Arg have not been recognized in the CNS. An additional

note of consciousness is necessary when considering that, as predicted from studies in other areas of body, L-citrulline might be derived from metabolic pathways other than brain NOS.

Another important finding supporting our hypothesis is that the supply of exogenous NO achieved by i.c.v. pretreatment with NO donors attenuates the pressure response to cadmium. The effect of NO donors on the pressor response evoked by cadmium largely exceeded the ability of these compounds to lower BP levels in basal conditions. In addition, selectivity of the effect of SIN-1 is documented by its failure to alter the response to other vasoconstrictors, such as angiotensin II and 5-HT.

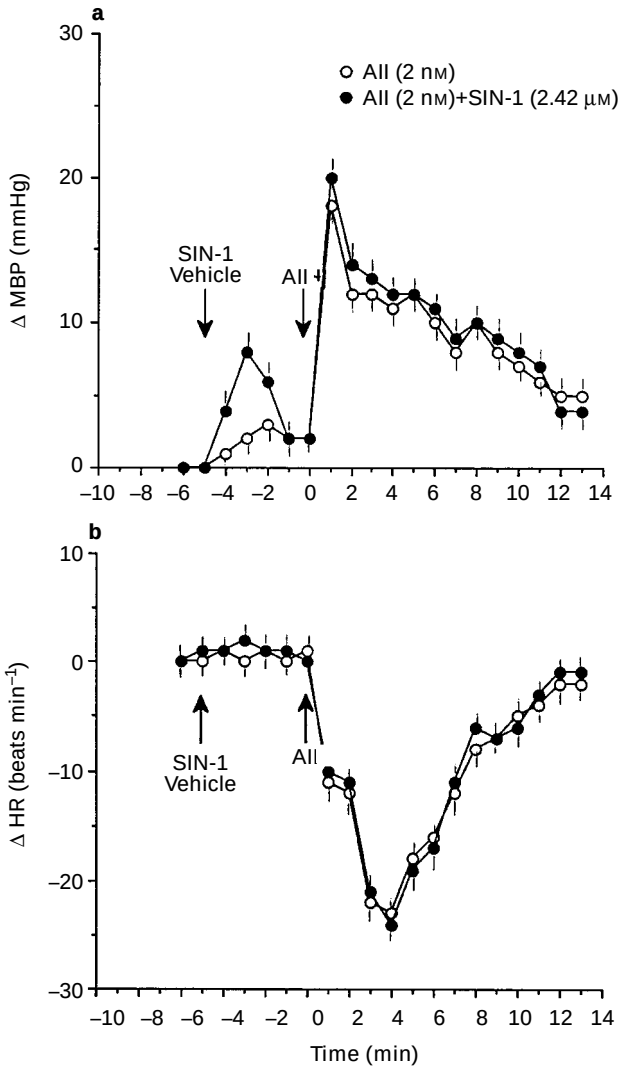

Figure 6 Effects of intracerebroventricular angiotensin II (AII, 2 nM) on (a) the mean blood pressure (MBP) and (b) heart rate (HR) of unanaesthetized Wistar rats pretreated with vehicle or SIN-1 (2.42 μM). Rats received vehicle or SIN-1 by the i.c.v. route ($n=6$ each group). Five minutes later, they were injected with angiotensin II. Values are expressed as mean and represent absolute changes from baseline values recorded at the end of stabilization period (time, −5 min); vertical lines show s.e.mean.

In the present study, calcium, a cation that simulates $cNOS_1$ to release NO, was also used as a probe to prevent the pressor response to cadmium. Antagonism of the response evoked by i.c.v. cadmium was directly proportional to the dose of calcium. This effect could be attributable to competition between calcium and cadmium for entering the neural cell as well as for interacting with calmodulin-NOS complex (Habermann *et al*., 1983; Suszkiw *et al*., 1984; Cheung, 1984). However, the effect of calcium was not selective since we found that the pressor change evoked by another centrally acting vasoconstrictor, 5-HT, was also abolished by calcium.

Availability of L-Arg could be a rate-limiting factor for NO biosynthesis in tissues that were deprived of substrate or during administration of substrate analogues (Hecker *et al*, 1990; Togashi *et al*., 1992; Tseng *et al*., 1996). In these conditions, administration of exogenous L-Arg normalizes the rate of NO formation and prevents or reverses the increase in BP induced by pharmacological inhibition of NOS. We determined if an excess of exogenous L-Arg can attenuate the pressor response to cadmium, using D-Arg as a control. Interestingly, two phases can be recognised in the pressor response to i.c.v. cadmium: a rapid increase in MBP, that peaks at 5 min, followed by a secondary pressor effect starting at 15 min (Madeddu *et al*., 1993). It is noteworthy that D-Arg (which is not a substrate for NOS) attenuated the main phase of the pressor response to cadmium. At variance with its D-isomer, L-Arg was able to affect both phases of the pressor response. Cadmium could interact with some regulatory site(s) of $cNOS_1$ to cause retardation of the catalytic site (Mittal *et al*., 1995). The administration of exogenous L-Arg, leading to a large excess of substrate, might have determined more favourable kinetic conditions, which could attenuate the delayed pressor response to cadmium. However, an interesting problem has arisen by the discrepancy between the clear increase in NOS activity (on average 69%) induced by i.c.v. L-Arg – as measured by L-citrulline levels in the brain – and the rather weak effect of L-Arg on the pressor response to cadmium, also with regard to the pronounced preventive effect of calcium and NO donors. It is possible that increases in NO release larger than that obtained by administration of exogenous L-Arg are necessary to prevent the pressure response evoked by cadmium, completely. Such large increases in brain NO levels could have been achieved following the administration of NO donors. Additional h.p.l.c. analysis, i.e.

Table 1 Effects of cadmium, L-arginine, D-arginine or L-NAME on citrulline levels in the brain

Group	*Pons*	*Hippocampus*	*Striatus*	*Hypothalamus*	*Cortex*
Vehicle	36.3 ± 1.7	39.2 ± 1.6	30.1 ± 3.6	32.9 ± 1.9	28.3 ± 1.8
Cadmium	19.7 ± 1.2^{a}	29.5 ± 0.7^{a}	14.7 ± 1.3^{a}	20.4 ± 1.4^{a}	18.7 ± 2.0^{a}
L-Arginine	$54.0\pm1.2^{a,b}$	$55.9\pm1.6^{a,b}$	$43.8\pm0.9^{a,b}$	$51.7\pm1.3^{a,b}$	$41.7\pm1.3^{a,b}$
D-Arginine	$35.2\pm1.1^{b,c}$	$42.4\pm0.5^{b,c}$	$33.7\pm1.4^{b,c}$	$34.4\pm2.0^{b,c}$	$31.4\pm1.8^{b,c}$
L-NAME	$10.2\pm0.9^{a,b,c,d}$	$14.0\pm1.1^{a,b,c,d}$	$8.47\pm0.9^{a,c,d}$	$10.6\pm1.1^{a,b,c,d}$	$6.7\pm0.6^{a,b,c,d}$
Cadmium + L-arginine	$40.8\pm0.9^{b,c,e}$	$50.3\pm1.3^{a,b,d,e}$	$39.1\pm0.5^{a,b,d,e}$	$42.1\pm1.4^{a,b,c,d,e}$	$36.6\pm1.7^{a,b,e}$
Cadmium + D-arginine	$19.5\pm0.4^{a,c,d,e,f}$	$27.4\pm0.9^{a,c,d,e,f}$	$17.4\pm1.3^{a,c,d,e,f}$	$20.8\pm1.2^{a,c,d,e,f}$	$17.4\pm1.4^{a,c,d,e,f}$

Values are mean ± s.e.mean and represent citrulline levels (pM mg^{-1} protein) in various parts of the brain in rats given an i.c.v. injection of vehicle, cadmium (88 nM), L-arginine (1 μM), D-arginine (1 μM), L-NAME (1 μM), cadmium + L-arginine or cadmium + D-arginine. Each group consists of 6 rats. $^{a}P<0.05$ versus vehicle; $^{b}P<0.05$ versus cadmium; $^{c}P<0.05$ versus L-arginine; $^{d}P<0.05$ versus D-arginine; $^{e}P<0.05$ versus L-NAME; $^{f}P<0.05$ versus cadmium + L-arginine.

measuring the levels of L-citrulline in the presence of D-Arg, cadmium plus L-Arg, and cadmium plus D-Arg, was performed to investigate whether NOS activity can be restored by pretreatment with the natural substrate or its D-isomer. The finding that L-Arg normalized L-citrulline levels in cadmium-treated rats, whereas it proved to be marginally active in reversing the BP response, strongly suggests that there is at least one other mechanism in addition to NOS inhibition which is responsible for the pressor effect evoked by cadmium. Such a possibility is supported by the findings that (1) D-Arg was able to attenuate the pressor action of cadmium, but it failed to alter the cadmium-induced reduction in brain L-citrulline levels, and (2) the pressor response to cadmium exceeded that obtained with central administration of NOS inhibitors, despite the fact that the latter compounds almost nullify NO formation. The discrepancy between functional and biochemical data implies that the central NO system is much more complex than anticipated.

The pressor response to cadmium was associated with reflex bradycardia, indicating integrity of baroreflex function in this model. These results may also suggest that the effect of cadmium is mediated by a pressor brain peptide not causing sympathoexcitation. Reflex bradycardia was less in magnitude in rats pretreated with $CaCl_2$ or SIN-1, possibly because of the ability of these agents to attenuate the pressor response to cadmium.

After the discovery that analogues of L-Arg increase BP by inhibiting brain NOS, our findings demonstrate a novel experimental model produced, at least in part, by interference with central NO production. Though the brain NO system has been implicated in the regulation of cardiovascular function, no data are available regarding a cause-effect relationship between alteration in central NO formation and hypertension, neither is it known if chronic low-level exposure to cadmium can affect the activity of NOS in endothelial cells and/or in the CNS. In this regard, we want to stress that the aim of the present study was to evaluate one of the mechanisms implicated in the central pressor response to cadmium and not its relevance in the pathogenesis of human essential hypertension.

In conclusion, our data indicate that the acute pressor effect of i.c.v. cadmium is due, at least in part, to reduced NO formation in the brain. Accumulation of cadmium in the CNS could interfere with various mechanisms (including NOS) implicated in the central regulation of cardiovascular function.

References

BALARAMAN, R., GULATI, O.D. & BHATT, J.D. (1989). Cadmium-induced hypertension in rats. *Pharmacology*, **38,** 226–234.

BREDT, D.S., HWANG, P.M. & SNYDER, S.H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature*, **347,** 768–770.

CABRERA, C.L. & BHOR, D.F. (1993). The role of nitric oxide in the central control of blood pressure. *Biochem. Biophys. Res. Commun.*, **206,** 77–81.

CHEUNG, W. (1984). Calmodulin: its potential role in cell proliferation and heavy metal toxicity. *Fedn. Proc.*, **43,** 2995–2999.

CURRÓ, D., VOLPE, A.R. & PREZIOSI, P. (1996). Nitric oxide synthase activity and non-adrenergic non-cholinergic relaxation in the rat gastric fundus. *Br. J. Pharmacol.*, **117,** 717–723.

DOMINICZAK, A.F. & BOHR, D.F. (1995). Nitric oxide and its putative role in hypertension. *Hypertension*, **25,** 1202–1211.

EL KARIB, A.O., SHENG, J., BETZ, A.L. & MALVIN, R.L. (1993). The central effects of a nitric oxide synthase inhibitor (N^w-nitro-L-arginine) on blood pressure and plasma renin. *Clin. Exp. Hypertens.*, **15,** 819–832.

FONTANA, S.A. & BOULOS, B.M. (1988). Blood cadmium level as affected by hypertension, smoking, occupation and body mass. *Am. J. Hypertens.*, **1,** S158–S160.

GLAUSER, S.C., BELLO, C.T. & GLAUSER, E.M. (1976). Blood cadmium levels in normotensive and untreated hypertensive humans. *Lancet*, **i,** 717–718.

HABERMANN, E., CROWELL, K. & JANICKI, P. (1983). Lead and other metals can substitute for Ca^{++} in calmodulin. *Arch. Toxicol.*, **323,** 31–46.

HARADA, S., TOKUNAGA, S., MOMOHARA, M., MASAKI, H., TAGAWA, T., IMAIZUMI, T. & TAKESHITA, A. (1993). Inhibition of nitric oxide formation in the nucleus tractus solitarius increases renal sympathetic nerve activity in rabbits. *Circ. Res.*, **72,** 511–516.

HECKER, M., SESSA, W.C., HARRIS, H.J., ANGGARD, E.E. & VANE, J.R. (1990). The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: Cultured endothelial cells recycle L-citrulline to L-arginine. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 8612–8616.

JOSHI, P. & DESAIAH, D. (1994). Inhibition of nitric oxide synthase activity in rat brain by metals. *Toxicologist*, **14,** 198.

KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1989). Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 5159–5162.

KRUKOFF, T.L., GEHLEN, F., GANTEN, D. & WAGNER, J. (1995). Gene expression of brain oxide synthase and soluble guanylyl cyclase in hypothalamus and medulla of two-kidney, one clip hypertensive rats. *Hypertension*, **25,** 171–176.

MADEDDU, P., DEMONTIS, M.P., VARONI, M.V., FATTACCIO, M.C., PALMIERI, A. & ANANIA, V. (1993). Verapamil prevents the acute hypertensive response to intracerebroventricular cadmium in conscious normotensive rats. *Am. J. Hypertens.*, **6,** 193–197.

MITTAL, C.K., HARREL, W.B. & MEHTA, C.S. (1995). Interaction of heavy metals toxicants with brain constitutive nitric oxide synthase. *Mol. Cell. Biochem.*, **149/150,** 263–265.

NORDBERG, M. (1984). General aspects of cadmium: transport, uptake and metabolism by the kidney. *Environ. Health Perspect*, **54,** 13–20.

PERRY, H.M., ERLANGER, M.W. & PERRY, E.F. (1977). Elevated systolic pressure following chronic low level cadmium feeding. *Am. J. Phyiol.*, **232,** H114–H121.

REVIS, N. (1977). A possible mechanism for cadmium induced hypertension in rats. *Life Sci.*, **22,** 479–488.

STAESSEN, J. & LAUWERYS, R. (1993). Health effects of environmental exposure to cadmium in a population study. *J. Human Hypertens*, **7,** 195–199.

SUSZKIW, J., THOT, G. & MURAWSKY, A. (1984). Effect of Pb^{++} and Cd^{++} on acetylcholine release and Ca^{++} movements in synaptosomes and subcellular fractions from rat brain and torpedo electric organ. *Brain Res.*, **323,** 31–46.

TEMPLETON, D.M. & CHERIAN, G.M. (1983). Cadmium and hypertension. *Trends Pharmacol. Sci.*, **4,** 501–503.

TODA, N., KITAMURA, Y. & OKAMURA, T. (1993). Neural mechanism of hypertension by nitric oxide synthase inhibitor in dogs. *Hypertension*, **2,** 13–18.

TOGASHI, H., SAKUMA, I., YOSHIOKA, M., KOBAYASHI, T., YASUDA, H., KITABATAKE, A., SAITO, H., GROSS, S.S. & LEVI, R. (1992). A central nervous system action of nitric oxide in blood pressure regulation. *J. Pharmacol. Exp. Ther.*, **262,** 343–347.

TSENG, C.J., LIU, H.Y., LIN, H.C., GER, L.P., TUNG, C.S. & YEN, M.H. (1996). Cardiovascular effects of nitric oxide in the brain stem nuclei of rats. *Hypertension*, **27,** 36–42.

WHITTEMORE, A.S., DICICCIO, Y. & PROVENZANO, G. (1991). Urinary cadmium and blood pressure: results from the NHANES II Survey. *Environ. Health Perspect*, **91,** 133–140.

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