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Direct effects of lead (Pb^{2+}) on the relaxation of in vitro cultured rat aorta to acetylcholine

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Abstract

Lead (Pb^{2+}) exposure is related to increased blood pressure or hypertension of human or animals. Abnormal vascular relaxant responses of low level Pb^{2+} exposed animals were reported by several studies. However, it is difficult to tell whether these effects were induced directly by Pb^{2+} or not. In this study we hypothesized that Pb^{2+} can directly affect the relaxation of vessels. Male Wistar rat aortae were removed and cultured in PMRI 1640 with 1 ppm Pb^{2+} (4.8 μM lead acetate) for 0.5, 6, 12 and 24 h, and then their responses to acetylcholine (ACh) and sodium nitroprusside (SNP) were examined. After incubated for 24 h, the relaxation induced by ACh was significantly decreased in Pb^{2+} exposed aortic rings. However, there was not significant difference in relaxation induced by SNP between Pb^{2+} exposed and control group. The nitrite in the culture media of aortic rings cultured for 24 h, measured with Griess method, was significantly decreased in the Pb^{2+} exposed group. The expression of endothelial NOS (eNOS) and isoform NOS (iNOS) in the homogenate of aortic rings cultured for 24 h was measured by Western blot. The expression of eNOS of the Pb^{2+} exposed group was significantly upregulated compared with that of the control group. However, there was no significant difference in the expression of iNOS in control and Pb^{2+} exposed group. In conclusion, Pb^{2+} was able to directly affect the relaxation of rat aorta. This effect may have some relation with the lower level of NO in the media, though the expression of eNOS was upregulated.

Keywords: Pb^{2+} toxicity; Aorta; Vascular reactivity; NO; eNOS; iNOS

1. Introduction

As a ubiquitous environmental and industrial pollutant, lead (Pb^{2+}) has been proved to have neurotoxicity (Goyer, 1996), nephrotoxicity (Sanchez et al., 2001), hepatotoxicity (Daggett et al., 1997) and reproductive toxicity (Ronis et al., 1996). Some evidences demonstrated that Pb^{2+} has been involved in the elevation of blood pressure (Webb et al., 1981; Skoczynska et al., 1986, 2001; Fenga et al., 2006; Marques et al., 2001) and arteriosclerosis (Revis et al., 1981; Preuss et al., 1994) in human and animals. The blood Pb^{2+} was able to significantly increase all-cause and cardiovascular mortality, even at substantially lower levels, such as below 0.48 μM (10 μg/dl) (Menke et al., 2006). Therefore, it

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is of importance to study the relation between the Pb^{2+} and hypertension.

Vascular abnormity, including the increased wall–lumen ratio, increased vascular responsiveness to vasoconstrictors and/or decreased responsiveness to vasodilators, can be found in almost all kinds of animal hypertension models and genetic hypertension patients (Mulvany, 1992; Gohlke et al., 1993; Intengan and Schiffrin, 2001). In Pb^{2+} induced hypertension rats, relaxation to both acetylcholine (ACh) and sodium nitroprusside (SNP) was reduced (Marques et al., 2001). However, whether the reduced relaxation was induced directly (at least partly) by Pb^{2+} or not is not known, because Pb^{2+} was proved to have the ability to change the functions of neurogenic components and humoral components in Pb^{2+} exposed rats, such as central sympathetic hyperactivity, barofeflex hyposensitivity, vagal parasympathetic hypotone (Boscolo and Carmignani, 1988; Carmignani et al., 2000), and increased activity of renin–angiotensin–aldosterone axis (Campbell et al., 1985). Most of these abnormities mentioned above can induce abnormal vascular responses. However, if adopting in vitro cultured vessels removed from normal rats to address this question, the confounding effects of systematic neurogenic and humoral components can be eliminated. Our previous study found that Pb^{2+} was able to directly enhance the contractile response of vessels to 5-hydroxytryptamine (5-HT) with in vitro cultured aortae (Zhang et al., 2005).

In this study, we assessed whether Pb^{2+} had direct effect on the relaxant response of in vitro cultured aorta. Since the relaxation of vessel to ACh has close relation to endothelium-derived relaxing factor (EDRF)-NO, we also assessed whether Pb^{2+} exposure was able to alter the level of NO, by measuring nitrite in the culture media, and the product of NO, by detecting the expressions of endothelial NOS (eNOS) and isoform NOS (iNOS) in aorta tissue.

2. Materials and methods

2.1. Drugs and reagents

Lead acetate, norepinephrine bitartrate, acetylcholine chloride, sodium nitroprusside sulphanilamide and naphthylethylenediamine dihydrochloride were purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA). All chemicals were made fresh on the day of use, and the amount of solvent added did not exceed 1% of the solution. The addition of these chemicals to tissue bath did not alter the pH of the solution in it. RPMI 1640 and fetal bovine serum were purchased from Gibco (Grand, NY, USA).

2.2. Preparation of rat aorta

Healthy male Wistar rats, 250–300 g, were obtained from Laboratory Animal Center in Beijing Institute of Pharmacology and Toxicology. After anesthetized by pentobarbital sodium (50 mg/kg) and killed by decapitation, thoracic aortae were carefully excised and placed into icy D-Hanks with 100 U/ml penicillin and 100 U/ml streptomycin. After being cleaned free of fat and other connective tissues, the thoracic aortae were cut into 3 mm long rings, then suspended into culture solution (RPMI 1640 containing 10% fetal bovine serum) with or without 1 ppm Pb^{2+} (4.8 μM lead acetate) at 37 °C for 0.5, 6, 12 and 24 h.

The Pb^{2+} concentration of 1 ppm in the present study was chosen on the basis of previous studies: 0.1–1 ppm Pb^{2+} were able to significantly reduce sGC-β1 subunit expression in a concentration dependent manner, and the maximal reduction in sGC-β1 subunit expression was achieved in rat aortic rings incubated with 1 ppm Pb^{2+} for 24 h (Courtois et al., 2003); 1 ppm Pb^{2+} was able to directly enhance the contractile response of vessels to 5-HT with in vitro cultured aortae (Zhang et al., 2005).

The studies were conducted in accord with the principles and procedures outlined in the NIH guide for the Care and Use of the Laboratory Animals (National Research Council, 1996).

2.3. Measurement of relaxant responses

After incubation, the reactivities of the aortic rings were evaluated in the vascular tissue baths. Each bath contained 8 ml Krebs–Henseleit (K–H) solution (containing in mM: NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 11.1; EDTA-Na_2, 0.026) bubbled with a mixture of 95% O_2 and 5% CO_2, and warmed to 37 °C by an equitherm heating circulation system. The aortic rings were mounted on a pair of stainless-steel △-shaped hooks, one of which was fixed to the bottom of the chamber and the other to an isometric force transducer (Xinhang Mechanical and Electronic Inc., Gaobeidian, China) which was connected to a polygraph (Meiyi Technological Inc., Nanjing, China). Tissues were allowed to equilibrate under an optimum final force of 1.5 g for a period of 60 min, renewing the buffer every 15 min. After stabilization, the preparations were depolarized twice with 40 mM KCl, precontracted by 1 μM NE, and then relaxed by cumulative addition of ACh (10 nM to 100 μM) or contracted by 40 mM KCl then relaxed by cumulative addition of SNP (1 nM to 10 μM). The aortic rings of Pb^{2+} exposure and control used in each experiment were removed from the same rat, and each ring was used only once. To check the integrity of the endothelium of the rings, two rings chosen randomly from each rat aorta without culture were processed with above procedure. If the rings precontracted by 1 μM NE then relaxed by 1 μM ACh both were able to show a relaxation more than 60% of the contraction induced by 1 μM NE, the rings from the same aorta cultured were chosen for this experiment.

2.4. Measurement of nitrite production as an assay of NO release

NO production by aorta was assayed by measuring the accumulation of nitrite in the culture medium using the Griess reaction (Green et al., 1982; Ding et al., 1988). Aortic rings were cultured in 24-well microtiter plates and exposed to 1 ppm Pb^{2+} for 24 h. At the end of the incubation, the medium was collected, treated with active carbon and 30% $ZnSO_4$, then mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% naphthyl-ethylenediamine dihydrochloride, 5% H_3PO_4). After 10 min at room temperature, the optical density of samples was read at 550 nm; sodium nitrite was used as standard. After removing the medium, the aortic rings was dried and weighted.

2.5. Western analysis of aortic eNOS and iNOS expression

Aortic eNOS and iNOS expression was determined by Western blot. After in vitro cultured for 24 h, aortae were ground to powder in liquid nitrogen, homogenized in homogenization buffer (50 mM Tris–Cl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% Nonidet P-40, 1% sodium deoxycholate and 1 μg/ml leupeptin), and centrifuged at 14,000 × *g* for 20 min at 4 °C. The total protein of supernatant was measured using the bicinchoninic acid method kit (Beyotime Biotech Co. Ltd., Hangzhou, China). Twenty micrograms of total protein was separated on 10% SDS-polyacrylamide gels and transferred onto polyvinylidine difluoride (PVDF) membranes. After blocked, the membranes were probed with anti-eNOS polyclonal antibody (Santa Cruz, sc-8311) or anti-iNOS polyclonal antibody (Santa Cruz, sc-649) at 1:500 overnight or 1.5 h, then incubated with the horseradish peroxidase conjugated secondary antibody at 1:2000 (Beijing Zhongshan Golden Bridge Biotechnology, China) for 1 h. After rinsed, blots incubated with enhanced chemiluminescence reagents to visualize bands. To compare the expression of iNOS with the expression of another protein, the expression of actin in a parallel gel with 15 μg samples was analyzed with the same experimental procedure. The first antibody for actin was actin polyclonal antibody (Santa Cruz, sc-1616) at 1:500.

2.6. Statistics

The results were expressed as mean ± S.E.M. The maximal effects (E_{max}) were calculated through Microcal Origin 7.0 (Microcal Software Inc.). The data were analyzed by paired *t*-test. $p < 0.05$ was considered statistically significant.

Based on our previous study, Pb^{2+} had no significant effects on the aortic contraction to NE and KCl (Zhang et al., 2005). In this study, the relaxation induced by ACh was expressed as a percentage of the contraction to 1 μM NE (taken as 100%). And the relaxation induced by SNP was expressed as a percentage of the contraction to 40 mM KCl (taken as 100%). The reasons to choose two references are the following. After cultured more than 12 h, the contraction induced by NE was significantly decreased, which was able to be relaxed to the baseline with very low level of SNP. We found that the relaxant response induced by ACh was also affected by incubation. After cultured more than 12 h, ACh was difficult to relax the contraction induced by 40 mM KCl.

3. Results

3.1. Effect of Pb^{2+} on the relaxation responses of cultured aorta to ACh and SNP

After the aortic rings were cultured with or without 1 ppm Pb^{2+} for 0.5 h, 6 h, 12 h or 24 h and treated as the procedure described in Section 2, they were precontracted by 1 μM NE to produce a maximal contraction (taken as 100% contraction). After the vascular tone reached a stable plateau, ACh (10 nM to 100 μM) was cumulatively added to baths at a volume of 0.1% of the bath volume. The relaxation induced by ACh was not significantly different between Pb^{2+} exposed group and control group when the rings cultured in vitro for 0.5, 6, 12 h. However, after cultured for 24 h, the relaxation induced by ACh in the Pb^{2+} exposed group was significantly lower than that of control group The maximum relaxation of Pb^{2+} exposed group was 51.03 ± 6.98% ($n = 6$), while the maximum relaxation of control group was 61.53 ± 7.46% ($n = 6$), with the $p < 0.05$ (Fig. 1D).

The relaxation responses to SNP (1 nM to 10 μM) of cultured aortic rings were precontracted by 40 mM KCl, and the other procedures were similar to the above assay. There was no significant difference in the relaxation induced by SNP between Pb^{2+} exposed aortic rings and control ones cultured for 0.5–24 h. Fig. 2 depicted the relaxation induced by SNP in the Pb^{2+} exposed aortic rings and control ones cultured for 24 h, with no significant difference between these two group ($n = 6$) (Fig. 2).

3.2. Effect of Pb^{2+} on the NO production of cultured aorta

NO production by the cultured aorta was assayed by measuring the accumulation of nitrite in the culture medium using the Griess reaction. The nitrite in the culture medium of Pb^{2+} exposed aorta was significantly lower than that of the control group (1.80 ± 0.47 and 2.54 ± 0.35 μM, respectively, $n = 6$, $p < 0.05$, Fig. 3).

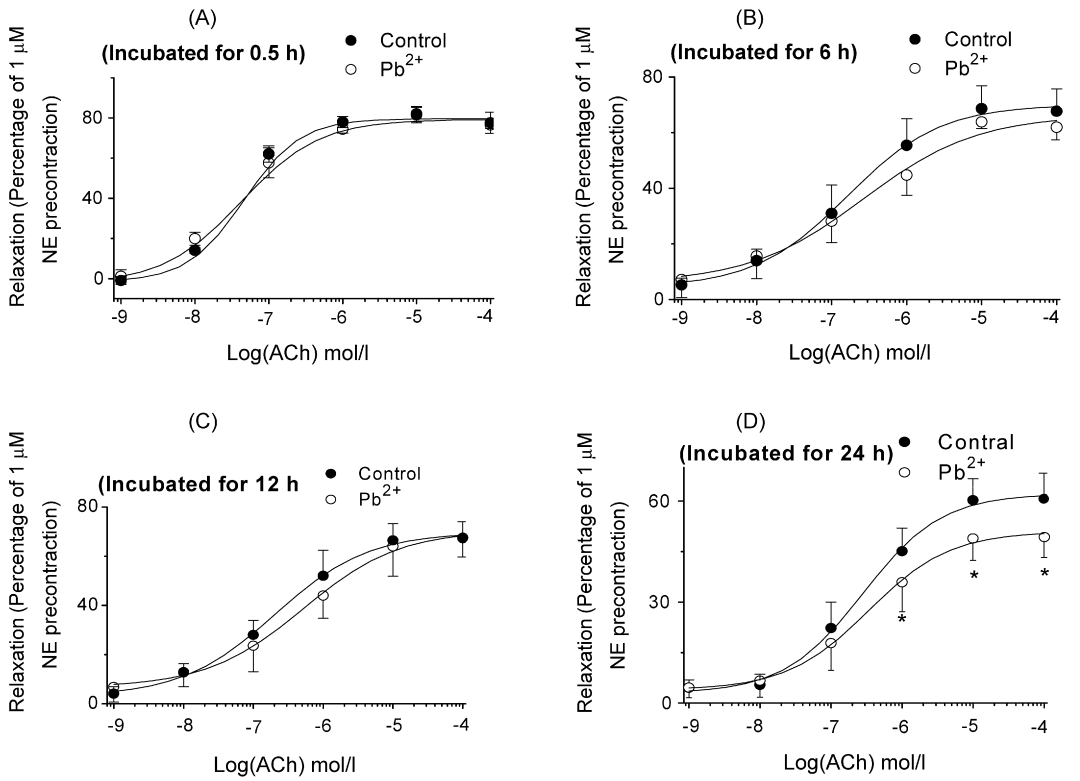

Fig. 1. Effects of Pb^{2+} on the concentration–response curves to ACh of aortic rings cultured for 0.5 h (A), 6 h (B), 12 h (C) and 24 h (D). Aortic rings cultured with or without 1 ppm lead acetate for 0.5, 6, 12 and 24 h were precontracted by 1 μM NE to produce a maximal contraction (taken as 100% contraction). After the vascular tone reached a stable plateau, ACh was added cumulatively. Points represent means ± S.E.M. ($n = 6$). *Statistically significant difference ($p < 0.05$) from control group.

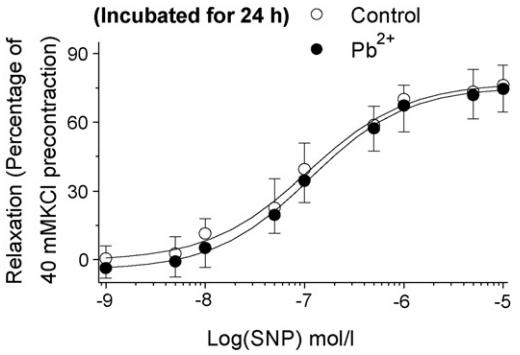

Fig. 2. Effects of lead on the concentration–response curves to SNP of aortic rings cultured for 24 h. Aortic rings cultured with or without 1 ppm lead acetate for 24 h were precontracted by 40 mM KCl to produce a maximal contraction (taken as 100% contraction). After the vascular tone reached a stable plateau, SNP was added cumulatively to baths at a volume of 0.1% of the bath volume. Points represent means ± S.E.M. ($n = 6$).

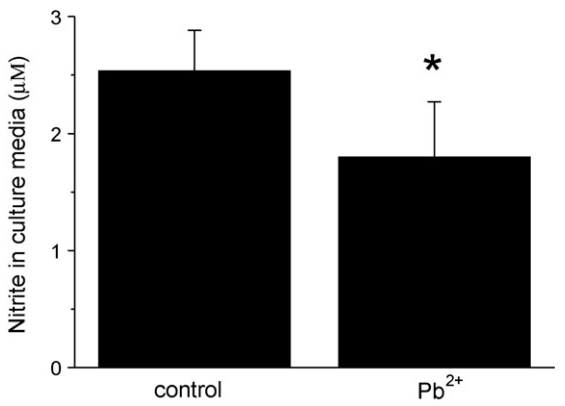

Fig. 3. Effects of Pb^{2+} on the accumulation of nitrite in the culture medium aorta cultured for 24 h. The accumulated nitrite in the culture medium of aortic rings with or without 1 ppm Pb^{2+} for 24 h was measured by the Griess reaction. Bars represent means ± S.E.M. ($n = 9$). *Statistically significant difference ($p < 0.05$) from control group.

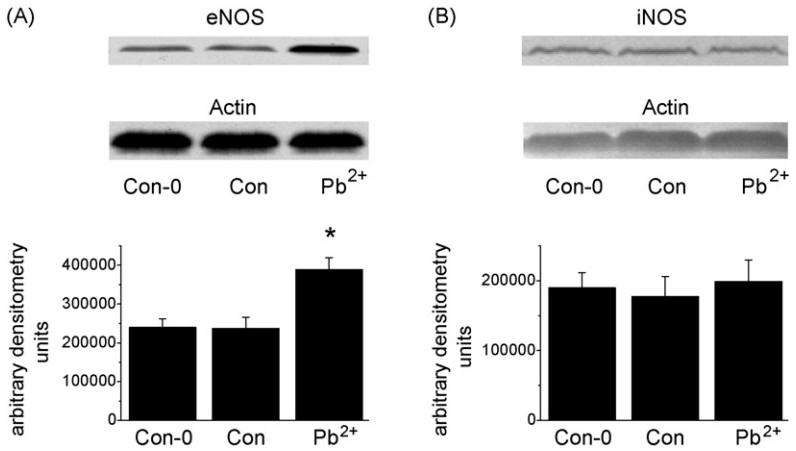

Fig. 4. Top, representative Western blots for eNOS (A), iNOS (B) and actin protein abundance in the uncultured aortae (Con-0), aortae incubated for 24 h without exposure of Pb^{2+} (Con) and aortae incubated for 24 h with 1 ppm Pb^{2+} (Pb^{2+}). Bottom, arbitrary optical densitometry units of eNOS and iNOS in aortic homogenates. Bars represent means ± S.E.M. ($n = 4$).

3.3. Effect of Pb^{2+} on the expression of eNOS and iNOS in the cultured aortae

Immunoblot analysis was performed on homogenates from control and Pb^{2+} exposed aortae incubated for 24 h to ascertain whether eNOS and iNOS protein expression was affected. Equivalent amounts of total protein were immunoblotted with an eNOS or iNOS antibody. The expression of eNOS of the Pb^{2+} group was marked increased ($n = 4$) (Fig. 4A). However, there was no significant difference in the iNOS immunoreactive bands between Pb^{2+} exposed aortae and control ones ($n = 4$) (Fig. 4B). These results demonstrated that there was a divergence between the decreased accumulation of nitrite in the culture medium of Pb^{2+} exposed aortae and the upregulated expression of eNOS in the homogenates of the Pb^{2+} exposed aortae.

4. Discussion

It was already reported by many researchers that Pb^{2+} was able to induce hypertension and arteriosclerosis in animals and human being. Though abnormal vascular relaxant responses in Pb^{2+} induced hypertensive animals were also reported by many (Webb et al., 1981; Marques et al., 2001; Karimi et al., 2002), none of them can tell us whether these abnormal vascular responses were induced, at least partly, by the direct interaction between Pb^{2+} and vessels or not, for the confounding effects of systematic neurogenic and humoral components were not able to eliminate.

In this study, adopting in vitro cultured aortae, we found Pb^{2+} was able to directly decrease the relaxant response of aorta to ACh after in vitro cultured for 24 h (Fig. 1D). However, Pb^{2+} had no significant effect on the relaxant response to SNP (Fig. 2), which was similar to the result of one previous report (Karimi et al., 2002). These results demonstrated that the adverse effects of Pb^{2+} on the in vitro cultured aorta were mainly located on the endothelium.

The ability of a blood vessel to relax to ACh is entirely upon the presence of an intact endothelium through an EDRF, which was late identified as NO (Furchgott and Zawadzki, 1980; Palmer et al., 1987). In this study, we found that the nitrite (breakdown products of NO) in the culture media of aorta exposed to Pb^{2+} was significantly decreased compared with that of the control group (Fig. 3). In animal studies, the level of stable NO metabolites (NO_x) in urinary excretion was also reported decreased significantly (Vaziri et al., 1997; Gonick et al., 1997; Ding et al., 1998). These results may reflect indirectly that the level of NO in serum available was decreased, which may be the cause (at least partly) of the decreased relaxant response to ACh of Pb^{2+} exposed cultured aorta or vessels of Pb^{2+} exposed animals.

NO is synthesized by the action of NO synthases (NOS). There are three isoforms of NOS: two are constitutively expressed (eNOS and neuronal NOS (nNOS), respectively) and one is an inducible iNOS (Cepinskas et al., 2002). In this study, we found that eNOS protein mass was significantly elevated while iNOS protein mass was at about the same level in the aortae exposed to 1 ppm Pb^{2+} in vitro for 24 h. The result of eNOS was in agreement with some results of animal studies which reported the eNOS and iNOS protein mass was marked raised both in aorta and kidney (Vaziri et al.,

1999). However, another paper reported that the eNOS protein mass in kidney cortex of Pb^{2+}-treated rats was not significantly different from that of control rats, whereas iNOS protein mass was significantly elevated in brain and kidney cortex (Gonick et al., 1997). Though there is divergence on the NOS protein mass in the references, till now no paper reported downregulation of NOS expression. Therefore, this is a paradox between the decreased level of NO and the upregulated expression of eNOS and/or iNOS. A plausible explanation for this paradox is that the NO was over-sequestrated, which is supported by many evidences: the reactive oxygen species (ROS), such as $O_2^{\bullet -}$ and the lipid peroxidation product, malondialdehyde (MDA), were marked elevated in Pb^{2+} exposed animals (Vaziri et al., 1997, 2003; Gonick et al., 1997; Ding et al., 1998) and in vitro cultured aortae (Courtois et al., 2003; Zhang et al., 2005); nitrotyrosine, the footprint of NO oxidation by ROS, was significantly increased in plasma, kidney, heart, liver, and brain of Pb^{2+} exposed rats (Vaziri et al., 1999); the Pb^{2+} induced hypertension, marked raised plasma MDA concentration and decreased urinary NO_x excretion was normalized or ameliorated by administrating antioxidants (Vaziri et al., 1997, 1999; Ding et al., 1998).

Although we found that Pb^{2+} was able to directly affect the relaxation of rat aorta to ACh with the in vitro cultured aortae, this model needs further emendation, for it has apparent disadvantages: after aortic rings cultured inertly for more than 12 h, the response to are significantly altered, which cannot represent the innate characteristics of aortae very well, for example, the contraction induced by NE or 5-HT was significantly decreased (Zhang et al., 2005), and the relaxation induced by ACh was also affected.

In summary, Pb^{2+} was able to directly affect the relaxation of rat aorta to ACh. This effect may be related to the decreased level of NO in the media. Though the eNOS was upregulated in cultured aorta, it was not able to eliminate this effect.

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