British Journal of Pharmacology (2002) 136, 1185–1193 
www.nature.com/bjp

# Effect of prolonged incubation with copper on endothelium-dependent relaxation in rat isolated aorta

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**1** We investigated the effects of prolonged exposure to copper ($Cu^{2+}$) on vascular functioning of isolated rat aorta.

**2** Aortic rings were exposed to $CuSO_4$ (3–24 h) in Dulbecco's modified Eagle medium with or without 10% foetal bovine serum (FBS) and then challenged with vasoconstrictors or vasodilators in the absence of $Cu^{2+}$.

**3** Exposure to 2 $\mu$M $Cu^{2+}$ in the absence of FBS did not modify the response to phenylephrine (PE) or acetylcholine (ACh) in aortic rings incubated for 24 h. Identical exposure in the presence of FBS increased the contractile response to 1 $\mu$M PE by 30% ($P<0.05$) and impaired the relaxant response to 3 $\mu$M ACh or 1 $\mu$M A23187 (ACh, from $65.7\pm7.1$ to $6.2\pm1.1$%, $n=8$; A23187, from $74.6\pm8.2$ to $12.0\pm0.8$%, $n=6$; $P<0.01$ for both). $Cu^{2+}$ exposure did not affect the relaxant response to NO-donors.

**4** Impairment of vasorelaxation appeared 3 h after incubation with 2 $\mu$M $Cu^{2+}$ and required 12 h to attain a steady state. Vasorelaxation to ACh was partially restored by 1 mM tiron (intracellular scavenger of superoxide ions; maximum relaxation $34.2\pm6.4$%, $n=10$, $P<0.01$ *vs* $Cu^{2+}$ alone), whereas catalase, superoxide dismutase or cycloheximide were ineffective.

**5** Twenty-four hour-exposure to 2 $\mu$M $Cu^{2+}$ did not affect endothelium integrity or eNOS expression, and increased the Cu content in arterial rings from $6.8\pm1.1$ to $18.9\pm2.9$ ng mg$^{-1}$ wet weight, $n=8$; $P<0.01$.

**6** Our results show that, in the presence of FBS, prolonged exposure to submicromolar concentrations of $Cu^{2+}$ impaired endothelium-dependent vasorelaxation in aortic rings, probably through an intracellular generation of superoxide ions.
*British Journal of Pharmacology* (2002) **136,** 1185–1193

**Keywords:** Copper; endothelium; nitric oxide; superoxide; eNOS; PECAM-1; tiron

**Abbreviations:** ACh, acetylcholine; CAT, catalase; $Cu^{2+}$, copper; DEANO, 1,1-diethyl-2-hydroxy-2-nitroso-hydrazine sodium; DMEM, Dulbecco's modified Eagle medium; eNOS, endothelial nitric oxide synthase; FBS, foetal bovine serum; ISDN, isosorbide dinitrate; LDH, lattic dehydrogenase; L-NOARG, $N^G$-nitro-L-arginine; NO, nitric oxide; $O_2^-$, superoxide ion; PE, phenylephrine; PECAM-1, platelet/endothelial cell adhesion molecule-1; SOD, superoxide dismutase

## Introduction

The involvement of copper ($Cu^{2+}$) in the pathogenesis of vascular dysfunction has been repeatedly proposed. Elevated serum levels of Cu are associated with atherosclerosis (Ferns *et al*., 1997), myocardial infarction and stroke (Reunanen *et al*., 1996) as well as pregnancy-induced hypertension (Wisdom *et al*., 1991). However, also the decreased availability of $Cu^{2+}$ is related to cardiovascular abnormalities such as cardiac enlargement, coronary artery thrombosis and myocardial infarction (Klevay, 2000). Studies examining the effect of $Cu^{2+}$ on vascular functioning *in vitro*, are also somehow conflicting. For instance, $Cu^{2+}$ reportedly enhances relaxation of rat aorta (Plane *et al*., 1997) and pulmonary artery (Ohnishi *et al*., 1997), probably by activating endothelial nitric oxide (NO) synthase (eNOS) (Demura *et al*., 1998). By contrast, the complex $Cu^{2+}$-ceruloplasmin inhibits acetylcholine (ACh)-induced vasorelaxation in rabbit aorta (Cappelli-Bigazzi *et al*., 1997) and eNOS activity in cultured endothelial cells (Bianchini *et al*., 1999). These contrasting views highlight the complex role of this metal ion in vascular homeostasis and suggest that both increase and decrease of its serum contents lead to vascular disorders.

$Cu^{2+}$ is an essential cofactor of numerous intracellular enzymes and its pharmacokinetic is not entirely understood.

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The absorption kinetics seems to be organ specific. Indeed, after intraperitoneal injection, the concentrations of $Cu^{2+}$ peak at 2 h in the liver, whereas up to 24 h are needed to attain the peak in heart, brain, striated muscle and kidney (Weiss & Linder, 1985). In serum, ceruloplasmin is classically the major $Cu^{2+}$ carrier protein, but other factors such as albumin and amino acids also bind $Cu^{2+}$ and favour its cellular uptake (Huffman & O'Halloran, 2001), leading to free metal concentrations in the order of $10^{-11}$ M. The intracellular uptake of $Cu^{2+}$ is tightly regulated by several chaperone proteins and at least two membrane ATPases (for review see Linder *et al*., 1998; Huffman & O'Halloran, 2001). In analogy with serum, inside the cell there is virtually no free $Cu^{2+}$ and all the metal ions are bound to proteins and amino acids (Rae *et al*., 1999). Thus, on the basis of the very low free concentrations and slow, chaperon-regulated pharmacokinetic of $Cu^{2+}$, we reasoned that the ion impact on vascular functioning *in vitro*, should be studied by adopting experimental paradigms of chronic exposure to low metal concentrations in the presence of appropriate carrier proteins. In this study, therefore, we investigated the effects of long-lasting exposure to micromolar concentrations of $Cu^{2+}$ on rat isolated aorta in the presence of foetal bovine serum (FBS), as a source of $Cu^{2+}$ carrier proteins.

Our results show that submicromolar concentrations of $Cu^{2+}$ reduced the endothelium-dependent relaxant response in rat aorta, without affecting vascular smooth muscle function and endothelium integrity.

## Methods

### *Aorta dissection and incubation*

Aortas from male Sprague–Dawley rats (Stefano Morini, S. Polo D'Enza, Italy; 280–350 g) were rinsed in cold modified Krebs-physiological solution (in mM): NaCl 118, KCl 4.6, $NaHCO_3$ 25, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $CaCl_2$ 2.5, glucose 10, EDTA 0.025, pH 7.4) and cut in rings (3–5 mm diameter). Each ring was then placed in a well of a 24-well plate containing 0.5 ml in Dulbecco's modified Eagle medium (DMEM) containing 100 u $ml^{-1}$ penicillin, 100 $\mu$g $ml^{-1}$ streptomycin. In some experiments foetal bovine serum (FBS, 10%) and different concentrations of $CuSO_4$ were added to DMEM. Aortic rings were incubated at 37°C in a 5% $CO_2$ incubator for a period of 24 h, unless otherwise stated.

### *Measurement of contractile response*

After incubation, arterial rings were suspended in 5 ml organ baths filled with physiological solution, bubbled with 95% $O_2$, 5% $CO_2$, at 37°C under a resting tension of 20 mN. Rings were isometrically connected to an isometric force transducer (Ugo Basile, Comerio Varese, Italy) and allowed to equilibrate for 60 min in physiological solution. A first contraction was induced with a $K^+$-rich depolarizing solution (in mM): NaCl 22.6, KCl 98.8, $NaHCO_3$ 25, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $CaCl_2$ 2.5, glucose 10, EDTA 0.025). After washout and 25 min-recovery in physiological solution, a second contraction was evoked by phenylephrine (PE, 1 $\mu$M) and when the response reached a plateau, relaxation was evoked by ACh (10 nM–10 $\mu$M) or A23187 (1 nM–1 $\mu$M). After washout and 30 min-incubation in the presence of $N^G$-nitro-L-arginine (L-NOARG, 0.1 mM), contractions were evoked again with PE, and relaxations to isosorbide dinitrate (ISDN, 100 nM–100 $\mu$M) and 1,1-diethyl-2-hydroxy-2-nitroso-hydrazine sodium (DEANO, 10 nM–10 $\mu$M) were measured. Relaxations to ACh, A23187, ISDN and DEANO were expressed as per cent of the steady-state contractile tone evoked by PE. Rings were then dried with filter paper, weighed and contractile responses were expressed as mN $mg^{-1}$ wet weight.

### *Measurement of lactate dehydrogenase activity*

Lactate dehydrogenase activity (LDH) in the culture medium was quantified with Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Monza, Italy). Briefly, 50 $\mu$l of medium were combined with reagents in a 96 well plate and the mixture incubated in the dark at room temperature (RT) for 20 min. Optical density was measured at 450 nm using a microplate reader (Dynatech, MR 4000). Optical density of DMEM/10% FBS (blank) was subtracted from all values. Results were normalized according to the wet weight of the tissue and expressed as per cent of control (not exposed to $Cu^{2+}$).

### *Western blot analysis*

Aortic rings were snap frozen in dry ice and stored at −80°C. Rings were then sonicated on ice in 100 $\mu$l of a buffer containing (mM): Tris pH 7.4 50, EDTA 1, EGTA 1, PMSF 1 and 4 $\mu$g $ml^{-1}$ aprotinin and 4 $\mu$g $ml^{-1}$ leupeptin. The suspension was centrifuged at $12{,}000 \times g$ for 10 min at 4°C and an aliquot of the supernatant was used to determine the protein content by the Pierce bicinchoninic acid assay kit according to the manufacturer's instructions. Laemmli buffer containing 100 mM 2-mercaptoethanol was then added to the samples and the mixture boiled for 10 min. Thirty $\mu$g of protein/lane were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE), in 6% acrylamide mini-gels. The separated proteins were transferred to nitrocellulose membranes (Hybond-ECL, Amersham Pharmacia Biotech, Little Chalfont, U.K.) by electroblotting. To estimate the levels of eNOS and $\beta$-actin expression, membranes were blocked by incubating 1 h at RT with phosphate buffered saline (PBS) containing 0.1% Tween-20 and 5% skimmed milk (TPBS/5% milk) and then incubated for 6 h at RT with a polyclonal anti-eNOS or monoclonal anti $\beta$-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) diluted 1 : 1000 in TBPS/5% milk. Membranes were then washed with TPBS (three-times/10 min each) and incubated for 1 h in TPBS/5% milk containing either an horseradish peroxydase conjugated anti-rabbit (eNOS) or an anti-mouse ($\beta$-actin) secondary antibody (1 : 2000). After three washes in TPBS, membranes were incubated for 1 min in a chemiluminescent reagent (ECL protein detection system, Amersham Pharmacia Biotech), and the peroxydase-coated protein bands were visualized on Hyperfilm-ECL (Amersham Pharmacia Biotech) after 1 min exposure. Intensities of eNOS bands were quantified by densitometry using the

NIH image software and then normalized to those of β-actin.

### *Immunohistochemical detection of platelet/endothelial cell adhesion molecule-1 (PECAM-1)*

Aortic rings were washed in PBS and immediately fixed with ethanol (1 h at −20°C). Transversal sections (30 μm) of the rings were cut with a cryostat, mounted on slides and air-dried. Sections were blocked (1 h) with PBS containing 10% horse serum and then incubated (3 h) with anti PECAM-1 polyclonal antibody (Santa Cruz Biotechnology) diluted 1 : 200 in PBS/2% horse serum at RT. After several washes with PBS, sections were incubated (1 h) with rhodamine-labelled anti-rabbit antibody (Jackson Laboratories, West Grove, PA, U.S.A.) diluted 1 : 100 in PBS/2% normal horse serum. After a further wash with PBS, microscopic examination was performed with a Nikon Labophot-2 inverted fluorescence microscope equipped with a rhodamine filter (Ex 520, Em >580). The specificity of the immune-detection assays was established by the omission of the primary antibody.

### *Atomic absorption*

After incubation, intact or endothelium-denuded (by gently scraping the inner surface) aortic rings were immersed 2 min in ice-cold physiological salt solution (composition as above, except for EDTA, 1 mM) to remove $Cu^{2+}$ from the extracellular space, dried on a filter paper and weighted. Dried rings were digested with $HNO_3$ and their Cu contents determined by flameless atomic absorption (Perkin Elmer 5000 spectrometer equipped with furnace Perkin Elmer IIGA 500) and expressed as ng $mg^{-1}$ tissue wet weight.

### *Drugs and chemicals*

PE, ACh, A23187, L-NOARG, ISDN, DEANO, cycloheximide (CXE), homocysteine, superoxide dismutase, catalase and tiron (1,2-dihydroxybenzene-3,5-disulfonic acid) were from Sigma (St. Louis, MO, U.S.A.); DMEM, FBS, penicillin and streptomycin were from Life Technologies (San Giuliano Milanese, Italy).

### *Statistical analysis*

Data are expressed as mean ± standard error of mean (s.e.mean). Statistical significance was determined by unpaired Student's *t*-test or by one-way analysis of variance (ANOVA) followed by Fisher test. *P* values lower than 0.05 were considered statistically significant.

## Results

### *$K^+$- or PE-evoked contraction and ACh-induced relaxation in rat aortic rings after prolonged* in vitro *incubation*

Contractions elicited by 100 mM $K^+$ or 1 μM PE in aortic rings incubated 24 h in DMEM with or without FBS did not differ from those of rings mounted in the organ baths immediately after dissection. Conversely, relaxation of PE-contracted rings to 3 μM ACh was reduced of about 25% ($P<0.01$, Table 1).

### *Effect of $Cu^{2+}$ on endothelium-dependent and independent relaxation of rat aortic rings*

Aortic rings incubated 24 h in DMEM containing 2 μM $Cu^{2+}$ and no FBS contracted to $K^+$ and PE and relaxed to ACh similarly to those incubated in the same medium without $Cu^{2+}$ (Table 1). All further incubations were carried out in the presence of FBS in DMEM. Under this condition, 2 μM $Cu^{2+}$ increased the contractile response to both $K^+$ and PE (although significantly only for the latter) and almost abolished ACh- and A23187-induced relaxations after 24 h incubation (Table 1 and Figure 1). When L-NOARG (100 μM) was included in the bathing physiological solution (30 min before KCl or PE stimulation), the contractile responses to either $K^+$ or PE in control preparations (incubated in the absence of $Cu^{2+}$) reached levels similar to those in $Cu^{2+}$-incubated preparations (control: $K^+$, 1.74 ± 0.18, PE 1.82 ± 0.12; $Cu^{2+}$: $K^+$, 1.68 ± 0.22, PE 1.76 ± 0.15; $n=6$ for both groups).

The minimum concentration of $Cu^{2+}$ able to inhibit the relaxation to ACh or A23187 was 600 nM. Conversely, the endothelium-independent relaxations to ISDN or DEANO were unaffected in rings exposed 24 h to 2 μM $Cu^{2+}$ (Figure 1). The inhibition of the vasorelaxant response to ACh by 2 μM $Cu^{2+}$ was present after 3 h-incubation, increased over time up to 12 h (Figure 2) and then reached the plateau (not shown). To investigate whether protein synthesis was involved in determining the effect of $Cu^{2+}$, aortic segments were exposed 24 h to the metal ion in the presence of 10 μM cycloheximide (CXE). This protein synthesis inhibitor did not affect the relaxation induced by 3 μM ACh (control, 66.6 ± 5.5%, $n=4$; CXE, 64.2 ± 8.4%, $n=4$) or its inhibition by 2 μM $Cu^{2+}$ ($Cu^{2+}$, 4.2 ± 2.0%, $n=4$; CXE + $Cu^{2+}$, 3.7 ± 1.1%, $n=4$).

**Table 1** Contractile response to KCl and phenylephrine (PE), and relaxant response to acetylcholine (ACh) in rat aortic rings mounted in organ baths after dissection (not incubated) or after 24 h incubation in DMEM with or without 10% FBS and 2 μM $Cu^{2+}$

| | *Contractile response* (mN $mg^{-1}$) | | *Relaxant response* (%) |
|---|---|---|---|
| | *KCl* (100 mM) | *PE* (1 μM) | *ACh* (3 μM) |
| *Not incubated* ($n=8$) | 1.69 ± 0.26 | 1.36 ± 0.21 | 91.9 ± 1.7 |
| *Incubated* | | | |
| −FBS, $-Cu^{2+}$ ($n=8$) | 1.55 ± 0.14 | 1.25 ± 0.13 | 64.3 ± 6.5** |
| −FBS, $+Cu^{2+}$ ($n=8$) | 1.50 ± 0.13 | 1.21 ± 0.08 | 61.5 ± 6.8** |
| +FBS, $-Cu^{2+}$ ($n=8$) | 1.62 ± 0.10 | 1.19 ± 0.11 | 65.7 ± 7.1** |
| +FBS, $+Cu^{2+}$ ($n=8$) | 1.73 ± 0.20 | 1.86 ± 0.09† | 4.8 ± 2.1**†† |

The relaxant response was assessed by adding 3 μM ACh on the contraction plateau evoked by 1 μM PE. **$P<0.01$ *vs* not incubated; †$P<0.05$, ††$P<0.01$ *vs* incubated with FBS, without $Cu^{2+}$; one-way ANOVA and Fisher test.

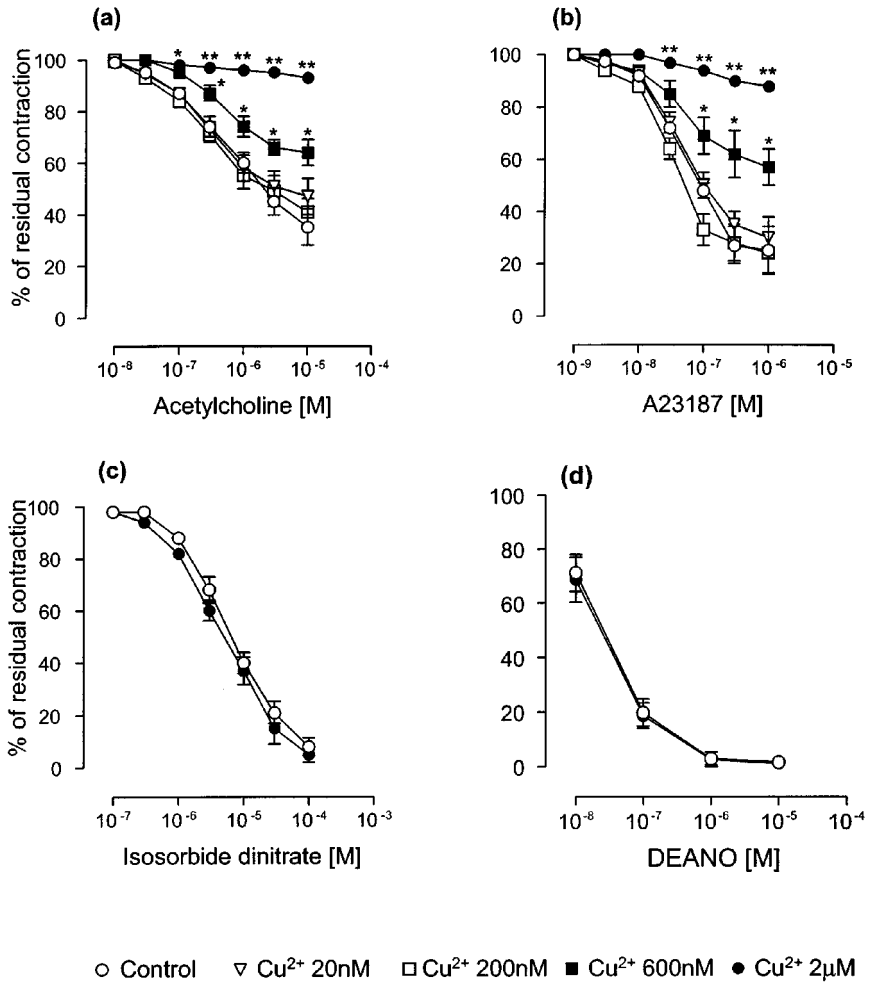


**Figure 1** Effect of Copper on endothelium-dependent vasodilatation. Relaxation to acetylcholine (a), A23187 (b), isosorbide dinitrate (c), and DEANO (d) in rat aortic rings bathed in Krebs physiological solution, after 24 h-incubation in Dulbecco's modified Eagle medium containing 10% foetal bovine serum and different concentrations of $Cu^{2+}$. The effect of vasodilators was evaluated in rings contracted with 1 $\mu$M phenyephrine. Relaxations to isosorbide dinitrate and DEANO have been obtained after 30 min incubation in the presence of $N^G$-L-nitroarginine. Each curve represents the mean$\pm$standard error of 6–8 different preparations. $^*P<0.05$; $^{**}P<0.01$ *vs* control (one-way ANOVA and Fisher test).

$Cu^{2+}$ is known to catalyse the formation of superoxide ion ($O_2^-$) and hydrogen peroxide either directly or through an interaction with homocysteine (Starkebaum & Harland, 1986; Loscalzo, 1996). We therefore added superoxide dismutase (SOD) or catalase (CAT) (scavengers of $O_2^-$ and hydrogen peroxide, respectively) in the incubation medium during $Cu^{2+}$ exposure. 150 u ml$^{-1}$ of superoxide dismutase or 1200 u ml$^{-1}$ of catalase did not prevent the effect of $Cu^{2+}$ on ACh-evoked relaxation (relaxation to 3 $\mu$M ACh: control, 58.5$\pm$7.5%; 2 $\mu$M $Cu^{2+}$+SOD, 4.9$\pm$2.7%; 2 $\mu$M $Cu^{2+}$+CAT, 3.4$\pm$1.4%; $n=6$ for all groups, $P<0.01$ for both SOD and CAT *vs* control). Conversely, 1 mM homocysteine and 10 $\mu$M diethyldithiocarbamate completely prevented the effect of the metal ion (relaxation to 3 $\mu$M ACh: 57.9$\pm$7% control, 64.4$\pm$7.6% homocysteine, 66.2$\pm$7.2% diethyldithiocarbamate, $n=6$ for all groups). Since both superoxide dismutase and catalase do not easily penetrate into the cell, we then incubated the aortic rings with $Cu^{2+}$ in the presence of tiron, a cell permeant $O_2^-$ scavenger (Mohazzab *et al*., 1994). As shown in Figure 3, 1 mM tiron restored in part the relaxant response to ACh (maximal relaxation to ACh: control, 70.8$\pm$6.9%; $Cu^{2+}$, 5.7$\pm$1.1%; tiron+$Cu^{2+}$, 34.2$\pm$6.4%; $n=10$ for all groups; $P<0.01$ for tiron *vs* control and *vs* $Cu^{2+}$). To establish whether the impaired vasorelaxant response was specific to $Cu^{2+}$ or shared by other divalent transition metals, we tested the effect of 24 h-exposure to iron ($Fe^{2+}$, 2 $\mu$M) and zinc ($Zn^{2+}$, 2 $\mu$M). A 24 h-incubation with these transition metals, however, did not change the relaxant response to ACh (response to 3 $\mu$M ACh: 64.8$\pm$8.1% control, 63.3$\pm$9.9% $Fe^{2+}$ and 64.0$\pm$7.3% $Zn^{2+}$, $n=6$ for all groups).

### *Effect of $Cu^{2+}$ on eNOS expression in the rat aortic rings*

In accordance with the molecular weight of eNOS, a band of approximately 130 kDa was recognised in lysates of both 24 h-incubated aortic rings and human umbilical vein endothelial cells (HUVEC). Notably, the presence of 2–20 $\mu$M $Cu^{2+}$ during the incubation did not affect eNOS expression, whereas a significant reduction in expression was found in aortic rings incubated with 200 $\mu$M $Cu^{2+}$ (Figure 4).

*Effect of $Cu^{2+}$ on cytotoxicity and structural integrity of aortic rings*

Two $\mu$M $Cu^{2+}$ did not increase LDH release from aortic rings as compared to control conditions after 24 h-exposure. However, a significant increase of LDH activity was found in those media containing 20–200 $\mu$M $Cu^{2+}$ (Figure 5).

As a parameter of endothelial integrity of the aortic rings, we evaluated immunoreactivity of PECAM-1, an adhesion molecule typically expressed by endothelial cells (Newman, 1997). In the lumen of aortic rings incubated 24 h in the presence or absence of 2 $\mu$M $Cu^{2+}$, fluorescence depicted an intact endothelium (Figure 6a–d). By contrast, at 20 $\mu$M the metal ion induced numerous endothelial ruptures (Figure 6e,f) and at 200 $\mu$M a detachment of the endothelium from the vessel wall as well as an almost complete loss of its integrity was found (Figure 6g,h).

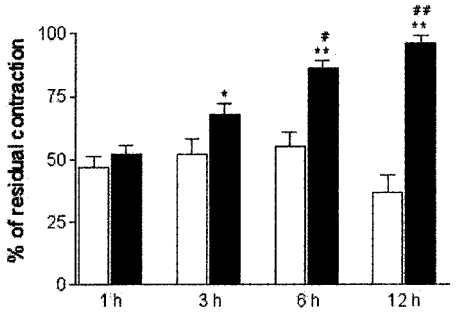


**Figure 2** Time course of the effect of copper on endothelium-dependent vasodilatation. Preparations were incubated in Dulbecco's modified Eagle medium containing 10% foetal bovine serum, with (black columns) or without (white columns) 2 $\mu$M $Cu^{2+}$ for 1, 3, 6 or 12 h. Thereafter, the preparations were mounted in organ baths containing Krebs physiological solution, and were contracted with 1 $\mu$M phenylephrine before receiving 3 $\mu$M ACh. Relaxations to ACh are expressed as per cent of the contractile tone evoked by phenylephrine. Each column represents the mean±standard error of six different preparations. $*P<0.05$, $**P<0.01$ *vs* preparations incubated without 2 $\mu$M $Cu^{2+}$; $\#P<0.05$, $\#\#P<0.01$ *vs* preparations incubated with 2 $\mu$M $Cu^{2+}$ for 3 h (one-way ANOVA and Fisher test).

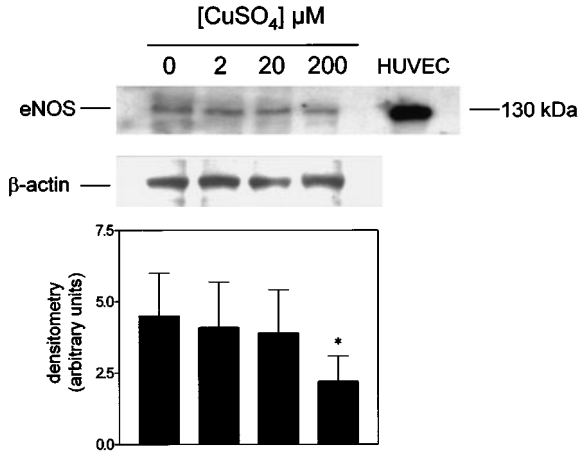


**Figure 4** Western-blot determination of eNOS expression in rat aortic rings incubated with different concentrations of copper. The aortic rings were incubated for 24 h with (2–200 $\mu$M) or without $Cu^{2+}$. Later on, the rings were homogenized and 30 $\mu$g protein/lane were loaded and subjected to SDS–PAGE. After transfer onto nitrocellulose membranes, eNOS and $\beta$-actin were revealed with a polyclonal and monoclonal antibody, respectively. Note that the 130 kDa band present in ring lysates co-migrated with that present in a whole homogenate of cultured human umbilical vein endothelial cells (HUVEC). As shown in the lower part of the figure, the ratio between the densitometry of eNOS and $\beta$-actin has been evaluated to normalize for the protein loading. Note that eNOS expression is reduced by the incubation with 200 $\mu$ $Cu^{2+}$. One blot representative of three is shown. Each column represents the mean±standard error from the three experiments. $*P<0.05$ *vs* preparations incubated without $Cu^{2+}$ (one-way ANOVA and Fisher test).

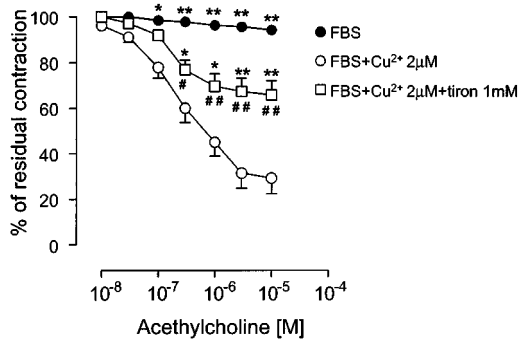


**Figure 3** Effect of tiron on copper-induced impairment of endothelium-dependent vasodilatation. Preparations were incubated for 24 h in Dulbecco's modified Eagle medium containing 10% foetal bovine serum (FBS), FBS and 2 $\mu$M $Cu^{2+}$ and FBS, 2 $\mu$M $Cu^{2+}$ and 1 mM tiron. Thereafter, relaxation to cumulative concentrations of acetylcholine was determined in preparations bathed in Krebs physiological solution. Each curve represents the mean±standard error of 10 different preparations. $*P<0.05$, $**P<0.01$ *vs* control; $\#P<0.05$, $\#\#P<0.01$ *vs* 2 $\mu$M $Cu^{2+}$ (one-way ANOVA and Fisher test).

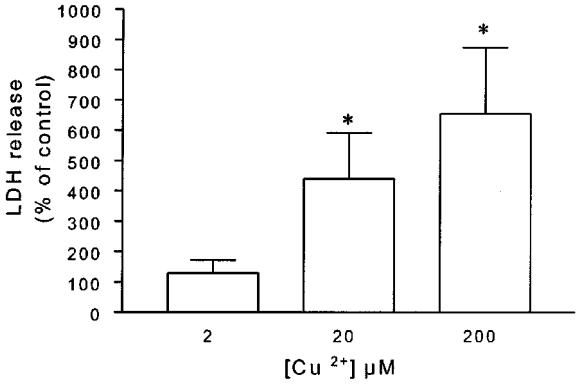


**Figure 5** LDH release from aortic rings incubated with different concentrations of copper. The aortic rings were incubated for 24 h with concentrations of $Cu^{2+}$ ranging from 2 to 200 $\mu$M. Thereafter, LDH activity was quantified in the incubating media and expressed as per cent of controls (no $Cu^{2+}$; see Methods). Note that the incubation with 2 $\mu$M $Cu^{2+}$ did not increase LDH release with respect to control conditions. Conversely, a significant increase of LDH was caused by the incubation with 20 and 200 $\mu$M $Cu^{2+}$. Each bar represents the mean±standard error of three experiments conducted in duplicate. $*P<0.05$ *vs* control (one-way ANOVA and Fisher test).

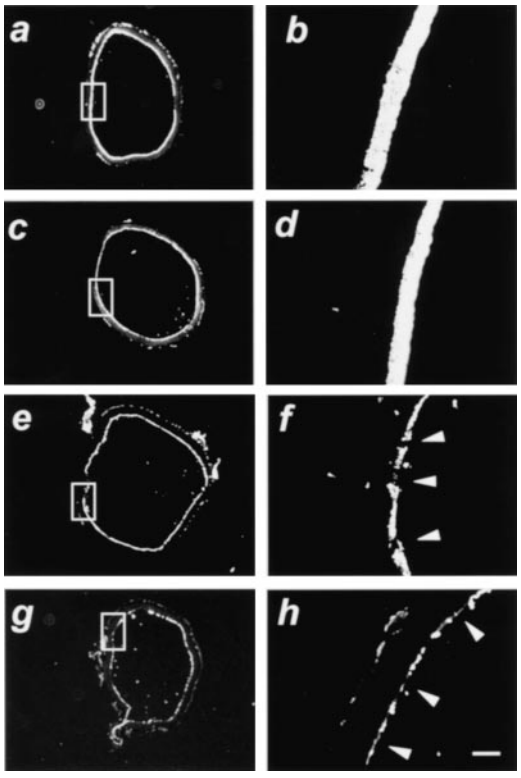


**Figure 6** PECAM-1 immunoreactivity in rat aortic rings exposed to different concentrations of copper. The immunoreactivity of PECAM-1, an adhesion molecule typically expressed by endothelial cells, was evaluated by immunofluorescence in aortic rings incubated for 24 h with different concentrations (2–200 $\mu$M) of $Cu^{2+}$. (a,b) Control, (c,d) 2 $\mu$M $Cu^{2+}$, (e,f) 20 $\mu$M $Cu^{2+}$, (g,h) 200 $\mu$M $Cu^{2+}$. Under control conditions (a), PECAM-1 immunofluorescence is mainly localized in the lumen. A higher magnification (b) shows the integrity of the endothelium (the preserved connections among the endothelial cells is also evident; note that, for a better evaluation of the endothelial surface, the aortic rings have been cut into 30 $\mu$m sections and the endothelium appears of an abnormal thickness). In aortic rings incubated with 2 $\mu$M $Cu^{2+}$ (c,d) the endothelium appears totally preserved. Conversely, 20 $\mu$M $Cu^{2+}$ alters the homogeneous distribution of the immunoreactivity (e) showing a widespread endothelial damage (arrowheads, f). 200 $\mu$M $Cu^{2+}$ causes a detachment of the endothelium from the vessel wall (g) and an almost complete loss of its integrity (arrowheads, h). Scale bar = 500 $\mu$m (a,c,e,g), 80 $\mu$m (b,d,f,h). One experiment representative of three is shown.

### *Cu uptake in aortic rings*

Cu was undetectable in DMEM, whereas in DMEM containing 10% FBS the concentrations of Cu raised up to 250 nM, 2.4 times lower than the threshold concentration (600 nM) found effective in reducing endothelium-dependent relaxation. Aortic rings incubated 24 h in DMEM had a Cu content of $6.8 \pm 1.1$ ng $mg^{-1}$ tissue. The presence of 2 $\mu$M $Cu^{2+}$ in DMEM led to a 3 fold increase of the metal content in the aortic rings, which did not change when they were incubated in DMEM containing 10% FBS or in the absence of endothelium (Table 2).

**Table 2** Effect of 24 h incubation with 2 $\mu$M $Cu^{2+}$ on Cu contents in rat aortic rings

| | *Endothelium* | *Cu content* (ng $mg^{-1}$ tissue wet weight) |
|---|---|---|
| Control (+FBS) ($n=8$) | Present | $6.8 \pm 1.1$ |
| 2 $\mu$M $Cu^{2+}$ (−FBS) ($n=8$) | Present | $20.2 \pm 3.7$** |
| 2 $\mu$M $Cu^{2+}$ (+FBS) ($n=8$) | Present | $18.9 \pm 2.9$** |
| 2 $\mu$M $Cu^{2+}$ (−FBS) ($n=8$) | Absent | $24.7 \pm 4.5$** |
| 2 $\mu$M $Cu^{2+}$ (+FBS) ($n=8$) | Absent | $20.1 \pm 3.2$** |

The Cu content in rat aortic rings were evaluated by atomic absorption as described in Methods. $^{**}P<0.01$ *vs* control; one-way ANOVA.

## Discussion

The present study shows that prolonged incubation of aortic rings with submicromolar concentrations of $Cu^{2+}$ impaired the relaxing response to ACh or A23187 and did not affect those elicited by the NO-donors ISDN and DEANO. Since relaxation of rat aorta to either ACh or A23187 entirely relies on NO release (Vargas *et al*., 1991) our findings suggest that $Cu^{2+}$ treatment selectively affected NO-dependent vasorelaxation. Furthermore, considering that the contractile response to PE was increased by the exposure to the metal ion, we reason that $Cu^{2+}$ also impaired basal NO release. This latter hypothesis is reinforced by the finding that, in the presence of L-NOARG, the contractile response to PE in control preparations (not incubated with $Cu^{2+}$) was enhanced up to the level of that in $Cu^{2+}$-incubated preparations. The evidence that homocysteine and diethyldithiocarbamate, two powerful $Cu^{2+}$ chelators (Agar *et al*., 1991; Smith & Reed, 1992), completely prevented the effect of the metal ion, indicates that the impairment of vasorelaxation was indeed mediated by $Cu^{2+}$.

In accordance with these results, it has been reported that when complexed with ceruloplasmin, $Cu^{2+}$ impairs the endothelium-dependent relaxation in isolated rabbit aorta (Cappelli-Bigazzi *et al*., 1997), an effect not reproduced by $Cu^{2+}$ salts, apoceruloplasmin or heat-denatured ceruloplasmin. Ceruloplasmin and other serum proteins along with intracellular chaperones finely regulate $Cu^{2+}$ uptake and its incorporation into prosthetic groups of several enzymes (Campbell *et al*., 1981; Percival & Harris, 1989; Harris, 1993; Culotta *et al*., 1997; Pufahl *et al*., 1997). In this regard, it is worth noting that in our model the presence of FBS in the incubation medium was a prerequisite for the effect of $Cu^{2+}$ on vascular functioning. Based on the importance of serum factors for $Cu^{2+}$ uptake, we reason that in our model FBS acted as a source of carriers that assisted the bioavailability of $Cu^{2+}$ to the endothelium. In keeping with our interpretation, Bianchini *et al*. (1999) report that eNOS is inhibited by $Cu^{2+}$ only when the metal is targeted to the cytoplasmic compartment by carriers typically present in the serum such as ceruloplasmin or histidine. In addition to ceruloplasmin and histidine, albumin is another important $Cu^{2+}$ transporter present in serum. Although we did not identify the serum component(s) promoting the endothelial effect of $Cu^{2+}$, we advance the following speculations. (i) The presence of 200 $\mu$M histidine in DMEM (a concentration almost double than that present in the serum) rules out that the amino acid *per se* was the active factor of FBS. However,

histidine directly interacts with albumin and induces the protein to release $Cu^{2+}$ as a histidine-$Cu^{2+}$ complex (Sarkar, 1980), suggesting that interplay between serum albumin and histidine could assist endothelial $Cu^{2+}$ uptake. (ii) Ceruloplasmin is another serum component that might have mediated the effect of FBS. Interestingly, maximum $Cu^{2+}$ delivery by ceruloplasmin occurs in the presence of ascorbate (Percival & Harris, 1989), this latter being present in serum but not in DMEM. Finally, the evidence that there is no difference in cellular $Cu^{2+}$ uptake when the metal ion is vehiculated by albumin, ceruloplasmin or histidine (Mas & Sarkar, 1992), suggests that FBS could represent a pool of interchangeable transporters acting in concert to assist endothelial $Cu^{2+}$ absorption.

Although our findings point to a role of FBS in $Cu^{2+}$ uptake by aortic rings, we were unable to detect a difference in the contents of the metal ion in aortic rings incubated in the presence or absence of FBS, leaving open the possibility that FBS regulated the effect of $Cu^{2+}$ through mechanism(s) unrelated to endothelial cell uptake. However, since the Cu content in aortic rings was unaffected by endothelium removal and functional data clearly indicate that $Cu^{2+}$ targeted endothelial cells, we infer that the amount of $Cu^{2+}$ contained in the endothelium was negligible when compared to that present in the entire vessel wall. Regardless of its quantitative aspects, the present findings underscore the significance of $Cu^{2+}$ in the endothelial functions.

Previous reports demonstrate that divalent cations such as $Mg^{2+}$, $Zn^{2+}$ and $Cu^{2+}$ itself inhibit NOS activity, probably by direct interaction with the enzyme and/or formation of reactive radicals (Howard *et al*., 1995; Persechini *et al*., 1995; Tian & Lawrence, 1996). However, $Zn^{2+}$ or $Fe^{2+}$ did not reproduce the effects of $Cu^{2+}$ in our model, suggesting different endothelial actions among the divalent cations, and a lack of involvement of the transition metal-dependent Haber–Weiss reaction in the vascular effect of $Cu^{2+}$. Nevertheless, the finding that tiron, a $O_2^-$ scavenger (Mohazzab *et al*., 1994), significantly reduced the effect of $Cu^{2+}$ indicates that $O_2^-$ was in part responsible for impairment of endothelial function by the metal ion. Although we cannot rule out a possible direct binding of $Cu^{2+}$ by tiron as an additional protective mechanism, this seems unlikely; indeed, strong intracellular $Cu^{2+}$ chelation, such that obtainable with millimolar concentrations of diethyldithiocarbamate, has been reported to inactivate SOD, leading to further intracellular generation of superoxide and endothelial dysfunction (Pagano *et al*., 1999). Our finding, along with the inefficacy of SOD, a cell impermeant $O_2^-$ scavenger, in reducing the effect of $Cu^{2+}$, indicates that the formation of $O_2^-$ ions in endothelial cells could be causative in the $Cu^{2+}$-induced impairment of vasodilatation. Taken as a whole, our results indicate that excessive production of $O_2^-$ triggered by low concentrations (0.6–2 $\mu$M) of $Cu^{2+}$ (Miller *et al*., 1990) resulted in altered NO activity and endothelial dysfunction. The evidence that the concentration of tiron used in the present study reduces NO scavenging by intracellular $O_2^-$ in endothelial cells (Zhang *et al*., 2001) is in keeping with our interpretation. However, although we did not obtain evidence of structural damage by 2 $\mu$M $Cu^{2+}$, we can not exclude the possibility that our measures of endothelial cell integrity may not detect small changes affecting endothelial function. As suggested by Western blotting, LDH release and PECAM immunohistochemistry, at 20–200 $\mu$M $Cu^{2+}$ led to higher cytotoxicity, presumably related to higher concentrations of intracellular $O_2^-$ (and other oxygen radicals). Notably, $O_2^-$ interacts with NO in endothelial cells, leading to the formation of toxic intermediates including peroxynitrite (Beckman *et al*., 1990).

In striking contrast with the present study, previous reports demonstrate that $Cu^{2+}$ induces endothelium-dependent vasorelaxation (Ohnishi *et al*., 1997; Plane *et al*., 1997). However, this effect occurs in the presence of $Cu^{2+}$ in the incubation media, under acute exposure paradigms and at concentrations two orders of magnitude higher than those we report here inhibiting the endothelium-dependent relaxation.

The evidence that tiron prevented only partially the $Cu^{2+}$ effects suggests that, in addition to oxidative stress, other mechanisms could participate to the impairment of vasorelaxation. Among these, it is possible that trapping and inactivation of NO by $Cu^{2+}$ contained in the prostetic group of enzymes such as cytochrome $b_0$ (Butler *et al*., 1997) and cytochrome *c* oxidase (Cooper *et al*., 1997; Cooper, 2002) might have contributed to the metal ion's effect. Interaction and ensuing inactivation between $Cu^{2+}$ and non-identified co-factor(s) essential for endothelial functioning is also possible. By contrast, we rule out the possibility that the $Cu^{2+}$-induced low-density lipoprotein oxidation induced endothelial dysfunction (Heinecke *et al*., 1984; Wagner & Heinecke, 1997; Tanner *et al*., 1991). In fact, in our system the final concentration of LDL approximated 0.3 $\mu$g ml$^{-1}$ (Quinn, Life Technologies, personal communication), which is 300–3000 times lower than those reported to affect endothelial functions (Tanner *et al*., 1991; Cox & Cohen, 1996; Hein & Kuo, 1998; Zhao & Tackett, 1998). Finally, despite 2 $\mu$M $Cu^{2+}$ impaired vasorelaxation only after long lasting exposure, gene expression and protein synthesis were apparently not involved, as suggested by both the inefficacy of CXE in reducing the effect of the metal ion and the unaffected eNOS expression in the aortic rings.

In conclusion, we show that prolonged incubation with submicromolar concentrations of $Cu^{2+}$ selectively impaired endothelium-dependent vasorelaxation probably because of intracellular generation of $O_2^-$. These findings suggest that slight increases of the serum levels of $Cu^{2+}$ could have profound pathophysiological outcomes. In this regard, it is worth of mention that high serum copper concentrations are significantly associated with an increased mortality from all cardiovascular diseases and from coronary heart disease in particular (Reunanen *et al*., 1996; Ford, 2000). These findings, along with the relationship between hypercupremia and pathologies such as hypertensive states, atherosclerosis, myocardial infarction and stroke (Reunanen *et al*., 1996; Ferns *et al*., 1997), point to the increased blood levels of $Cu^{2+}$ as a risk factor for cardiovascular mortality and underscore the pathophysiological significance of our findings. The present study, therefore, furthers our understanding of the mechanisms by which subtle alterations in $Cu^{2+}$ homeostasis may affect vascular functions, and suggests that regulation of the blood concentrations of $Cu^{2+}$ may be a potential therapeutic target for prevention and treatment of cardiovascular diseases.

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*(Received March 22, 2002*
*Revised May 13, 2002*
*Accepted May 30, 2002)*