British Journal of Pharmacology (1998) 125, 1180–1187
http://www.stockton-press.co.uk/bjp

The activation of nitric oxide synthase by copper ion is mediated by intracellular Ca^{2+} mobilization in human pulmonary arterial endothelial cells

[1,3]Yoshiki Demura, [1]Takeshi Ishizaki, [1]Shingo Ameshima, [1]Seitarou Okamura, [1]Takio Hayashi, [2]Shigeru Matsukawa & [1]Isamu Miyamori

[1]Third Department of Internal Medicine, [2]Central Research Laboratories, Fukui Medical University, Matsuoka-cho, Yoshida-gun, Fukui 910-11, Japan

1 The aim of the study was to elucidate the vasodilatory mechanism due to Cu^{2+} by assessing nitric oxide (NO) production as determined by NOx (NO, NO_2^-, and NO_3^-) that is released from human pulmonary arterial endothelial cell (HPAEC) monolayers using a NO chemiluminescence analyzer, and also to assess Ca^{2+} movement using ^{45}Ca and fura 2 in HPAEC.

2 Cu^{2+} (10^{-6}–10^{-4} M) significantly increased NO production in a dose-dependent manner when extracellular Ca^{2+} was present.

3 ^{45}Ca influx into the adherent cells was dose-dependently enhanced by Cu^{2+} (10^{-6}–10^{-4} M), but not by Mn^{2+}, Zn^{2+} or Fe^{2+}.

4 $[Ca^{2+}]_i$, measured by monitoring the fluorescence changes of fura 2, was significantly elevated in the presence of Cu^{2+}.

5 The increase in $[Ca^{2+}]_i$ induced by Cu^{2+} was inhibited by either diethyldithiocarbamate (DDC) or the depletion of extracellular Ca^{2+}.

6 The dihydropyridine receptor agonist, BayK8644, significantly attenuated the Cu^{2+}-induced increase in $[Ca^{2+}]_i$ in a dose dependent manner and nitrendipine or nifedipine, the dihydropyridine receptor antagonists, dose-dependently inhibited a Cu^{2+}-induced increase in $[Ca^{2+}]_i$.

7 These results suggest that Cu^{2+} activates eNOS through the mechanism of $[Ca^{2+}]_i$ elevation due to Ca^{2+} influx into HPAEC and that the Cu^{2+}-induced $[Ca^{2+}]_i$ elevation in HPAEC is likely due to activation of the dihydropyridine-like receptors.

Keywords: Copper; nitric oxide; nitric oxide synthase; calcium mobilization; endothelial cell; dihydropyridine

Introduction

Essential trace elements such as copper, zinc and manganese take part in various enzyme reactions necessary for the antioxidant defense system in cells. It is not clear whether metabolism of these trace elements and related enzymes are specifically altered in hypertensive animals and humans. In hypertensive rats, the levels of copper in tissues such as the liver, kidney and heart decreased while serum copper reciprocally increased (Clegg *et al*., 1987). Klevay (1987) found that adult rats developed hypertension after being fed a copper-deficient diet. It has been reported that dietary copper deficiency reduces the relaxation responses to acetylcholine, histamine, or sodium nitroprusside in rat aortic rings probably by disturbing the interaction between the endothelium-derived relaxing factor (EDRF) and smooth muscle (Saari, 1992). Further, it has been suggested that the inhibition of vasodilatation associated with copper deficiency could involve a cyclic GMP mediated mechanism. Therefore, the effect of Cu^{2+} on EDRF may suggest a regulatory role for trace elements in the vascular tone. In a previous study (Gryglewski *et al*., 1986), superoxide dismutase (SOD) and Cu^{2+} were reported to cause relaxation of the rabbit aorta, suggesting that Cu^{2+} like Cu-, Zn-SOD dismutes superoxide anion (O_2^-), and/or prolongs the half life of EDRF as manganese ion (Mn^{2+}) does (Kasten *et al*., 1994). Ohnishi *et al*. (1997) reported that Cu^{2+} elevated the NO level in rat pulmonary arterial rings not by prolonging the half life of NO, but by activating endothelial nitric oxide synthase. It has been conceptualized that the major pathway for NO production involves Ca^{2+} mobilization which results in elevating intracellular free Ca^{2+} and an activation of NOS (Buckley *et al*., 1995), and that the increased NO production in endothelial cells coincides with the elevation of $[Ca^{2+}]_i$. The mechanism of eNOS activation by Cu^{2+} in pulmonary arterial endothelial cells proposed by Ohnishi *et al*. (1997), however, needs to be further clarified. In the present study, we examined the effect of Cu^{2+} on NO production and the intracellular Ca^{2+} level by measuring ^{45}Ca uptake, and on the dynamism of the intracellular free Ca^{2+} level using fura 2-AM and on the prostaglandine metabolite (since prostaglandine is also calcium-dependently produced) in cultured human pulmonary arterial endothelial cells (HPAEC). Herein, we report that Cu^{2+} (10^{-6}–10^{-4} M) dose-dependently enhances NO production due to an increase in intracellular free Ca^{2+} concentration by causing Ca^{2+} influx into HPAEC through the mechanism in association with dihydropyridine. We also refer to the physiological role of Cu^{2+} in activating eNOS and relaxing the rat pulmonary arterial ring, the mechanisms involved being different from those in Mn^{2+}.

Methods

Cell culture

Human pulmonary arterial endothelial cells (HPAEC) from an established cell line (Endocell-PA) and E-GM u.v. medium

[3] Author for correspondence.

were obtained from Kurabou Ltd. (Osaka, Japan). HPAEC were grown in E-GM UV medium containing 2% foetal bovine serum, 50 μg ml^{-1} gentamycin, 50 ng ml^{-1} amphotericin-B, 10 ng ml^{-1} recombinant human EGF, 1 mg ml^{-1} hydrocortisone, and 0.4% bovine brain extract (BBE). Cells were cultured in 75 cm^2 tissue culture flasks (Corning, NY, U.S.) and used upon reaching confluence at the fifth to tenth passage, after trypsinization.

Measurement of NOx concentration from HPAEC

For quantification of the NO produced by cells grown in monolayers, cells in T-25 flasks were washed three times with Krebs-Henseleit buffer and incubated in the same buffer in 5% CO_2 at 37°C. Cells were stimulated with Cu^{2+} (10^{-7}–10^{-4} M) or thapsigargin (1 μM) in 2 ml Krebs-Henseleit buffer containing 1 mM L-arginine, pH 7.4, for 5 min. Then, 1 ml of aliquot was collected and assayed for NOx (nitric oxide, nitrite and nitrate) using a catalytic method for the reduction of oxidation products of NO to NO gas. The samples were injected into a refluxing glass reaction chamber containing vanadium (III)-HCl (2 N) at 85°C and NO gas was detected by a NO chemiluminescence analyzer (FES-450 NO analyzer, Scholartec. Osaka) (Ishizaki *et al*., 1995; Whorton *et al*., 1997). Mn^{2+}, Zn^{2+}, Fe^{2+} (10^{-4} M) were also examined in the same fashion. The protein concentration of the cells per flask was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

$^{45}Ca^{2+}$ uptake in HPAEC

$^{45}Ca^{2+}$ uptake measurements were performed by the method of Galizzi *et al*. (1987). Cells were suspended in E-GM u.v. medium to a density of 2×10^5ml^{-1}, and 1 ml of the cell suspension was added to each well of a 12-well flat-bottom culture plate (Corning, NY, U.S.A.). After 24 h, almost all of the cells were adhered to the bottoms, having reached confluency. The adherent cells were washed twice with 1 ml of buffer A: 10 mM HEPES buffer, pH 7.4, containing 135 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM glucose at 37°C, and were then preincubated for 15 min in the same buffer at 37°C. To initiate $^{45}Ca^{2+}$ uptake, the preincubation solution was replaced by 500 μl of buffer A containing 1.5 μCi^{45} Ca^{2+} with or without metal ion – either Cu^{2+}, Mn^{2+}, Zn^{2+} or Fe^{2+}. A low Ca^{2+} concentration (0.1 mM) was used to increase the radioactive $^{45}Ca^{2+}$ incorporation into the cells because Ca^{2+} and $^{45}Ca^{2+}$ were introduced into the cells proportionally as described previously. At the indicated time, the incubation was terminated by aspirating the $^{45}Ca^{2+}$ uptake solution and washing each well three times with 1 ml of ice-cold buffer A containing 3 mM $LaCl_3$. The cells were solubilized with 500 μl of 0.2% (wt/vol) SDS, and an aliquot of the SDS lysate was measured for ^{45}Ca radioactivity. The protein concentration of the cells per well was determined by the Bradford method.

Ca^{2+} uptake in the cells was calculated as follows:

$$Ca^{2+}\ \text{uptake} = [^{45}Ca^{2+}]_i \times [Ca^{2+}]_o / [^{45}Ca^{2+}]_o$$

In this assay, $[^{45}Ca^{2+}]_i$ is the radioactivity of $^{45}Ca^{2+}$ retained in the cells, $[^{45}Ca^{2+}]_o$ is the radioactivity in the incubation solution, and $[Ca^{2+}]_o$ is the amount of nonradioactive Ca^{2+} in the incubation solution (0.1 mM) (Hayashi *et al*., 1991). Nifedipine (10 μM) was used for a 20 min pretreatment, and the effect of the drug on Cu^{2+}-induced $^{45}Ca^{2+}$ uptake was investigated.

Measurement of $[Ca^{2+}]_i$ in HPAEC by Fura 2 method

$[Ca^{2+}]_i$ levels were monitored by measuring the fluorescence of fura 2 (Colden-Stanfield *et al*., 1987). Cells were suspended in E-GM u.v. medium containing 4 μM fura 2-AM and incubated for 30 min at 37°C. After that, the cells were centrifuged, washed and then incubated in E-GM u.v. medium without fura 2-AM for 20 min. The cells were then centrifuged, washed with 20 ml of PBS and resuspended in Krebs/HEPES buffer (130 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 11.5 mM glucose, 10 mM HEPES adjusted to pH 7.4) or in Ca^{2+} free-Krebs/HEPES buffer (130 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 11.5 mM glucose, 10 mM HEPES adjusted to pH 7.4) to a density of 2×10^6 ml^{-1}. An aliquot (500 μl) of the cell suspension in a cylindrical glass cuvette (7 mm ø × 50 mm; MC Medical, Tokyo, Japan) was warmed at 37°C by circulation of thermostated water and the cells were kept in suspension by a magnetic stirrer. The ratio of fluorescence at an emission wavelength of 490 nm of cells alternately excited at 340 nm and 380 nm was continuously monitored on a fluorescence spectrophotometer (RF5000; Shimadzu, Kyoto, Japan) and was used to calculate $[Ca^{2+}]_i$ (Grynkiewicz *et al*., 1985). The metal ion – either Cu^{2+}, Mn^{2+}, Zn^{2+} or Fe^{2+}, or drugs – thapsigargin (1 μM) or (±)BayK8644 (1–10 μM) were added and the effects of these metal ions or drugs on $[Ca^{2+}]_i$ were evaluated. Further, the cell suspensions were preincubated at 37°C with thapsigargin (1 μM) or (±)BayK8644 (1–10 μM) for 15 min. Then the cells were stimulated with Cu^{2+} (10^{-6}–10^{-4} M). Dihydropyridine, nitrendipine or nifedipine (1–10 μM), were also used for a 20 min pretreatment (after 20 min, basal $[Ca^{2+}]_i$ levels were not changed), and the effect of these drugs on Cu^{2+}-induced $[Ca^{2+}]_i$ changes were investigated.

Measurement of prostaglandin metabolite in HPAEC

6-keto prostaglandin F 1α (6-keto PGF 1α), a prostaglandin metabolites was measured in the presence/absence of Cu^{2+} in HPAEC. Cells were incubated either with the control vehicle or Cu^{2+} (10^{-4} M) in Krebs-Henseleit buffer at 37°C for 20 min. Then the cells were homogenated in the presence of 100 μM indomethacin and 10 mM EDTA and were stored at −80°C until the assay of 6-keto prostaglandin F 1α (6-keto PGF 1α). Cells were assayed by radioimmunoassay. The 6-keto PGF 1α [^{125}I]-RIA KIT was obtained from NEN Life Science Products.

Cell viability

We examined whether copper (and the other metal ions) affected the viability of the cells using a trypan blue dye exclusion test (Chun-Lan *et al*., 1992). HPAEC monolayer cultures were incubated with either Cu^{2+}, Mn^{2+}, Zn^{2+} or Fe^{2+} (10^{-4} M, each concentration) for 1 h (in the current study, all of the experimental times were within 1 h). After trypsinization, the cells were suspended in Hanks' solution (1 ml) and 0.4% trypan blue was added to the cells. A drop of the suspension was placed on a hemocytometer and a viability count was made 4 min after dispersing the cells. The number of stained cells and nonstained cells in a given area on the hemocytometer was counted. The percentage of viable cells was equal to the nonstained cells/(stained + nonstained cells) × 100.

None of the metal ions significantly affected the viability of the cells (control = 94.93 ± 0.67%, Cu^{2+} = 94.53 ± 0.82%,

$Mn^{2+} = 94.27 \pm 1.2\%$, $Zn^{2+} = 95.3 \pm 1.36\%$ and $Fe^{2+} = 96.63 \pm 1.1\%$, means $\pm$ s.e.mean, $n = 3$).

We also examined cell viability using an MTT assay (Mosmann, 1983). HPAEC monolayer culures were incubated with either Cu^{2+}, Mn^{2+}, Zn^{2+} or Fe^{2+} (10^{-4} M, each concentration) for 1 h. None of the metal ions significantly affected the viability of the cells in the MTT assay as well ($Cu^{2+} = 105.98 \pm 6.94\%$, $Mn^{2+} = 98.54 \pm 5.05\%$, $Zn^{2+} = 112.45 \pm 5.74\%$ and $Fe^{2+} = 111.13 \pm 5.15\%$, results are ratio of means $\pm$ s.e.mean expressed as per cent of control values. Per cent control $= 100 \pm 5.28\%$, $n = 7$).

Drugs

N^G-monomethyl L-arginine (L-NMMA) was purchased from Calbiochem. (La Jolla, CA, U.S.A.). Ethylenediamine-tetra-acetic acid disodium (EDTA·Na_2), $MnCl_2$, $ZnCl_2$, $CaCl_2$, fura 2, fura 2-acetoxymethylester (fura 2-AM), nifedipine, nitrendipine, thapsigargin, BayK8644, and the other reagents were purchased from Wako Pure Chemical Ltd. (Osaka, Japan). $CuCl_2$, diethyldithiocarbamic acid (DDC) and Krebs-Henseleit buffer were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) was obtained from Dojindo Laboratories (Kumamoto, Japan). $^{45}Ca^{2+}$ (1.6 mCi $mmol^{-1}$) was obtained from New England Nuclear (Wilmington, DE, U.S.A.).

Statistical analysis

Results are expressed as means $\pm$ s.e.mean. Statistical analysis was performed with the use of a one-way analysis of variance with Bonferroni for multiple comparisons. Results of the curves were compared by using a two-way analysis of variance for repeated measures. Comparisons were considered statistically significant at the $P < 0.05$ level.

Results

The effect of Cu^{2+} on NO production in HPAEC

To assess NO synthesis by Cu^{2+} in HPAEC, we measured NOx released by adherent HPAEC. In this assay, 1 mM L-arginine was added to the buffer in order to support full eNOS activity while maintaining its stability. Figure 1A shows that Cu^{2+} dose-dependently increased the production NOx in HPAEC. Figure 1B shows that thapsigargin also increased NOx and the effect of Cu^{2+} on NO production in HPAEC was inhibited by 500 μM DDC and 200 μM L-NMMA. Further, NO synthesis by Cu^{2+} was not observed in the absence of extracellular Ca^{2+}. Mn^{2+}, Zn^{2+} and Fe^{2+} (10^{-4} M) did not increase NO production in HPAEC ($Mn^{2+} = 105.28 \pm 18.08$, $Zn^{2+} = 74.52 \pm 11.76$, $Fe^{2+} = 69.92 \pm 12.91$, [pmol mg^{-1} protein], respectively) compared with that (93.63 ± 9.26 pmol mg^{-1} protein) of the control (results are means $\pm$ s.e.mean, $n = 6$).

The effect of copper ion on $^{45}Ca^{2+}$ uptake into adherent HPAEC

Figure 2A shows the time courses of $^{45}Ca^{2+}$ uptake in adherent cells treated with various concentrations (10^{-7}–10^{-4} M) of Cu^{2+}. The Ca^{2+} uptake of the cells treated with Cu^{2+} increased dose-dependently with a significant elevation observed from 10^{-6} M at 2 min (Figure 2B). This result indicates that Cu^{2+} evoked an influx of Ca^{2+} into HPAEC. Of the other divalent metal ions, neither Mn^{2+}, Zn^{2+} nor Fe^{2+} increased the $^{45}Ca^{2+}$ uptake of HPAEC. (Figure 2C) As shown in Table 1, the Cu^{2+} (10^{-4} M)-induced $^{45}Ca^{2+}$-influx was significantly inhibited by nifedipine (10^{-5} M) in HPAEC.

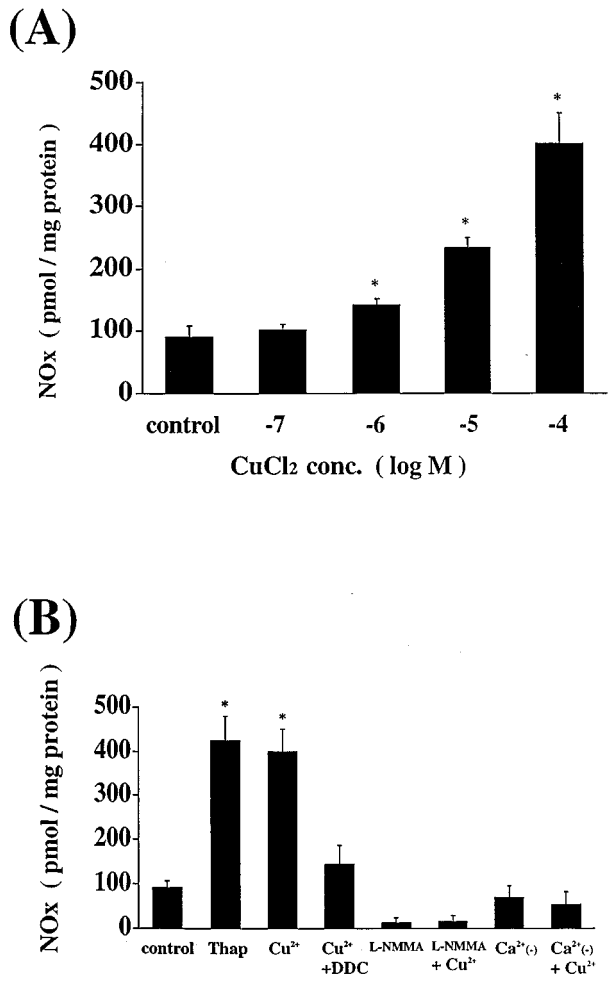


Figure 1 Cu^{2+}-stimulated NOx production by HPAEC. Confluent monolayers of endothelial cells grown on T-25 flasks were used. (A) Cells were stimulated with Cu^{2+} (10^{-7}–10^{-4} M) in Krebs-Henseleit buffer containing 1 mM L-arginine for 5 min. The samples were collected and assayed for NO using a NO chemiluminescence analyzer. NOx production was compared with basal (unstimulated) production (control). (B) Cells were incubated in Ca^{2+}-complete Krebs-Henseleit (K-H) buffer (2.5 mM) containing L-arginine (1 mM) either with thapsigargin (1 μM), or Cu^{2+} (10^{-4} M). 5×10^{-4} M of diethyldithiocarbamic acid (DDC) was also incubated with 10^{-4} M of Cu^{2+}. In some experiments, cells were incubated in Ca^{2+}-free Krebs-Henseleit buffer containing L-arginine (1 mM) with/without Cu^{2+}. After 5 min, samples were collected for analysis. Using K-H buffer containing L-NMMA (200 μM) without L-arginine (1 mM), the inhibitory capacity of NO synthesis was examined. NOx production was compared with basal (unstimulated) production (control). Values are means $\pm$ s.e.mean ($n = 6$). *$P < 0.05$ vs control.

The effect of copper and other metal ions on the mobilization of $[Ca^{2+}]_i$ in HPAEC

We next investigated whether Cu^{2+} actually elevated intracellular Ca^{2+}. $[Ca^{2+}]_i$ levels were measured by spectrofluorometry using the Ca^{2+}-sensitive dye, fura 2, as previously described (Grynkiewicz *et al.*, 1985). Stimulation with 10^{-7}–10^{-4} M Cu^{2+}, increased $[Ca^{2+}]_i$ from the basal level as follows (Figure 3A): at 10^{-7} M the levels increased faintly, and at

Table 1 The effect of dihydropyridine derivatives on the Cu^{2+}-induced $^{45}Ca^{2+}$ uptake into HPAEC

$^{45}Ca^{2+}$ *uptake* (pmoles mg^{-1} protein)	Cu^{2+} (−)	Cu^{2+} 10^{-4} M
Control	100.16±6.76	914.88±27.92*
Nifedipine 10^{-5} M	94.54±11.41	267.37±33.11**

$^{45}Ca^{2+}$ uptake of monolayers of human pulmonary arterial endothelial cells for 10 min. Nifedipine (10 μM) was used for a 20 min pretreatment and the effect of the drug on Cu^{2+}-induced $^{45}Ca^{2+}$ uptake was shown. Values are expressed by means±s.e.mean ($n=5$). *$P<0.05$ vs control. **$P<0.05$ vs Cu^{2+} (10^{-4} M).

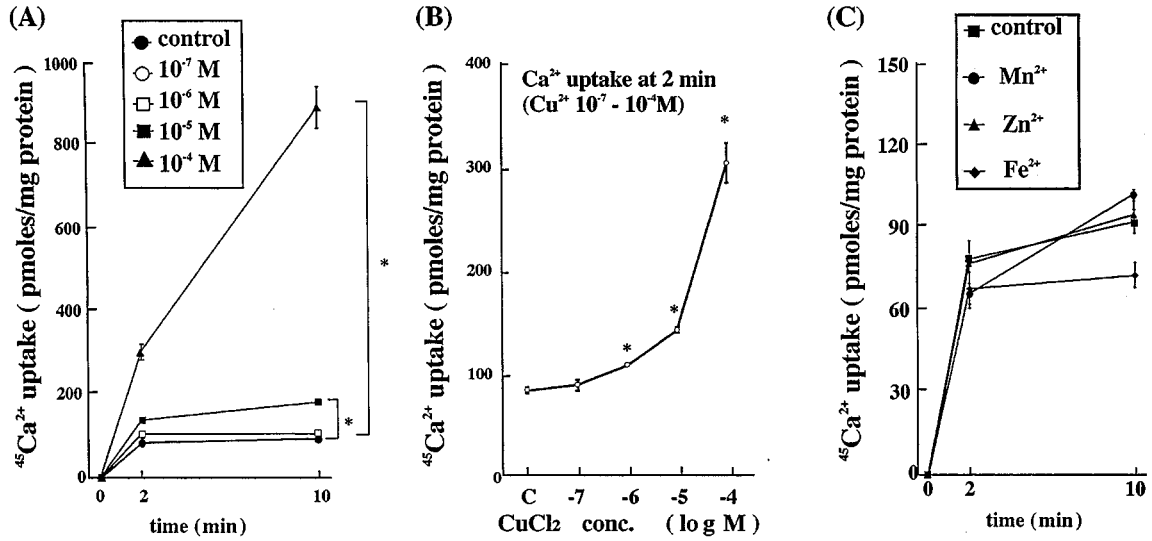


Figure 2 The effect of Cu^{2+} and other divalent metal ions on the kinetics of Ca^{2+} uptake into HPAEC. (A) The effect of different concentrations of copper ion (Cu^{2+}) on the kinetics of Ca^{2+} uptake into HPAEC. Adhering HPAEC (2×10^5 cells) were incubated with buffer A containing 1.5 μCi ml^{-1} $^{45}Ca^{2+}$ in the absence or presence of 10^{-7}–10^{-4} M copper ion (Cu^{2+}) at times of 2–10 min at 37°C (see Methods). (B) Concentration dependency of copper ion (Cu^{2+}) on Ca^{2+} uptake into HPAEC. HPAEC were incubated in 1 ml of buffer A with various concentrations of Cu^{2+} for 2 min. Two min after the addition of Cu^{2+} the maximal dilatation was observed in rat pulmonary arterial rings as described elsewhere (Ohnishi *et al*., 1997). (C) The effect of various divalent metal ions on Ca^{2+} uptake into HPAEC. In the presence of either 10^{-4} M Mn^{2+}, Zn^{2+} or Fe^{2+}, $^{45}Ca^{2+}$ uptake into HPAEC was measured according to the method used for A. Values are means±s.e.mean ($n=6$). *$P<0.05$ vs control.

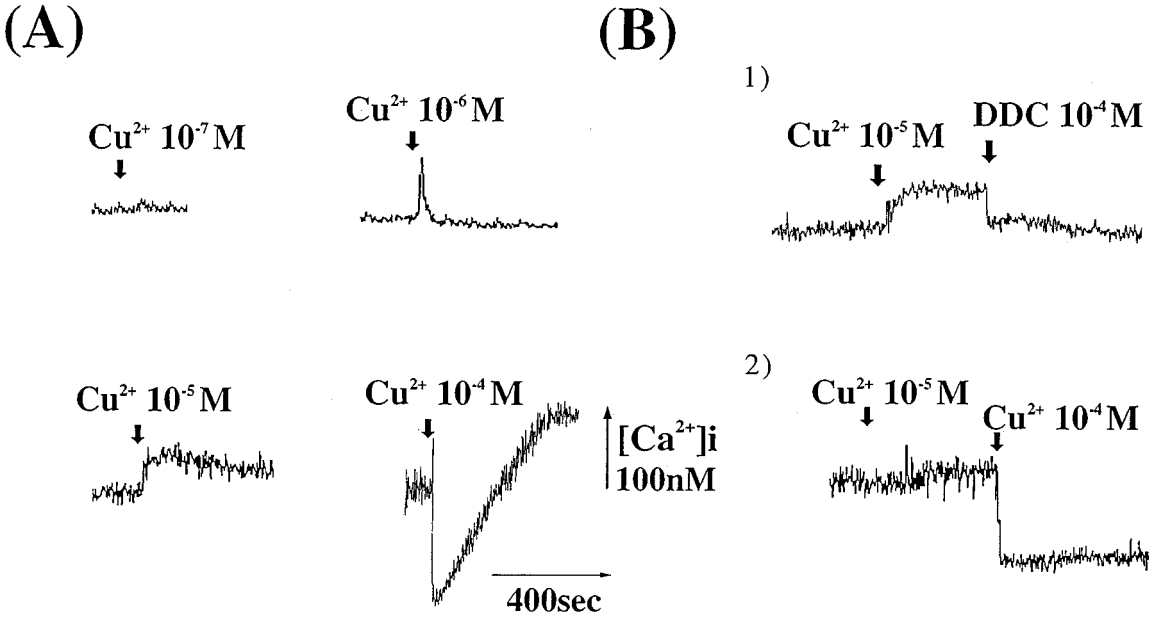


Figure 3 The effect of copper ion (Cu^{2+}) on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in HPAEC. HPAEC (1×10^6 cells) were incubated in 0.5 ml of Krebs-HEPES buffer containing 4 μM fura 2-AM and $[Ca^{2+}]_i$ was measured fluorimetrically. The traces represent the changes in $[Ca^{2+}]_i$ of HPAEC loaded with fura 2-AM that were suspended in 0.5 ml of Krebs-HEPES buffer. (A) Dynamism of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in HPAEC stimulated with various concentrations of copper ion (10^{-7}–10^{-4} M). At the time, indicated by the arrow, $CuCl_2$ was added to the cell suspension which was continuously stirred and temperature-controlled at 37°C. (B) (1) The effect of diethyldithiocarbamate (DDC) on $[Ca^{2+}]_i$ dynamism after stimulation with Cu^{2+}. DDC (10^{-4} M) known as a copper chelator was added after the effect of 10^{-5} M Cu^{2+} reached a plateau. (B) (2) The effect of Cu^{2+} on $[Ca^{2+}]_i$ dynamism in the absence of extracellular Ca^{2+}.

10^{-6} M they elevated transiently, while at $10^{-5}-10^{-4}$ M Cu^{2+}, the elevation of $[Ca^{2+}]_i$ was sustained. When 10^{-4} M Cu^{2+} was added, $[Ca^{2+}]_i$, as estimated by the F340/F380 ratio, was immediately reduced, then increased to a level higher than that induced by 10^{-5} M Cu^{2+} (Figure 3A). The $[Ca^{2+}]_i$ elevation caused by 10^{-5} M Cu^{2+} was restored by 10^{-4} M DDC, a copper chelater, but abolished in the case of no extracellularly added Ca^{2+} (Figure 3B). Cu^{2+} at 10^{-4} M caused an immediate drop in fluorescence intensity at 490 nm with excitation at 340, 360 nm, and 380 nm (Figure 4A). After that, with excitation at 340 nm, a delayed rise in fluorescence intensity at 490 nm was evoked, though there was no change in intensity with excitation at 360 nm. With excitation at 380 nm, the fluorescence intensity was lessened even further. The early phase of the event indicated that Cu^{2+}, which entered the endothelial cells, quenched the fluorescence due to the intracellular fura 2-Ca^{2+} complex. The later phase indicated that Cu^{2+} stimulated a Ca^{2+} influx into endothelial cells (Sage *et al*., 1989).

The initial decline of the ratio of fluorescence intensity at 490 nm with excitation at 340 nm and 380 nm (F340/380 ratio) was assumed to be due to the Cu^{2+} that quenched the fluorescence of the fura 2-Ca^{2+} complex. The influence of Cu^{2+} on the F340/380 ratio is shown in Figure 4B. Figure 3A shows that when 10^{-4} M Cu^{2+} was added, the quenching of fluorescence of fura 2-Ca^{2+} complex was observed at approximately 80 nM of intracellular Ca^{2+}. According to the standard titration curve (Figure 4B), it was estimated that approximately 800 nM of Cu^{2+} entered the endothelial cells and quenched the fura 2-Ca^{2+} complex. Cu^{2+} ($10^{-6}-10^{-4}$ M) significantly increased $[Ca^{2+}]_i$ in HPAEC in a dose-dependent manner (Figure 5).

Pretreatment with thapsigargin evoked $[Ca^{2+}]_i$ elevation over 400 nM and a successive addition of Cu^{2+} after 15 min further elevated $[Ca^{2+}]_i$ (Figure 6A), indicating that the Cu^{2+}-induced increase in $[Ca^{2+}]_i$ was not affected by thapsigargin. The Cu^{2+}-induced increase in $[Ca^{2+}]_i$ was significantly attenuated by the pretreatment with the dihydropyridine receptor agonist, BayK8644, in a dose-dependent manner (Figure 6B). Furthermore, it was also inhibited by the dihydropyridine receptor antagonists, nitrendipine and nifedipine, dose-dependently ($10^{-6}-10^{-5}$ M) (Figure 7). Thus, the Cu^{2+}-induced $[Ca^{2+}]_i$ elevation in HPAEC is likely due to the activation of dihydropyridine-like receptors.

The effect of copper on prostaglandin metabolite in HPAEC

Cu^{2+} (10^{-4} M) significantly increased 6-keto PGF 1α production (vehicle control = 65.20 ± 5.65 vs Cu^{2+} (10^{-4} M) = 93.00 ± 5.44 [pg/10^6 cells], results are means ± s.e.-mean, $n = 5$. $P < 0.05$ vs vehicle control.). Cu^{2+} appeared to elevate the prostacyclin level in HPAEC.

Discussion

We showed that Cu^{2+} dose-dependently increased NO production in which extracellular Ca^{2+} was a prerequisite for eNOS activation by Cu^{2+} is in keeping with the concept of a

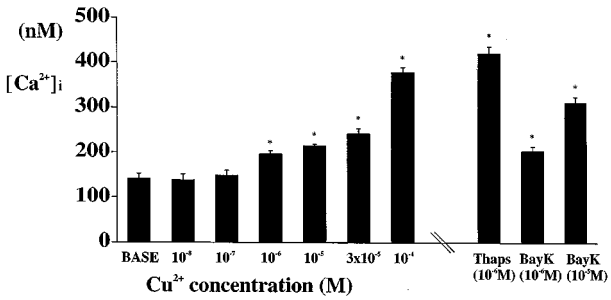


Figure 5 Concentration dependent effect of copper ion (Cu^{2+}) on intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in HPAEC. Fura 2-AM loaded cells were stimulated with various concentrations of Cu^{2+} ($10^{-8}-10^{-4}$ M). The effects of thapsigargin (Thaps) (10^{-6} M) or (±)BayK8644 (BayK) (10^{-6} or 10^{-5} M) on $[Ca^{2+}]_i$ were also examined. The respective peak levels of $[Ca^{2+}]_i$ were measured and plotted. Data are expressed by means ± s.e.mean ($n = 6$).

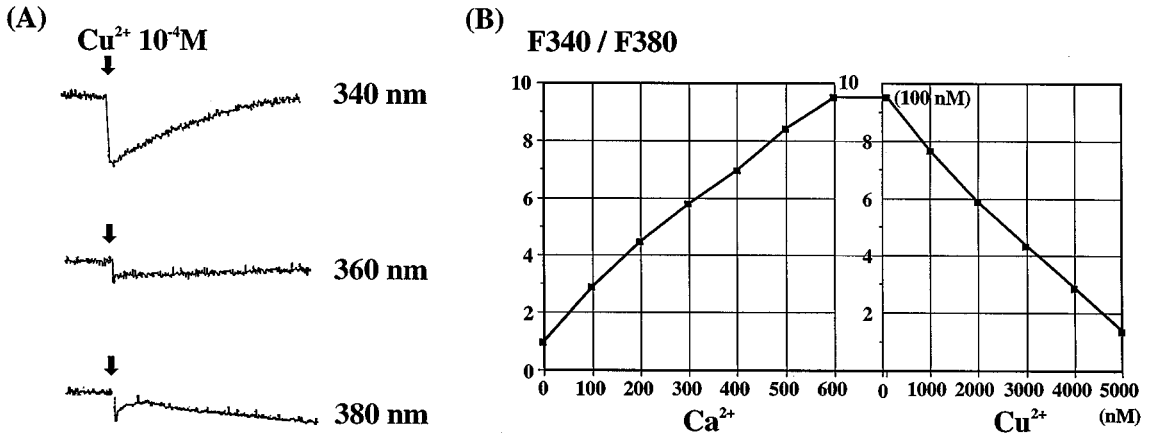
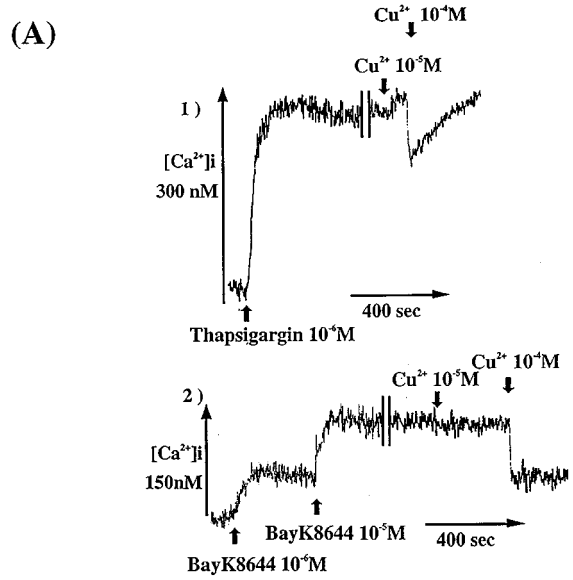


Figure 4 The changes in fluorescence of fura 2-AM loaded HPAEC in the presence of Cu^{2+} and the influence of Cu^{2+} on fluorescence of fura 2. (A) HPAEC were stimulated with 10^{-4} M Cu^{2+} in the presence of extracellular Ca^{2+} and fluorescence emission intensities at 490 nm were measured with excitations at 340, 360 and 380 nm, respectively. The recorded traces show the time courses of fluorescence intensity at 490 nm excited at 340, 360 and 380 nm. At the time indicated by the arrow, 10^{-4} M Cu^{2+} was added to the cell suspension which was continuously stirred at 37°C. (B) The influence of Cu^{2+} on the ratio of fluorescence intensity at 490 nm with excitation at 340 and 380 nm (F340/F380) was determined. The change in the F340/F380 ratio by titration with a dose of 100–600 nM Ca^{2+} and the quenching of fluorescence by successive addition of Cu^{2+} (from 100–5000 nM) in 0.1 M potassium phosphate buffer, pH 7.4 containing 4 μM fura 2 was measured and recorded.



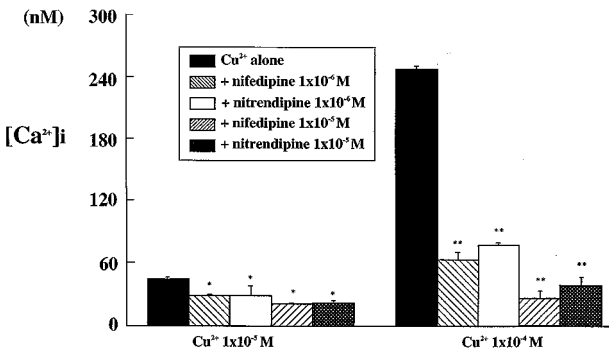


Figure 7 The effect of dihydropyridine derivatives on the Cu^{2+}-induced $[Ca^{2+}]_i$ increase in HPAEC. The effect of nifedipine or nitrendipine (10^{-6} or 10^{-5} M) was tested on Cu^{2+}-induced $[Ca^{2+}]_i$ elevation in HPAEC. After pretreatment with each type of dihydropyridine for 20 min, endothelial cells were stimulated with Cu^{2+} (10^{-5} or 10^{-4} M). The peak level of $[Ca^{2+}]_i$ attained with each concentration of Cu^{2+} was compared with that of the vehicle control. Each value indicated is subtracted from the level before the addition of Cu^{2+}. Values are expressed by means ± s.e.mean ($n=6$). *$P<0.05$ vs vehicle control.

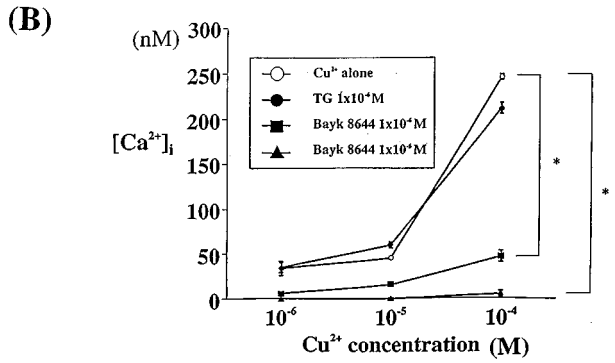


Figure 6 The effect of thapsigargin or (±)BayK8644 on $[Ca^{2+}]_i$ in HPAEC (A) and inhibition by (±)BayK8644, (the optical isomers, a dihydropyridine receptor agonist – (S−) and a dihydropyridine receptor antagonist – (R+)), of the Cu^{2+}-induced $[Ca^{2+}]_i$ increase in HPAEC (B). (A) Traces show the effects of thapsigargin and BayK8644 on Cu^{2+}-induced elevation of $[Ca^{2+}]_i$. Fura 2-AM loaded HPAEC were pretreated with thapsigargin (10^{-6} M) or with BayK8644 (10^{-6} or 10^{-5} M) for 15 min, and after the changes in $[Ca^{2+}]_i$ caused by these reagents reached a plateau, 10^{-5} and 10^{-4} M Cu^{2+} was added. (B) Fura 2-AM loaded HPAEC were pretreated with thapsigargin (10^{-6} M) or BayK8644 (10^{-6} or 10^{-5} M) for 15 min and then subjected to measurement of Cu^{2+} (10^{-6}–10^{-4} M)-induced $[Ca^{2+}]_i$ elevations. Each value indicated is subtracted from the level before the addition of Cu^{2+}. Values are expressed by means ± s.e.mean ($n=6$). *$P<0.05$ vs vehicle control.

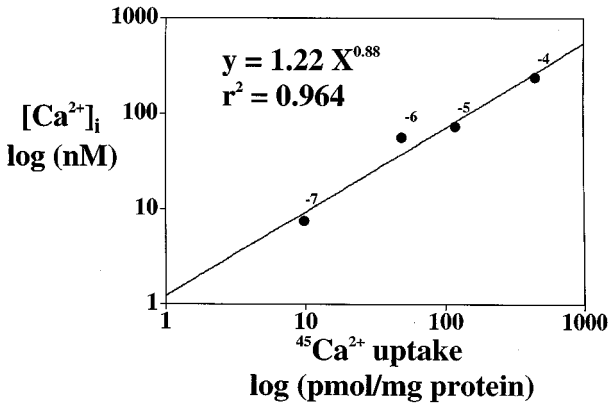


Figure 8 A close correlation between ^{45}Ca uptake into HPAEC and changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), both stimulated with various concentrations of copper. This figure was constructed with the data from Figures 2B and 5. In ^{45}Ca uptake experiments, the value of ^{45}Ca radioactivity at each copper concentration minus that of the vehicle control was plotted as the data point for ^{45}Ca uptake against $[Ca^{2+}]_i$ at the respective copper concentration added.

close correlation between cytoplasmic Ca^{2+} levels and the release of nitric oxide from endothelial cells (Korenaga *et al*., 1993; Buckley *et al*., 1995; Lothar *et al*., 1995; Wang *et al*., 1996). The result is in accordance with our previous report noting vasodilatation of the pulmonary arterial ring by Cu^{2+} (Ohnishi *et al*., 1997) and clarified a novel NOS activation by Cu^{2+}.

To measure the $[Ca^{2+}]_i$ movement we used the ^{45}Ca uptake method for evaluating Ca^{2+} influx and fura 2, a fluorescent Ca^{2+} indicator, for evaluating the increase in $[Ca^{2+}]_i$. Cu^{2+} preferentially augmented the Ca^{2+} influx into HPAEC in a dose-dependent (10^{-7}–10^{-4} M) manner, though Mn^{2+}, Zn^{2+} and Fe^{2+} had no such effect. Interestingly, 10^{-7}–10^{-6} M Cu^{2+} increased $[Ca^{2+}]_i$ transiently and 10^{-5}–10^{-4} M Cu^{2+} sustained the $[Ca^{2+}]_i$ elevation. The increase in $[Ca^{2+}]_i$ due to Cu^{2+} was not observed in the absence of extracellular Ca^{2+}, or in the presence of the copper chelator, DDC, indicating that a Ca^{2+} release from the intracellular Ca^{2+} store was not involved in the effect of Cu^{2+}. This notion is compatible with the experimental results whereby thapsigargin (1 μM), a specific inhibitor of the Ca^{2+} pump on endoplasmic reticulum, elevated $[Ca^{2+}]_i$ but did not alter the Cu^{2+}-induced increase in $[Ca^{2+}]_i$.

Additionally, a dihydropyridine sensitive L-type calcium channel antagonist, nifedipine or nitrendipine, dose-dependently suppressed the $[Ca^{2+}]_i$ increase induced by Cu^{2+}, suggesting that the Cu^{2+}-induced increase in $[Ca^{2+}]_i$ is largely due to Ca^{2+} entry through an L-type voltage-sensitive calcium channel in HPAEC (Carlson *et al*., 1993; Smart *et al*., 1995). Although there is very little evidence for voltage-opened calcium channels in endothelial cells, our study using Ca^{2+}-agonist, and antagonists, implies the existence of such channels

in endothelial cells. Along with our assumption, BayK8644 has been reported to stimulate the release of EDRF in porcine coronary arteries (Williams *et al*., 1987) and BayK8644 and (+)202, 791 (dihydropyridine Ca^{2+} agonists) did so in the case of canine femoral arteries. Nitrendipine, a calcium channel antagonist, suppressed the latter effect by BayK8644 and (+)202, 791 (Rubanyi *et al*., 1985). Further, the release of nitrogen oxides from bovine aortic endothelial cells was reported to be inhibited by nifedipine (1 μM) (Mügge *et al*., 1991). One can suppose that the dose of dihydropyridines might be non-selective in HPAEC. However, our results showing that BayK8644 could cause $[Ca^{2+}]_i$ elevation in HPAEC, and that BayK8644 or the dihydropyridine receptor antagonists, nifedipine or nitrendipine, actually suppressed Cu^{2+}-induced increase in $[Ca^{2+}]_i$, and that the Cu^{2+}-induced $^{45}Ca^{2+}$-influx was significantly inhibited by nifedipine (10^{-5} M) in HPAEC (Table 1), all indicate that Ca^{2+}-influx may be associated with dihydropyridine-like receptor(s). Regarding a Ca^{2+} antagonist, Hirosumi *et al*. (1998) reported that nifedipine suppressed the increase in $[Ca^{2+}]_i$ provoked by linoleate hydroperoxide and superoxide radicals in pig aortic endothelial cells, indicating another possibility that the Cu^{2+}-induced increase in $[Ca^{2+}]_i$ in HPAEC might be linked with oxygen radicals and peroxidation, since Cu^{2+} could catalyze the oxidative reaction and the Fenton reaction of hydroxyl radical production.

Our result (Figure 6A,B) negates another possible mechanism that Cu^{2+} modulates Ca^{2+}-ATPase activity, since thapsigargin has been reported to elevate $[Ca^{2+}]_i$ by inhibiting the Ca^{2+}-ATPase activity of the endoplasmic reticulum (ultimately depleting intracellular Ca^{2+} stores with an influx of extracellular Ca^{2+}) (Thastrup *et al*., 1990; Dolor *et al*., 1992). In this point, it is interesting that Colombani-Vidal & Barnea (1986) noted that chelated copper stimulates the release of LHRH from explants of the median eminence area (MEA) *via* mobilization of calcium from intracellular stores, and further that Abramson *et al*. (1983) also demonstrated that copper was a potent releaser of calcium from isolated sarcoplasmic reticulum vesicles isolated from skeletal muscle. The effect of Cu^{2+} may be differently exerted when applied to different tissue or cells. One can argue that the Cu^{2+}-induced increase in $[Ca^{2+}]_i$ solely affects cellular NOS activation. We focused on the calcium-dependent function of endothelial cells by measuring the prostacyclin level, another biologically important arachidonate metabolite of endothelial cells. Cu^{2+} actually increased the PGI_2 level in endothelial cells, suggesting that the effect of Cu^{2+} on $[Ca^{2+}]_i$ elevation can affect several distinct calcium-dependent functions of endothelial cells.

While we used fura 2 to evaluate the $[Ca^{2+}]_i$ movement in the presence of Cu^{2+}, it is not clear whether Cu^{2+} itself interacts with the fura 2-Ca^{2+} complex. Cu^{2+} attenuated the intensity of fluorescence at 490 nm when excited at 340, 360 and 380 nm, and lessened the ratio of F340/380. Mn^{2+} and Fe^{2+} also attenuated the ratio of fluorescence when excited at 340 and 380 nm, while Zn^{2+} did not (data not shown). The initial attenuation of the F 340/380 ratio observed in the presence of 10^{-4} M Cu^{2+} provided evidence that Cu^{2+} transiently entered the cells and remarkably quenched the fluorescence, presumably because of the intracellular fure 2-Ca^{2+} complex. The attenuation of fluorescence in the initial phase indicates a quenching by Cu^{2+} but not the $[Ca^{2+}]_i$ response, because this was observed with excitation at 340, 360 and 380 nm. The changes in intensity of fluorescence accompanied by the rise at 340 nm and fall at 380 nm in the second phase reflected the increase in $[Ca^{2+}]_i$ because there was no change in fluorescence at 360 nm (Sage *et al*., 1989). Further, in order to determine the concentration at which Cu^{2+} entered the cells, we examined the change in fura 2 fluorescence by the addition of Ca^{2+} or Cu^{2+} in a cell-free model system. When 100–600 nM Ca^{2+}, which is within the physiological $[Ca^{2+}]_i$ range, was added to the fura 2 solution, the F 340/380 ratio increased almost linearly (Figure 4). The addition of 10^{-7} M Cu^{2+} did not change the ratio but a successive addition of 10^{-6} M Cu^{2+} decreased it in a dose-dependent manner. The initial attenuation of the F 340/380 ratio was estimated to be roughly equal to a concentration of approximately 100 nM $[Ca^{2+}]_i$, and equal to a Cu^{2+} concentration of approximately 1 μM. We also confirmed that Cu^{2+} at 100 nM did not affect $[Ca^{2+}]_i$, and Cu^{2+} at 10^{-8}–10^{-4} M (used for this assay) did not affect the levels of Fmax and Fmin that were necessary to calculate $[Ca^{2+}]_i$. Therefore, when 10^{-4} M Cu^{2+} was added to the culture of HPAEC, Cu^{2+} might have entered the endothelial cells, and the intracellular concentration of Cu^{2+} could have reached 800 nM. Furthermore, this $[Ca^{2+}]_i$ measured by fura 2 correlates closely with ^{45}Ca uptake into HPAEC (Figure 8). This relationship implies that the Cu^{2+}-induced $[Ca^{2+}]_i$ elevation is due to a Ca^{2+} influx into cells. Our results thus clarified that the major mechanism of Cu^{2+}-induced NOS activation was $[Ca^{2+}]_i$ elevation due to an enhanced Ca^{2+} influx into endothelial cells.

A high serum or tissue level of copper is known to be a risk factor of cancer and cardiovascular disease (Kok *et al*., 1988). It is also noted that several mutual actions occur between reactive oxygen species, copper and copper-containing proteins such as SOD or ceruloplasmin (Ookawara *et al*., 1992; Swain *et al*., 1994; Gordge *et al*., 1995). Furthermore, the Cu/Zn ratio increases in the shocked lung as Ming *et al*. (1991) described. Thus, copper is considered to affect the human body *via* reactive oxygen species such as NO, O_2^- and OH^-. With this background, we assume that Cu^{2+} released from SOD or ceruloplasmin may contribute to the increase in NO production in the pathological state in which too much oxidant stress exists, such as in acute respiratory distress syndrome (ARDS) (Tsan, 1993; Mucord *et al*., 1994).

In conclusion, this study demonstrates that Cu^{2+}, but not Mn^{2+}, Zn^{2+} or Fe^{2+}, specifically activates eNOS through a mechanism of $[Ca^{2+}]_i$ elevation due to a Ca^{2+} influx into endothelial cells.

This work was supported by a grant-in-aid for the Encouragement of Young Scientists (No. 07770420) and by a grant from the Ministry of Education, Science and Culture of Japan (No. 06670605 and 07457145).

References

ABRAMSON, J.J., TRIMM, J.L., WEDEN, L. & SALAMA, G. (1983). Heavy metals induce rapid calcium release from sarcoplasmic reticulum vesicles isolated from skeletal muscle. *Proc. Natl. Acad. Sci. U.S.A.,* **80,** 1526–1530.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram. Quantities of protein utilizing the principle of protein-dyebinding. *Anal. Biochem.,* **72,** 248–254.

BUCKLEY, B.J., MIRZA, Z. & WHORTON, A.R. (1995). Regulation of Ca^{2+}- dependent nitric oxide synthase in bovine aortic endothelial cells. *Am. J. Physiol.,* **269,** C757 – C765.

CARLSON, R.O., MASCO, D., BROOKER, G. & SPIEGEL, S. (1993). Endogenous ganglioside GM1 modulates L-type calcium channel activity in N18 neuroblastoma cells. *J. Neurosci.,* **14,** 2272 – 2281.

CHUN-LAN, Z., LYNGMO, V. & NORDØY, A. (1992). The effects of saturated fatty acids on endothelial cells. *Tromb. Res.,* **65,** 65 – 75.

CLEGG, M.S., FERRELL, F. & KEEN, C.L. (1987). Hypertension-induced alterations in copper and zinc metabolism in Dahl rats. *Hypertension,* **9,** 624 – 628.

COLDEN-STANFIELD, M., SCHILLING, W.P., RITCHIE, A.K., ESKIN, S.G., NAVARRO, L.T. & KUNZE, D.L. (1987). Bradykinin- induced increases in cytosolic calcium and ionic currents in cultured bovine aortic endothelial cells. *Circ. Res.,* **61,** 632 – 640.

COLOMBANI-VIDAL, M. & BARNEA, A. (1986). Copper stimulation of LHRH release from median eminence explants. *Neuroendocrinology,* **43,** 664 – 669.

DOLOR, R.J., HURWITZ, L.M., MIRZA, Z., STRAUSS, H. & WHORTON, A.R. (1992). Regulation of extracellular calcium entry in endothelial cells: role of intracellular calcium pool. *Am. J. Physiol.,* **262,** C171 – C181.

GALIZZI, J.P., QAR, J., FOSSET, M., VAN RENTERGHEM, C. & LANZDUNSKI, M. (1987). Regulation of calcium channels in aortic smooth muscle cells by protein kinase C activators (diacyglycerol and phorbol esters) and by peptides (vasopressin and bombesin) that stimulate phosphoinositide breakdown. *J. Biol. Chem.,* **262,** 6947 – 6950.

GORDGE, M.P., MEYER, D.J., HOTHERSALL, J., NIELD, G.H., PAYNE, N.N. & NORONHA, D.A. (1995). Copper chelation-induced reduction of the biological activity of S-nitrosothiols. *Br. J. Pharmacol.,* **114,** 1083 – 1089.

GRYGLEWSKI, R.J., PALMER, R.M.J. & MONCADA, S. (1986). Superoxide anion plays a role in the breakdown of endothelium - derived relaxing factor. *Nature,* **320,** 454 – 456.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). New generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440 – 3450.

HAYASHI, T., NAKAI, T. & MIYABO, S. (1991). Glucocorticoids increase Ca^{2+} uptake and [3H] dihydropyridine binding in A7r5 vascular smooth muscle cells. *Am. J. Physiol.,* **261,** C106 – C114.

HIROSUMI, J., OUCHI, Y., WATANABE, M., KUSUNOKI, J., NAKAMURA, T. & ORIMO, H. (1988). Effect of superoxide and lipid peroxide on cytosolic free calcium concentration in cultured pig aortic endothelial cells. *Biochem. Biophys. Res. Commun.,* **152,** 301 – 307.

ISHIZAKI, T., SHIGEMORI, K., YAMAMURA, Y. & MATSUKAWA, S. (1995). Increased nitric oxide biosynthesis in Leukotoxin, 9, 10-epoxy-12-octadecenoate injured lung. *Biochem. Biophys. Res. Commun.,* **210,** 133 – 137.

KASTEN, T.P., SETTLE, S.L., MISKO, T.P. & CURRIE, M.G. (1994). Manganese potentiation of nitric oxide-mediated vascular relaxation. *Eur. J. Pharmacol.,* **253,** 35 – 43.

KLEVAY L.M. (1987). Hypertension in rats due to copper deficiency. *Nutr. Rep. Int.,* **35,** 999 – 1005.

KOK, F.J., DUIJN, C.M.V., HOFMAN, A., VAN DER VOET, G.B., DE WOLFF., F.A., PAAYS, C.H.C.H. & VALKENBURG, H.A. (1988). Serum copper and zinc and the risk of death from cancer and cardiovascular disease. *Am. J. Epidem.,* **128,** 352 – 359.

KORENAGA, R., ANDO, J., OHTSUKA, A., SAKUMA, I., YANG, W., TOYOOKA, T. & KAMIYAMA, A. (1993). Close correlation between cytoplasmic Ca^{2+} levels and release of an endothelium-derived relaxing factor from cultured endothelial cells. *Cell Struct. Funct.,* **18,** 95 – 104.

LOTHAR, A.B., ZIAD, T., STEFAN, M., PHILIP, S.S., WITHROW, G.W. & TADEUSZ, M. (1995). Simultaneous measurement of Ca^{2+} and nitric oxide in bradykinin-stimulated vascular endothelial cells. *Circ. Res.,* **76,** 922 – 924.

MING, Y., YIHLING, L., SHANSHENG, H., SHUQING, G. & ZHENXING, Y. (1991). Changes in pulmonary Cu-Zn contents in superior mesenteric artery occlusion shock of rabbit. *Biol. Trace Elem. Res.,* **29,** 281 – 288.

MOSMANN, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods,* **65,** 55 – 63.

MUCORD, J.M., GAO, B., LEFF, J. & FLORES, S.C. (1994). Neutrophil-generated free radicals : possible mechanisms of injury in adult respiratory distress syndrome. *Environ. Health. Perspect.,* **102,** 57 – 60.

MÜGGE, A., PETERSON, T. & HARRISON, D.G. (1991). Release of nitrogen oxides from cultured bovine aortic endothelial cells is not impaired by calcium channel antagonists. *Circulation,* **83,** 1404 – 1409.

OHNISHI, T., ISHIZAKI, T., SASAKI, F., AMESHIMA, S., NAKAI, T., MIYABO, S. & MATSUKAWA, S. (1997). The effect of Cu^{2+} on rat pulmonary arterial rings. *Eur. J. Pharmacol.,* **319,** 49 – 55.

OOKAWARA, T., KAWAMURA, N., KITAGAWA, Y. & TANIGUCHI, N. (1992). Site - specific and random fragmentation of Cu, Zn-superoxidedismutase by glycation reaction. *J. Biol. Chem.,* **267,** 18505 – 18510.

RUBANYI, G.M., SCHWARTZ, A. & VANHOUTTE, P.M. (1985). The calcium agonists Bay K 8644 and (+)202,791 stimulate the release of endothelial relaxing factor from canine femoral arteries. *Eur. J. Pharmacol.,* **117,** 143 – 144.

SAARI, J.T. (1992). Dietary copper deficiency and endothelium-dependent relaxation of rat aorta. *Proc. Soc. Exp. Biol. Med.,* **200,** 19 – 24.

SAGE, S.O., MERRITT, J.E., HALLAM, T.J. & RINK, T.J. (1989). Receptor-mediated calcium entry in fura 2-loaded human platelets stimulated with ADP and thrombin. *Biochem. J.,* **258,** 8923 – 8926.

SMART, D., WANDLESS, A. & LAMBERT, D.G. (1995). Activation of phospholipase C in SH-SY5Y neuroblastoma cells by potassium-induced calcium entry. *Br. J. Pharmacol.,* **116,** 1797 – 1800.

SWAIN, J.A., DARLEY-USMAR, V. & GUTTERIDGE, J.M. (1994). Peroxynitrite releases copper from caeruloplasmin: implications for atherosclerosis. *FEBS lett.,* **342,** 49 – 52.

THASTRUP, O., CULLEN, P.J., DROBAK, B.K., HANLEY, M.R. & DAMSON, A.P. (1990). Thapsigargin a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 2466 – 2470.

TSAN, MF. (1993). Superoxide dismutase and pulmonary oxygen toxicity. *Proc. Soc. Exp. Biol. Med.,* **203,** 286 – 290.

WANG, Y., SHIN, W.S., KAWAGUCHI, H., INUKAI, M., KATO, M., SAKAMOTO, A., UEHARA, Y., MIYAMOTO, M., SHIMAMOTO, N., KORENAGA, R., ANDO, J. & TOYOOKA, T. (1996). Contribution of sustained Ca^{2+} elevation for nitric oxide production in endothelial cells and subsequent modulation of Ca^{2+} transient in vascular smooth muscle cells in coculture. *J. Biol. Chem.,* **271,** 5647 – 5655.

WHORTON, A.R., SIMONDS, D.B. & PIANTADOSI, C.A. (1997). Regulation of nitric oxide synthesis by oxygen in vascular endothelial cells. *Am. J. Physiol.,* **272,** L1161 – L1166.

WILLIAMS, J.S., BAIK, Y.H., KOCH, W.J. & SCHWARTZ, A. (1987). A possible role for the endothelium in porcine coronary smooth muscle responses to dihydropyridine calcium channel modulators. *J. Pharmacol. Exp. Ther.,* **241,** 379 – 386.

(Received January 23, 1998
Revised August 28, 1998
Accepted September 1, 1998)