Low Micromolar Concentrations of Copper Augment the Impairment of Endothelium-Dependent Relaxation of Aortae From Diabetic Rabbits

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Both diabetes mellitus (DM) and elevated plasma copper concentrations are risk factors for cardiovascular disease (CVD). DM is associated with impaired endothelial nitric oxide (NO) and with excess superoxide ($O_2^{\cdot-}$) formation. Copper is also elevated in DM and is also associated with the generation of $O_2^{\cdot-}$. To explore possible interactions between DM and copper, the effect of exogenous copper ($CuCl_2$) on endothelium-dependent relaxation and cyclic guanosine monophosphate (GMP) formation was investigated in aortae from diabetic rabbits. Rabbits were rendered diabetic by intravenous injection of alloxan. Six months after induction of DM, the aortae were excised, cut into rings, and mounted in an organ bath for isometric measurement of acetylcholine (Ach)-evoked relaxation in rings precontracted with phenylephrine (PE). In parallel studies, cyclic (c)GMP formation by aortic rings following stimulation with Ach, calcium ionophore A23187 (A23187) and sodium nitroprusside (SNP) was assessed using radiommunoassay. The effect of copper on these parameters was then studied using the same methods. Ach-evoked relaxation and Ach- and A23187-evoked cGMP formation were significantly impaired in aortae from diabetic rabbits compared to controls, effects that were reversed with superoxide dimutase (SOD) and catalase (CAT). In contrast, there were no significant differences in SNP-stimulated relaxation or cGMP formation in aortae from diabetic rabbits compared to controls. Copper (1 to 10 μmol/L) promoted a further significant inhibition of Ach-stimulated relaxation in aortae from diabetic but not control rabbits. This reduction by copper was again reversed by SOD and CAT. We conclude that copper augments the reduction of NO bioavailability, which is already impaired in aortae from diabetic rabbits due to excess production of $O_2^{\cdot-}$ and H_2O_2. These results indicate that patients with DM may be susceptible to copper-mediated vasculopathy at much lower concentrations than those that promote vasculopathy in nondiabetic patients.

BOTH diabetes mellitus (DM)[1,2] and elevated plasma concentrations of copper[3,4] are independent risk factors for cardiovascular disease (CVD). Although there are several reports that plasma copper (and its carrier protein ceruloplasmin [CP]) are elevated in patients with both type 1 and type 2 DM,[5-10] the etiological role of copper in diabetic angiopathy is unknown.

It is well established, however, that DM is associated with increased vascular superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) formation.[8-11] $O_2^{\cdot-}$ elicits a number of pro-atherogenic effects, including the oxidation of lipids, the promotion of vascular smooth muscle cell (VSMC) proliferation, and the reaction with NO to produce peroxynitrite ($ONOO^-$), which reduces the bioavailability of NO.[12,13] In this latter context, there is now increasing evidence that reduced NO formation is an important etiological factor in diabetic angiopathy.[14,15] Similarly, copper is a potent pro-oxidant that catalyzes the generation of reactive oxygen species, including $O_2^{\cdot-}$, hydroxyl free radicals ($OH^{\cdot}$) and H_2O_2, through Fenton-type and Haber-Wiess reactions.[16-18] In a relevant study, Daimon et al[19] found a positive correlation between elevated serum levels of CP (a copper-binding protein) and decreased serum NO levels in patients with type 2 DM, indicating a pathological relationship between increased plasma copper and diminished NO formation in DM.

It is therefore proposed that in DM, copper may augment the generation of reactive oxygen species from arterial tissue, in particular $O_2^{\cdot-}$, which would lead to reduced NO formation and therefore impaired endothelial function. To test this proposal, the effect of exogenous copper (± superoxide dismutase [SOD]) on endothelium-dependent relaxation and cyclic guanosine monophosphate (cGMP) formation (index of endothelial NO bioactivity) in aortae from hyperglycemic, nonketotic, diabetic rabbits was studied. It has recently been established that this rabbit model of DM promotes a significant impairment of NO-mediated relaxation and cGMP formation.[20,21]

MATERIALS AND METHODS

Drugs and Materials

Acetylcholine (ACH), alloxan, calcium ionophore A23187 (A23187), catalase (CAT), copper chloride ($Cu\ Cl_2$), isobutyl methyl xanthine, phenylephrine (PE), sodium nitroprusside (SNP), CuZn SOD, CAT, and all buffer components were purchased from Sigma Chemical Co (Poole, UK). Dual-range [^{125}I] cGMP kits were purchased from Amersham Radiochemicals (Amersham, UK).

Induction of Diabetes

Male New Zealand white rabbits (3 kg) were injected intravenously with alloxan (Sigma) via the lateral ear vein at a stat dose of 65 mg/kg[20,21] and age-matched controls with saline alone. The diabetic rabbits were fed ad libitum with SDS standard rabbit chow (SDS, Whitham, UK) and allowed free access to water. Blood was sampled for plasma glucose when the rats were killed. Urine was also monitored over the duration of diabetes for glucose, ketone bodies, and proteins with Multistix (Ames Division, Miles Laboratories, Stoke Poges, UK) as previously described.[20,21] Any animals displaying ketonuria or proteinuria were excluded from the study.

Organ Bath Studies

Six months after the induction of DM, rabbits were killed by cervical dislocation and the aortae rapidly excised and placed in cold oxygenated

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Kreb's solution (KRB). Adventitial tissue was removed and aortae cut into 2-mm rings and mounted vertically in a 15-mL capacity organ attached to a Grass FTO3 force displacement transducer (Linton Instruments, Norfolk, UK) and data recorded on disc using MacLab (Linton Instruments). In each experiment, 4 rings were set up simultaneously. An initial tension of 2 g was applied to the suspended rings, which were then equilibrated for 30 minutes, after which time tension was reset. The experiment commenced after a further equilibration time of 30 minutes. Divalent $CuCl_2$ (±SOD or CAT) was then added to the organ bath chamber at different concentrations and left to equilibrate for 30 minutes. Contraction was then elicited with PE (1 to 3 μmol/L), with the concentration of PE being adjusted to give the same level of tension (2 g) in all rings. The rings were then relaxed with a cumulative concentrations of the endothelium-dependent vasodilator, Ach (0.01 to 10 μmol/L) or the endothelium-independent vasodilator, SNP (0.001 to 10 μmol/L).

cGMP Formation

Aortae from control and diabetic rabbits were cut into 2-mm segments as described above. Rings were then placed in polypropylene tubes containing 250 μmol/L of the phosphodiesterase (PDE) inhibitor, isobutylmethylxanthine (IBMX) in KRB and various concentrations of copper and/or CuZn SOD or CAT. After a 30-minute incubation at 37°C, cGMP formation was stimulated with: (1) ACH (generates NO through a receptor-mediated increase in Ca^{2+} and NOS activation), (2) A23187 (activates NO synthase through elevation of cytosolic Ca^{2+}), and (3) SNP (an NO donor that activates guanylyl cyclase directly). Tubes were incubated for a further 20 minutes at 37°C. Reactions were stopped by the addition of 1 mol/L perchloric acid and the tissues sonicated (3 × 30 seconds; Soniprep, MSE, Bucks, UK). Following centrifugation at 1,000 × *g* for 15 minutes, supernatants were taken and neutralized with 1 mol/L K_3PO_4. Aliquots were then taken and acetylated with triethylamine/acetic anhydride (1/2, vol/vol) and diluted with phosphate-buffered saline (PBS; pH 7.4). To these, cGMP standards (0 to 256 fmol) 200 μL diluted antisera against cGMP antisera containing [^{125}I] cGMP was added. Following overnight incubation at 4°C, antisera against rabbit globulins in phosphate buffer was added to each tube and incubated on melting ice for 15 minutes. Tubes were then centrifuged at 2,500 rpm for 10 minutes. Supernatants were decanted into vials and scintillation fluid added and counted in a gamma particle counter. Standard curves were compiled and unknown values calculated.

Data Analysis and Statistics

Comparisons of weights, plasma glucose and plasma lipids between the 6 month diabetic groups and the age-matched controls were performed using the Mann-Whitney *U* paired test. For the cGMP measurements, data are related to mg tissue/min (wet weight). Each data point is expressed as the mean ± SD. Responses of isolated aortic rings to Ach and SNP are expressed as percent relaxation of PE-induced contraction. Statistical analyses were performed using 1-way repeated-measures analysis of variance (ANOVA) followed by Bonferroni's test for comparisons among multiple groups. Differences among means was considered significant at $P < .05$. Data were analyzed using Graphpad (San Diego, CA).

RESULTS

Animal Weights, Serum Glucose, and Cholesterol Concentrations

The starting weights in both the control and diabetic rabbit groups were similar (control: median, 3.0 kg; range, 2.7 to 3.5, n = 6; diabetic: median, 3.1; range, 2.8 to 3.6, n = 6). The diabetic rabbits were significantly ($P < .03$) lighter than the control group (control: median, 4.1 kg; range, 3.5 to 4.35, n = 6; diabetic: median, 3.6; range, 3.0 to 3.9, n = 6). Serum glucose concentrations (nonfasting) were significantly elevated ($P < .009$) in the 6-month diabetic group (median, 32.2; range, 18.3 to 41.1, n = 6) compared to controls (median, 6.4 mmol/L; range, 6.1 to 7.5 mmol/L, n = 6). Serum cholesterol and serum triglycerides were not significantly different between control and diabetic groups, as previously described.[20,21] All animals displayed an absence of ketone bodies in the urine when assessed with Multistix.

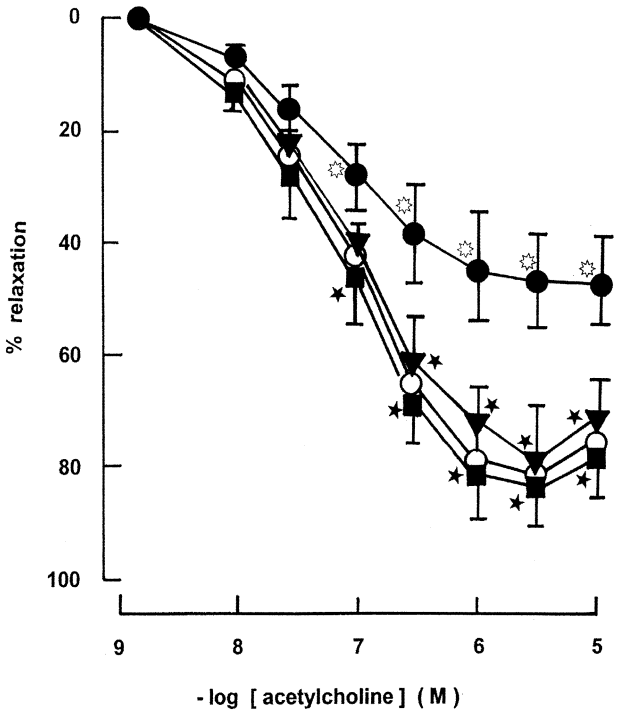

Fig 1. ACH-mediated relaxation of aortae from control (○) and diabetic (●) rabbits of 6 months duration and effect of 500 U/mL SOD (■) and 100 U/mL CAT (▼) on impaired relaxation in diabetic aortae only (SOD and CAT were without significant effect on relaxation in control aotric rings). Each point represents the mean ± SD, n = 6. ☆*P* < .0001 when comparing relaxation in aortae from diabetic compared to control rabbits control and ★*P* < .001 when comparing the effect of addition of SOD or CAT on relaxation in diabetic aortae.

Organ Bath Studies and cGMP Levels

Ach-stimulated relaxation was significantly impaired in aortae from diabetic rabbits compared to controls (Fig 1). The impairment of Ach-stimulated relaxation in diabetic animals was reversed with SOD (500 U/mL) and CAT (100 U/mL) in aortae from diabetic rabbits (Fig 1), but had no effect on relaxation in aortae from nondiabetic, control rabbits (Table 1).

Following preincubation for 30 minutes with $CuCl_2$ (at 0.1 μmol/L, 1 μmol/L, and 10 μmol/L), there was a further significant inhibition of Ach-stimulated relaxation in aortae from diabetic rabbits, an effect reversed with 500 U/mL SOD and 100 U/mL CAT (Fig 2). In contrast, incubation for 30 minutes with copper (0.1 μmol/L to 10 μmol/L), had no significant effect on ACH-stimulated relaxation in aortae from control rabbits (Table 1).

Table 1. Effect of 30-Minute Preincubation of Aortae From Control Nondiabetic Rabbits With $CuCl_2$ With or Without SOD or CAT on ACH-Evoked Relaxation

	% Maximal ACH-Evoked Relaxtion (concentration of acetylcholine, mol/L)					
	1×10^{-8}	3×10^{-8}	1×10^{-7}	3×10^{-7}	1×10^{-6}	3×10^{-6}
No $CuCl_2$	10 ± 1	24 ± 3	42 ± 7	65 ± 8	76 ± 9	80 ± 10
1 μmol/L $CuCl_2$	13 ± 3	25 ± 3	43 ± 5	63 ± 14	76 ± 15	82 ± 11
10 μmol/L $CuCl_2$	11 ± 2	24 ± 3	42 ± 6	66 ± 10	78 ± 8	88 ± 9
No $CuCl_2$ + SOD	13 ± 4	26 ± 4	42 ± 3	64 ± 8	74 ± 7	87 ± 8
1 μmol/L $CuCl_2$ + SOD	12 ± 3	25 ± 3	41 ± 5	62 ± 9	76 ± 9	85 ± 11
10 μmol/L $CuCl_2$ + SOD	11 ± 1	24 ± 3	44 ± 3	60 ± 10	79 ± 11	85 ± 9
1 μmol/L $CuCl_2$ + CAT	13 ± 3	23 ± 3	46 ± 4	58 ± 5	72 ± 6	80 ± 9
10 μmol/L $CuCl_2$ + CAT	9 ± 1	20 ± 4	39 ± 3	60 ± 10	75 ± 8	82 ± 12

NOTE. Values are means ± SD, n = 6. No statistically significant effects of $CuCl_2$ with or without SOD or CAT compared to zero controls (ie, with no additions) were detected.

Ach-stimulated and A23187-stimulated cGMP formation was significantly impaired in aortae from diabetic rabbits compared to controls (Fig 3). The similarity of the impairment of cGMP formation to that of relaxation in response to Ach consolidates that the reduction of relaxation in diabetic aortae is due to an impairment of the NO-cGMP axis rather than other pathways (eg, endothelium-dependent hyperpolarizing factor [EDHF] or prostaglandin I_2). This impairment of cGMP formation was reversed by 500 U/mL SOD and partially by 100 U/mL CAT (Fig 3).

There were no significant differences in SNP-stimulated relaxation (Fig 4) or on SNP-stimulated cGMP formation (Tables 2 and 3) by aortae from diabetic rabbits compared to controls.

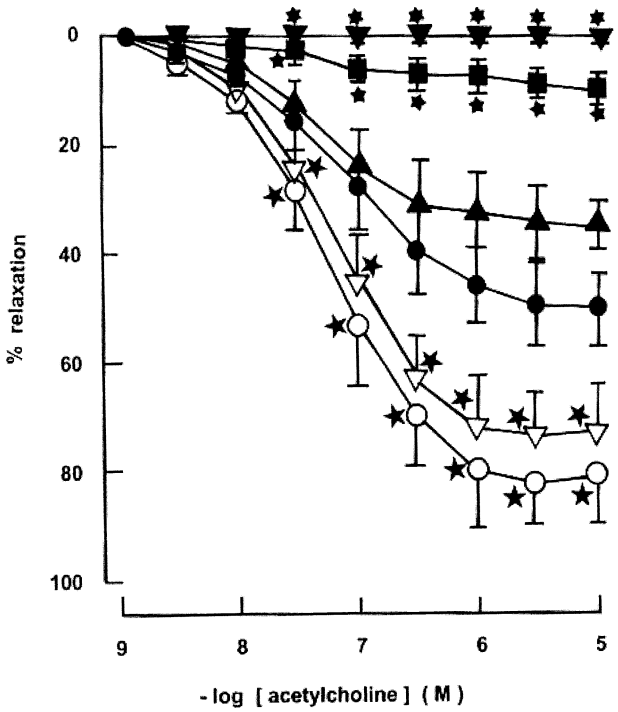

Fig 2. Effect of $CuCl_2$ on ACH-evoked relaxation of aortic rings from diabetic rabbits (6 months duration). (●) Diabetic aorta without additions, (▲) diabetic aorta plus 0.1 μmol/L $CuCl_2$, (■) diabetic aorta plus 1 μmol/L $CuCl_2$, (▼) diabetic aorta plus 10 μmol/L $CuCl_2$, (▽) diabetic aorta plus 1 μmol/L $CuCl_2$ plus calatase (100 U/mL), (○) diabetic aorta plus 1 μmol/L $CuCl_2$ plus SOD (500 U/mL). Each point represents the mean ± SD, n = 6. ✱P < .0001 when comparing the effect of copper with control and ★P < .001 when comparing the effect of SOD or CAT on $CuCl_2$-mediated inhibition of relaxation.

DISCUSSION

The present study demonstrates that low micromolar concentrations of copper augment an already impaired endothelium-

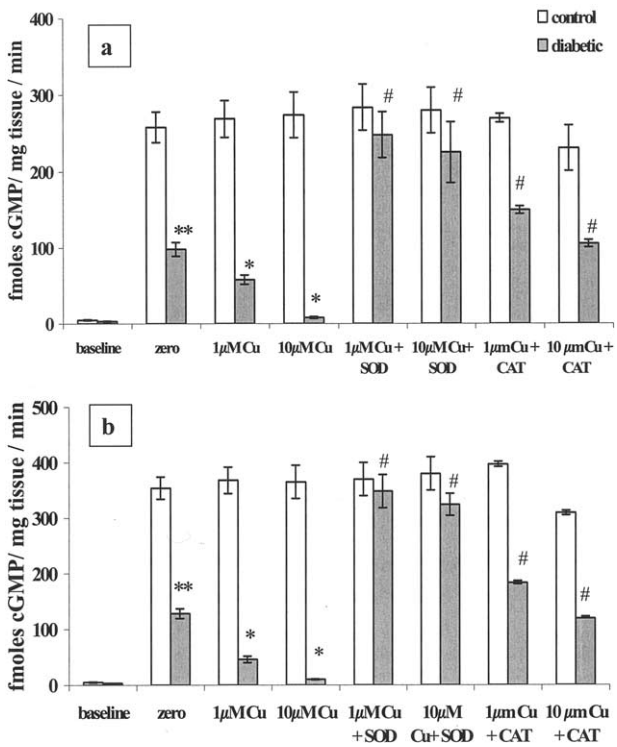

Fig 3. Effect of $CuCl_2$ (Cu) (±500 U/mL SOD or ± 100 U/mL calatase [CAT]) on cGMP formation simulated with (a) ACH and (b) A23187 in aorta from control and diabetic rabbits (mean ± SD; n = 6 animals). ✱✱P < .001 comparing control with diabetics (no additions); ✱P < .001 comparing Cu additions with zero additions; #P < .01 comparing Cu additions with SOD or CAT.

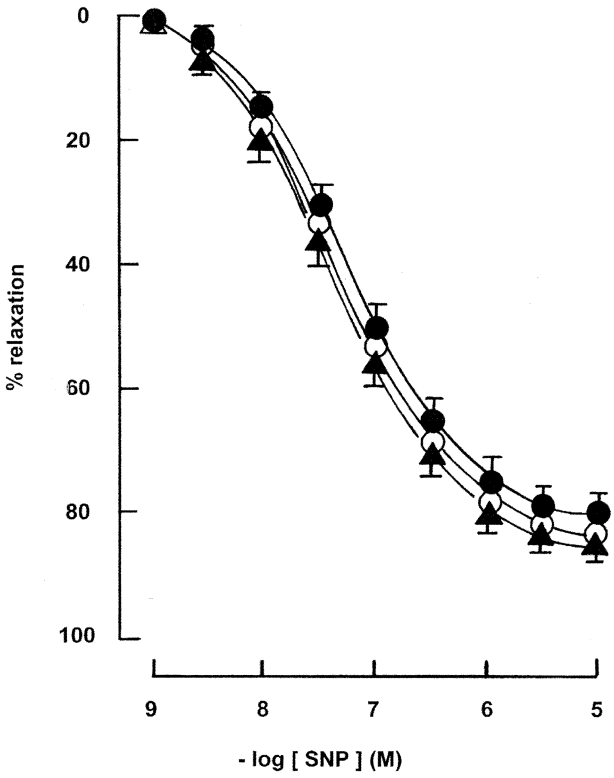

Fig 4. SNP-stimulated relaxation aortae from control (○) and diabetic (●) rabbits of 6 months duration and effect of $CuCl_2$ (▲). Data are means ± SD, n = 6.

dependent relaxation and cGMP formation in aortae from rabbits with hyperglycemic DM but not from nondiabetic rabbits. The inhibition of these effects with SOD and CAT demonstrates that copper is exerting this effect through an augmentation of both $O_2^{\cdot-}$ and H_2O_2 formation

In a previous study, it was demonstrated that copper (at 20 μmol/L and greater) actually promotes the formation of NO in aortae from nondiabetic rats.[22] However, O'Brien et al[23] demonstrated that low micromolar concentrations of copper impaired ACH-evoked relaxation in mesenteric arteries from insulin-resistant rats (nonhyperglycemic), an effect also blocked by SOD. Chiarugi et al[24] also recently demonstrated that low micromolar concentrations copper impair ACH-evoked relaxation in aortae from normal (ie, nondiabetic rats) but only after prolonged (>3 hours) incubation with the cation and in the presence of fetal bovine serum (FBS). However, this effect was not SOD-inhibitable.[24]

In diabetic animal models, the apparent excess formation of both $O_2^{\cdot-}$ and H_2O_2 by arterial tissues, as assessed by the use of inhibitors, has been documented.[8-11,25] In diabetic patients, an impairment of forearm blood flow (an index of endothelial NO formation) and its reversal with antioxidants has also been reported.[26-28] These (and many other) studies have led to the paradigm that oxidative stress is central to the etiology of diabetic angiopathy.[29] The excess production of $O_2^{\cdot-}$ by the vasculature of diabetic animals may also be attributable to the overexpression of endogenous $O_2^{\cdot-}$ generating enzymes, including NADPH oxidase, xanthine oxidase, cyclooxgenase, and lipoxygenase, as well as the auto-oxidation of glucose and mitochondrial respiration.[30] The overproduction of $O_2^{\cdot-}$ in DM has also been ascribed, in part, to a reduction in endogenous vascular SOD activity, which has been demonstrated in the diabetic rabbit model.[31] A reduction of SOD activity has been reported for the aorta from diabetic rats[32,33] and in human erythrocytes.[34,35] However, other reports indicate there is no change of SOD[36-38] or increased SOD,[39] and therefore whether there is a reduction of SOD in DM remains equivocal.

In the present study, CAT also partially reversed the inhibitory effect of copper on relaxation and cGMP formation, indicating that H_2O_2 also plays a role in reducing NO bioavailability. Increased H_2O_2 in diabetic rats has been demonstrated[10,11] and decreased vascular CAT activity has been reported in the aorta of diabetic rabbits.[31] H_2O_2 has been shown to react with NO to produce a singlet oxygen, thereby reducing NO bioavailability.[40] Copper also catalyses Fenton-type reactions that generate $O_2^{\cdot-}$ from H_2O_2 as a precursor:[16,17]

$$Cu^{2+} + H_2O_2 \rightarrow Cu^{+} + 2H^{+} + O_2^{\cdot-} \quad (1)$$

Furthermore, reduced copper (Cu^{+}) catalyzes hydroxyl radical ($OH^{\cdot}$) formation,[16,17] which in turn can generate even more $O_2^{\cdot-}$ via Haber-Weiss reactions[18]:

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\cdot} \quad (2)$$

$$OH^{\cdot} + H_2O_2 \rightarrow H_2O + O_2^{\cdot-} + H^{+} \quad (3)$$

Table 2. Effect of $CuCl_2$ on SNP-Evoked Relaxation in Aortae from control or Diabetic Rabbits

	% Maximal SNP-Evoked Relaxation (concentration of SNP, mol/L)					
	1×10^{-8}	3×10^{-8}	1×10^{-7}	3×10^{-7}	1×10^{-6}	3×10^{-6}
Controls						
No $CuCl_2$	2 ± 0.2	3 ± 0.3	12 ± 1.3	49 ± 5	85 ± 8	99 ± 7
1 μmol/L $CuCl_2$	3 ± 0.2	2 ± 0.1	9 ± 1.4	50 ± 5	86 ± 5	93 ± 6
10 μmol/L $CuCl_2$	3 ± 0.2	2 ± 0.1	11 ± 1.4	48 ± 4	84 ± 8	90 ± 6
Diabetic rabbits						
No $CuCl_2$	2 ± 0.2	3 ± 0.3	12 ± 1.3	50 ± 5	84 ± 8	95 ± 7
1 μmol/L $CuCl_2$	3 ± 0.2	2 ± 0.1	10 ± 1.4	47 ± 5	79 ± 7	88 ± 6
10 μmol/L $CuCl_2$	3 ± 0.2	2 ± 0.1	14 ± 1.4	53 ± 5	82 ± 8	87 ± 6

NOTE. Values are means ± SD, n = 6. No significant effects of $CuCl_2$ compared to zero controls (ie, with no additions) were detected.

Table 3. Effect of $CuCl_2$ With or Without SOD or CAT on SNP-Stimulated cGMP Formation in Aortae From Diabetic Rabbits

cGMP Formation (fmol/mg tissue/min)	Basal	1 μmol/L $CuCl_2$	10 μmol/L $CuCl_2$	1 μmol/L $CuCl_2$ + 500 U/mL SOD	10 μmol/L $CuCl_2$ + 500 U/mL SOD	1 μmol/L $CuCl_2$ + 100 U/mL CAT	10 μmol/L $CuCl_2$ + 100 U/mL CAT
Control	4 ± 46	480 ± 46	470 ± 43	490 ± 47	475 ± 58	486 ± 83	475 ± 58
Diabetic	3 ± 46	475 ± 44	460 ± 46	456 ± 49	443 ± 67	482 ± 71	443 ± 67

NOTE. Values are means ± SD, n = 6.

The Cu^{2+} generated in (2) could feed back into reaction (i), promoting further $O_2^{\cdot-}$ formation. Since $O_2^{\cdot-}$ react readily with NO to reduce NO bioavailability, these reactions would readily explain why copper augments the impairment in NO-dependent relaxation and GMP observed in aortae from diabetic rabbits. An impairment of CAT activity reported in rabbits would also provide fuel for further $O_2^{\cdot-}$ production via these reactions.

These observations may be of clinical significance since several studies have now identified elevated plasma levels of copper and its carrier protein CP in both type 1 and type 2 DM.[4-6] Walter et al[6] reported that diabetic subjects with retinopathy, hypertension, or microvascular disease had higher plasma copper concentrations compared with both diabetic subjects without complications and with nondiabetic control subjects. Daimon et al[19] found a correlation between elevated plasma copper levels and diminished NO production in type 2 diabetic patients, a relationship that could be explained by enhanced endogenous $O_2^{\cdot-}$ formation. Sajithlal et al proposed a role for copper in mediating the formation of advanced glycation end-products (AGEs), major factors for diabetic angiopathy.[41,42] In this context, the glycation of SOD (and a concomitant reduction in its activity) has also been demonstrated in DM,[34] which would also result in increased $O_2^{\cdot-}$ formation. Other studies have demonstrated an enhanced oxidation of low-density lipoprotein in DM that is attributable to copper.[43] Copper also augments the formation of $O_2^{\cdot-}$ from thiols, in particular, homocysteine,[44] which is itself associated with diabetic angiopathy and with a reduction of NO formation in DM.[21]

As mentioned, copper in blood is bound almost entirely to CP, which itself is elevated in DM.[19] Indeed, the elevation of plasma copper levels in DM may be due to an elevation of CP. In its own right, elevated plasma levels of CP is a risk factor for CVD.[2,3] It has been demonstrated that CP promotes the destruction of NO through $O_2^{\cdot-}$ formation, as well as endothelial cell toxicity through the generation of H_2O_2.[45,46] Thus, copper may not need to be dissociated from its binding sites for it to have a pathological impact.

In the present study we investigated the effect of free copper only and found that copper as low as 1 μmol/L had an emphatic inhibitory effect on endothelium dependent relaxation in aortic rings from diabetic but not control rabbits. However, in healthy individuals, free copper (ie, not bound to proteins) in blood is almost zero as it is bound to CP and other proteins, including albumin.[47] However, a large proportion of copper associated with blood proteins is "loosely bound."[47-51] Gutteridge[48] demonstrated that approximately 40% of total copper (8 μmol from a total of 19 μmol) was dissociable from serum. It is implicit that copper be exchangeable or loosely bound for it to be uptaken by tissues for their copper requisites. Thus, the copper concentrations at which we observed effects are within this concentration range (ie, low micromolar).

Perhaps more importantly and of relevance to the present work, several studies have demonstrated that reactive oxygen species readily dissociate copper from CP and other copper-binding proteins. $ONOO^-$ (the byproduct of the reaction between NO and $O_2^{\cdot-}$) also dissociates copper from CP,[49] which has been proposed as a self-perpetuating cascade that would further promote oxidant stress.[44] H_2O_2 causes a conformational change in CP that releases copper from its binding sites eliciting DNA damage.[50] In tissues, copper is bound largely to metallothioneins.[51] In turn, it has been demonstrated that glucose increase endothelial metallothionein levels[52] and that H_2O_2 can release copper from the protein,[53,54] as for CP. As discussed above, H_2O_2 appears to be generated by aortic tissue from diabetic animals, which may constitute a mechanism by which free copper is released and made available for the effects documented here.

Although little is known about the degree of dissociation of copper from blood proteins or from binding sites in tissue in patients with DM, Eaton and Qian[55] demonstrated that there is twice the amount of exchangeable copper in the plasma of diabetic rats compared to control animals. In another study it was found that in diabetic patients with advanced nephropathy that copper is dissociated from both CP and albumin, apparently within the kidney.[56] Whether this is the case in other tissues, in particular within the vasculature, warrants investigation.

To summarize, the present study demonstrates that low micromolar concentrations of copper promote the formation of $O_2^{\cdot-}$ in aortae from diabetic rabbits. In DM there is an a priori increase in $O_2^{\cdot-}$ and H_2O_2 formation in arterial tissue, which is mediated through an upregulation of $O_2^{\cdot-}$ and H_2O_2 generating enzymes and/or a downregulation of antioxidant systems. Although copper exerts no effect in normal arterial tissues, DM renders the vasculature susceptible to the prooxidant attack by copper. The possibility that reactive oxygen species liberate copper from its binding sites would also appear to worthy of further study. It is suggested that intervention with drugs that inhibit the formation of $O_2^{\cdot-}$ (or prevent the interaction between copper and the oxygen free radicals [eg, copper chelators]) may be therapeutically useful in reducing oxidative stress in DM. These data also predict that other factors that can augment the Fenton and Haber-Weiss reactions (eg, other transition metals and thiols) may also promote oxidant stress in DM. However, due to the limitations of available tissue, we were unable to explore this possibility in the present study.

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