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# Neocuproine inhibits the decomposition of endogenous *S*-nitrosothiol by ultraviolet irradiation in the mouse gastric fundus

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**Abstract**

In the present study, we investigated whether copper ions are involved in the decomposition of endogenous *S*-nitrosothiols by ultraviolet (UV) light irradiation in the mouse gastric fundus. The effects of copper ions and chelators of copper(I) and copper(II), neocuproine and cuprozine, respectively, were studied on relaxations in response to *S*-nitrosoglutathione, UV irradiation, exogenous nitric oxide (NO), added as acidified $NaNO_2$, and isoproterenol. UV irradiation of smooth muscle strips induced fast and transient relaxations which were mimicked by exogenous NO. *S*-Nitrosoglutathione induced concentration-dependent relaxations, which were more sustained than those elicited by UV irradiation or NO. $CuCl_2$ did not affect relaxations elicited by UV irradiation, exogenous NO and isoproterenol but enhanced those elicited by *S*-nitrosoglutathione. $CuSO_4$ but not $FeSO_4$ mimicked the effect of $CuCl_2$ on relaxations elicited by *S*-nitrosoglutathione. Neocuproine, the copper(I)-specific chelator, inhibited both photorelaxation and *S*-nitrosoglutathione-induced relaxation, and this inhibition was prevented by $CuCl_2$. In contrast, neocuproine significantly enhanced the relaxations in response to exogenous NO, without affecting the relaxations elicited by isoproterenol. Cuprizone, a specific copper(II) chelator, did not affect relaxations in response to *S*-nitrosoglutathione, UV irradiation, exogenous NO and isoproterenol. These results suggest that copper(I) and not copper(II) may play a role in the NO release evoked by the light-induced decomposition of endogenous *S*-nitrosothiols in mouse gastric fundus. Also, results with the selective copper(I) chelator, neocuproine, confirmed our recent findings that the endogenous "store" of *S*-nitrosoglutathione, rather than NO, acts as an intermediate in photorelaxation of the mouse gastric fundus, and that photorelaxation may be a suitable model to elucidate the nature of endogenous *S*-nitrosothiols.


*Keywords:* Gastric fundus, mouse; Photorelaxation; Nitric oxide (NO); *S*-nitrosoglutathione; $CuCl_2$; Neocuproine; Cuprizone

## 1. Introduction

We previously reported that mouse gastric fundus smooth muscle strips relax when exposed to ultraviolet (UV) light and that this photorelaxation was inhibited by haemoglobin, hydroxocobalamin and methylene blue, whereas $N^G$-monomethyl-L-arginine had no effect (Ögülener et al., 1996). These observations led to the suggestion that the release of nitric oxide (NO) by UV light causes relaxation. Furchgott et al. (1955) observed that UV light causes relaxation of vascular smooth muscle. In addition, Matsunaga and Furchgott (1989) indicated that photorelaxation might also be due to endogenous NO, liberated from a photodegradable molecular "store" of NO contained within the vessel wall. Subsequently, it was suggested that a light-activated, depletable and replenishable NO-yielding store was responsible for the photorelaxation of vascular smooth muscle (Venturini et al., 1993; Kubaszewski et al., 1994; Megson et al., 2000). Recently, we reported that there might be a store of photosensitive compounds yielding NO in smooth muscle strips of the mouse gastric fundus, similar to the photodegradable molecular store of NO in the vascular system, and suggested that photorelaxation is, at least in part, due to NO release by catalysis of the decomposition of an endogenous store of *S*-nitrosothiols such as *S*-nitrosoglutathione (Ögülener and Ergün, 2002).

It is known that endogenous thiols are able to bind NO with concomitant formation of *S*-nitrosothiols, and *S*-nitrosothiols have therefore been proposed to function as reservoirs of NO in cells. *S*-Nitrosothiols, which are also intermediates in the vasodilator actions of organic nitrites

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(Ignarro et al., 1981), are potent relaxants of gastrointestinal smooth muscle, including the mouse gastric fundus (Barbier and Lefebvre, 1994; De Man et al., 1996, 2001; Ögülener and Ergün, 2002). Possible pathways for the intracellular formation of *S*-nitrosothiols have been proposed but the cellular components that are involved in the endogenous release of NO from *S*-nitrosothiols remain unclear. *S*-Nitrosothiols can decompose photochemically and thermally to liberate NO and the corresponding disulphide (Williams, 1983; Sexton et al., 1994; Singh et al., 1995). Also, some studies showed a copper(I)-dependent mechanisms to be responsible for the release of NO from *S*-nitrosothiols (Askew et al., 1995; Gordge et al., 1995; Al-Sa'doni et al., 1997). The major argument in favour of *S*-nitrosothiol decomposition is the fact that the selective bidentant copper(I) chelator, neocuproine, exerts a blocking effect on this process. Selective chelators of the copper(I) ion, neocuproine and bathocuproine disulphonate, but not the chelator of copper(II), cuprizone, block the ability of *S*-nitroso-*N*-acetyl-D,L-penicillamine to inhibit human blood platelets (Gordge et al., 1995, 1996). Moreover, neocuproine blocks the ability of *S*-nitroso-*N*-acetyl-D,L-penicillamine and *S*-nitrosogluathione to relax rat vascular smooth muscle (Al-Sa'doni et al., 1997), rat and mouse gastric fundus (De Man et al., 1999, 2001) and mouse corpus cavernosum (Göçmen et al., 2000). These results prompted us to investigate whether a similar copper-dependent mechanism contributes to photorelaxation. Although it is well known that the decomposition of *S*-nitrosothiols is catalysed by copper ions, to our knowledge, the role of copper ions in photorelaxation has not been studied. In the present study, we investigated the effects of copper and selective chelators of copper(I) and copper(II),neocuproine and cuprizone, respectively, to determine whether this metal ion participates in the release of NO following the light-induced decomposition of endogenous *S*-nitrosothiols in mouse gastric fundus. For comparison, we studied their effects on the relaxation evoked by *S*-nitrosoglutathione, since the role of copper in the liberation of NO from this compound is well established (Dicks et al., 1996; Gorren et al., 1996; Noble et al., 1999), and, also, we recently reported that intracellular *S*-nitrosogluathione may be a photosensitive store of NO in mouse gastric fundus (Ögülener and Ergün, 2002).

## 2. Materials and methods

### 2.1. Tissue preparation

Swiss albino mice of either sex, weighing 25–30 g, were used in these experiments. They were fasted for 24 h with free access to water, then killed by stunning and cervical dislocation. The stomach was removed and longitudinal muscle strips (approximately 10 mm long and 2 mm wide) were mounted in 10-ml organ baths filled with Tyrode solution (in mM: NaCl 136.75, KCl 2.68, $CaCl_2$ 1.80, $MgCl_2 \cdot 6H_2O$ 0.95, $NaH_2PO_4 \cdot 2H_2O$ 0.4166, $NaHCO_3$ 11.904, glucose 5.05). The bath medium was maintained at 37 °C and gassed with a mixture of 95% $O_2$ and 5% $CO_2$. Muscle strips were allowed to equilibrate for 60 min, during which the medium was changed every 15 min. Over the first 30 min of incubation, the strips were stretched to obtain an initial tension of 0.5 g and remained approximately at this level throughout the experiments without addition of exogenous contractile agonist. Changes in muscle length were recorded isometrically via an isometric transducer (Ugo Basile 7006, Varese, Italy) connected to an ink-writer (Ugo Basile "Gemini" 7070, Varese, Italy).

### 2.2. Light source

The radiation source for photorelaxation was a 6-W UV lamp with peak intensity at 366 nm (VL 6 LC; Vilber Lourmat, Cedex, France). The UV lamp was placed next to the outer wall of a jacketed glass incubation chamber. The distance from the lamp to the preparation during irradiation was about 3 cm. The UV lamp, organ bath and solution flasks were wrapped with aluminium foil to block out light.

### 2.3. Experimental protocols

Once a stable basal tone was obtained, two series of control relaxant responses were obtained according to one of two experimental protocols. In the first protocol, irradiation (60 s), exogenous NO (10 μM; administered as acidified $NaNO_2$) or isoproterenol (5 nM) was applied without rinsing the tissue between each individual application to a single tissue. In the second protocol, concentration–response curves for *S*-nitrosoglutathione were constructed.

After the relaxant responses had been obtained, the tissues were rinsed and incubated for at least 30 min with the drug under study and the second series of responses were recorded in the same manner. At the end of the experimental protocol, sodium nitroprusside (10 μM) was added to the organ bath to achieve maximal relaxation.

In the first series of experiments, the effect of $CuCl_2$ (0.01, 0.1, 1, 5 and 10 μM) was examined on the response to *S*-nitrosoglutathione. Also, the effect of $CuCl_2$ (0.01, 0.1, 1 and 10 μM) was investigated on the relaxant responses to UV irradiation, exogenous NO and isoproterenol. After the first control responses were obtained, $CuCl_2$ was added and UV irradiation and the above-mentioned relaxant substances were applied for the second time. The tissue was incubated with $CuCl_2$ for 10 min. Furthermore, to clarify whether the action was due to copper, the influence of $CuSO_4$ and $FeSO_4$ was studied on the relaxant responses to *S*-nitrosoglutathione.

In a second series of experiments, the effects of the copper(I)-specific chelator, neocuproine, (10, 50 and 100

μM) and the copper(II)-specific chelator, cuprizone (10 and 50 μM), were investigated on relaxations induced by *S*-nitrosoglutathione, UV irradiation, exogenous NO and isoproterenol. The tissue was incubated with neocuproine and cuprizone for 10 min. The effects of neocuproine (50 μM) plus $CuCl_2$ (0.01μM) also were investigated on the relaxant responses to *S*-nitrosoglutathione, UV irradiation, exogenous NO and isoproterenol. None of the drugs used in this investigation influenced the basal tone of the tissue under study.

### 2.4. Drugs and solutions

The following drugs were used: copper(II) chloride, copper sulphate 5-hydrate, iron sulphate (Merck, Darmstadt, Germany); cuprizone, isoproterenol, *S*-nitrosoglutathione, sodium nitrite (Sigma, St. Louis, MO, USA); neocuproine (ICN Biomedicals, Aurora, OH, USA). Acidified sodium nitrite, which was used as exogenous NO source, was obtained by diluting sodium nitrite in deaerated water acidified to pH 2 with HCl, was stored at −4 °C and used in its original concentration of 10 μM (Cocks and Angus, 1990). All drugs were dissolved in distilled water.

### 2.5. Presentation of results and statistical analysis

Relaxations are expressed as percentages of the relaxation induced by 10 μM sodium nitroprusside at the end of the experiment. The results are expressed as means ± S.E.M. and *n* refers to the number of animals used for each experiment. Differences in results between tissues were tested by analysis of variance (ANOVA) and *t* test corrected for multiple comparisons (Bonferroni correction). *P* values of less than 0.05 were considered to be significant.

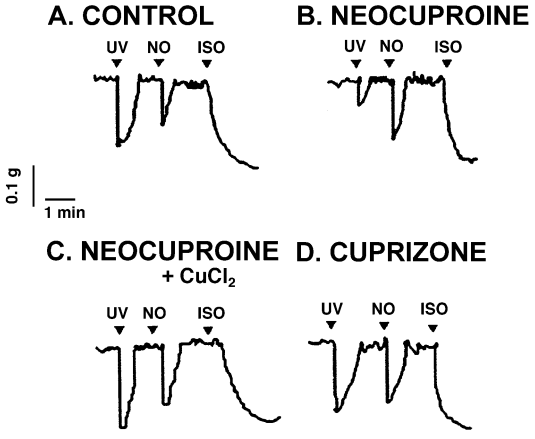


Fig. 1. Representative traces showing (A) the relaxations in response to UV irradiation (UV; 366 nm, 60 s), exogenous NO (NO; 10 μM) and isoprotrenol (ISO; 5 nM); (B) the effects of neocuproine (50 μM); (C) neocuproine (50 μM) plus $CuCl_2$ (0.01 μM) ; (D) cuprizone (50 μM) on the relaxations elicited by UV irradiation (366 nm, 60 s), exogenous NO (10 μM) and isoprotrenol (5 nM) in longitudinal strips of the mouse gastric fundus.

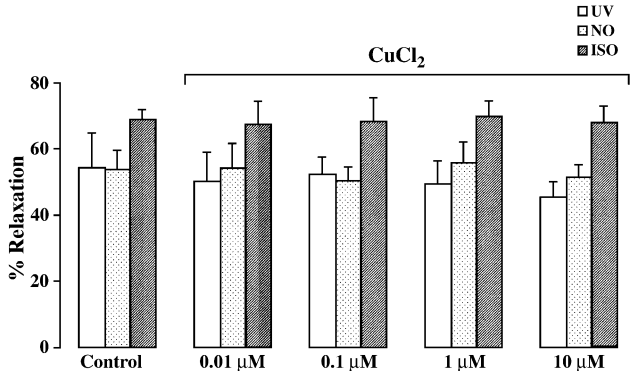


Fig. 2. Effects of $CuCl_2$ (0.01, 0.1, 1 and 10 μM) on the relaxations induced by UV irradiation (UV; 366 nm, 60 s), exogenous NO (NO; 10 μM) and isoprotrenol (ISO; 5 nM) in longitudinal strips of the mouse gastric fundus. Values are means ± S.E.M. for $n=6$ experiments. One-way ANOVA followed by Bonferroni multiple comparison *t* test for values did not reveal any significant differences.

## 3. Results

### 3.1. Effect of $CuCl_2$ on relaxation evoked by UV irradiation, exogenous NO and isoproterenol

To investigate the involvement of copper ions in the mechanism of photorelaxation, the effect of $CuCl_2$ was studied on the relaxant responses to UV irradiation. UV irradiation (366 nm, 60 s) of the mice gastric fundus induced a relaxation in a reversible manner. This photorelaxation was fast and transient, and after the light was switched off the tone of strips returned to the initial level within 1 min. Bolus injection of exogenous NO (10 μM) caused a fast and transient relaxation while the relaxation induced by isoproterenol (5 nM) was slow in onset and was sustained ($n=6$, Fig. 1A). Pretreatment with $CuCl_2$ (0.01–10 μM) did not affect the relaxation evoked by UV irradiation, exogenous NO (10 μM) and isoproterenol (5 nM), as compared to control conditions ($n=6$, Fig. 2).

### 3.2. Effect of $CuCl_2$ on relaxations evoked by S-nitrosoglutathione

*S*-Nitrosoglutathione at 0.01 μM did not induce relaxations, whereas higher concentrations (0.1, 0.5 and 1 μM) induced concentration-dependent relaxations ($n=6$). Pretreatment with $CuCl_2$ at 0.01 μM did not affect the relaxations evoked by *S*-nitrosoglutathione, but higher concentrations of $CuCl_2$ (0.1–10 μM) significantly enhanced the amplitude of the relaxations evoked by *S*-nitrosoglutathione ($n=6$), and the start of the relaxation induced by *S*-nitrosoglutathione became much more rapid after addition of $CuCl_2$ (1, 5 and 10 μM). $CuSO_4$ but not $FeSO_4$

mimicked the effect of $CuCl_2$ on relaxations elicited by *S*-nitrosoglutathione. $CuSO_4$ (10 μM) enhanced the *S*-nitrosoglutathione-induced relaxations whereas $FeSO_4$ (10 μM) had no effect on the relaxations evoked by *S*-nitrosoglutathione (data not shown).

### *3.3. Effects of neocuproine and cuprizone on relaxation evoked by UV irradiation, exogenous NO and isoproterenol*

To investigate the involvement of copper ions in the mechanism of photorelaxation, we studied the effects of neocuproine and cuprizone on the relaxant response to UV irradiation, exogenous NO (10 μM) and isoproterenol (5 nM). Neocuproine (10, 50 and 100 μM), after an incubation period of 10 min, significantly inhibited the photorelaxation in a concentration-dependent manner without affecting the basal tone ($n=6$; Figs. 1B and 3). The inhibitory effect of neocuproine (50 μM) on the photorelaxation was prevented by addition of $CuCl_2$ in concentrations of 0.01 and 0.1 μM, which had no effect on the photorelaxation ($n=6$; Figs. 1C and 3). In contrast to photorelaxation, the relaxations elicited by exogenous NO (10 μM) were significantly enhanced by neocuproine (10, 50 and 100 μM). The relaxations elicited by isoproterenol (5 nM) were not affected by neocuproine (10, 50 and 100 μM) ($n=6$; Fig. 3).

The copper(II)-specific chelator cuprizone (10 and 50 μM) did not affect relaxations elicited by UV irradiation, exogenous NO (10 μM) and isoproterenol (5 nM) ($n=6$; Fig. 1D).

### *3.4. Effects of neocuproine and cuprizone on relaxations elicited by S-nitrosoglutathione*

The effects of the copper(I)-specific chelator, neocuproine, and the copper(II)-specific chelator, cuprizone, were studied on the relaxant response to *S*-nitrosoglutathione (0.1, 0.5 and 1 μM). Neocuproine (10 and 50 μM) significantly inhibited the relaxations induced by *S*-nitrosoglutathione ($n=6$; Fig. 4). The inhibitory effect of neocuproine was prevented by addition of $CuCl_2$ at 0.01 and 0.1 μM ($n=6$; Fig. 4). In contrast to neocuproine, cuprizone at 10 and 50 μM did not affect *S*-nitrosoglutathione-induced relaxations ($n=6$; data not shown).

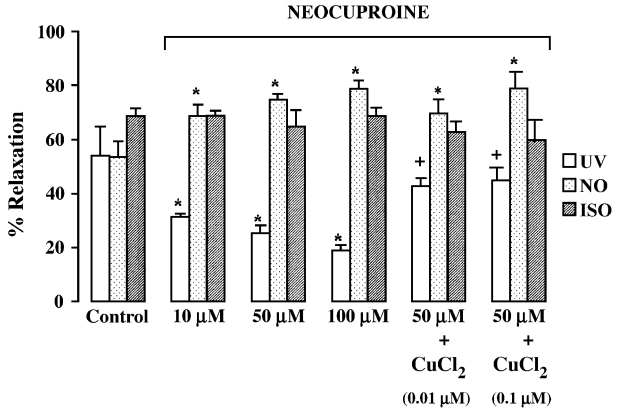


Fig. 3. Effects of neocuproine (10, 50 and 100 μM) and neocuproine (50 μM) plus $CuCl_2$ (0.01 and 0.1 μM) on the relaxations induced by UV irradiation (UV; 366 nm, 60 s), exogenous NO (NO; 10 μM) and isoprotrenol (ISO; 5 nM) in longitudinal strips of the mouse gastric fundus. Values are means ± S.E.M. for $n=6$ experiments. $^{*}P<0.05$ is considered as significantly different from control; $^{+}P<0.05$, significantly different from neocuproine (50 μM), One-way ANOVA followed by Bonferroni multiple comparison *t* test.

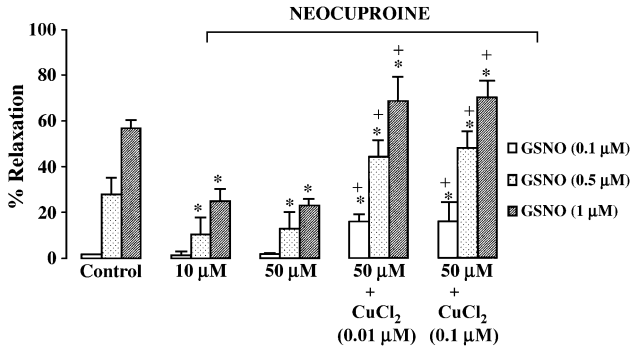


Fig. 4. Effects of neocuproine (10 and 50 μM) and neocuproine (50 μM) plus $CuCl_2$ (0.01 and 0.1 μM) on the relaxations induced by 0.1, 0.5 and 1 μM *S*-nitrosoglutathione (GSNO) in longitudinal strips of the mouse gastric fundus. Values are means ± S.E.M. for $n=6$ experiments. $^{*}P<0.05$ is considered as significantly different from control; $^{+}P<0.05$, significantly different from neocuproine (50 μM), One-way ANOVA followed by Bonferroni multiple comparison *t* test.

## 4. Discussion

In this study, we investigated the effects of copper ions and chelators of copper, neocuproine and cuprizone, selective chelators of copper(I) and copper(II), respectively, on the relaxations elicited by UV irradiation, *S*-nitrosoglutathione and exogenous NO to determine whether copper ions are involved in the release of NO by the decomposition of endogenous *S*-nitrosothiols induced by UV irradiation in the mouse gastric fundus. The present results suggest that copper(I) and not copper(II) may play a role in the NO release subsequent to the light-induced decomposition of an endogenous store of *S*-nitrosoglutathione. Also, the results obtained with the selective copper(I) chelator, neocuproine, confirmed our recent findings that the endogenous "store" of *S*-nitrosoglutathione, rather than NO, acts as an intermediate in photorelaxation in mouse gastric fundus.

It is known that *S*-nitrosothiols are sensitive to both photolytic and transition metal ion-dependent breakdown (Singh et al., 1996). Photolytic and transition metal ion (specially copper)-mediated decomposition of *S*-nitrosothiols liberates NO and results in the formation of disulphides. In the present study, we found that copper ions had a different effect on relaxations induced by UV light and relaxations induced by *S*-nitrosoglutathione. Although $CuCl_2$ concentration dependently enhanced the relaxations evoked by *S*-nitrosoglutathione, it did not affect those

induced by UV light. Indeed, *S*-nitrosoglutathione-evoked relaxations, which were less rapid in onset and more sustained than those evoked by UV irradiation, became much more rapid when the tissue was pre-incubated with 0.1 μM $CuCl_2$, implying that copper ions catalyse the release of NO from *S*-nitrosoglutathione. Our results obtained in the present experiments are consistent with those of De Man et al. (1996), who showed that $CuSO_4$ or $CuCl_2$ enhanced the relaxations evoked by *S*-nitrosothiols in rat gastric fundus. Possibly, the contact time of $CuCl_2$ with exogenous *S*-nitrosoglutathione will be longer than the contact time of $CuCl_2$ with endogenous *S*-nitrosoglutathione, and this may partially explain the discrepant effect. Another possible explanation for this discrepancy is that endogenous tissue copper levels in the mouse gastric fundus are high enough to catalyse of endogenous *S*-nitrosothiols since concentrations of copper ions as low as 1 μM are sufficient to effect the decomposition of *S*-nitrosothiols with the production of NO (Butler and Rhodes, 1997). The ineffectiveness of $CuCl_2$ on the relaxation evoked by exogenous NO and isoproterenol shows that these agents do not require the activity of a copper-dependent mechanism to exert their effects. The effect of $CuCl_2$ was mimicked by $CuSO_4$ but not by $FeSO_4$, showing that the action was due to copper. These results are in agreement with the observation that copper ions but not the iron ions and $SO_4^-$ ions catalyse the release of NO from *S*-nitrosothiols (Askew et al., 1995; De Man et al., 1996).

To further investigate the role of copper ions in the relaxation induced by UV irradiation, we made use of selective chelators of copper ions. Previous chemical studies have shown that the decomposition of *S*-nitrosothiols to release NO is catalysed by copper(I) ions. The strongest evidence to support copper(I) as the catalytic species is the inhibition caused by the selective copper(I) inhibitor, neocuproine. It has been reported that the biological activity of *S*-nitrosothiols can be modulated by copper (Askew et al., 1995; Gordge et al., 1995). Also, Dicks et al. (1996) observed that neocuproine inhibits the copper-induced liberation of NO from *S*-nitrosothiols in a cell-free system. Moreover, neocuproine blocks the ability of *S*-nitroso-*N*-acetyl-D,L-penicillamine and *S*-nitrosoglutathione to relax the rat isolated perfused tail artery and a copper(I)-dependent mechanism is responsible for the liberation of NO from endogenous *S*-nitrosothiols (Al-Sa'doni et al., 1997). Subsequently, Göç men et al. (2000) showed that a copper(I)-dependent mechanism may play a role in the relaxation induced by endogenous relaxant factor as well as by *S*-nitrosoglutathione in mouse cavernosal tissue. Similarly, experiments with neocuproine and cuprizone, selective chelators of copper(I) and copper(II), respectively, in rat and mouse gastric smooth muscle provided evidence that copper(I) and not copper(II) modulates the biological activity of *S*-nitrosothiols (De Man et al., 1999, 2001). In keeping with the conclusion from these studies that endogenous copper(I) plays a role in the generation of NO from *S*-nitrosothiols, we found that the copper(I) chelator, neocuproine, but not the copper(II) chelator, cuprizone, significantly inhibited the relaxations induced by both UV-irradiation and *S*-nitrosoglutathione, suggesting that copper(I) and not copper(II) modulated the biological activity of endogenous *S*-nitrosothiols in the mouse gastric fundus. The inhibitory effect of neocuproine that resulted from chelation of copper(I) ions was prevented by addition of $CuCl_2$. The relaxations evoked by isoproterenol were not affected by neocuproine, ruling out a non-specific inhibitory action of specific copper(I) chelator on smooth muscle. In contrast, neocuproine enhanced the relaxation in response to exogenous NO in a different manner to that of relaxations evoked by *S*-nitrosothiols and UV irradiation, indicating that the activity of the NO released by UV irradiation in this tissue resembles that of *S*-nitrosoglutathione but not that of free NO. This augmentation of the relaxation elicited by NO may be dependent on a prejunctional protective effect of neocuproine. Also, De Man et al. (2001) showed that neocuproine potentiated relaxations elicited by the nitrergic neurotransmitter and by exogenous NO in mouse gastric fundus, suggesting that neocuproine protects the biological activity of the nitrergic neurotransmitter by acting as an antioxidant both at a prejunctional and a postjuctional level.

In summary, in the mouse gastric fundus, neocuproine but not cuprizone inhibited the relaxations evoked by UV irradiation and *S*-nitrosoglutathione and enhanced those evoked by free NO. In the presence of copper, the neocuproine-induced inhibition was prevented. The present results suggest that copper(I) and not copper(II) may partly mediate the release of NO by the light-induced decomposition of an endogenous store of *S*-nitrosoglutathione. But additional studies are required to fully understand the transition metal ion-dependent decomposition chemistry of endogenous *S*-nitrosothiols by UV light. Also, results obtained with the selective copper(I) chelator, neocuproine, confirmed our recent findings that the endogenous "store" of *S*-nitrosoglutathione, and not NO, acts as an intermediate in photorelaxation of the mouse gastric fundus, and we propose that photorelaxation is a suitable model to elucidate the nature of endogenous *S*-nitrosothiols.

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