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*Asian J Androl 2008; 10 (6): 905–913*
DOI: 10.1111/j.1745-7262.2008.00380.x

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·*Original Article*·

# Homocysteine and copper interact to promote type 5 phosphodiesterase expression in rabbit cavernosal smooth muscle cells

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**Abstract**

**Aim:** To study the effects of homocysteine and copper on type 5 phosphodiesterase (PDE5) expression in cavernosal vascular smooth muscle cells (CVSMCs) and to investigate superoxide ($O_2^{\cdot-}$) derived from nicotinamide adenine dinucleotide phosphate oxidase as homocysteine and copper generate $O_2^{\cdot-}$, and $O_2^{\cdot-}$ upregulates PDE5 expression. **Methods:** CVSMCs derived from rabbit penis were incubated with homocysteine or copper chloride with or without superoxide dismutase (SOD), catalase, sildenafil citrate, or apocynin (nicotinamide adenine dinucleotide phosphate inhibitor) for 16 h. The expression of PDE5 and of glyceraldehyde-3-phosphate dehydrogenase (internal standard) was assessed using Western blot analysis. In parallel, $O_2^{\cdot-}$ was measured spectrophotometrically. **Results:** $CuCl_2$ alone (up to 10 μmol/L) and homocysteine alone (up to 100 μmol/L) had no effect on $O_2^{\cdot-}$ formation in CVSMCs compared to controls. In combination, however, homocysteine and $CuCl_2$ markedly increased $O_2^{\cdot-}$ formation, an effect blocked by SOD, catalase, apocynin, and sildenafil (1 μmol/L) when co-incubated over the same time course. PDE5 expression was also significantly increased in CVSMCs incubated with homocysteine and $CuCl_2$, compared to controls. This effect was also negated by 16-h co-incubation with SOD, catalase, apocynin and sildenafil. **Conclusion:** This represents a novel pathogenic mechanism underlying ED, and indicates that the therapeutic actions of prolonged sildenafil use are mediated in part through inhibition of this pathway. *(Asian J Androl 2008 Nov; 10: 905–913)*

**Keywords:** erectile dysfunction; superoxide; PDE5; sildenafil

## 1 Introduction

The nitric oxide (NO)–cyclic guanosine monophosphate (cGMP) system, by eliciting relaxation of cavernosal smooth muscle and pudendal arteries, is central to penile erection [1]. NO is released from non-adrenergic non-cholinergic fibers that innervate the penis and pudendal arteries and from the endothelium within the corpus cavernosum and pudendal vasculature [1]. NO then activates guanylyl cyclase, which catalyses the conversion of guanosine triphosphate to cGMP, which then induces vascular smooth muscle relaxation, principally through reducing calcium mobilisation [1]. Impairment of NO bioavailability is associated with erectile dysfunc-

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Received 2007-07-03 Accepted 2007-11-25

tion (ED) [2, 3].

Risk factors for ED associated with reduced NO include diabetes mellitus (DM), hypertension, dyslipidemia, smoking, and more recently with hyperhomocysteinemIa (HHC), all of which are also associated with increased formation of superoxide ($O_2^{\cdot-}$) [4, 5]. $O_2^{\cdot-}$ reacts with NO leading to reduced "NO drive" [4, 5]. A principal intravascular source of $O_2^{\cdot-}$ is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [5]. Many factors associated with ED increase the expression of NADPH oxidase. These include thromboxane $A_2$, cytokines, angiotensin II, hypoxia, and $O_2^{\cdot-}$ itself [5, 6]. NADPH oxidase is also linked to the risk factors for ED listed above [5]. Furthermore, sildenafil inhibits the activity and expression of NADPH oxidase (and therefore $O_2^{\cdot-}$) in vascular smooth muscle cells through a cGMP–protein kinase G (PKG)-mediated mechanism [7–11], exemplifying the possible central role of NADPH oxidase in the etiology of ED.

The bioeffectiveness of the NO–cGMP system is also determined by the inactivation of cGMP by type 5 phosphodiesterases (PDE5) [12–15]. PDE5 hydrolyses cGMP to inactive guanosine monophosphate (GMP) [16], thereby stopping the dilator effects of NO. The importance of PDE5 in erection is exemplified by the therapeutic benefit of sildenafil (Viagra®; Pfizer, NY, USA), a PDE5 inhibitor, in treating ED [12–15]. A recent study showed that nicotine and tumor necrosis factor-α increase the expression of PDE5 through a mechanism triggered by $O_2^{\cdot-}$ derived from NADPH oxidase [17] and suggested that risk factors for ED might entail an upregulation of PDE5 by oxidative stress.

Experimental HHC, a risk factor for cardiovascular disease, also promotes ED in rabbit through augmentation of $O_2^{\cdot-}$ formation and negation of NO bioactivity, an effect mediated by an upregulation of NADPH oxidase [18]. *Ex vivo*, homocysteine alone at concentrations far above those seen in HHC has little effect on cavernosal relaxation, indicating that homocysteine alone is not an independent risk factor for ED [19]. At concentrations seen in HHC, however, homocysteine interacts with physiological concentrations of copper to inhibit NO-mediated relaxation of cavernosal and arterial tissue [19, 20]. Penicillamine, a copper chelator, reverses ED elicited by HHC [9]. It has therefore been suggested that the erectopathic impact of HHC is mediated by an interaction of homocysteine with copper that augments $O_2^{\cdot-}$ formation and reduces NO bioavailability. As $O_2^{\cdot-}$ upregulates the expression of PDE5, it is reasonable to suggest that homocysteine and copper might interact to promote ED through increased intracavernosal PDE5 expression. In order to test this possibility, we studied the interactive effect of homocysteine and copper on $O_2^{\cdot-}$ formation, determined its source, and studied its effects on PDE5 expression. The effect of sildenafil on these systems was also studied, as sildenafil inhibits the formation of $O_2^{\cdot-}$ through suppression of NADPH oxidase activity [7–11] and, as such, would be expected to block these hypothetical sequelae.

## 2 Materials and methods

New Zealand White rabbits were used for this study, and given humane care in compliance with Bristol University and the UK Home Office rules and regulations. They were killed with a lethal dose of barbiturates, and the penile tissue promptly excised. The corpora cavernosae were dissected from the surrounding tunica albuginea, cut into 2 $mm^2$ segments, and placed in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (both from Gibco BRL, Paisley, UK) and incubated at 37ºC in a 95% air, 5% $CO_2$ incubator for 7 days, during which time cavernosal vascular smooth muscle cells (CVSMCs) grew from the explant. The explants were removed and adherant CVSMCs trypsinized and placed in culture plates until confluent. These cells stained positively for alpha-actin but not von Willebrand Factor (vWF) or lectin (markers of endothelial cells). CVSMCs were subsequently incubated at 37ºC in a 95% air, 5% $CO_2$ incubator as previously described [6]. When confluent, CVSMCs were quiesced in low glucose DMEM (containing 100 units/mL penicillin and 100 μg/mL streptomycin; Gibco BRL). After 72-h quiescence, the following experiments were undertaken.

### *2.1 Effects of homocysteine and copper on $O_2^{\cdot-}$ formation*

CVSMCs were incubated with homocysteine (1 μmol/L–1 mmol/L; Sigma Chemical, Poole, Dorset, UK), or copper chloride (0.1 μmol/L–10 μmol/L; Sigma Chemical) or homocysteine plus copper chloride in 4.5 g/L DMEM for 16 h. $O_2^{\cdot-}$ formation was measured using the reduction of ferricytochrome c method [21, 22]. Supernatants were discarded, and cells washed with sterile Dulbecco's phosphate-buffered saline (Bio Whittacker, Bio Science, Erembodegen, Belgium). The cells were equilibrated with DMEM without phenol red (Bio

Whittaker, Bio Science) with or without copper–zinc superoxide dismutase (SOD; 500 U/mL; Sigma Chemical). Following 30 min incubation at 37ºC in a 95% air, 5% $CO_2$ incubator (Heraeus Equipment, Essex, UK), 20 μmol/L horse heart cytochrome c (Sigma Chemical) was added, and incubated for a further 1 h. The amount of cytochrome c reduced by SOD was determined at 550 nm using an Anthos Lucy 1 spectrometer (Laboratory-tech International, East Sussex, UK) and converted to μmol/L of $O_2^{\cdot-}$ (using the equation $\Delta E = 550\ nm = 21.1\ mM^{-1}\ cm^{-1}$ as the extinction coefficient). This reflects the actual $O_2^{\cdot-}$ release [9]. Following determination of the optimal concentration at which homocysteine plus copper induced $O_2^{\cdot-}$ production, the experiment was repeated with co-incubation for 16 h with SOD (500 U/mL; converts $O_2^{\cdot-}$ to $H_2O_2$; Sigma Chemical)) and catalase (100 U/mL; converts $H_2O_2$ to $H_2O$; Sigma Chemical). In order to ascertain the source of increased $O_2^{\cdot-}$ release from NADPH oxidase, the experiment was repeated with the NADPH oxidase inhibitor, apocynin (1 μmol/L; Sigma Chemical). The effects of sildenafil citrate (1 μmol/L for 16 h; Schering Pharmaceuticals, Newbury, UK) on $O_2^{\cdot-}$ release were assessed through $O_2^{\cdot-}$ experiments as previously described [21, 22].

*2.2 Effects of homocysteine and copper on PDE5 expression using Western blot analysis*

CVSMCs were incubated with homocysteine plus copper chloride with or without SOD (500 U/mL), catalase (100 U/mL), and sildenafil citrate (1 μmol/L) in 4.5 g/L DMEM for 16 h at 37ºC in a 95% air, 5% $CO_2$ incubator. For Western blots, the supernatant was discarded, and the cells were washed with Dulbecco's phosphate-buffered solution (Gibco BRL). Following the addition of protein lysis buffer (Tris buffer, 100 mmol/L, pH 6.8) containing 1% glycerol and 1% sodium dodecyl sulphate, the cell lysates were stored at –20ºC. Protein content on the lysates was quantified using the MicroBCA kit (Pierce, Rockford, IL, USA). Samples of equal protein concentration were loaded to 10% Tris-glycine sodium dodecyl sulphate gel, as well as rainbow markers (14–220 kDa; Amersham International, Little Chalfont, UK) to assess molecular weight, and electophoresed. Samples were transferred to a Hybond-C nitro-cellulose-*pure* membrane (Amersham International). After blocking in 5% Marvel (Spalding, UK) the membranes were incubated with 1:1 000 rabbit anti-PDE5 antibody (Cell Signalling Technology, Hitchin, UK) in 5% bovine serum albumin (Sigma Chemical) and 0.1% sodium azide at 5ºC, with constant agitation, for 16 h. Following incubation with the secondary antibody, goat anti-mouse antibody conjugated with horseradish peroxidase (1:5 000; Dako, Ely, UK) for 1 h, the blots were developed using enhanced chemiluminescence (Amersham International) on X-ray film, and bands scanned using Quantity One® 1-D analysis software (Bio-Rad Laboratories, Hercules, CA, USA).

*2.3 Data analysis and expression*

Data were expressed as mean ± SEM of six separate studies. The paired *t*-test was used to compare effects of homocysteine and $CuCl_2$ with the control. Multiple group comparisons were made using the repeated measures ANOVA test. Data was calculated using Instat Graphpad software (Graphpad Software, San Diego, CA, USA). Significance was accepted at $P < 0.05$.

## 3 Results

Homocysteine alone and copper chloride alone elicited no effects on $O_2^{\cdot-}$ formation by CVSMCs following a 16-h incubation compared to controls (Figure 1). In contrast, when combined together, homocysteine and copper chloride markedly augmented the formation of $O_2^{\cdot-}$ (Figure 1). In all subsequent studies, 100 μmol/L homocysteine and 10 μmol/L copper chloride were used, as this combination elicited maximal effects. Incubation of CVSMCs with 100 μmol/L homocysteine and 10 μmol/L copper chloride with diphenylene iodonium chloride (DPI), apocynin, or allopurinol for 16 h reversed $O_2^{\cdot-}$ production (Figure 2), indicating that the source of $O_2^{\cdot-}$ is NADPH oxidase, by xanthine oxidase. Co-incubation with SOD (500 U/mL), catalase (100 U/mL) and sildenafil citrate (1 μmol/L) also completely inhibited formation of $O_2^{\cdot-}$ in CVSMCs induced with 100 μmol/L homocysteine and 10 μmol/L copper chloride (Figures 2, 3). Penicillamine inhibited the formation of $O_2^{\cdot-}$ induced by 16-h incubation with 100 μmol/L homocysteine and 10 μmol/L copper chloride (Figure 4). PDE5 expression in rabbit CVSMCs was increased by 100 μmol/L homocysteine and 10 μmol/L copper chloride, compared to control, following 16 h incubation (Figures 5, 6). This effect was inhibited by co-incubation with SOD, catalase, allopurinol, apocynin, and sildenafil citrate (Figure 5). Co-incubation of 100 μmol/L homocysteine and 10 μmol/L copper chloride with penicillamine for 16 h reduced PDE5 expression (Figure 6).

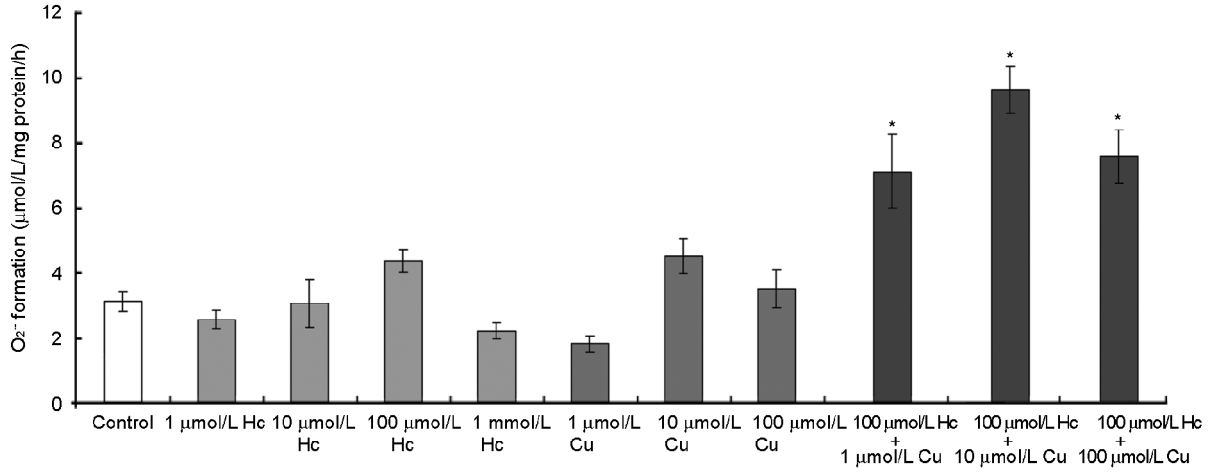


Figure 1. Effect of homocysteine (Hc) alone, copper chloride (Cu) alone, and combinations of Hc and Cu on superoxide ($O_2^{\cdot-}$) formation in rabbit isolated corpus cavernosal smooth muscle cells following 16-h incubation. Each point represents mean ± SEM ($n$ = 6). $^{*}P < 0.01$, significantly greater than control.

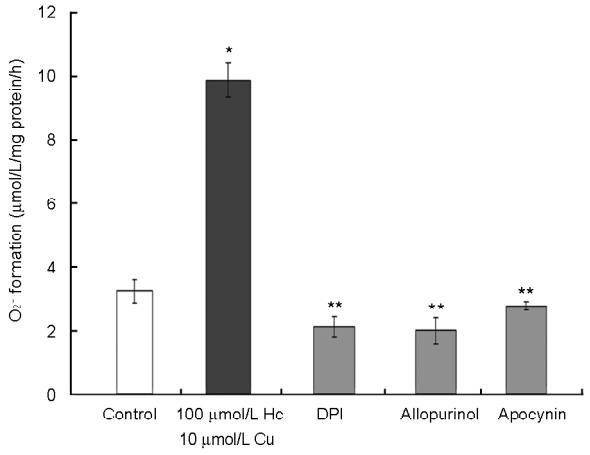


Figure 2. Effect of homocysteine (Hc; 100 μmol/L)/copper chloride (Cu; 10 μmol/L) and co-incubation with diphenylene iodonium chloride (DPI), allopurinol and apocynin on superoxide ($O_2^{\cdot-}$) formation in rabbit isolated corpus cavernosal smooth muscle cells following 16 h incubation. Each point represents mean ± SEM ($n$ = 6). $^{*}P = 0.0003$, significantly greater than control; $^{**}P < 0.01$, significantly less than Hc (100 μmol/L)/Cu (10 μmol/L) alone.

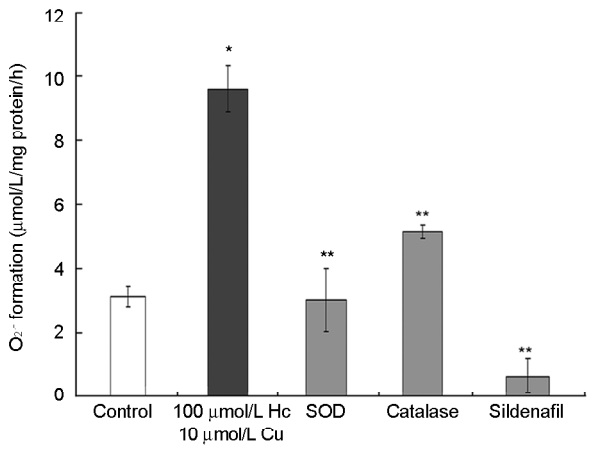


Figure 3. Effect of homocysteine (Hc; 100 μmol/L)/copper chloride (Cu; 10 μmol/L) and co-incubation with superoxide dismutase (SOD), catalase, and sildenafil citrate (1 μmol/L) on superoxide ($O_2^{\cdot-}$) formation in rabbit cavernosal smooth muscle cells following 16-h incubation. Each point represents mean ± SEM ($n$ = 6). $^{*}P < 0.0008$, significantly greater than control; $^{**}P < 0.01$, significantly less than Hc (100 μmol/L)/Cu (10 μmol/L) alone.

## 4 Discussion

The present study shows that homocysteine alone or copper alone at (patho)physiological concentrations have no significant effect on $O_2^{\cdot-}$ formation in rabbit cavernosal smooth muscle cells, following 16-h incubation. Plasma concentrations of copper are between 10 μmol/L and 20 μmol/L [23]. A plasma concentration of homocysteine above 14.5 μmol/L is considered a risk factor for vasculopathy [24]. By contrast, homocysteine and copper, in combination, markedly augment the formation of $O_2^{\cdot-}$. These effects were inhibited by apocynin

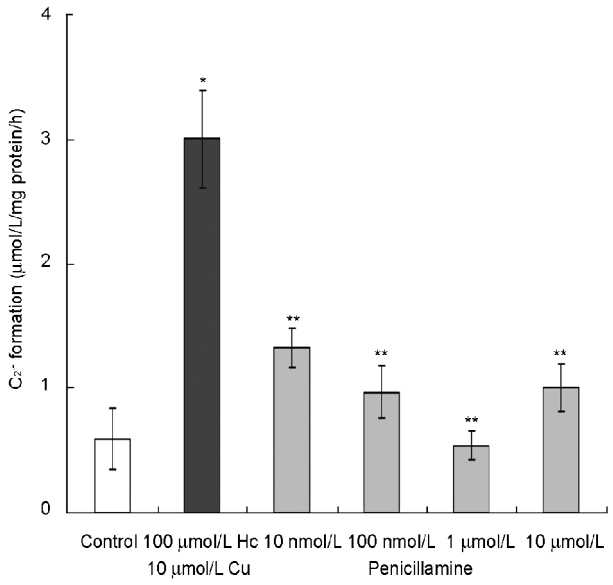


Figure 4. Effect of homocysteine (Hc; 100 μmol/L) with copper chloride (Cu; 10 μmol/L), and co-incubation with penicillamine (10 nmol/L–10 μmol/L), on superoxide ($O_2^{\cdot-}$) formation in rabbit cavernosal smooth muscle cells, following 16-h incubation. Each point represents mean ± SEM ( $n$ = 6). $^{*}P$ = 0.0008, significantly greater than control; $^{**}P$<0.01, significantly less than Hc/Cu alone.

and DPI, specific inhibitors of NADPH oxidase, and by allupurinol, a xanthine oxidase inhibitor [21–22, 25], after incubation, indicating that the inducible sources of $O_2^{\cdot-}$ are both NADPH oxidase and xanthine oxidase. Co-incubation of homocysteine and copper with SOD or catalase over the 16-h incubation phase significantly reduced $O_2^{\cdot-}$ formation, indicating that the endogenous formation of $O_2^{\cdot-}$ and $H_2O_2$ mediates this upregulation of NADPH oxidase and xanthine oxidase. This is consistent with a recent study in pulmonary endothelial cells in which $O_2^{\cdot-}$ was shown to directly augment the expression of NADPH oxidase, thereby generating even more $O_2^{\cdot-}$ through a positive feedback loop [26].

In a previous study, we showed that nicotine and tumor necrosis factor-α promoted the expression of PDE5 through an *a priori* augmentation of NADPH oxidase and $O_2^{\cdot-}$ formation [17]. The present study also shows that homocysteine + copper together elicited an increase in PDE5 protein expression in cavernosal cells following 16-h incubation. This PDE5 expression was blocked by the co-incubation of cells with SOD, catalase, and apocynin, indicating that the effect is mediated through both $O_2^{\cdot-}$ and $H_2O_2$, the source of which is NADPH oxidase. As PDE5 hydrolyses cGMP to inactive GMP, and cGMP mediates NO-elicited erection, these data indicate that homocysteine and copper together, but not alone, might contribute to ED through an upregulation of PDE5. Although increased PDE5 activity has yet to be shown in cavernosal tissue in risk factor models for ED, it has been shown that both cGMP and cyclic adenosine monophosphate (cAMP) are reduced in the cavernosal tissue from diabetic animals [27, 28]. As DM is a major risk factor for ED [29], this indicates that there is an increase in PDE5 and possibly PDE4 activity in conditions associated with ED. In the context of the present study and DM, homocysteine has been shown to augment $O_2^{\cdot-}$ formation from arterial tissue of diabetic rabbits [30]. Due to the enormous complexity of the possible systems involved, the intracellular mechanisms underlying the effect of $O_2^{\cdot-}$ on PDE5 expression was not addressed in this study. However, possible candidates include activation of the kinases that modulate transcription and translation of proteins, including rho and tyrosine kinases, as well as PKG itself. This area warrants further study.

Penicillamine, a copper chelator, inhibited the upregulation of $O_2^{\cdot-}$ formation and PDE5 expression. However, the concentrations at which this was effective were well below the levels required to chelate copper. This indicates that penicillamine is exerting an antioxidative action independently of copper chelation. As penicillamine reverses ED in the hyperhomocysteinemic rabbit [9], these other possible mechanisms warrant further investigation.

Several *in vitro* studies have indicated that homocysteine alone has little effect on NO formation and mmol/L concentrations need to be used to elicit inhibitory effects [18, 19, 31–34]. Even 1 mmol/L is significantly higher than the concentrations of homocysteine seen even in severe HHC, which range from 15 μmol/L to 50 μmol/L [24]. The present study consolidates that homocysteine alone is not an independent risk factor for ED, as 1 mmol/L homocysteine had little effect on $O_2^{\cdot-}$ and PDE5 expression. By contrast, there are several reports that physiological concentrations of copper interact with physiological concentrations of homocysteine to reduce NO formation, an effect that is dependent on the formation of $O_2^{\cdot-}$ [19, 20]. Under normal circumstances, copper is tightly bound to plasma proteins, in particular, ceruloplasmin [31]. However, $O_2^{\cdot-}$, as well as homocysteine itself, dissociates copper from its protein binding sites on ceruloplas-

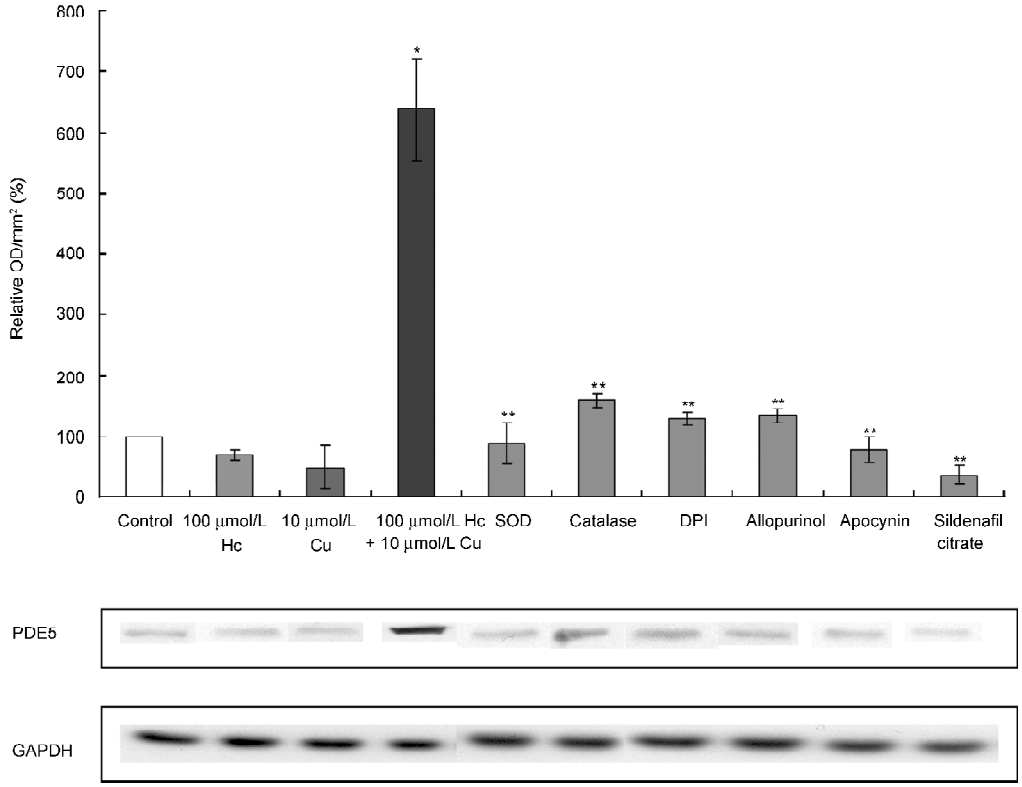


Figure 5. Western blot analysis of the effect of homocysteine (Hc; 100 μmol/L) with copper chloride (Cu; 10 μmol/L), and co-incubation with superoxide dismutase (SOD), catalase, diphenylene iodonium chloride (DPI), allopurinol, apocynin, and sildenafil citrate, on type 5 phosphodiesterase (PDE5) expression in rabbit cavernosal smooth muscle cells, after 16-h incubation. Data are expressed as percentage optical density (OD) relative to controls (control being 100%). Representative Western blots of PDE5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression are shown beneath these data. Each point represents mean ± SEM ($n = 6$). $^{*}P = 0.0014$, significantly greater than control; $^{**}P < 0.01$, significantly less than Hc/Cu alone.

min and fragments the protein [31], which might augment the powerful pro-oxidant effects of copper and its interaction with copper [31]. Furthermore, both homocysteine and copper alone augment oxidative stress in arteries from diabetic rabbits but have no effect on arteries from non-diabetic controls [30, 35].

The present study also indicates that sildenafil citrate, at therapeutic circulating levels of the drug [11], significantly reduces PDE5 expression in response to homocysteine plus copper. Sildenafil also inhibits the activity and expression of NADPH oxidase in cavernosal and arterial endothelial cells, through augmentation of the cGMP–PKG axis [7–11]. Because $O_2^{\cdot-}$ derived from NADPH oxidase upregulates PDE5, we suggest that sildenafil suppresses PDE5 expression through an *a priori* inhibition of NADPH oxidase. This would restore the NO–cGMP pathway, improving the erectile response. PDE5 inhibitors also potentiate endogenous increases in cGMP by inhibiting its breakdown at the catalytic site. Phosphorylation of PDE5 increases its enzymatic activity as well as the affinity of its allosteric sites for cGMP. Binding of cGMP to the allosteric site further stimulates enzymatic activity [36, 37]. Thus, phosphorylation of PDE5 and binding of cGMP to the noncatalytic sites mediates negative feedback regulation of the cGMP pathway. Binding of substrate or substrate analogs, such as tadalafil and vardenafil, to the catalytic site converts a fast (low-affinity) inhibitor dissociation component of the PDE5 catalytic site to a slow (high-affinity) inhibitor dissociation component. This effect is predicted to improve the substrate affinity or inhibitory potencies of these compounds in intact cells [36, 37].

From a therapeutic perspective, the present data point to reducing homocysteine–copper interactions as a means

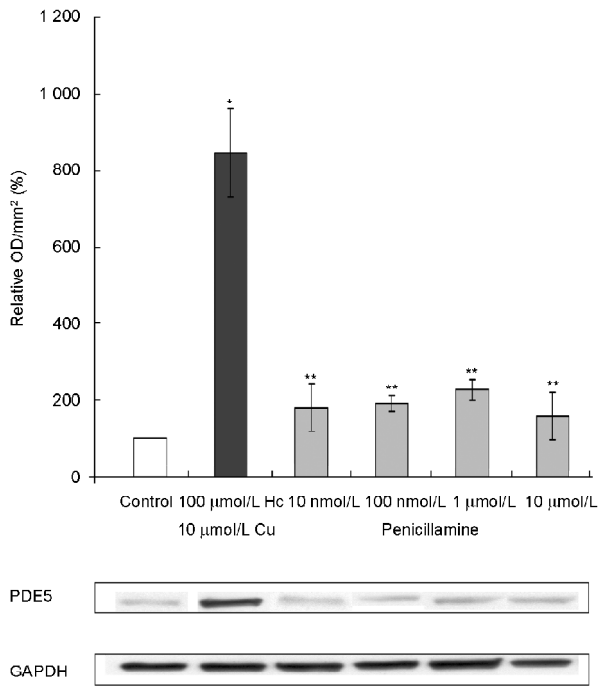


Figure 6. Western blot analysis of the effect of homocysteine (Hc; 100 μmol/L) with copper chloride (Cu; 10 μmol/L), and co-incubation with penicillamine (10 nmol/L–10 μmol/L) on type 5 phosphodiesterase (PDE5) expression in rabbit cavernosal smooth muscle cells, after 16 h incubation. Data are expressed as percentage optical density (OD) relative to controls (control being 100%). Representative Western blots of PDE5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression are shown beneath these data. Each point represents mean ± SEM ($n$ = 6). $^{*}P = 0.0057$, significantly greater than control; $^{**}P < 0.01$, significantly less than Hc/Cu alone.

of improving erectile function. Penicillamine is used to treat Wilsons's disease, systemic sclerosis, cysteine stones, and intractable rheumatoid arthritis [38, 39]. However, due to side-effects, it is unlikely that penicillamine would be an advisable treatment in patients with ED. In contrast, the folic acid reliably reduces plasma homocysteine and improves endothelium-dependent relaxation, not only in HHC patients but also in patients with DM, dyslipidemia, and hypertension [40], all risk factors for ED. It is reasonable to suggest, therefore, that folic acid would improve erectile function in patients with ED. Importantly, there is now a school of thought that the therapeutic benefit of folic acid is independent of its homocysteine-lowering effect and that folate and its active metabolite, 5-methyltetrahydrofolate exerts direct protective effects on blood vessels that include a reduction of $O_2^{\cdot-}$ formation and prevention of endothelial nitric oxide synthase uncoupling [41]. Furthermore, the use of NO donors, which augment cGMP formation, is also a possible therapeutic strategy for treating ED in pateints who do not respond favourably to PDE5 inhibitors [42].

In conclusion, this study shows that homocysteine and copper interact to induce $O_2^{\cdot-}$ formation from NADPH oxidase in rabbit CVSMCs that, in turn, leads to an upregulation of PDE5. Both $O_2^{\cdot-}$ formation and PDE5 upregulation would impair normal erectile responses. Sildenafil blocks both of these events, indicating that prolonged use of sildenafil might be beneficial in treating ED through inhibition of intra-penile oxidative stress. Although long-term effects of PDE5 inhibitors on erectile function in man are not yet published, recent studies have indicated that long-term therapy with PDE5 inhibitors improves erectile function in laboratory animals. Therapeutically, the co-administration of folic acid might improve the therapeutic impact of PDE5 inhibitors in patients who do not respond favourably, as they have a common site of action at the tissue level. Clinical studies with folic acid are therefore warranted.

## Acknowledgment

We thank the Ralph Shackman Trust for financial support.

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