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## **Tetrahydrobiopterin protects soluble guanylate cyclase against oxidative inactivation**

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**Running Title:** Protection of soluble guanylate cyclase by tetrahydrobiopterin

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**Abbreviations:** BAY 58-2667, 4-[[[(4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl}amino)methyl]benzoic]acid; BAY 60-2770, 4-[[[(4-carboxybutyl){2-(5-fluoro-2-[(4'-(trifluoromethyl)biphenyl-4-yl]methoxy)phenyl)ethyl]amino]methyl]benzoic acid; BH<sub>2</sub>, 7,8-dihydro-L-biopterin; BH<sub>4</sub>, (6R)-5,6,7,8-tetrahydro-L-biopterin; DEA/NO, 1,1-diethyl-2-hydroxy-2-nitroso-hydrazine; deoxyHb, deoxyhemoglobin; DHFR, dihydrofolate reductase; eNOS, endothelial nitric oxide synthase; GTN, glycerol nitrate (nitroglycerin); metHb, methemoglobin; MnTBAP, Mn(III)tetrakis(4-benzoic acid)porphyrin; MTX, methotrexate; NH<sub>2</sub>, 7,8-dihydro-D-neopterin; NH<sub>4</sub>, (6RS)-5,6,7,8-tetrahydro-D-neopterin; ODQ, 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxaline-1-one; oxyHb, oxyhemoglobin; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; SOD, superoxide dismutase

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## Abstract

Tetrahydrobiopterin (BH4) is a major endogenous vasoprotective agent that improves endothelial function by increasing NO synthesis and scavenging of superoxide and peroxynitrite. Accordingly, administration of BH4 is considered as a promising therapy of cardiovascular diseases associated with endothelial dysfunction and oxidative stress. Here we report on a novel function of BH4 that might contribute to the beneficial vascular effects of the pteridine. Treatment of cultured porcine aortic endothelial cells with nitroglycerin (GTN) or 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxaline-1-one (ODQ) resulted in heme oxidation of soluble guanylate cyclase (sGC), as evident from diminished NO-induced cGMP accumulation that was paralleled by increased cGMP response to a heme- and NO-independent activator of soluble guanylate cyclase (BAY 60-2770). While scavenging of superoxide and/or peroxynitrite with superoxide dismutase, Tiron, Mn(III)tetrakis(4-benzoic acid)porphyrin and urate had no protective effects, supplementation of the cells with BH4, either by application of BH4 directly or of its precursors dihydrobiopterin or sepiapterin, completely prevented the inhibition of NO-induced cGMP accumulation by GTN and ODQ. Tetrahydrobiopterin had the same effect and virtually identical results were obtained with RFL-6 fibroblasts, suggesting that our observation reflects a general feature of tetrahydropteridines that is unrelated to NO synthase function and not limited to endothelial cells. Protection of sGC against oxidative inactivation may contribute to the known beneficial effects of BH4 in cardiovascular disorders associated with oxidative stress.

## Introduction

Tetrahydrobiopterin (BH4) plays an important role in the vascular system, where it is synthesized in endothelial cells and - to lesser extent - in smooth muscle cells and fibroblasts via *de novo* and salvage pathways (Nichol et al., 1983). Guanosine triphosphate cyclohydrolase I is the first and rate-limiting enzyme in the *de novo* biosynthetic pathway. It catalyzes hydrolysis of GTP to 7,8-dihydroneopterin triphosphate, which is then converted to BH4 by the consecutive action of 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase. In addition to its *de novo* biosynthesis, BH4 is enzymatically regenerated from its oxidation products quinonoid 6,7[8H]-dihydrobiopterin and BH2 by dihydropteridine reductase and DHFR, respectively. Latter reaction is part of the so-called salvage pathway, in which sepiapterin is reduced by sepiapterin reductase to BH2, and further by DHFR to yield BH4 (Werner et al., 2011).

As an essential cofactor of eNOS, BH4 promotes NO synthesis by facilitating the transfer of electrons from the reductase domain to the heme group for reductive activation of molecular O<sub>2</sub>, which is required for oxidation of the amino acid substrate L-arginine. At subsaturating concentrations of L-arginine or BH4, autoxidation of the ferrous-oxy and ferrous-superoxy complexes results in release of superoxide anion and hydrogen peroxide, respectively, at the expense of the productive cycle yielding L-citrulline and NO (Gorren and Mayer, 2007). This phenomenon, referred to as eNOS uncoupling, leads to simultaneous formation of NO and superoxide which rapidly combine to form peroxynitrite, an endogenous cytotoxin causing cellular dysfunction through oxidation of various biomolecules, in particular proteins and DNA (Calcerrada et al., 2011).

Superoxide/peroxynitrite formation by uncoupled eNOS has been implicated in endothelial dysfunction associated with a variety of cardiovascular diseases including atherosclerosis, hypertension and diabetes. In accordance with the role of BH4 as "coupling switch", a causal

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link between BH<sub>4</sub> deficiency and endothelial dysfunction was found in many (though not all) animal models of vascular diseases. Moreover, administration of BH<sub>4</sub> has been shown to restore endothelial function in patients with hypertension, diabetes mellitus, hypercholesterolemia, atherosclerosis, coronary artery disease, chronic heart failure, and in chronic smokers, suggesting that BH<sub>4</sub> deficiency, resulting in eNOS uncoupling and limited NO bioavailability, may play a prominent role in the pathogenesis of cardiovascular diseases (Schmidt and Alp, 2007; Katusic et al., 2009; Vasquez-Vivar, 2009; Förstermann, 2010).

In addition to preventing eNOS uncoupling, BH<sub>4</sub> is also a powerful antioxidant, reacting with superoxide, H<sub>2</sub>O<sub>2</sub>, and peroxynitrite. Accordingly, the beneficial effects of BH<sub>4</sub> could also be ascribed to the antioxidant properties of the pteridine. Some studies have focused on this issue by using NH<sub>4</sub>, a compound that exhibits similar antioxidative properties as BH<sub>4</sub> but does not bind to eNOS, and the results are ambiguous. In caveolin-1-deficient mice, an animal model for endothelial dysfunction, vascular superoxide production was diminished after oral application of BH<sub>4</sub> but not NH<sub>4</sub> (Wunderlich et al., 2008). Similarly, in human studies performed with chronic smokers, endothelial-dependent vasodilation measured as forearm blood flow response to acetylcholine, was improved upon intra-arterial infusion of BH<sub>4</sub> but not NH<sub>4</sub> (Heitzer et al., 2000). However, when endothelial dysfunction was induced by ischemia reperfusion injury in the forearm of healthy human subjects, both BH<sub>4</sub> and NH<sub>4</sub> exerted protective effects (Mayahi et al., 2007). These data demonstrate that at least under certain conditions of oxidative stress, the beneficial effect of BH<sub>4</sub> supplementation is not explained by the reversal of eNOS uncoupling.

In a recent study aimed at clarifying whether exposure of vascular tissue to GTN results in BH<sub>4</sub> depletion, we showed that long-term treatment of cultured endothelial cells with GTN markedly diminished NO-induced cGMP formation in cells and cell extracts (Schmidt et al., 2010). Here we provide conclusive evidence that the apparent dysfunction of sGC results from GTN-triggered oxidation of enzyme-bound heme and demonstrate that the oxidative

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inactivation of sG C is prevented by supplementation of cells with BH4, revealing a novel protective mechanism that might contribute to the beneficial vascular effects of BH4.

## Materials and Methods

### *Materials*

BAY 60-2770 (Bayer AG, Wuppertal, Germany) was dissolved in DMSO. The stock solution (1 mM) was diluted with 50 % DMSO (v/v) in water to yield a BAY 60-2770 concentration of 0.1 mM. All further dilutions were made with water. The solvent (final concentration of DMSO in the assay  $\leq$  5 %) did neither affect basal nor NO-induced cGMP accumulation. DEA/NO (Sigma-Aldrich, Vienna, Austria) was dissolved and diluted in 10 mM NaOH. Nitropohl<sup>®</sup> ampoules (G. Pohl-Boskamp GmbH. & Co, Hohenlockstedt, Germany), containing 4.4 mM GTN in 250 mM glucose, were obtained from a local pharmacy and diluted in water. Glucose vehicle controls had no effects. Sepiapterin, BH<sub>2</sub>, BH<sub>4</sub>, NH<sub>2</sub>, and NH<sub>4</sub> were from Schircks Laboratories (Jona, Switzerland). Antibiotics and fetal calf serum were purchased from PAA Laboratories (Linz, Austria). Culture media and other chemicals were from Sigma-Aldrich (Vienna, Austria).

### *Culture and treatment of cells*

Porcine aortic endothelial cells were isolated as described (Schmidt et al., 1989) and cultured at 37 °C, 5 % CO<sub>2</sub>, in Dulbecco's modified Eagle's medium, supplemented with 10 % (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1.25 µg/ml amphotericin B. Rat lung fibroblasts (RFL-6 cells) were obtained from ATCC (CCL-192) and cultured in Ham's F-12 medium (37 °C, 5 % CO<sub>2</sub>), containing 20 % (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1.25 µg/ml amphotericin B. Where indicated, cells were pretreated in culture medium containing GTN, methotrexate and/or pteridines.

### *Determination of cGMP formation*

Accumulation of intracellular cGMP was determined as previously described (Schmidt et al., 1999). Briefly, endothelial or RFL-6 cells grown in 24-well plates were washed and preincubated for 15 min at 37 °C in 50 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 5

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mM KCl, 1 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub>, 1 mM 3-isobutyl-1-methylxanthine, 1 μM indomethacin and, where indicated, ODQ (10 nM - 10 μM). Reactions were started by addition of DEA/NO (1 μM) or BAY 60-2770 (10 nM - 10 μM) and terminated after 4 min by removal of the incubation medium and addition of 0.01 N HCl. Within 1 h, intracellular cGMP was completely released into the supernatant and measured by radioimmunoassay. Data were normalized to maximal cGMP formation induced by DEA/NO under control conditions.

*Determination of sGC activity in cytosolic fractions of endothelial cells*

Endothelial sGC activity was determined as previously described (Schmidt et al., 2010). Briefly, endothelial cells grown in petri dishes (90 mm diameter) were incubated for 24 h at 37 °C in DMEM in the absence and presence of 0.1 mM GTN and/or 0.1 mM sepiapterin. Then cells were harvested and lysed by three cycles of freeze/thawing. Cytosolic fractions were obtained by centrifugation at 10,000 g for 10 min, and protein concentration was determined with the Bradford assay. For measuring sGC activity, cytosolic fractions (~0.2 mg of protein) were incubated for 10 min at 37 °C in a total volume of 0.1 ml of 50 mM triethanolamine/HCl buffer, pH 7.4, containing 0.5 mM [ $\alpha$ -<sup>32</sup>P]GTP (~300,000 cpm), 3 mM MgCl<sub>2</sub>, 1 mM cGMP, 1 mM 3-isobutyl-1-methylxanthine, 10 mM phosphocreatine, 152 U/ml creatine phosphate kinase, 1 mM EGTA, and DEA/NO or protoporphyrin IX (10 μM, each). Reactions were terminated by addition of 450 μl zinc acetate (120 mM) and 450 μl sodium bicarbonate (120 mM), followed by isolation of <sup>32</sup>P-cGMP by chromatography over Al<sub>2</sub>O<sub>3</sub> columns.

*Determination of intracellular BH4 and BH2 levels*

Cells grown in petri dishes (diameter 90 mm) were harvested (~5 x 10<sup>6</sup> per petri dish), washed, and resuspended in 0.1 ml of either PBS containing 1 mM dithiothreitol (for quantifying biopterin), alkaline oxidant solution (0.02 M KI/I<sub>2</sub> in 0.1 M NaOH; for quantifying biopterin plus BH<sub>2</sub>), or acidic oxidant solution (0.02 M KI/I<sub>2</sub> in 0.1 M HCl; for quantifying biopterin plus BH<sub>2</sub> plus BH<sub>4</sub>). Subsequent to lysis of the cells by sonication, 20-μl aliquots



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were removed for protein determination, and the remaining samples were incubated for 1 h in the dark at room temperature. Then, samples oxidized under alkaline or acidic conditions were mixed with 10  $\mu$ l of 1 M HCl or water, respectively, and centrifuged. The resulting supernatants were mixed with 10  $\mu$ l of 0.2 M ascorbic acid and neutralized by addition of 1 M phosphate buffer, pH 8.0. Non-oxidized samples were mixed with 30  $\mu$ l of water and centrifuged. For HPLC analysis, samples were loaded on a LiChrospher 100 RP 18 HPLC column, 5  $\mu$ m particle size (Merck, Darmstadt, Germany) and eluted with  $\text{NaH}_2\text{PO}_4$  (20 mM, pH 3) containing 5 % (v/v) methanol at a flow rate of 1 ml/min. Biopterin was quantified with a fluorescence detector at excitation and emission wavelengths of 350 and 440 nm, respectively, by interpolation in calibration curves that were established daily with authentic  $\text{BH}_2$  (3 - 300 nM) oxidized under acidic conditions.  $\text{BH}_4$  and  $\text{BH}_2$  levels were calculated from the amounts of biopterin measured after oxidation in acid (biopterin +  $\text{BH}_2$  +  $\text{BH}_4$ ), after oxidation in base (biopterin +  $\text{BH}_2$ ), or in non-oxidized samples (biopterin).

*Spectral measurements*

Stock solutions of oxyHb were freshly prepared each day by dissolving 17 mg of bovine hemoglobin in 1 ml of 50 mM potassium phosphate buffer, pH 7.4. Subsequent to addition of sodium dithionite, the solution was passed over a Sephadex G-25 column, and oxyHb was eluted with phosphate buffer. Before the start of each experiment, an aliquot of the oxyHb solution was mixed with 1 ml of phosphate buffer (see above) to yield a concentration of 10  $\mu$ M, transferred into an air-tight cuvette, and bubbled for 10 min with argon to convert oxyHb to deoxy Hb. Reactions were started by addition of ODQ and/or  $\text{BH}_4$ , and light absorbance spectra were recorded at 37°C with a Hewlett–Packard 8452A Diode Array spectrophotometer. Conversion of deoxyHb to metHb and vice versa was measured by monitoring the 430 nm deoxyHb absorption peak.

## Results

Treatment of cultured endothelial cells with 0.1 mM GTN resulted in a time-dependent decrease in cGMP accumulation induced by the NO donor DEA/NO (**Fig. 1A**). The inhibitory effect of GTN became significant after 30 min, was maximal (~ 75 % inhibition) after 4 - 6 hours, and was completely prevented when the cells were exposed to GTN in the presence of the BH4 precursor sepiapterin (0.1 mM). The concentration-dependent effects of sepiapterin on cGMP formation and intracellular BH4 levels are shown in **Fig. 1B**. In the absence of sepiapterin, GTN (0.1 mM, 24 h) diminished DEA/NO-induced cGMP accumulation to  $29.0 \pm 0.7$  %, whereas BH4 levels remained unchanged ( $7.8 \pm 1.0$  vs.  $7.6 \pm 0.6$  pmol/mg protein in untreated cells, not shown). Upon coincubation of the cells with GTN and 1 to 100  $\mu$ M sepiapterin, BH4 levels were increased up to  $240 \pm 35$  pmol/mg, and the cGMP response to DEA/NO was restored. Incubation of the cells with sepiapterin alone (0.1 mM, 24 h) did not substantially alter DEA/NO-induced cGMP formation ( $106.3 \pm 2.2$  %, see **Fig. 5A**).

BH4 is formed intracellularly from sepiapterin by the consecutive action of sepiapterin reductase and DHFR, which catalyze the reduction of sepiapterin to BH2 and further reduction of BH2 to BH4, respectively. Accordingly, treatment of the cells with sepiapterin usually results in an increase of both BH2 and BH4. To see whether sGC protection requires the fully reduced biopterin, we studied the effect of the DHFR inhibitor MTX (0.1 mM), which blocks BH2-to-BH4 conversion. MTX led to accumulation of BH2 (from  $104 \pm 30$  to  $554 \pm 23$  pmol/mg) that was accompanied by a decrease in BH4 levels close to that of untreated cells ( $13.3 \pm 2.6$  vs.  $7.6 \pm 0.6$  pmol/mg). As shown in **Fig. 2A**, the effect of sepiapterin on DEA/NO-induced cGMP formation was virtually abolished in the presence of MTX, indicating that sGC is protected against oxidative inactivation by BH4 but not by BH2. To substantiate these results, we incubated the cells with BH2, added to the culture medium every 8 hours (final concentration of each application 0.1 mM) to account for the oxidative loss of the pteridine during the 24-h incubation. As observed with sepiapterin, exogenously added BH2

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prevented the effect of GTN in the absence but not in the presence of MTX, demonstrating that reduction to BH<sub>4</sub> was essential to confer sGC protection. In contrast to BH<sub>2</sub>, NH<sub>2</sub> added every 8 h to the cells had no effect<sup>1</sup>. Similarly, scavenging of superoxide and/or peroxynitrite with SOD (1000 U/ml), Tiron (10 mM), MnTBAP (0.1 mM) or urate (1 mM) did not prevent the effect of GTN on DEA/NO-induced cGMP accumulation (**Fig. 2B**), excluding scavenging of reactive oxygen species as the underlying mechanism.

To confirm that the observed effects on endothelial cGMP levels do indeed reflect changes in sGC function rather than changes in the rates of cGMP hydrolysis or other indirect effects, we measured sGC activity in cytosolic fractions of endothelial cells that had been incubated for 24 h with GTN in the absence or presence of sepiapterin. As shown in **Fig. 3**, the data were identical to those obtained with intact cells. Specific sGC activity was decreased by about 50 % upon exposure of the cells to GTN and the effect of the organic nitrate was prevented by coincubation of the cells with GTN and sepiapterin. Activation of sGC by protoporphyrin IX, which acts through binding to heme-free sGC, was not affected by pretreating the cells with GTN, indicating that the effect of the organic nitrate is due to oxidation of ferrous sGC and subsequent loss of the ferric heme, as suggested previously (Schröder et al., 1988; Schrammel et al., 1996; Artz et al., 2002; Gorren et al., 2005; Schmidt et al., 2010). Thus, the present data suggest that BH<sub>4</sub> protects sGC-bound heme against oxidation.

To address this intriguing possibility we used BAY 60-2770 (Knorr et al., 2008), a close chemical analog of BAY 58-2667 (Cinaciguat) with a similar pharmacological profile. Apparently acting as protoporphyrin IX mimetics, these drugs potently activate heme-free sGC without affecting the NO-sensitive ferrous enzyme (Roy et al., 2008; Hoffmann et al., 2009). In the absence of GTN, BAY 60-2770 increased cGMP formation to ~50 % of the rate achieved by DEA/NO, indicating that untreated endothelial cells may already contain a substantial amount of heme-free sGC (**Fig. 4A**). Exposure of the cells for 24 h to

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increasing concentrations of GTN led to increased cGMP formation in the presence of BAY 60-2770, whereas stimulation by DEA/NO was inhibited. The concentrations of GTN causing half-maximal stimulation (with BAY 60-2770) or inhibition (with DEA/NO) of cGMP accumulation were virtually identical ( $3.5 \pm 0.6 \times 10^{-5}$  M and  $2.5 \pm 0.6 \times 10^{-5}$  M, respectively).

The data obtained with GTN were confirmed with ODQ, a potent sGC inhibitor that acts through heme oxidation (Schrammel et al., 1996). As shown in **Fig. 4B**, preincubation of the cells for 15 min with ODQ had the same effect as long-term exposure to GTN, i.e. BAY 60-2770-induced cGMP accumulation was increased and the effect of DEA/NO was inhibited. As evident from **Fig. 4C**, BAY 60-2770 increased cGMP levels in GTN- and ODQ-treated cells with identical  $EC_{50}$  values of  $7.3 \pm 1.8 \times 10^{-8}$  M and  $7.8 \pm 2.8 \times 10^{-8}$  M, respectively.

In view of the striking similarity between the cGMP responses in GTN- and ODQ-treated cells, we studied the effect of BH4 supplementation on ODQ-triggered sGC inactivation. As shown in **Fig. 5A**, pretreatment of endothelial cells with the BH4 precursor sepiapterin (0.1 mM, 24 h) markedly diminished the inhibitory effect of ODQ on DEA/NO-induced cGMP formation, evident as a more than 25-fold rightward shift of the ODQ concentration-response curve. A similar, albeit less pronounced effect was observed with cells pretreated for 1 h with 0.1 mM BH2 or 0.1 mM BH4 (**Fig. 5B**). Of note, short-term incubation with BH2 and BH4 resulted in intracellular BH4 levels significantly lower than those achieved by long-term incubation with sepiapterin ( $36.7 \pm 6.0$  and  $12.7 \pm 0.8$  pmol/mg protein with BH2 and BH4, respectively, vs.  $240 \pm 35$  pmol/mg protein). As observed with GTN-treated cells (see **Fig. 2A**), preincubation with 0.1 mM NH2 for 1 h had no effect, whereas NH4 caused a 6-fold rightward shift of the ODQ concentration-response curve (**Fig. 5C**), suggesting insufficient uptake and/or reduction of NH2 by endothelial cells.

Some key experiments were repeated with RLF-6 cells to see whether the observed effects of the reduced pteridines reflect a specific feature of endothelial cells. The data shown in

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**Fig. 6** indicate that this not the case. Again, inhibition of cGMP accumulation by ODQ was antagonized by sepiapterin (**panel A**), BH2 and BH4 (**panel B**), as well as NH4, but not by NH2 (**panel C**).

To investigate whether BH4 also interacts with other hemoproteins, we monitored the UV/Vis absorption spectra of deoxyHb, which contains a similar heme group as sGC, after administration of ODQ and BH4. As shown in **Fig. 7**, addition of ODQ (10  $\mu\text{M}$ ) to deoxyHb (10  $\mu\text{M}$ ) caused rapid oxidation to methHb, which was reversed upon addition of 100  $\mu\text{M}$  BH4. These results demonstrate that BH4 is able to regenerate oxyHb from methHb, implying that heme reduction by BH4 is not specific for sGC. The reduction of methHb by BH4 was concentration-dependent (not shown), with an apparent second-order rate constant of  $9.80 \pm 0.07 \text{ M}^{-1} \cdot \text{s}^{-1}$ , in close agreement to a previously published value for the reduction of methHb by 6,7-dimethyltetrahydropterin (Capeillere-Blandin et al., 2005).

## Discussion

There is a large body of evidence indicating that BH4 improves endothelial function in cardiovascular diseases by two mechanisms that may operate in a synergistic manner (Förstermann, 2010). As an essential cofactor of eNOS, BH4 prevents uncoupling of reductive O<sub>2</sub> activation from L-arginine oxidation and thereby ensures maximal NO synthesis without concomitant formation of superoxide and peroxynitrite (Gorren and Mayer, 2007). In addition, BH4 is a fairly efficient scavenger of superoxide and peroxynitrite and, therefore, reduces oxidative stress as endogenous antioxidant. Surprisingly, the protection of sGC against oxidative inactivation described here appears to reflect a novel effect of BH4 that is unrelated to both eNOS uncoupling and superoxide/peroxynitrite scavenging.

The prosthetic heme group of sGC represents the intracellular receptor for NO. Upon binding to the ferrous heme, NO induces a change in heme geometry resulting in enzyme activation and enhanced conversion of GTP to cGMP (Ignarro, 1992; Denninger and Marletta, 1999; Koesling et al., 2004). Oxidation of the heme iron to its ferric form prevents NO binding and renders the enzyme insensitive to activation by NO. Moreover, heme oxidation promotes dissociation of the heme from sGC (Roy et al., 2008) and induces degradation of the protein by the ubiquitin-proteasome pathway (Hoffmann et al., 2009; Meurer et al., 2009). In contrast to NO, which requires ferrous heme iron for binding, the NO- and heme-independent sGC activators, e.g. BAY 58-2667 or BAY 60-2770, bind with high affinity to the heme-binding pocket of the apoenzyme (Schmidt et al., 2004; Martin et al., 2010). Initially it was proposed that these drugs activate sGC if either the heme iron is oxidized or the heme group is missing (Stasch et al., 2002), but recent data suggest that oxidation results in dissociation of the ferric heme group, with heme-free sGC being the sole target for this class of sGC activators (Roy et al., 2008). Based on their unique mechanism of action, BAY 58-2667 and analogs are useful tools to determine the fraction of heme-free sGC in cells and tissues under various physiological or pathophysiological conditions (Stasch et al., 2006; Roy et al., 2008; Ahrens et al., 2011; Hoffmann et al., 2011).

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Both functional and spectroscopic data published previously clearly indicate that GTN and ODQ oxidize the ferrous heme iron of sGC, resulting in loss of NO sensitivity of the enzyme (Schröder et al., 1988; Schrammel et al., 1996; Artz et al., 2002; Gorren et al., 2005; Schmidt et al., 2010). Early studies by Ignarro and coworkers showed that the heme-free enzyme is activated by protoporphyrin IX (Ignarro et al., 1982), and the new sGC activators, including BAY 60-2770 (Knorr et al., 2008) appear to act as highly potent protoporphyrin IX mimetics that activate heme-free sGC formed subsequent to oxidation by ODQ (Roy et al., 2008; Hoffmann et al., 2011). Our results showing that exposure of cells to both GTN or ODQ led to converse effects on the cGMP response to DEA/NO and BAY 60-2770 agree well with these previous reports, but there were notable differences between the effects of GTN and ODQ. While ODQ caused rapid inhibition of cGMP accumulation within 15 min, the effect of GTN occurred with a lag time of about 30 min, with maximal inhibition requiring 6 - 8 h of incubation (see Fig. 1A). Considering the high lipophilicity of GTN and oxidation of purified sGC within minutes (Gorren et al., 2005), this slow mode of action is hardly explained by delayed cellular uptake or slow binding of the drug to sGC. Based on literature reports on oxidation of sGC-bound heme by reactive oxygen species (Stasch et al., 2006; Boerrigter et al., 2007; Zhou et al., 2008) and a recent study from our laboratory showing that exposure of endothelial cells to GTN led to slow formation of superoxide (Schmidt et al., 2010), we speculated that the effect of GTN could be mediated by superoxide or peroxynitrite generated by uncoupled eNOS or other sources. However, neither NO synthase inhibition nor ascorbate (Schmidt et al., 2010), nor scavenging of superoxide and/or peroxynitrite with SOD, Tiron, MnTBAP or urate (see Fig. 2B) prevented inhibition of DEA/NO-induced cGMP accumulation by GTN, most likely excluding a role of oxidative stress in this experimental setup. Alternatively, enzymatic or non-enzymatic reduction of the organic nitrate to NO could counteract and thereby slow down heme oxidation. Endothelial and RLF-6 cells show slight but significant increases in cGMP levels when exposed to GTN (K. Schmidt, unpublished observation), indicating that GTN metabolism results in formation of small amounts of NO,

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which has been shown to protect sGC-bound heme against oxidation by ODQ (Schrammel, 1996). Further work is necessary to clarify this issue.

The observation that BH4 supplementation of cells protects sGC against inactivation by GTN and ODQ independently of its known antioxidative functions was unexpected. The BH4 precursor sepiapterin proved to be ideal for long-term incubations. The compound is fairly stable in aqueous solution under aerobic conditions (Dantola et al., 2008) and efficiently taken up by endothelial cells (Ohashi et al., 2011). Intracellular BH4 levels are rapidly increased and remain constantly high for up to 2 days after addition of sepiapterin to the cell culture medium (K. Schmidt, unpublished observation). In contrast to sepiapterin, BH2 and BH4 are prone to autoxidation (Davis et al., 1988; Mayer et al., 1995; Dantola et al., 2008) and according to our own data, 10 - 20 % of BH2 and ~99 % of BH4 are lost within 1 h incubation in culture medium (K. Schmidt, unpublished observations). Consequently, the increases in intracellular BH4 observed upon exogenous addition of BH2 or BH4 were transient and less pronounced than those observed with sepiapterin. Accordingly, we used BH2/BH4 supplementation only in short-term experiments or - in the case of long-term studies - re-added BH2 to the culture medium every 8 h. To test for a potential role of endogenous BH4, we inhibited BH4 biosynthesis by treating endothelial cells for 24 h with the guanosine triphosphate cyclohydrolase I inhibitor, 2,4-diamino-6-hydroxypyrimidine. Despite pronounced reduction of intracellular BH4 levels from  $7.6 \pm 0.6$  to  $0.3 \pm 0.1$  pmol/mg protein, the response to GTN remained unaltered (Supplemental Fig. 1), indicating that basal levels of BH4 are not sufficient to protect sGC against inactivation. Using the same experimental protocol that was established for supplementation of cells with reduced biopterins, we observed that NH4 mimicked the effect of BH4 on both endothelial cells and fibroblasts, further confirming that the protective effects of the pteridines are unrelated to the function of eNOS, which does not accept NH4 as a cofactor (Gorren et al., 1998). However, in contrast to BH2, NH2 had no effect, indicating inefficient uptake and/or reduction to the corresponding tetrahydro derivative by DHFR. The mechanisms of cellular uptake and



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metabolism of reduced neopterin are poorly understood, mainly due to the lack of analytical techniques for reliable quantification of these compounds and their oxidation products in tissues.

The protection of the heme by BH4 is not peculiar to sGC, since BH4 is able to reverse the oxidation of deoxy Hb by ODQ (this study) and to reduce ferric cytochrome c (Capeillere-Blandin et al., 2005). The reaction apparently involves a direct, one electron transfer from BH4 to the heme iron, with intermediate formation of a BH4 cation radical (Mathieu et al., 2004). Using 6,7-dimethyltetrahydropterin, which exhibits electrochemical properties very similar to those of BH4 (Gorren et al., 2001), Mansuy and coworkers demonstrated that only Fe(III)-hemoproteins having a redox potential more positive than -160 mV are reduced to their ferrous form, with a clear correlation between reduction rate and redox potential of the heme (Capeillere-Blandin et al., 2005). Based on these data and the midpoint potential published for sGC ( $E_m = 187$  mV, (Makino et al., 2011)), an expected reduction rate constant of  $\sim 134 \text{ M}^{-1}\cdot\text{s}^{-1}$  can be estimated, which would yield a half-life of  $\sim 17$  min for oxidized sGC in the presence of  $5 \mu\text{M}$  BH4. Of note, the basal intracellular BH4 concentration of endothelial cells is  $\sim 1 \mu\text{M}$  and increased by sepiapterin up to  $\sim 25 \mu\text{M}^2$ , enabling efficient reduction of sGC in BH4-supplemented cells.

Preclinical and clinical data obtained with BAY 58-2667 suggest that the relative amount of heme-free vascular sGC is increased in cardiovascular disorders like coronary artery disease, pulmonary hypertension and chronic heart failure (Boerrigter et al., 2007; Lapp et al., 2009; Ahrens et al., 2011). In view of the essential role of oxidative stress in most cardiovascular diseases (Strobel et al., 2011), this is probably due to oxidation by reactive oxygen species and subsequent dissociation of sGC-bound heme. Partial dysfunction of sGC may cause NO resistance of the diseased blood vessels, resulting in increased blood pressure and atherosclerosis. The present data clearly demonstrate that BH4 protects sGC

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against oxidative inactivation. This hitherto unrecognized action of the reduced pteridine might contribute to the beneficial vascular effects of BH4 supplementation.

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### **Authorship contributions**

*Participated in research design:* Schmidt, Gorren and Mayer

*Conducted experiments:* Neubauer and Kolesnik

*Contributed new reagents or analytical tools:* Stasch

*Performed data analysis:* Schmidt, Neubauer, Kolesnik and Gorren

*Wrote or contributed to the writing of the manuscript:* Schmidt, Werner, Gorren and Mayer

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### Footnotes

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<sup>1</sup>We did not treat cells for the 24 h period with the labile tetrahydropteridines, since more than 99% of BH4 was found to be lost within the first hour of incubation.

<sup>2</sup>Intracellular BH4 concentrations were derived from the data shown in Fig. 1B, assuming a cell volume of 1 pl and a protein content of 100 pg/cell.



## Figure legends

### **Figure 1 Effects of GTN and sepiapterin on NO-induced cGMP accumulation and BH4 levels in endothelial cells.**

(A) Endothelial cells were pretreated in culture medium in the presence of 0.1 mM GTN and, where indicated, 0.1 mM sepiapterin (which was added to the medium 1 h prior to addition of GTN). At the time points indicated, cells were washed and DEA/NO-induced cGMP formation determined as described in the methods section. (B) Endothelial cells were pretreated for 24 h in culture medium in the absence and presence of 0.1 mM GTN and, where indicated, 1 to 100  $\mu$ M sepiapterin (which was added to the medium 1 h prior to addition of GTN). Then, cells were washed and DEA/NO-induced cGMP formation and intracellular BH4 levels determined as described in the methods section. Data are mean values  $\pm$  S.E.M. of 3-5 independent experiments.

### **Figure 2 Effects of GTN, pteridines, and scavengers of superoxide/peroxynitrite on NO-induced cGMP accumulation in endothelial cells.**

(A) Endothelial cells were pretreated for 24 h in culture medium in the absence and presence of 0.1 mM GTN. Where indicated, MTX (final concentration 0.1 mM) was added to the medium 2 h prior to addition of GTN, and sepiapterin (Sep), BH2, or NH2 (final concentration 0.1 mM, each) 1 h prior to addition of GTN. To account for the oxidative loss of BH2 and NH2, the two pteridines were re-added every 8 h to the culture medium. (B) Endothelial cells were pretreated for 24 h in culture medium in the absence and presence of 0.1 mM GTN and, where indicated, SOD (1000 U/ml), Tiron (10 mM), MnTBAP (0.1 mM), or urate (1 mM). Following pretreatment, cells were washed and DEA/NO-induced cGMP formation determined as described in the methods section. Data are mean values  $\pm$  S.E.M. of 3-5 independent experiments.

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**Figure 3 Effects of GTN and sepiapterin on sGC activity in cytosolic fractions of endothelial cells.**

Endothelial cells were pretreated for 24 h in culture medium in the absence and presence of 0.1 mM GTN and/or 0.1 mM sepiapterin (which was added to the medium 1 h prior to addition of GTN). Following washing and lysis of the cells, cytosolic fractions were prepared and activity of sGC was determined as described in the methods section in the absence or presence of protoporphyrin-IX (PP-IX) or DEA/NO (10  $\mu$ M each). The data are mean values  $\pm$  S.E.M. of 3 independent experiments.

**Figure 4 Effects of DEA/NO and BAY 60-2770 on cGMP accumulation in GTN- and ODQ-treated endothelial cells.**

Endothelial cells were pretreated for 24 h in culture medium with increasing concentrations of GTN (A) or for 15 min in assay buffer with increasing concentrations of ODQ (B), followed by determination of cGMP formation in response to DEA/NO or BAY 60-2770 (1  $\mu$ M, each). (C) Endothelial cells were pretreated for 24 h in culture medium with 0.1 mM GTN or for 15 min in assay buffer in the absence (control) and presence of 1  $\mu$ M ODQ, followed by determination of cGMP formation in response to increasing concentrations of BAY 60-2770. Data are mean values  $\pm$  S.E.M. of 3-5 independent experiments.

**Figure 5 Effect of pteridine supplementation on NO-induced cGMP accumulation in ODQ-treated endothelial cells.**

Endothelial cells were pretreated in culture medium for 24 h in the absence (control) or presence of 0.1 mM sepiapterin (A), for 1 h with 0.1 mM BH2 or BH4 (B), or for 1 h with 0.1 mM NH2 or NH4 (C). Then, cells were washed, preincubated in assay buffer for 15 min with increasing concentrations of ODQ, and cGMP formation was determined in response to 1  $\mu$ M DEA/NO. Data are mean values  $\pm$  S.E.M. of 3-5 independent experiments.

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**Figure 6 Effect of pteridine supplementation on NO-induced cGMP accumulation in ODQ-treated RFL-6 cells.**

RFL-6 cells were pretreated in culture medium for 24 h in the absence (control) or presence of 0.1 mM sepiapterin (A), for 1 h with 0.1 mM BH<sub>2</sub> or BH<sub>4</sub> (B), or for 1 h with 0.1 mM NH<sub>2</sub> or NH<sub>4</sub> (C). Then, cells were washed, preincubated in assay buffer for 15 min with increasing concentrations of ODQ, and cGMP formation was determined in response to 1  $\mu$ M DEA/NO. Data are mean values  $\pm$  S.E.M. of 3-5 independent experiments.

**Figure 7 Reduction of methemoglobin by BH<sub>4</sub>**

DeoxyHb (10  $\mu$ M) was prepared as described in the methods section, and light absorbance was recorded at 430 nm under anaerobic conditions. Reactions were started by addition of ODQ (10  $\mu$ M final concentration) to convert deoxyHb into metHb. At the indicated time points, BH<sub>4</sub> (final concentration 100  $\mu$ M) or vehicle was added. An original trace representative of three experiments is shown.

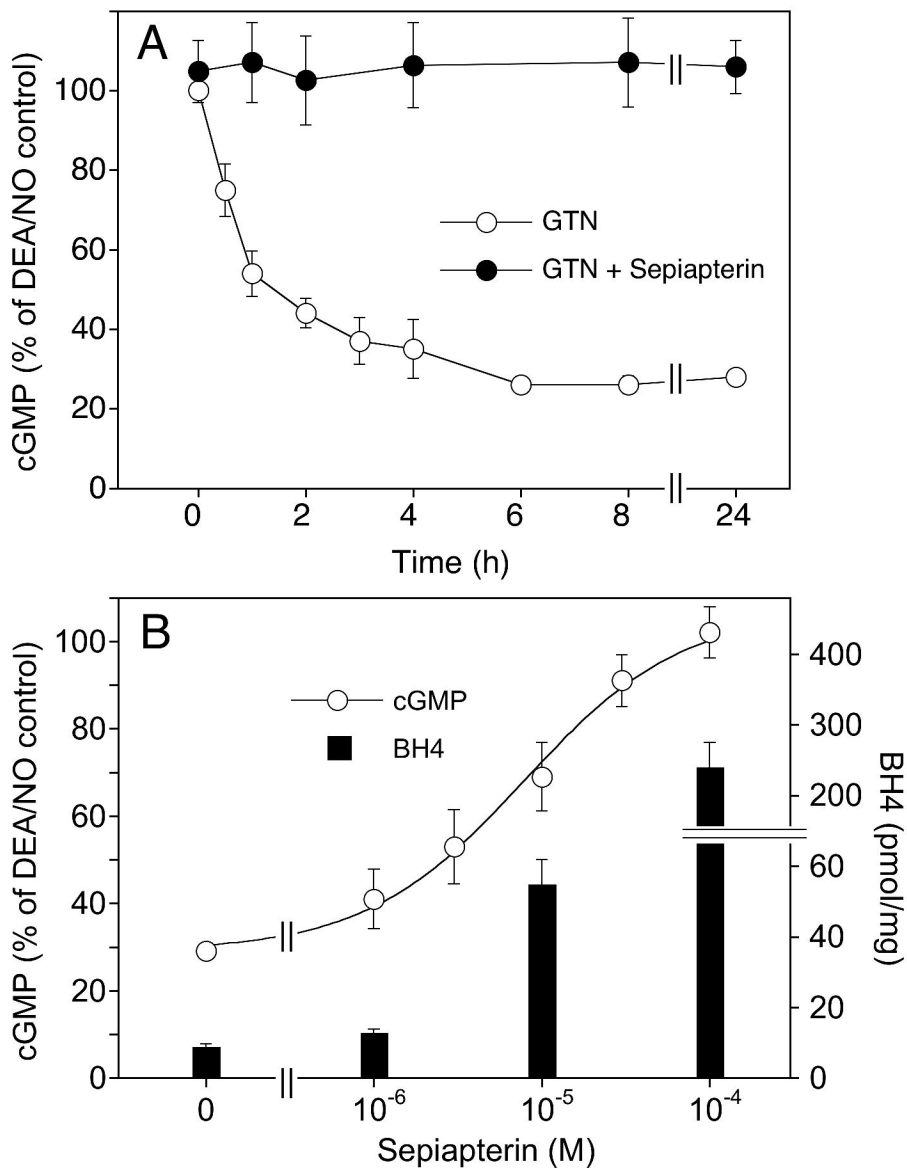


Figure 1

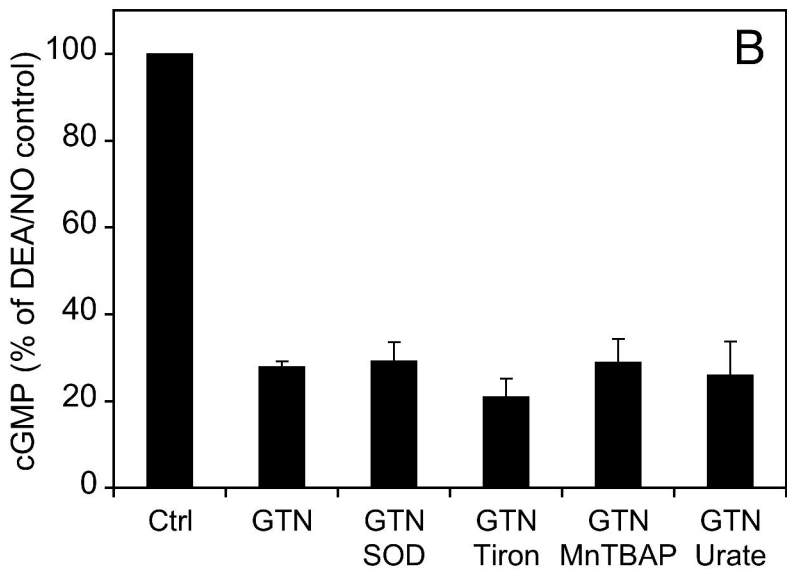
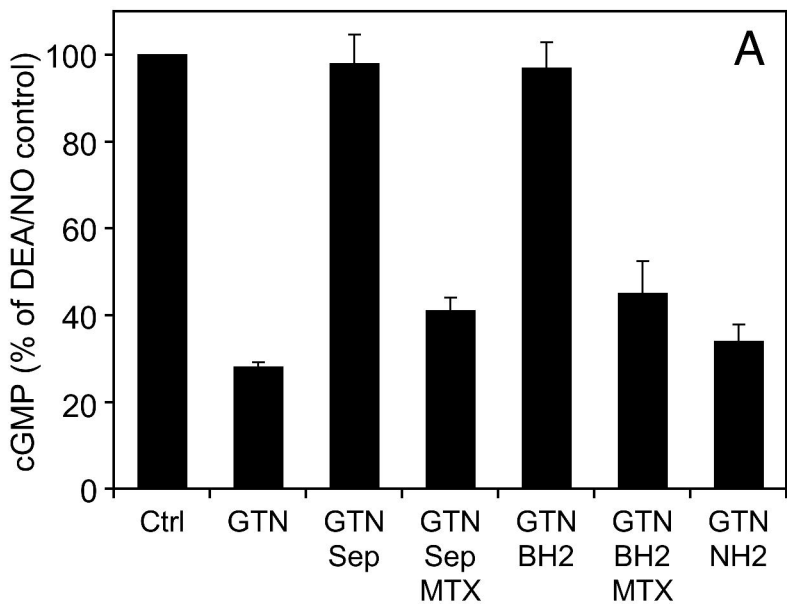


Figure 2

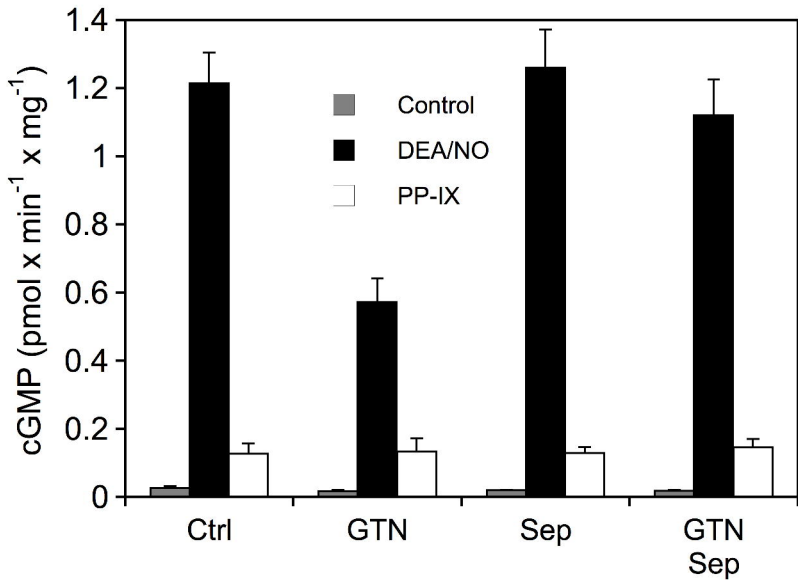


Figure 3

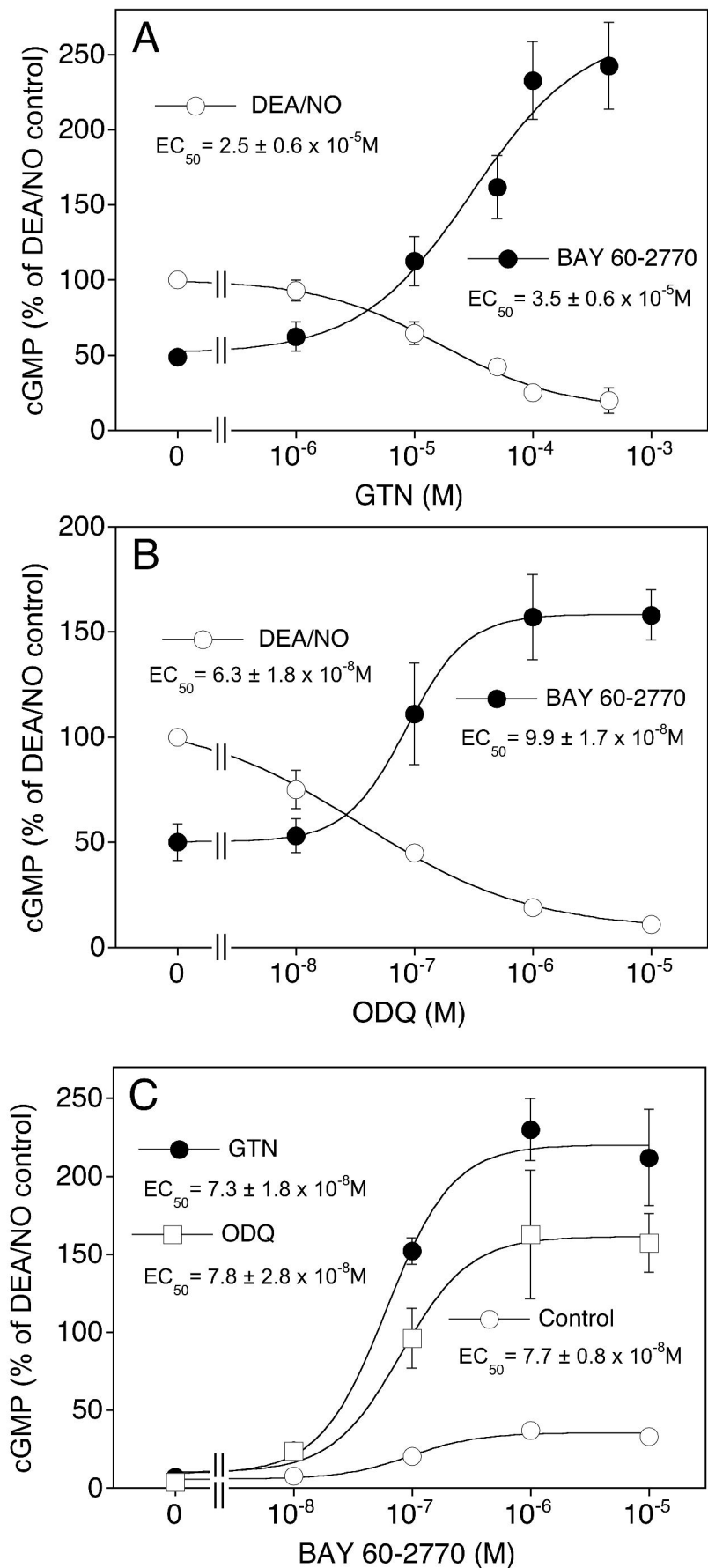


Figure 4

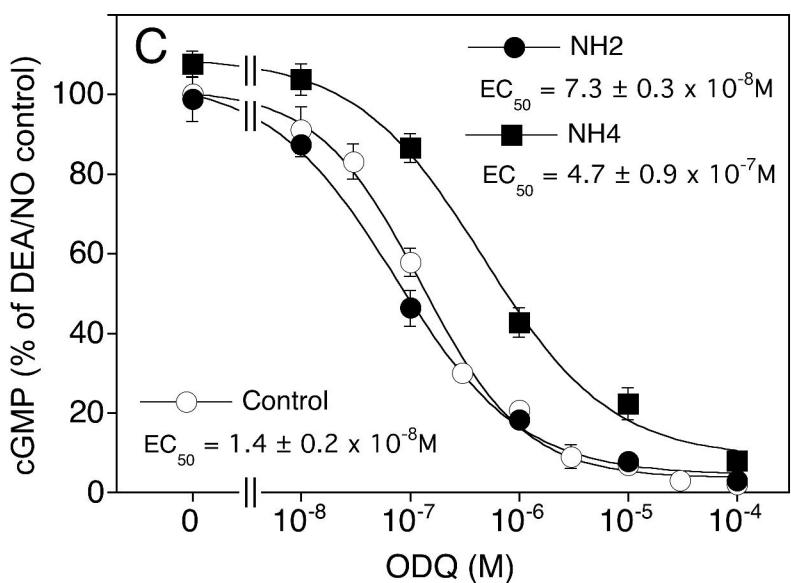
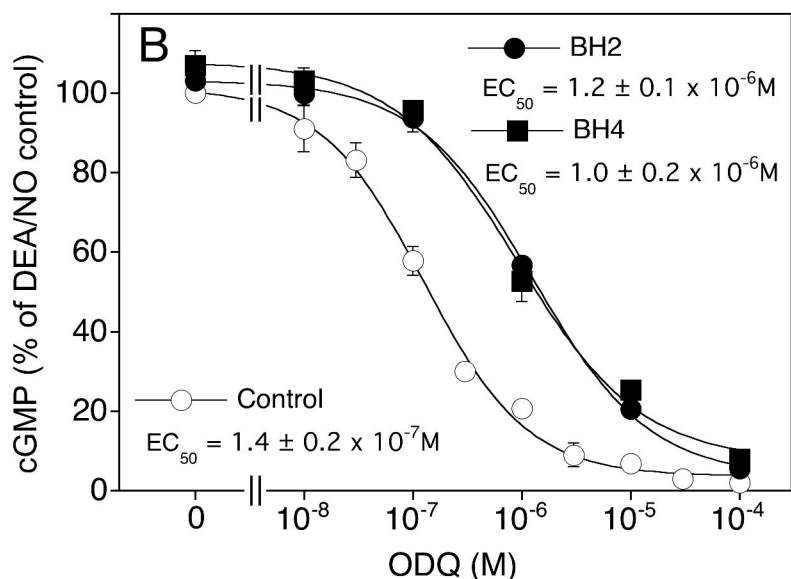
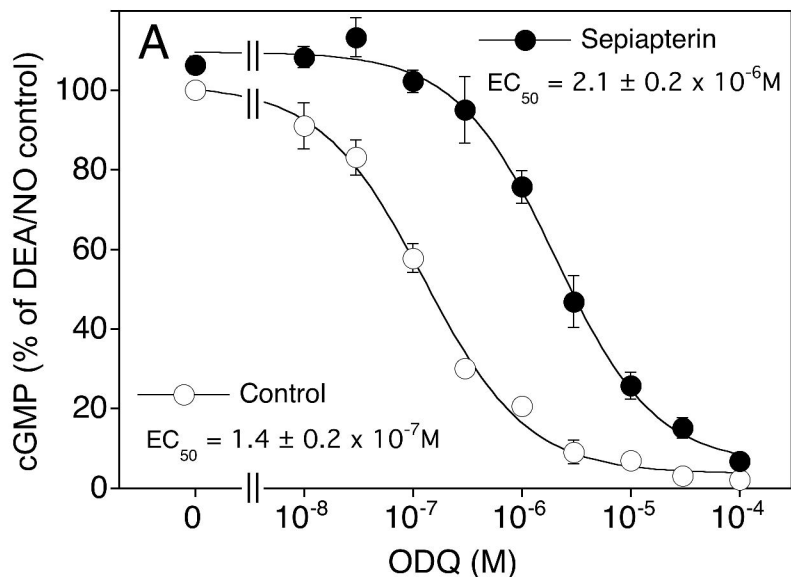


Figure 5



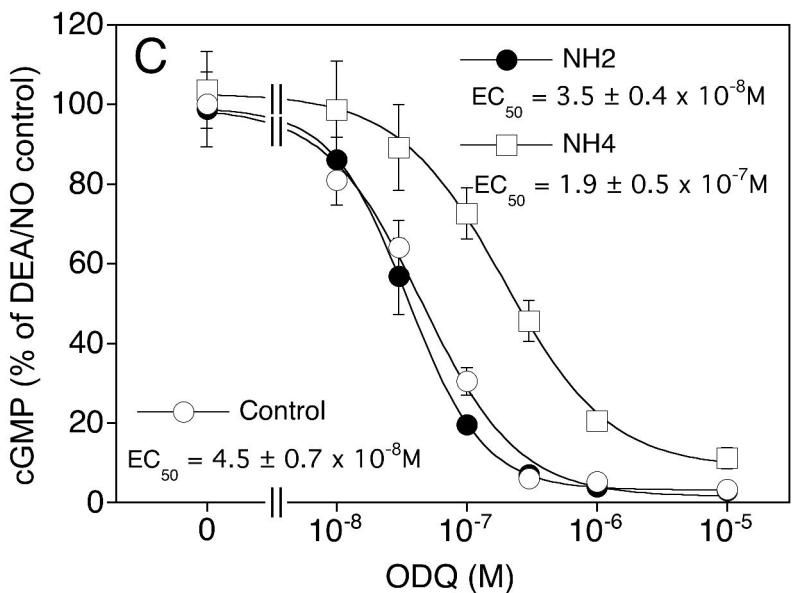
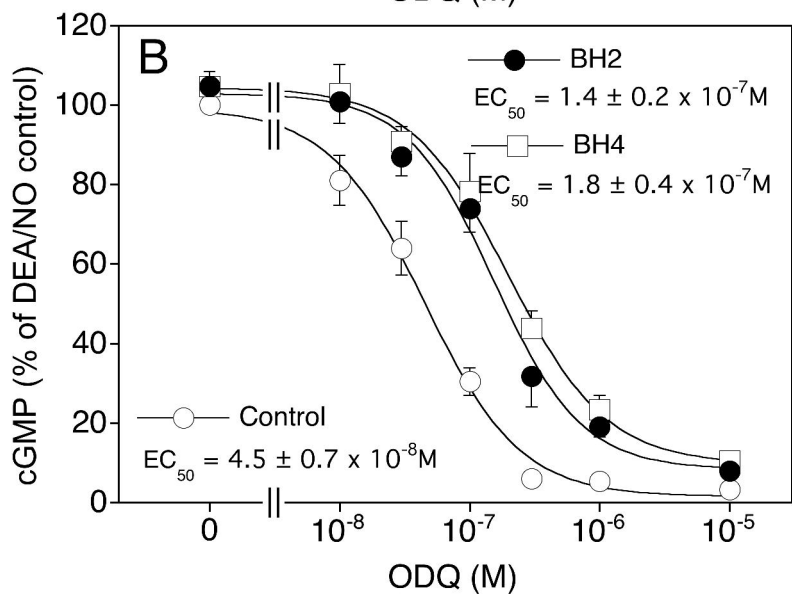
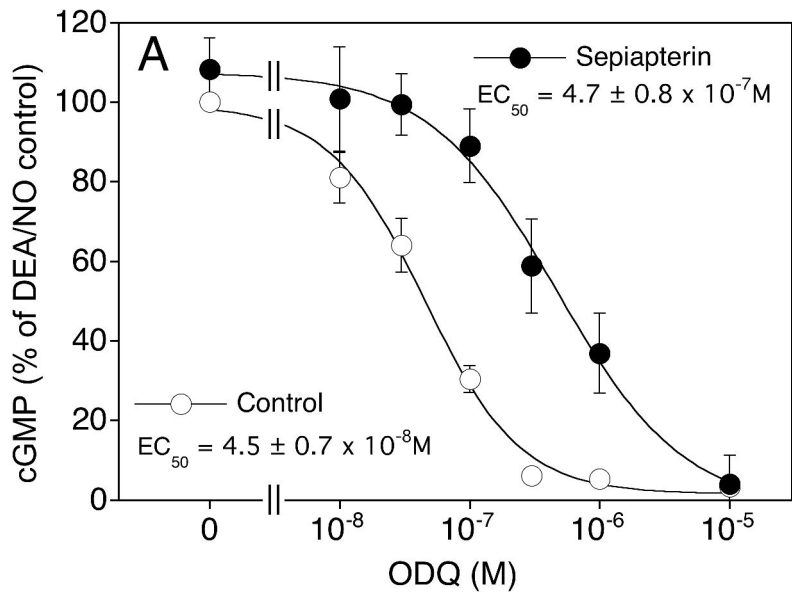


Figure 6

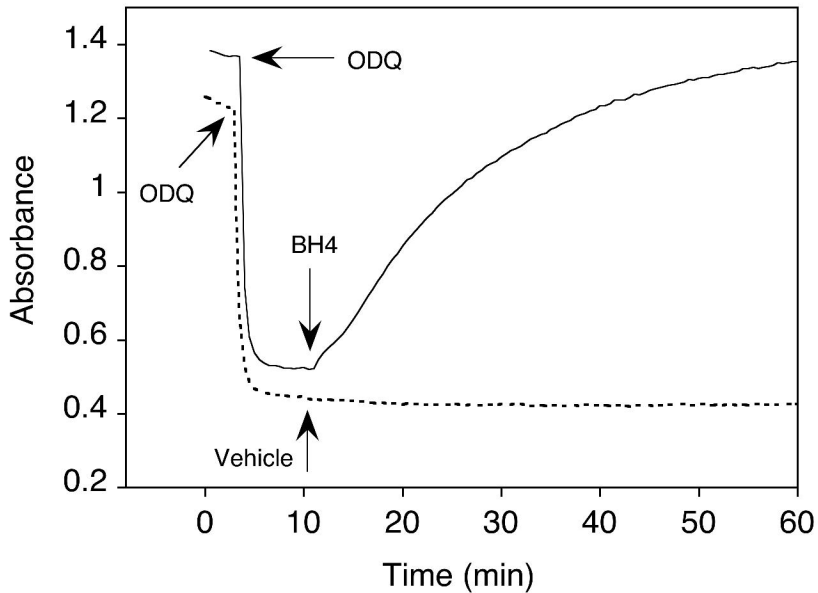


Figure 7