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RESEARCH PAPER

Effect of oral organic nitrates on expression and activity of vascular soluble guanylyl cyclase

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Background and purpose: The regulation of vascular soluble guanylyl cyclase (sGC) expression by nitric oxide (NO) is still under discussion. *In vitro*, NO has been shown to downregulate the expression of sGC but it is unclear if this mechanism is operative *in vivo* and occurs during nitrate treatment.
Experimental approach: We investigated whether high dose isosorbide mononitrate (ISMN) or pentaerythrityl tetranitrate (PETN) treatment changes vascular sGC expression and activity *in vivo*. New Zealand White rabbits received a standard diet, 2 or 200 mg ISMN kg^{-1} d^{-1} for 16 weeks, and C57BL/6 mice received a standard diet, 6, 60 or 300 mg PETN kg^{-1} d^{-1} for four weeks. Absorption was checked by measuring the plasma levels of the drug/metabolite.
Key results: Western blots of rabbit aortic rings showed similar protein levels of sGC α1- ($P=0.2790$) and β1-subunits ($P=0.6900$) in all groups. Likewise, ANOVA showed that there was no difference in the expression of sGC in lungs of PETN-treated mice ($P=0.0961$ for α1 and $P=0.3709$ for β1). The activities of isolated sGC in response to SNAP (1 μM–1 mM) were identical in aortae of ISMN-treated rabbits ($P=0.0775$) and lungs of PETN-treated mice ($P=0.6348$). The aortic relaxation response to SNAP slightly decreased at high ISMN but not at high PETN.
Conclusions and implications: These data refute the hypothesis that therapeutic treatment with long acting NO donors has a significant impact on the regulation of vascular sGC expression and activity *in vivo*.
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Keywords: organic nitrates; nitric oxide; soluble guanylyl cyclase; endothelium; nitric oxide synthase; chronic treatment

Abbreviations: eNOS, endothelial nitric oxide synthase; ISMN, isosorbide mononitrate; NO, nitric oxide; PETN, pentaerythrityl tetranitrate; sGC, soluble guanylyl cyclase; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; VASP, vasodilator-stimulated phosphoprotein

Introduction

Organic nitrates are commonly used drugs for prevention and acute treatment of coronary artery disease symptoms. They are activated to nitric oxide (NO), which is involved in physiological processes such as smooth muscle relaxation, neurotransmission, platelet aggregation, host defence mechanisms and apoptosis and has antioxidative effects (Moncada and Higgs, 1993). The effects of NO greatly contribute to the physiological vascular functions and probably protect the vascular wall from vasotoxic compounds such as reactive oxygen species (Gewaltig and Kojda, 2002). In the vasculature, the majority of the effects of NO are mediated by the activation of soluble guanylyl cyclase (sGC), generation of cyclic guanosine monophosphate (cGMP), activation of protein kinase G (PKG) and phosphorylation of various cellular proteins regulating calcium haemostasis (Ignarro *et al*., 1999). The sGC enzyme is composed of two subunits, α and β, and a prosthetic haem group. The vast majority of vascular sGC is formed by the subunits α1 and β1 (Buechler *et al*., 1991; Russwurm and Koesling, 2004).

A disturbance of the NO–cGMP pathway induced by changes of the expression of sGC has been previously observed. The expression of vascular sGC is downregulated in spontaneously hypertensive rats (Bauersachs *et al*., 1998; Ruetten *et al*., 1999), in smooth muscle cells of Fischer 344 rats (Chen *et al*., 2000) and in aged Wistar Kyoto rats (Kloss *et al*., 2000; Friebe and Koesling, 2003). Other studies have shown a downregulation of sGC mRNA and protein expression by endotoxin, cAMP, cytokines and oestradiol (Shimouchi *et al*., 1993; Papapetropoulos *et al*., 1996; Kojda *et al*., 1998a; Ruetten *et al*., 1999; Krumenacker *et al*., 2001; Takata *et al*., 2001; Friebe and Koesling, 2003). In contrast, hypercholesterolaemia increases the expression of rabbit aortic vascular sGC, particularly in atherosclerotic plaques

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(Laber *et al*., 2002), and a qualitatively similar but smaller effect has been observed in experimental chronic myocardial infarction and in nitrate tolerance (Bauersachs *et al*., 1998; Mulsch *et al*., 2001). In late-stage atherosclerosis, this may be different, in particular in the neointima (Melichar *et al*., 2004).

The mechanisms underlying these changes of sGC expression are currently unknown. Studies in cultured smooth muscle cells have suggested a crucial role of vascular NO generation, which may downregulate sGC expression in a negative feedback manner (Filippov *et al*., 1997; Weber *et al*., 2001). Likewise, transfection of HEK 293 cells with an endothelial nitric oxide synthase (eNOS)-containing plasmid and incubation of rat aortic rings with the NO-donor *N*-[4 [1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine (SPER/NO) reduced mRNA expression of the β1 subunit (Schmidt *et al*., 2001; Weber *et al*., 2001).

To investigate whether alterations of sGC protein expression and activity may be involved in oral therapy with long-acting NO donors such as isosorbide mononitrate (ISMN) and pentaerythrityl tetranitrate (PETN), we sought to determine the effect of nitrate treatment on vascular smooth muscle sGC *in vivo*. To accomplish this, we measured the vascular sGC protein expression and activity as well as NO effects in aortic segments after treatment with different doses of ISMN and in mice treated with different doses of PETN.

Methods

Test systems used

A total of 30 New Zealand white rabbits (10–12 weeks old) with a mean body weight of 2105 ± 47 g were housed individually as previously described (Kojda *et al*., 1995). The rabbits were randomly assigned to three groups of 10 animals and were fed a standard diet (ISMN-0) and a diet supplemented with ISMN to achieve a daily dose of ISMN of 2 mg kg^{-1} (ISMN-2) or 200 mg kg^{-1} (ISMN-200) for 16 weeks. The dose of ISMN was given in two identical portions in the morning at 0800 hours and in the early afternoon at 1500 hours. Body weight was determined weekly and the animals were supervised by a veterinerian. Previous studies have shown that 200 mg kg^{-1} per day of ISMN induces a nitrate tolerance as indicated selectively by reduced vasodilator activity of ISMN, both in normal and in hypercholesterolaemic rabbits (Muller *et al*., 2003, 2004).

C57BL/6 mice (male, 5-month old) were divided into four groups and were then randomly allocated to receive placebo ($n = 8$) or PETN treatment ($n = 12$ per group) for 4 weeks. The groups were treated with placebo (PETN-0) or 6 (PETN-6), 60 (PETN-60) or 300 mg kg^{-1} per day (PETN-300) of PETN, according to an average body weight of 25 g and an assumed daily amount of 5 g of consumed food. According to the manufacturer's recommendations, PETN was given continuously.

Permission for the animal studies was provided by the regional government of Germany (AZ 23.05-230-3-77/99, AZ 23.05-230-3-52/99, AZ 50.05-230-3-65/99, AZ 50.05-230-3-94/00 AZ 50.05-230-18/06). The experiments were performed according to the guidelines for the use of experimental animals, as given by the German 'Tierschutzgesetz' and the '*Guide for the Care and Use of Laboratory Animals*' of the US National Institutes of Health.

Measurements of plasma levels of drug/metabolite

Plasma concentrations of ISMN, pentaerythrityl dinitrate and mononitrate were determined by ACC GmbH (Leidensbach, Germany) with gas chromatography/mass spectrometry (GC/MS, HP6890, Hewlett-Packard, Germany) after liquid–liquid extraction with ethyl acetate as previously described (Muller *et al*., 2004).

Measurement of blood pressure

Systolic blood pressure and heart rate were measured in awake male C57BL/6 ($n = 4$ of each PETN treatment group) at 3–4 months of age using an automated tailcuff system as previously described (Kojda *et al*., 1999). On day 7, the PETN diet was started and blood pressure measurement was continued for a maximum of 7 days.

Vasorelaxation studies, sGC activity and western blots

Preparation of rabbit thoracic ring segments, equilibration and relaxation to the NO-donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) as well as preparation of cytosols from rabbit aorta and mouse lung were performed as previously described (Kojda *et al*., 1995; Laber *et al*., 2002). Specific activity of sGC was measured as described by Kojda *et al*. (1998a) and Schultz and Böhme (1984). Western blots for sGC α1 and β1 subunits were performed in rabbit aorta and mouse lung using specific antibodies. In addition, blots for vasodilator-stimulated phosphoprotein (VASP) were performed in lung and heart homogenates of PETN-treated mice. Please refer to Supplementary information for detailed protocols.

Data analyses and statistical procedures

All data were analysed by a standard computer programme (GraphPad Prism PC software, version 3.03) and are expressed as mean ± s.e.m. of n individual samples. Statistical comparisons between groups were performed by Newman–Keuls multiple comparisons test following analysis of variance (ANOVA) for pD_2 values and protein expression or two-way ANOVA for concentration–response curves. $P < 0.05$ was considered statistically significant.

Drugs, chemical reagents and other materials

[α-^{32}P]-GTP was obtained from PerkinElmer (Rodgau, Germany). Antibodies against sGC α1 subunit (catalogue no. G4280) and actin (no. A2066) were from Sigma (Munich, Germany); against sGC β1 subunit (no. 160897) from Cayman (Biozol, Eching, Germany); against rabbit immunoglobulin G (IgG) (no. 401315) from Calbiochem (Darmstadt, Germany); against phosphorylated and total VASP (no. 804–240 and no. 210–880) from Alexis Biochemicals (Lörrach, Germany) and against mouse IgG (no. 170–6516) from Bio-Rad (Munich, Germany). Polyvinylidene fluoride membranes were from

Millipore (Schwalbach, Germany). SNAP was synthesized in our laboratory as previously described (Kojda *et al*., 1996). ISMN was provided by Schwarz Pharma (Monheim, Germany); PETN by Actavis (Langenfeld, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany) or from Sigma in analytical grade. The stock solutions of acetylcholine (10 mM) and phenylephrine (10 mM) and ISMN (100 mM) were prepared in distilled water. Solutions of SNAP (200 mM) were prepared in dimethylsulphoxide. All stock solutions were prepared daily, diluted with Krebs buffer as required, kept on ice and protected from daylight until use. The blood pressure measurement system was from Visitech Systems (Apex, NC, USA).

The molecular target nomenclature in this paper conforms with the BJP's *Guide to Receptors and Channels* (Alexander *et al*., 2008).

Results

ISMN-treated rabbits

Treatment with ISMN resulted in plasma ISMN concentrations of 6.2 ± 1.9 ng mL^{-1} ($n = 10$) in ISMN-2 and 1.77 ± 0.469 μg mL^{-1} ($n = 9$) in ISMN-200. Densitometric analyses of the α1 subunit revealed that its expression in the ISMN-2 and ISMN-200 groups (each $n = 10$) was not different from that in the ISMN-0 group (Figure 1a). Expression of β1 in the ISMN-2 and ISMN-200 groups also showed no difference from that in the ISMN-0 group (Figure 1b). In addition, we measured sGC protein expression using actin protein as a standard. These data showed protein levels of $77.4 \pm 21.0\%$ ($n = 10$) in ISMN-2 and $97.5 \pm 18.9\%$ ($n = 8$) in ISMN-200 for α1 ($P = 0.1598$, ANOVA) and $105.1 \pm 17.9\%$ (ISMN-2) or $136.7 \pm 26.9\%$ (ISMN-200) for β1 ($P = 0.3072$, each $n = 10$, ANOVA). Thus, the method of standardization had no influence on the main result.

Measurement of sGC activity in response to increasing concentrations of SNAP (1 μM–1 mM) in aortic cytosols showed comparable pD_2 values (half-maximal effective concentrations in −logM) in ISMN-2 (3.41 ± 0.24, $n = 6$), ISMN-200 (3.05 ± 0.39, $n = 7$) and ISMN-0 (3.39 ± 0.26, $n = 6$, $P = 0.6524$, ANOVA). Likewise, the dose–response curves showed no significant differences between all three groups, as determined by two-way ANOVA (Figure 1c).

The dose–response curves for SNAP-induced vasorelaxation in rabbit aortic rings were slightly shifted to the right in the ISMN-2 and ISMN-200 groups. However, the shift was very small and the maximal response to SNAP was not changed. The corresponding pD_2 values were significantly less in the treated groups compared to the untreated group (Figure 2a). Measurements after removal of the endothelium did not significantly change this SNAP response pattern or pD_2 values for ISMN-0 (Figure 2b), ISMN-2 (Figure 2c) and ISMN-200 (Figure 2d). Again, significant differences occurred between treatment groups and ISMN-0 ($P = 0.0206$, ANOVA) but not between ISMN-2 and ISMN-200.

PETN-treated mice

The plasma concentrations of the PETN dinitrate (PEDN) and mononitrate (PEMN) metabolites, which were detectable by

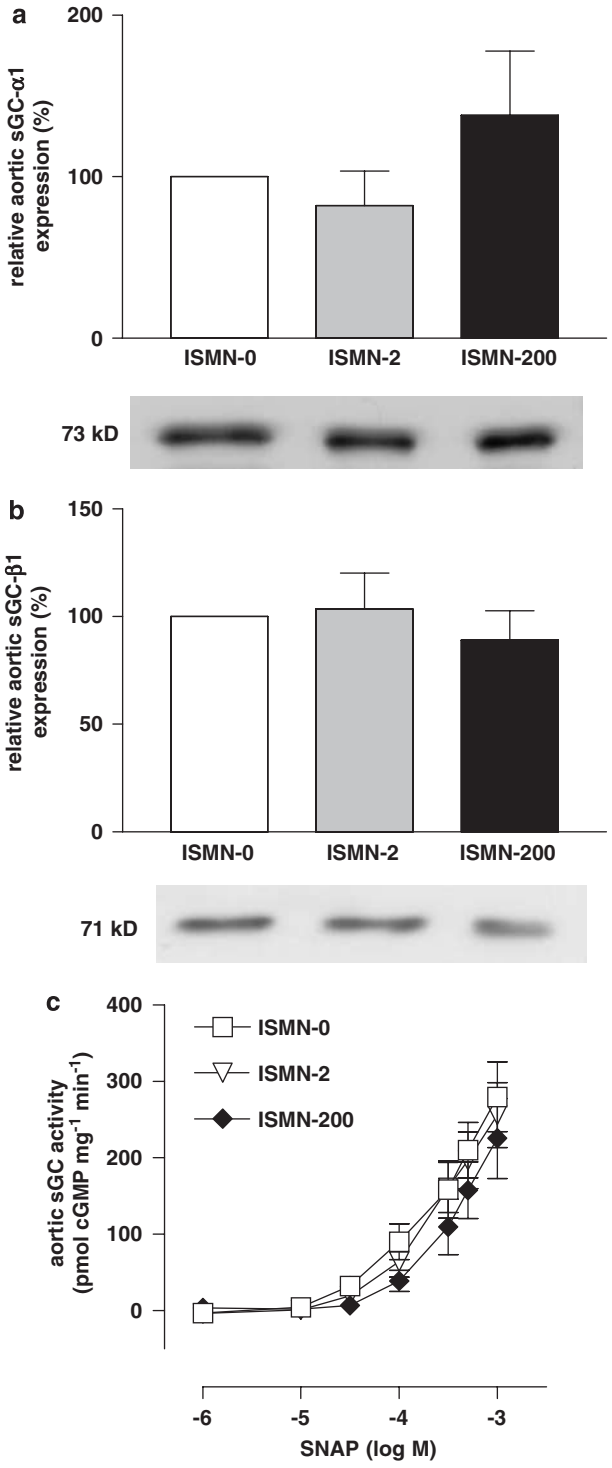

Figure 1 Effect of isosorbide mononitrate (ISMN) treatment on soluble guanylyl cyclase (sGC) in rabbits. Protein expression of (**a**) sGC α1 subunit ($P = 0.2790$) and (**b**) sGC β1 subunit in aortae of ISMN-treated rabbits measured by western blot densitometry. Neither the low-dose (ISMN-2) nor the high-dose (ISMN-200, each $n = 10$, $P = 0.6900$, analysis of variance (ANOVA)) group showed a significant change in protein expression. (**c**) Activity of sGC protein measured in pmol cGMP per mg protein per min in ISMN-treated rabbits. There was no significant difference among all three groups ($n = 6$–7, $P = 0.0775$, ANOVA). Results shown represent mean ± s.e.m.

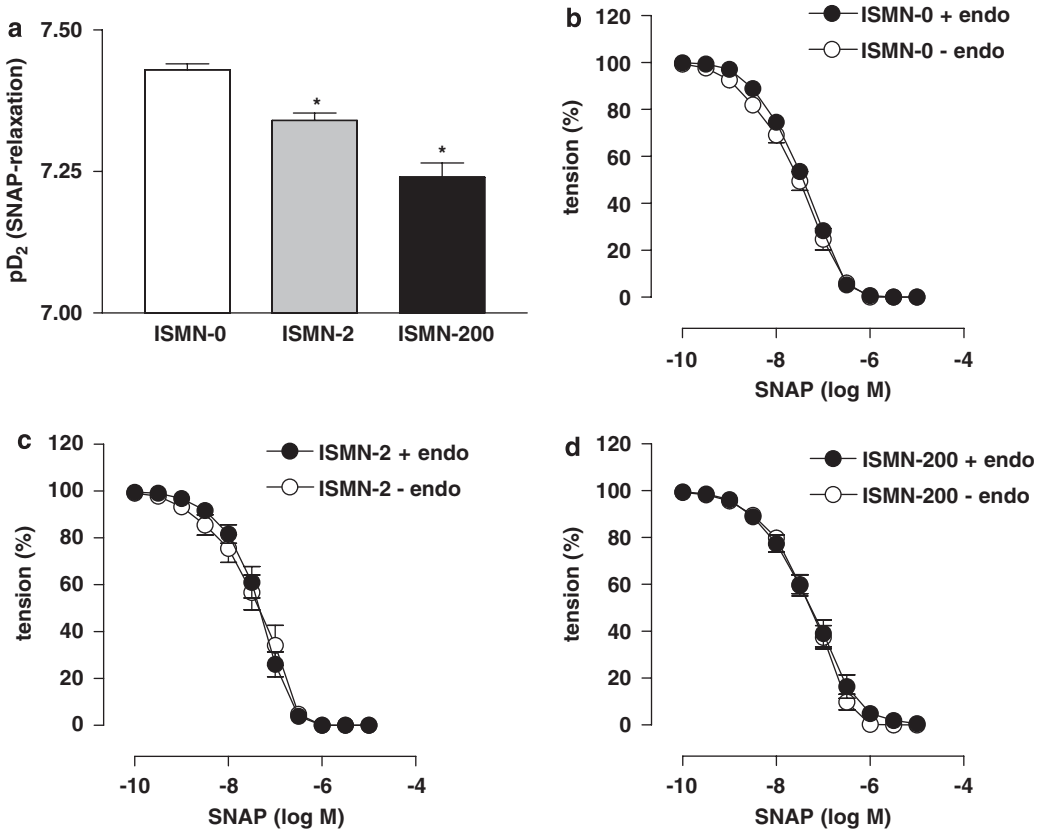

Figure 2 Vasorelaxation experiments with rabbit aorta. (**a**) The nitric oxide (NO)-sensitivity of aortic rings progressively declined with increasing isosorbide mononitrate (ISMN) dosage ($n=9$–10, $P=0.0161$, analysis of variance (ANOVA)). (**b**–**d**) Removal of the endothelium (–endo) in all three groups did not change the responses to *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) in any of the groups ($P=0.1999$ for ISMN-0, $P=0.3706$ for ISMN-2 and $P=1.000$ for ISMN-200, $n=9$–10, *t*-tests). All values represent mean ± s.e.m.

GC/MS, increased with the daily oral dose of PETN. The concentrations of both metabolites increased in a directly proportional way (Figure 3).

Western blot analyses of the sGC α1 subunit standardized by total protein revealed that its expression in the lungs of PETN-treated mice did not differ significantly from that in the untreated (PETN-0) group (Figure 4a). Likewise, expression of the β1 subunit showed no significant difference between the groups (Figure 4b). Additional western blots for β1 in aortic tissues showed an aortic expression of 109.8 ± 25.8% in PETN-6 and 144.9 ± 25.3% in PETN-60 group ($n=5$, $P=0.3135$, ANOVA).

Phosphorylation of VASP at serine 239 relating to total VASP showed no difference induced by PETN treatment, either. In lung tissue, phosphorylation was 103 ± 47.4% (PETN-6, $n=6$) and 121 ± 34.0% (PETN-60, $n=5$, $P=0.8996$, ANOVA) compared to PETN-0 ($n=6$), in hearts 112.3 ± 32.0% (PETN-6, $n=7$) and 99.7 ± 42.8% (PETN-60, $n=7$, $P=0.9460$, ANOVA).

The maximal activities of sGC in response to 1 mM SNAP in lung cytosols (in pmol cGMP per mg protein per min) did not differ significantly between the groups (Figure 4c). Likewise, the pD_2 values (in −logM) for SNAP in PETN-6 (3.84 ± 0.07), PETN-60 (3.89 ± 0.15) and PETN-300 (3.73 ± 0.14) were not significantly different from PETN-0 (3.86 ± 0.31, each $n=7$, $P=0.9412$, ANOVA), as well as the dose–response curves ($P=0.6348$, two-way ANOVA).

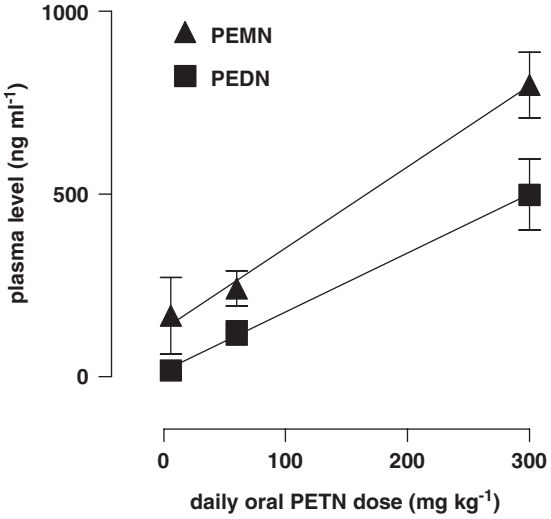

Figure 3 Plasma levels of the pentaerythrityl tetranitrate (PETN) metabolites PETN dinitrate (PEDN) and mononitrate (PEMN) detected in PETN-treated mice. The concentrations of both metabolites increased significantly with increased PETN dose in a directly proportional manner; r^2 is 0.5606 for PEDN (significant deviation from 0, $P=0.0013$) and 0.7374 for PEMN ($P<0.0001$, $n=7$). All values represent mean ± s.e.m.

The NO-dependent vasorelaxation was determined in organ bath experiments using the NO-donor SNAP in concentrations from 1 nM up to 10 mM. The dose–response

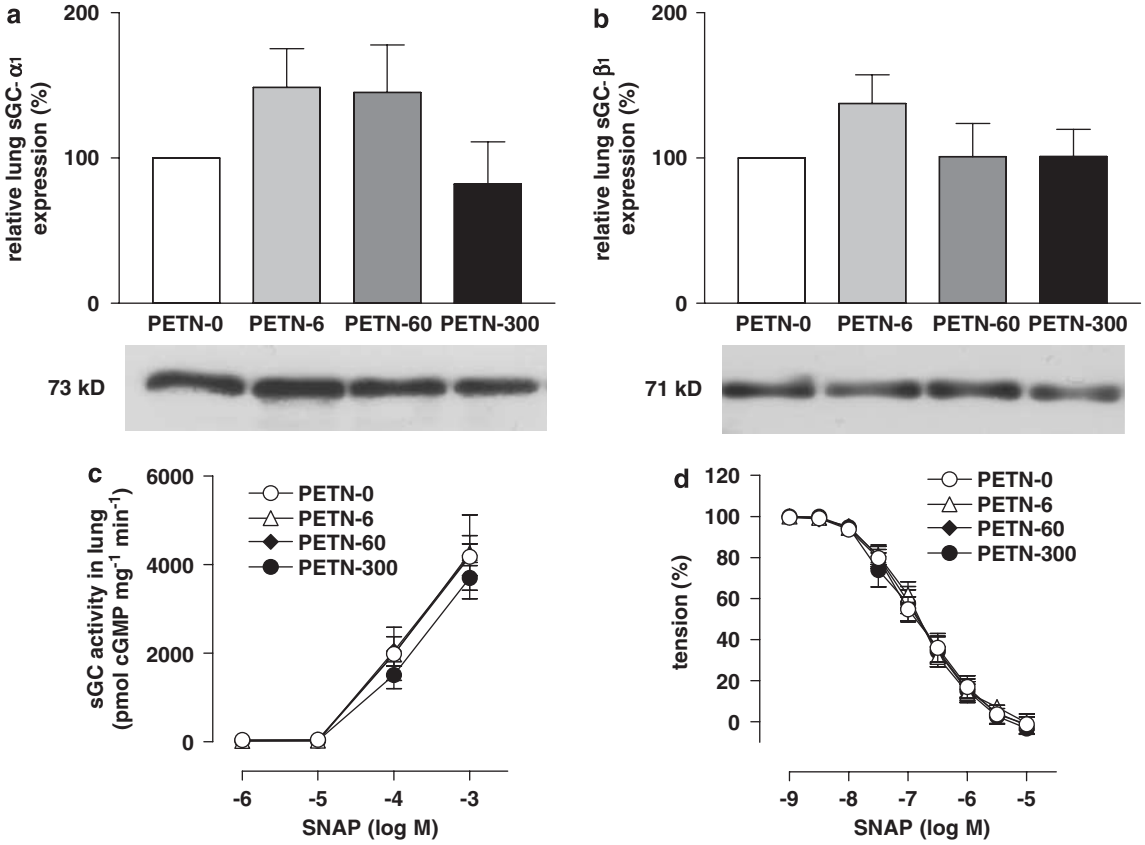

Figure 4 Effects of pentaerythrityl tetranitrate (PETN) treatment on soluble guanylyl cyclase (sGC) in mice. Protein expression of (**a**) sGC α1 subunit ($P=0.0961$, $n=8$) and (**b**) sGC β1 subunit ($P=0.3709$, $n=8$, analysis of variance (ANOVA)) in lungs of PETN-treated mice measured by western blot densitometry. None of the groups treated with different doses of PETN showed a significant change in protein expression. (**c**) Activity of sGC protein measured in pmol cGMP per mg protein per min in PETN-treated mice. There was no significant difference among all four groups ($P=0.6348$, $n=7$, two-way ANOVA). (**d**) NO-dependent vasorelaxation in aortic rings of PETN-treated mice induced by the NO-donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP). Again, there was no significant difference between the groups ($P=0.8865$, two-way ANOVA). All values represent mean ± s.e.m.

patterns did not differ between the groups (Figure 4d), and the half-maximal concentrations of SNAP in this experiment (−logM) were similar in all four groups (PETN-0: 6.85 ± 0.08, PETN-6: 6.82 ± 0.06, PETN-60: 6.81 ± 0.07 and PETN-300: 6.84 ± 0.09, $P=0.7217$, ANOVA).

PETN had no effect on systolic blood pressure, either on the first day (acute effect) or on days 5–7 of treatment (chronic effect) in all three groups as compared to pretreatment values (Figure 5a). Measurements of heart rate in the three PETN-treated groups also showed no significant differences between the groups and from the pretreatment values (Figure 5b).

Discussion and conclusions

The aim of this study was to determine the influence of pharmacological nitrate treatment on the expression and function of vascular sGC *in vivo*. Our main finding is that none of the conditions changed the protein expression of sGC α1 and β1 subunits and sGC activity. In addition, the functional efficacy of the vascular NO–cGMP pathway was maintained in all experimental models used. These data suggest that oral therapy with long-acting nitrates does not impair the vascular NO–cGMP pathway.

Studies in cultured rat aortic smooth muscle cells have provided evidence for an NO-dependent downregulation of sGC protein expression, suggesting a negative feedback loop where NO acts as a signalling molecule regulating sGC expression (Filippov *et al*., 1997). In another study, rat pulmonary artery smooth muscle cells responded to treatment with lipopolysaccharide, which is known to induce the expression of inducible NOS, with a downregulation of sGC mRNA levels (Scott and Nakayama, 1998). In eNOS-transfected cells showing a strong western blot signal for eNOS, we found an approximately fourfold downregulation of sGC mRNA expression and a reduction of sGC activity (Schmidt *et al*., 2001). Likewise, incubation of rat aortic rings with the NO-donor SPER/NO at NO generation levels of approximately $2\,\mu mol\,L^{-1}\,min^{-1}$ resulted in both, a reduction of activity and of protein expression (Weber *et al*., 2001). Therefore, it appears that our *in vivo* findings contradict the results obtained in cultured cells (Filippov *et al*., 1997; Scott and Nakayama, 1998) and isolated rat aortic rings (Weber *et al*., 2001).

In contrast, our data are consistent with reports that failed to show the existence of an NO-dependent feedback loop

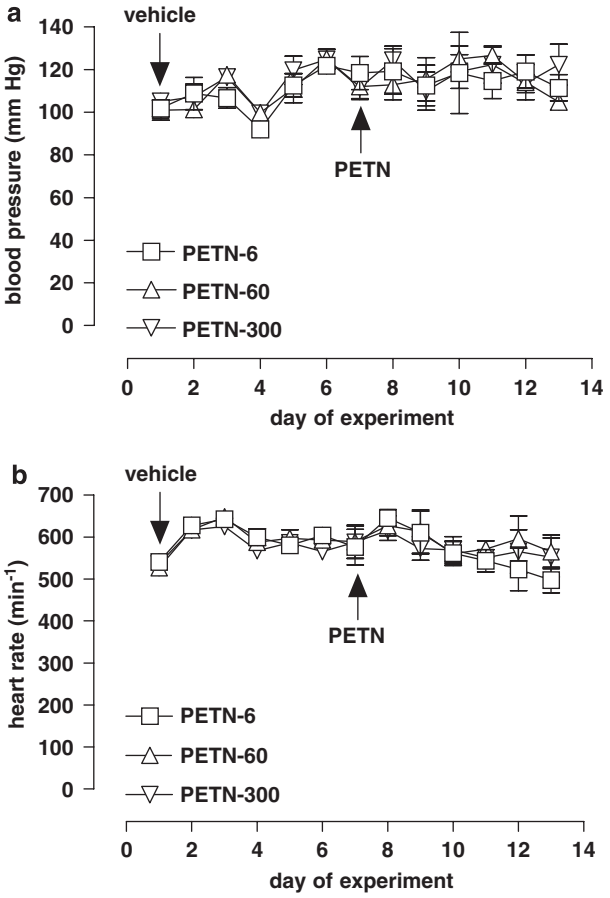

Figure 5 Effects of pentaerythrityl tetranitrate (PETN) treatment on blood pressure and heart rate. (**a**) Blood pressure measurements before and during PETN treatment of C57BL/6 mice. On day 7, vehicle food was replaced by diets enriched with 6, 60 or 300 mg kg^{-1} per day of PETN. None of these doses showed a significant acute or chronic effect on the blood pressure compared to pretreatment measurements ($P=0.5314$, $n=4$, two-way analysis of variance (ANOVA)). (**b**) Heart rate of PETN-treated mice before and during treatment. The different PETN doses had no significant effect on the heart rate compared to pretreatment measurements ($P=0.4416$, $n=4$, two-way ANOVA). All values represent mean ± s.e.m.

controlling sGC expression *in vivo*. Mice overexpressing eNOS in the vasculature show a resistance to endothelium-dependent and NO-induced vasodilatation but not a decrease of sGC expression (Yamashita *et al*., 2000). Instead, the authors describe a 50% reduction of basal unstimulated sGC activity and a 20% reduction of PKG expression. However, other groups have found that changes of PKG activity do not occur in eNOS knockout mice and that there is no change of sGC protein expression in this animal model (Hussain *et al*., 1999; Brandes *et al*., 2000). Inhibition of NOS by chronic treatment of mice with the NOS inhibitor L-NAME has been shown to have no effect on vascular sGC expression but potentiate the aortic cGMP response to the NO-donor sodium nitroprusside (Mullershausen *et al*., 2003).

There is some debate as to whether NO is indeed the pharmacologically active principle of organic nitrates (Kleschyov *et al*., 2003), although spin trap-based NO analyses in rabbits have demonstrated vascular NO formation from glyceryl trinitrate in both venous and arterial vessels (Mülsch *et al*., 1995). Detailed investigations identified a novel reductase activity of mitochondrial aldehyde dehydrogenase as the most important pathway for glyceryl trinitrate bioactivation, whereas nitrite but no NO was detectable as an intermediate of this reaction (Chen *et al*., 2002). It has been shown that PETN and its trinitrate metabolite PETriN is effectively metabolized by ALDH-2 (Wenzel *et al*., 2007), whereas the PEDN and PEMN metabolites as well as ISMN are probably bioactivated by other pathways, for example, in a cytochrome P 450-dependent manner to generate NO or at least an sGC stimulating intermediate compound (McGuire *et al*., 1998; Minamiyama *et al*., 1999). These data suggest that vascular generation of such a compound or NO from PEDN, PEMN and ISMN is a prerequisite for the therapeutic efficacy of PETN and ISMN.

The apparent contradiction between the *in vitro* and *in vivo* results (Francois and Kojda, 2004) might be caused by various mechanisms, such as the difference between bioactive concentrations of vascular NO, the time of exposure and possibly associated compensatory changes of the NO–cGMP pathway. In this study, increasing doses of the NO-donor ISMN in rabbits causing a 280-fold increase of maximal ISMN plasma concentrations in ISMN-200 compared to ISMN-2 and a moderate nitrate tolerance (Muller *et al*., 2003) had no effect on sGC activity and protein expression, whereas the NO sensitivity of aortic rings progressively declined with increasing ISMN dosage. Although, the overall effect on SNAP-induced vasodilatation was very small. These data suggest that the bioactive concentration of vascular NO even in the ISMN-200 group did not reach a concentration threshold necessary to induce inhibition of sGC expression.

To confirm these results and to avoid species- or treatment-specific artefacts, we repeated our experiments using a different species (mice) and various doses of a different nitrate (PETN). Although plasma concentrations of PEDN and PEMN were detectable in a dose-dependent manner, PETN treatment did not have an effect on sGC activity, sGC expression, NO-dependent vasorelaxation and blood pressure. However, our VASP measurements in the lung and in the heart showed no difference as well, although the plasma concentrations of PEDN and PEMN at 60 mg kg^{-1} per day of PETN largely exceed any therapeutic plasma concentration. So far, there is no other report available showing blood pressure reduction in mice receiving long-term treatment with PETN and just one paper describes a blood pressure drop 3 h after oral bolus application of 20 mg kg^{-1} of ISMN, a dose exceeding the human dose by approximately 80-fold (Momi *et al*., 2007), whereas another newly developed nitrate had no effect. Of the several possible explanations for the lack of blood pressure reduction in response to PETN, physiological adaptation to any initial arterial vasodilator effect appears to be the most likely. Furthermore, PETN has been shown to dilate venous vessels predominantly (Mullenheim *et al*., 2001). The supine position of the animals may blunt the blood pressure response as well.

Nevertheless, our data suggest that the bioactive concentration of vascular NO even in the PETN-300 and ISMN-200

groups did not reach a concentration threshold necessary to induce a negative feedback signalling on sGC expression that has been described to occur *in vitro*. One explanation for this phenomenon might be that vascular metabolism of organic nitrates to NO is decreased by NO itself (Kojda *et al*., 1998b). Using ^{14}C-glyceryl trinitrate, we have previously provided data to support this hypothesis.

In another study, chronic glyceryl trinitrate treatment induced severe nitrate tolerance and elicited a small upregulation of sGC that could be related to a negative feedback signalling between NO and sGC expression (Mulsch *et al*., 2001), but the results of this study and those previously obtained *in vivo* (Laber *et al*., 2002) and *in vitro* (Weber *et al*., 2001) rather suggest that this effect was mediated by the increase of vascular superoxide production associated with nitrate tolerance. Taken together, these results suggest that treatment with NO donors does not increase vascular bioavailable NO enough to induce a negative feedback signalling on sGC expression.

Previous *in vitro* observations in cultured smooth muscle cells expressing either sGC or PKG showed that transfection of PKG-deficient smooth muscle cells with the catalytic domain of PKG-I reduced protein expression of the β1 subunit, whereas transfection of sGC-deficient smooth muscle cells with both α1 and β1 subunits reduced protein expression of PKG after a 48 h treatment with the NO-donor 2,2′-(hydroxynitrosohydrazono)-bis-ethanimine (DETA/NO) (Browner *et al*., 2004). The effect of NO on the expression of sGC appears to involve a post-transcriptional regulation (Filippov *et al*., 1997), which might be caused by decreased binding of the sGC-mRNA-stabilizing family protein HuR (Kloss *et al*., 2003). In view of our *in vivo* results we suggest such inhibition by NO of sGC expression occurs at concentrations of NO exceeding normal *in vivo* concentrations and which are not even achievable with extremely high nitrate doses.

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Conflict of interest

GK received an unrestricted small educational grant from Actavis GmbH, Germany.

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