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# Pharmacological induction of vascular extracellular superoxide dismutase expression *in vivo*

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## Abstract

Pentaerythritol tetranitrate (PETN) treatment reduces progression of atherosclerosis and endothelial dysfunction and decreases oxidation of low-density lipoprotein (LDL) in rabbits. These effects are associated with decreased vascular superoxide production, but the underlying molecular mechanisms remain unknown. Previous studies demonstrated that endogenous nitric oxide could regulate the expression of extracellular superoxide dismutase (ecSOD) in conductance vessels *in vivo*. We investigated the effect of PETN and overexpression of endothelial nitric oxide synthase ($eNOS^{++}$) on the expression and activity of ecSOD. C57BL/6 mice were randomized to receive placebo or increasing doses of PETN for 4 weeks and $eNOS^{++}$ mice with a several fold higher endothelial-specific eNOS expression were generated. The expression of ecSOD was determined in the lung and aortic tissue by real-time PCR and Western blot. The ecSOD activity was measured using inhibition of cytochrome C reduction. There was no effect of PETN treatment or eNOS overexpression on ecSOD mRNA in the lung tissue, whereas ecSOD protein expression increased from 2.5-fold to 3.6-fold ($P < 0.05$) by 6 mg PETN/kg body weight (BW)/day and 60 mg PETN/kg BW/day, respectively. A similar increase was found in aortic homogenates. $eNOS^{++}$ lung cytosols showed an increase of ecSOD protein level of 142 $\pm$ 10.5% as compared with transgene-negative littermates ($P < 0.05$), which was abolished by $N^{\omega}$-nitro-L-arginine treatment. In each animal group, the increase of ecSOD expression was paralleled by an increase of ecSOD activity. Increased expression and activity of microvascular ecSOD are likely induced by increased bioavailability of vascular nitric oxide. Up-regulation of vascular ecSOD may contribute to the reported antioxidative and anti-atherosclerotic effects of PETN.

**Keywords:** endothelial nitric oxide synthase • extracellular superoxide dismutase • nitric oxide • pentaerythritol tetranitrate

## Introduction

Nitric oxide exhibits a variety of anti-atherogenic effects such as vasodilation, anti-aggregation, anti-apoptosis, anti-adhesion, anti-proliferation and antioxidation [1]. In common conditions such as hypertension, coronary artery disease and type 2 diabetes, the bioavailability of nitric oxide is reduced, as demonstrated by blunted endothelium-dependent vasodilation [2]. Such endothelial dysfunction is likely a consequence of increased vascular oxidative stress, a condition characterized by a misbalance of endogenous production of vascular reactive oxygen species (ROS) and the vascular antioxidative capacity. Although a variety of mediators have been described to contribute to vascular oxidative stress, both the generation and the detoxification of superoxide most likely play a major role in this process.

Superoxide is generated as a metabolic by-product, for example, in the mitochondria, by cytochrome P450 (CYP) oxygenases, by xanthine oxidase, by nitric oxide synthases (NOS) and particularly by NAD(P)H-oxidases [3]. It is also produced as a signalling molecule for certain cellular processes such as hypertrophy, smooth muscle cell growth, Ras signalling and NFκB activation [4]. There are highly specific mechanisms to rapidly detoxify superoxide and thereby prevent unwanted oxidative processes in

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cells and tissues. Of these, the superoxide dismutases I (CuZnSOD), II (MnSOD) and III (extracellular SOD [ecSOD]) are of utmost importance. Although CuZnSOD and MnSOD provide intracellular and intramitochondrial protection against superoxide, respectively, ecSOD does so in the interstitium. It is expressed in vascular smooth muscle cells and is subsequently secreted to the interstitium [5]. It binds to polyanionic sites such as heparin sulphates at the outer membrane of endothelial cells and likely protects endothelial nitric oxide while it traverses to the smooth muscle cell layer of the vascular wall [6]. The highest ecSOD concentrations are found between the endothelial cell layer and the smooth muscle, and 99% of ecSOD is tissue-bound.

Previous investigations have shown that endogenous nitric oxide is an essential stimulus of ecSOD expression in large conductance vessels such as the aorta, and that an increase of endothelial (e)NOS expression by exercise results in a consecutive overexpression of ecSOD [7]. Hence, driving the expression of ecSOD appears to be an important mechanism underlying the antioxidative effects of nitric oxide. We have also found in several studies that the nitric oxide donors such as pentaerythritol tetranitrate (PETN) [8, 9] and isosorbide mononitrate [10] exert substantial anti-atherosclerotic effects in rabbits, as demonstrated by a reduction in aortic plaque load, an improvement of endothelial function and a reduction of vascular superoxide production. One experimental study reported that PETN increases vascular haemoxygenase expression by approximately 1.5-fold, and it is likely that such a mechanism may play a role in the anti-atherosclerotic effects of PETN [11].

The aim of this study was to investigate whether PETN can increase ecSOD expression and activity in the microvasculature and whether such effects are induced by endogenous nitric oxide, as generated by endothelial-specific overexpression of eNOS as well.

# Materials and methods

## C57BL/6 mice

A total of 39 C57BL/6 male mice were randomly assigned to three groups and were treated with placebo (PETN-0) and 6 mg (PETN-6) or 60 (PETN-60) mg oral PETN/kg BW/day for 4 weeks. PETN was provided by Actavis, Langenfeld, Germany. Plasma concentrations of pentaerythritol dinitrate (PEDN) and pentaerythritol mononitrate (PEMN) were determined by gas chromatography/mass spectrometry (GC/MS; HP6890; Hewlett-Packard, Boelingen, Germany) after liquid–liquid extraction with ethyl acetate, as described previously [10].

## Transgenic eNOS$^{++}$ mice

Bovine eNOS complementary DNA (cDNA; 4.1 kb) was inserted between the murine Tie-2 promoter (2.1 kb) cDNA and a 10-kb Tie-2 intron fragment, designated as Tie-2-enhancer, and this construct was used to target eNOS gene expression to the vasculature, as described previously [12, 13]. Founder mice showing high eNOS expression (eNOS$^{++}$) compared with controls were crossed eight times to C57BL/6 mice to generate a C57BL/6 background. The mice were used at 12–16 weeks of age. Transgene-negative littermates (eNOS$^{n}$) served as controls. Permission for the animal studies was provided by the regional government of Germany, and 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 U.S. National Institutes of Health.

## Measurement of blood pressure

Systolic blood pressure and heart rate were measured in awake male eNOS$^{++}$ ($n = 6$) and eNOS$^{n}$ ($n = 6$) mice at 3–4 months of age using an automated tailcuff system (Visitech Systems, Apex, NC, USA), as described previously [14]. On day 6, the mice received N$^{\omega}$-nitro-L-arginine (L-NA, Sigma, Munich, Germany) with the drinking water (100 mg L-NA/kg BW/day), and blood pressure measurement was continued for a maximum of 30 days. Blood pressure of PETN-treated mice was measured from the day before treatment until 6 days after treatment.

## Determination of ecSOD mRNA concentration

Total RNA was isolated from snap-frozen lung tissue with the QIAshredder and RNeasy kit (Qiagen, Hilden, Germany), including an on-column DNAse digestion. Complementary DNA was synthesized from 1 μg total RNA using the QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's protocol. A primer/probe set directed to amplify an 82-bp fragment overlapping the exon2/exon3 boundary was designed with the online software provided by Qiagen (http://www.qiagen.com) and it revealed the following primers: sense: 5′-CTGACAGGTGCAGAGAA-3′, antisense: 5′-ACATGGTGACAGAGCCACA-3′, probe: 5′-CTACGGCTTGC-TACTGG-3′. For normalization purposes, coamplification of hypoxanthine guanine phosphoribosyl transferase (HPRT) cDNA was performed. The probes for ecSOD and HPRT were labelled with 6-carboxy-fluorescein and Yakima Yellow, respectively. The relative ecSOD expression was determined using the $\Delta\Delta C_t$ method. A second set of normalization experiments was based on 18S rRNA or smooth muscle actin (SMA) using 18S rRNA: 5′-ATACAGGACTCTTTCGAGGCCC-3′ and 5′-CGGGACACTC AGCTAAGAG-CAT-3′ annealing at 61°C; SMA-U: 5′-AGAGCAAGAGAGGGATCCTGA-3′ and SMA-L: 5′-GTCGTCCCAGTTGGTGATGAT-3′ annealing at 57°C. All real-time PCR experiments were carried out on an ABI PRISM 7900HT Sequence Detector System (Applied Biosystems, Weiterstadt, Germany).

## Western blot

Preparation of cytosols from mouse lungs was performed as described previously [15]. The tissues were flash frozen in liquid nitrogen and stored until use at –80°C. The supernatants of mouse lung and aortic homogenates (10 μg of total protein per lane) as well as blood plasma (10 and 20 μg) were fractionated on denaturing 12% polyacrylamide gels, blotted on PVDF membranes (Protrans; Schleicher & Schuell, Berlin, Germany) and stained with a monoclonal antibody (R&D Systems, Wiesbaden, Germany) directed against ecSOD. Actin staining served as a loading control (polyclonal anti-actin antibody; Sigma, Munich, Germany), and autoradiographs were analysed by densitometry. For confirmation


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purpose, Western blot analyses were repeated with polyclonal antibodies (Biomol GmbH, Stressgen, Hamburg, Germany; and R&D Systems, Wiesbaden, Germany). Western blots for eNOS in mouse crude aortic homogenates were done with a monoclonal eNOS antibody (Transduction Lab, BD Biosciences, Heidelberg, Germany).

### Measurement of ecSOD activity

The lungs were homogenized in 50 mM potassium phosphate (pH 7.4) containing 0.3 mol/l potassium bromide, as described previously [16]. Chromatography of homogenates on concanavalin A sepharose (GE Healthcare, Uppsala, Sweden) has been used to separate ecSOD from other SOD isoenzymes [16]. The SOD activity was determined spectrophotometrically by monitoring the inhibition of the rate of xanthine oxidase-mediated reduction of cytochrome C, as described previously [17]. The effectiveness of ecSOD separation was confirmed by Coomassie native gel staining and Western blot analysis. The lungs from 6 individual animals were pooled and two separate pools were analysed.

### Statistics

All data were analysed by a standard computer program (GraphPad Prism PC software, version 3.03, Graphpad Software, La Jolla, CA, USA) and are expressed as mean ± S.E.M. of *n* individual samples. Statistical comparisons between the groups were performed by either the t-tests or the Newman–Keuls multiple comparisons test following one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

## Results

### PETN metabolites and blood pressure data

The plasma concentrations of PETN metabolites detectable by GC/MS, PEDN and PEMN, rose with the daily oral dose of PETN. PEMN increased from 167.0 ± 104.8 ng/ml (PETN-6, $n = 3$) to 242.0 ± 48.4 ng/ml (PETN-60, $n = 4$, $P = 0.5059$), and PEDN from 17.3 ± 3.3 ng/ml (PETN-6, $n = 3$) to 120.8 ± 32.7 ng/ml (PETN-60, $n = 4$, $P = 0.0444$), indicating successful PETN resorption. Six days of treatment with PETN had no effect on blood pressure in awake mice. In PETN-6 mice ($n = 4$), the initial blood pressure of 121.8 ± 1.4 mmHg remained unchanged after treatment (119.1 ± 7.7 mmHg, $P = 0.7414$). Similar results were obtained before (125.0 ± 4.6 mmHg) and after (114.1 ± 8.3 mmHg, $P = 0.2915$) treatment in PETN-60 mice ($n = 4$; Fig. 1A). These data suggest that PETN, even at doses exceeding therapeutical doses by 20-fold, had no effect on resting blood pressure in mice.

### Blood pressure and eNOS expression in eNOS-overexpressing mice

Systolic blood pressure in $eNOS^{++}$ mice was reduced (Fig. 1B; $P = 0.0126$, $n = 4$). Treatment with the eNOS-inhibitor L-NA increased blood pressure in both $eNOS^{n}$ and $eNOS^{++}$ mice, and the difference between the two groups disappeared after 25 days of L-NA treatment ($P = 0.4407$, $n = 4$). Western blot analysis showed increased expression of aortic eNOS protein in $eNOS^{++}$ mice of 2.8 ± 0.5-fold ($P = 0.0369$, $n = 4$; Fig. 1C).

### ecSOD mRNA levels

The mRNA expression of lung ecSOD relative to HPRT did not show significant changes in the PETN-treated groups compared with the PETN-0-treated groups (each $n = 6$, $P = 0.7558$, ANOVA; Fig. 2A). In $eNOS^{++}$ mice, ecSOD mRNA expression (0.522 ± 0.080) was not significant different from $eNOS^{n}$ mice (0.637 ± 0.156, $n = 6$, $P = 0.5238$; Fig. 2B). Likewise, $eNOS^{++}$ aortic ecSOD mRNA levels were unchanged. Similar results on aortic ecSOD mRNA were obtained after treatment with L-NA and when SMA and 18S rRNA were used as standards (Fig. 2C).

### ecSOD protein expression

PETN increased ecSOD protein expression in the lungs to 251 ± 91.0% ($n = 5$) in the PETN-6 group and to 362 ± 84.9% ($n = 6$) in the PETN-60 group ($n = 6$, $P = 0.0479$ *versus* PETN-0; Fig 3A). A comparable result was obtained with a polyclonal ecSOD antibody (data not shown). A *post-hoc* analysis revealed statistical significance for PETN-60 *versus* PETN-0 but not for PETN-6 *versus* PETN-0 (Fig. 3A). Similar data were obtained in the aortas of the PETN-treated mice (Fig. 3B). The expression in PETN-6 mice was 152 ± 18.9% ($n = 6$) and that in PETN-60 was 207 ± 34.7% ($n = 5$) as compared with PETN-0 mice ($P = 0.0116$, for ANOVA; * = $P < 0.01$ for PETN-0 *versus* PETN-60, *post-hoc* analysis).

The lung tissue of $eNOS^{++}$ mice showed increased ecSOD protein as well (142 ± 10.5%, $n = 5$, $P < 0.05$ *versus* $eNOS^{n}$). Treatment with L-NA strongly reduced ecSOD expression ($P = 0.0003$, ANOVA; Fig. 3C) to 59.8 ± 18.6% in $eNOS^{n}$ mice ($n = 6$, $P < 0.001$ *versus* untreated) and to 44.4 ± 12.0% in $eNOS^{++}$ mice ($n = 6$, $P < 0.001$ *versus* untreated). Furthermore, the difference in ecSOD protein expression disappeared (Fig. 3C; $P > 0.05$ for $eNOS^{++}$/L-NA *versus* $eNOS^{n}$/L-NA). Additionally, $eNOS^{++}$ mice showed increased ecSOD blood plasma levels of 122 ± 5.6% ($n = 3$, $P = 0.0021$) compared with $eNOS^{n}$ mice (Fig. 3D).

### ecSOD activity

Changes in ecSOD activity paralleled changes in ecSOD expression ($n = 7$, $P < 0.0001$, ANOVA; Fig 4A). In the lungs of the PETN-treated mice, the ecSOD activity was 24.2 ± 2.23 U/mg (PETN-6, $n = 7$, $P < 0.05$, *post-hoc* analysis) and 37.3 ± 2.77 U/mg (PETN-60, $n = 6$, $P < 0.01$, *post-hoc* analysis) compared with the ecSOD activity of 16.1 ± 1.95 U/mg in PETN-0 mice. Likewise, the ecSOD activity was significantly increased in $eNOS^{++}$ mice (63.5 ± 4.24 U/mg, $n = 6$) compared with $eNOS^{n}$ mice (51.3 ± 2.95 U/mg, $n = 6$, $P = 0.0401$; Fig. 4B).

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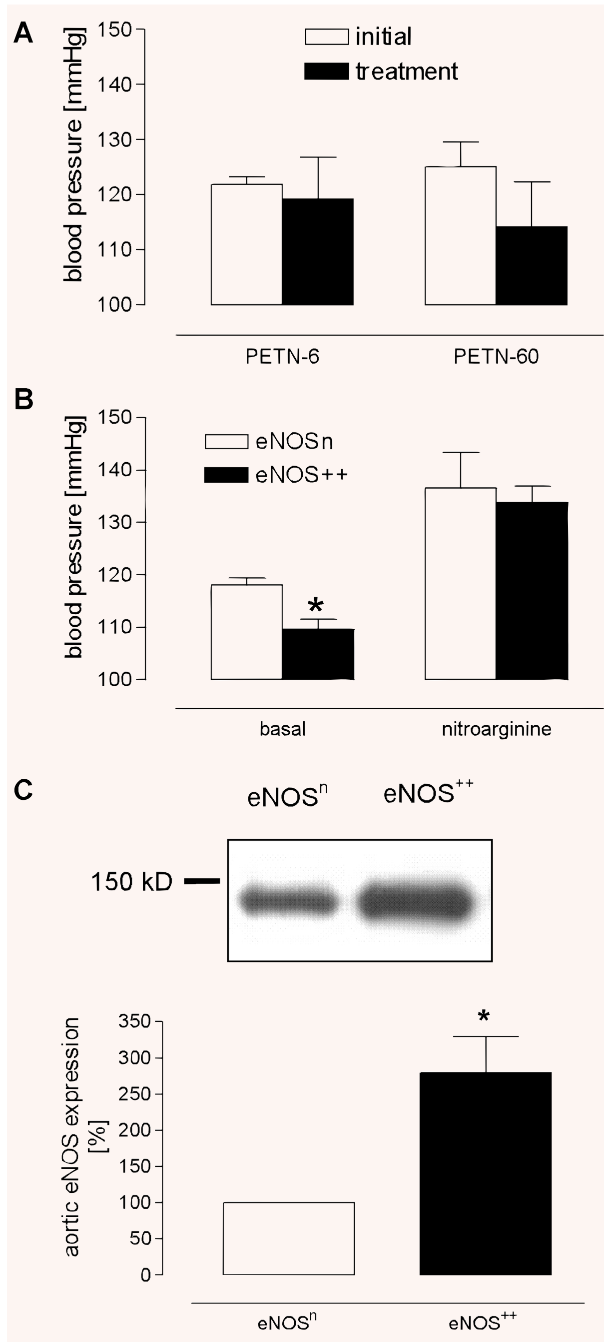


**Fig. 1** eNOS and blood pressure in eNOS-overexpressing mice. (**A**) Blood pressure in C57Bl/6 mice before (initial) and after treatment with PETN (not significant). (**B**) Untreated eNOS$^{++}$ mice have a significant lower blood pressure than eNOS$^{n}$ (* = $P < 0.05$). After 25 days of treatment with the eNOS inhibitor L-NA, this difference disappeared ($P > 0.05$). (**C**) Upper panel: Western blot for eNOS in aortic homogenates of eNOS$^{++}$; lower panel ($n = 4$): a mean of 2.8 ± 0.5-fold greater eNOS expression than in eNOS$^{n}$ ($n = 4$, * = $P < 0.05$).

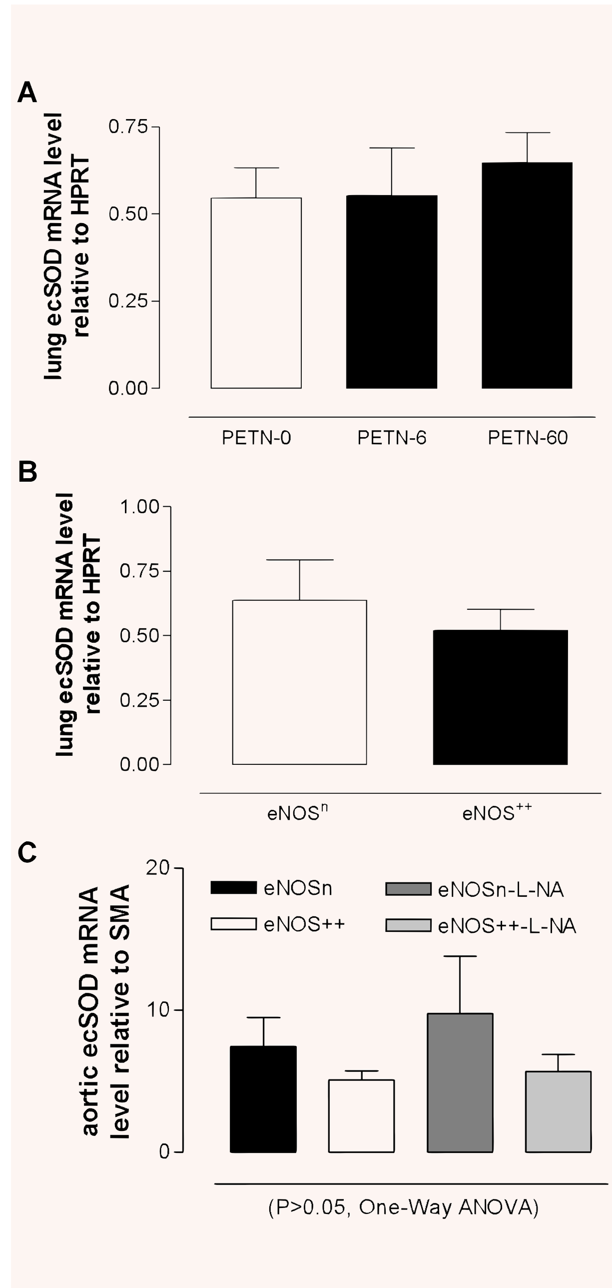


**Fig. 2** ecSOD mRNA levels. (**A**) Treatment of C57Bl/6 mice with 6 mg/kg/day (PETN-6) or 60 mg/kg/day (PETN-60) PETN induced no changes in lung ecSOD mRNA expression relative to HPRT ($P > 0.05$ *versus* PETN-0). (**B**) eNOS$^{++}$ mice induced no changes in lung ecSOD mRNA expression relative to HPRT ($P > 0.05$ *versus* eNOS$^{n}$). (**C**) eNOS$^{++}$ mice induced no changes in aortic ecSOD mRNA expression relative to SMA ($P > 0.05$ *versus* eNOS$^{n}$), irrespective of oral treatment with L-NA.


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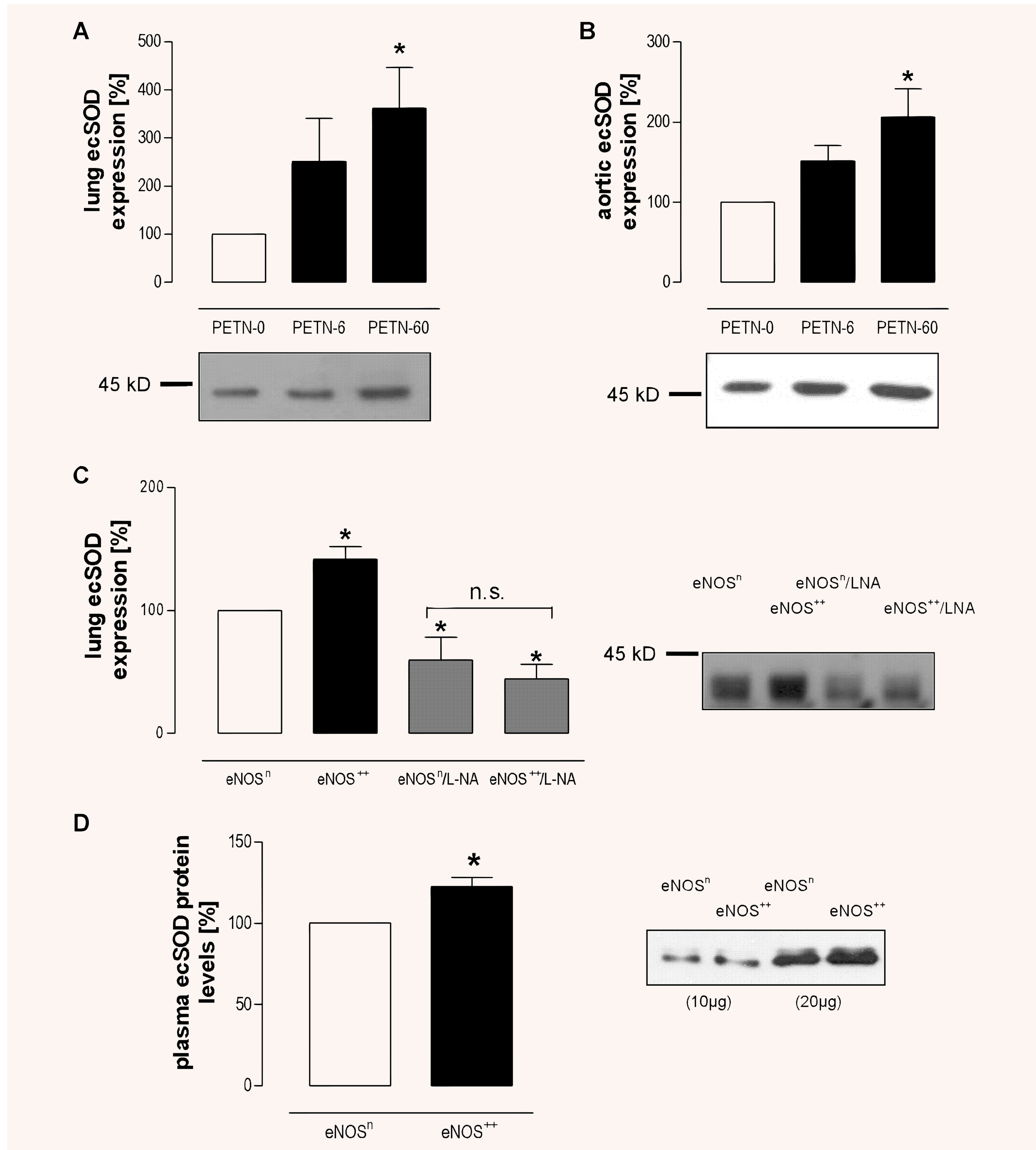


**Fig. 3** ecSOD protein expression. (**A**) Increased ecSOD protein expression in lung cytosolic fractions of C57Bl/6 mice treated with 0, 6 or 60 mg/kg/day PETN (PETN-0, PETN-6 or PETN-60, respectively, $* = P < 0.05$ for PETN-60 *versus* PETN-0). (**B**) Increased ecSOD protein expression in aortic homogenates of PETN-treated mice ($* = P < 0.01$ *versus* PETN-0). (**C**) Increased ecSOD protein expression in lung cytosols of eNOS$^{++}$ *versus* eNOS$^n$ mice ($P < 0.05$). Treatment with L-NA significantly lowered ecSOD expression in both groups (eNOS$^n$/L-NA and eNOS$^{++}$/L-NA; each $* = P < 0.05$ *versus* eNOS$^n$) and blunted the difference between both groups (N.S. = $P > 0.05$). (**D**) ecSOD protein in blood plasma of eNOS$^{++}$ mice was significantly increased compared with transgene-negative littermates (eNOS$^n$, $* = P < 0.01$).

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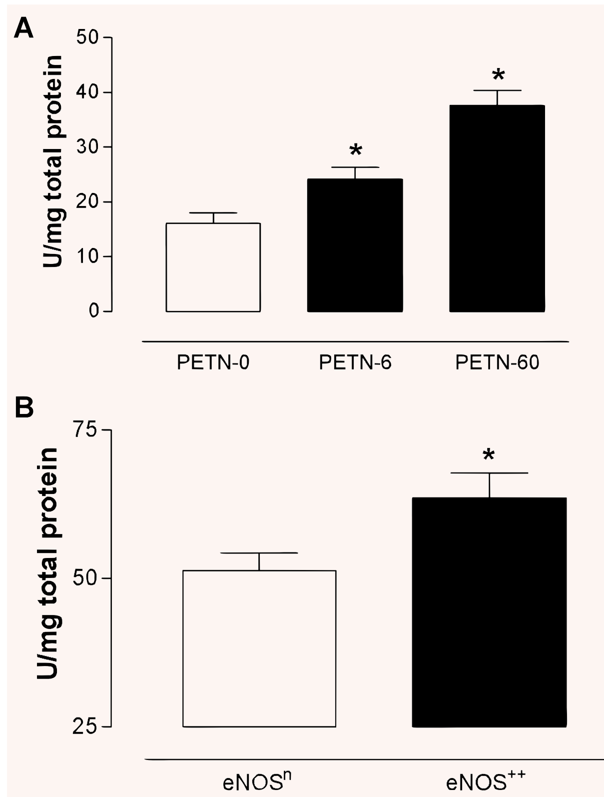


**Fig. 4** ecSOD activity. (**A**) The activity of extracellular superoxide dismutase (ecSOD) in lung tissue of $eNOS^{++}$ mice was significantly increased compared with $eNOS^{n}$ mice ($* = P < 0.05$). (**B**) Treatment of C57Bl/6 mice with 6 or 60 mg/kg/day pentaerythritol tetranitrate (PETN-6 or PETN-60, respectively) similarly resulted in increased activities compared with control mice (PETN-0). $* = P < 0.05$ *versus* PETN-0.

# Discussion

The aim of this study was to investigate whether vascular expression of ecSOD is subject to genetic and pharmacological regulation. Our findings are that the organic nitrate PETN increases the expression and activity of microvascular and macrovascular ecSOD in mice *in vivo*, and that a similar increase was observed in untreated transgenic mice with an endothelium-specific overexpression of bovine eNOS. These data extend previous observations and suggest that increased bioavailability of vascular nitric oxide regulates ecSOD expression, and that this effect might contribute to the antioxidative and anti-atherosclerotic effects of PETN observed previously.

This is the first study that demonstrates that PETN can induce the expression of ecSOD. PETN is an organic nitrate that is known to exert its anti-anginal effect by nitric oxide-dependent activation of soluble guanylyl cyclase (sGC), subsequent cyclic guanosine monophosphate (cGMP)-induced activation of protein kinase G and vasodilation of conductive arteries [18]. There is some debate whether nitric oxide is indeed the pharmacologically active principle of organic nitrates [19], although spin-trap-based nitric oxide analyses in rabbits have demonstrated vascular nitric oxide formation from glyceryl trinitrate in both venous and arterial vessels [20]. Detailed investigations identified a novel reductase activity of mitochondrial aldehyde dehydrogenase as the most important pathway for glyceryl trinitrate bioactivation, whereas nitrite but no nitric oxide was detectable as an intermediate of this reaction [21]. It has been shown that PETN and its trinitrate metabolite PETriN is effectively metabolized by aldhyde dehydrogenase (ALDH)-2 [22], whereas PEDN and PEMN are likely bioactivated by other pathways, for example, in a cytochrome P 450-dependent manner [23, 24]. Thus, there is neither evidence nor reasons to conclude that PETN increases vascular superoxide generation *in vivo*.

There is evidence that PEMN and PEDN generate nitric oxide. PETN and all metabolites are able to release nitric oxide in a cysteine-dependent manner. Likewise, the activation of sGC occurs in a cystein-dependent fashion. Furthermore, there are excellent correlations between cystein-dependent nitric oxide release and *in vitro* vasodilation expressed as pD2 values [18]. Neither PETN nor PETriN appears in blood plasma, suggesting that PETN and PETriN undergo extensive hepatic metabolism following intestinal absorption, the so-called first-pass effect [25]. Furthermore, intragastral PETN reduced the mean aortic pressure in anaesthetized rabbits by 20%, and the same quantitative effect was measured by intravenous PEDN, suggesting that PEDN is the major vasoactive metabolite [26]. Furthermore, the plasma half-lives of PEDN and PEMN are closely correlated with the duration of the anti-anginal action of PETN [25]. These data strongly suggest that vascular nitric oxide formation from PEDN and PEMN is a prerequisite for the therapeutical efficacy of PETN.

According to the metabolism pathways discussed previously, it seems likely that the up-regulation of ecSOD protein expression was mediated not directly by PETN but rather by nitric oxide released from its metabolites PEDN and PEMN. To further substantiate this, we used our transgenic mouse model with an endothelial overexpression of eNOS. In these mice, overexpression of eNOS was functionally active, as demonstrated by a significant reduction of blood pressure, which was completely blunted by oral treatment with the NOS inhibitor L-NA. We found a significant up-regulation of ecSOD protein expression and activity in cytosols of the lungs and aorta, suggesting that nitric oxide is the active mediator. To further challenge this hypothesis, we treated both $eNOS^{n}$ and $eNOS^{++}$ mice with the NOS inhibitor L-NA and observed a strong down-regulation of ecSOD protein expression. This result provides additional evidence for a regulatory role of vascular nitric oxide for ecSOD protein expression. Likewise, this result demonstrates that the increase of ecSOD in $eNOS^{++}$ mice is not an unspecific consequence of genetic manipulation. Finally, the reduction of blood pressure itself unlikely influenced the expression of ecSOD. This up-regulation was evident either at reduced blood pressure in $eNOS^{++}$ mice or at normal blood pressure following PETN treatment.

Our earlier observations with spontaneous nitric oxide donors in smooth muscle cells and aortic organoid cultures and with


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exercise in mice *in vivo* [7] are consistent with the suggestion that nitric oxide is an important mediator driving ecSOD expression. In that same study, it was shown that the effect of nitric oxide on ecSOD expression seems to be cGMP-dependent, as it was prevented by the selective sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and was mimicked by a cGMP analogue. So far, it is not known whether other nitric oxide/superoxide interactions such as suppression of thioredoxin-interacting protein [27] or alterations in superoxide release by neutrophils [28] may be involved in or modify ecSOD up-regulation.

In human vascular smooth muscle cells, nitric oxide was shown to increase the transcription but not the half-life of ecSOD mRNA, and the effect on transcription was critically dependent on the activity of p38 mitogen activated protein (MAP) kinase. In contrast to these findings in cultured human cells, we were not able to detect a change in mRNA levels in murine lung and aortic tissue after PETN treatment and in $eNOS^{++}$ mice as well. One alternative pathway could be that ecSOD is produced in other organs or tissues and reaches the lungs by travelling through the circulation. In this case, one would expect an increase of circulating ecSOD protein, which was indeed measurable. However, it remains unclear whether the small increase of circulating ecSOD is sufficient to account for the increase of ecSOD found in the aorta and lungs. Alternatively, a yet unresolved, solely post-translational *in vivo* regulation of ecSOD protein expression might occur. Peng *et al.* recently showed a nitric oxide-induced inhibition of the ubiquitin–proteasome system in murine primary cortical neurons [29]. Given this, one could speculate that the increase of ecSOD protein expression and activity *in vivo* could be a yet undiscovered effect of nitric oxide that needs to be substantiated further. However, other studies on this matter have suggested that nitric oxide rather increases proteasomal degradation of proteins by nitric oxide-dependent protein modification such as S-nitrosylation [30]. Taken together, established mechanisms of ecSOD regulation like the influence of proteolytic removal of the heparin-binding domain [31] and inflammatory cytokines [32] might extend to a yet unknown nitric oxide-dependent mechanism that deserves further interest and investigation.

Up-regulation of ecSOD likely appears to be a mechanism that contributes to the antioxidative and anti-atherosclerotic effects of PETN observed previously. PETN was shown to reduce the development and progression of experimental atherosclerosis [8], and this effect was evident even in established atherosclerosis [9]. Further activities of PETN such as inhibition of endothelial dysfunction and LDL oxidation are consistent with an inhibition of superoxide formation as well [9], although it is not sure that the observed effect is active in a similar manner in pathological settings. Additionally, PETN does not induce the development of *in vivo* nitrate tolerance [18], which strikingly contrasts the effect of glyceryl trinitrate, which is a well-known inducer of nitrate tolerance associated with increased vascular oxidative stress [33]. The lack of nitrate tolerance induction by PETN was shown in experimental [26, 34] and clinical studies [35]. Hence, the increased expression of ecSOD might contribute to the lack of nitrate tolerance induction by PETN as well.

By induction of ecSOD expression, PETN likely increases the bioavailability of endogenous nitric oxide, as superoxide and nitric oxide rapidly form peroxynitrite, a strong oxidant contributing to vascular oxidative stress and a source for further reactive radicals. These related products involve peroxynitrous acid or decomposition products of the carbon dioxide adduct of peroxynitrite such as the nitrogen dioxide radical [36–38]. However, superoxide is rapidly detoxified to hydrogen peroxide, which has been shown to induce both the expression and the activity of eNOS [39]. Thus, a higher activity of ecSOD will not only reduce extracellular superoxide but also protect readily formed nitric oxide. By increasing hydrogen peroxide, it most likely increases endothelial nitric oxide synthesis as well. This effect of hydrogen peroxide has been shown to contribute to the up-regulation of vascular nitric oxide synthesis induced by exercise training [12].

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