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RESEARCH PAPER

Vascular biotransformation of organic nitrates is independent of cytochrome P450 monooxygenases

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Background and Purpose: Organic nitrates such as nitroglycerin (NTG) or pentaerythritol tetranitrate (PETN) have been used for over a century in the treatment of angina or ischaemic heart disease. These compounds are prodrugs which release their nitrovasodilators upon enzymic bioactivation by aldehyde dehydrogenase (ALDH2) or cytochromes P450 (CYP). Whereas ALDH2 is known to directly activate organic nitrates in vessels, the contribution of vascular CYPs is unknown and was studied here.

Experimental Approach: As all CYPs depend on cytochrome P450 reductase (POR) as electron donor, we generated a smooth muscle cell-specific, inducible knockout mouse of POR (smcPOR$^{-/-}$) to investigate the contribution of POR/CYP to vascular biotransformation of organic nitrates.

Key Results: Microsomes containing recombinant CYPs expressed in human vascular tissues released nitrite from NTG and PETN with CYP2C9 and CYP2C8 being most efficient. SFK525, a CYP suicide inhibitor, blocked this effect. smcPOR$^{-/-}$ mice exhibited no obvious cardiovascular phenotype (normal cardiac weight and endothelium-dependent relaxation) and plasma and vascular nitrite production was similar to control (CTL) animals. NTG- and PETN-induced relaxation of isolated endothelium-intact or endothelium-denuded vessels were identical between CTL and smcPOR$^{-/-}$. Likewise, nitrite release from organic nitrates in aortic rings was not affected by deletion of POR in smooth muscle cells (SMCs). In contrast, inhibition of ALDH2 by benomyl (10 μM) inhibited NTG-induced nitrite production and relaxation. Deletion of POR did not modulate this response.

Conclusions and Implications: Our data suggest that metabolism by vascular CYPs does not contribute to the pharmacological function of organic nitrates.

Abbreviations: ALDH2, mitochondrial aldehyde dehydrogenase; BAC, bacterial artificial chromosome; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CYP, cytochrome P450; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; H&E, haematoxylin and eosin; HT, HEPES Tyrode buffer; ISDN, isosorbide dinitrate; ISMN, isosorbide mononitrate; Mhy11, smooth muscle myosin heavy chain; NBT, nitro blue tetrazolium; NOA, NO analyser; NTG, nitroglycerin; PETN, pentaerythritol tetranitrate; PFA, paraformaldehyde; POR, cytochrome P450 reductase; sGC, soluble GC; SKF525, proadifen; SMC, smooth muscle cell; VSMC, vascular smooth muscle cell.

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KEYWORDS
ALDH2, cytochrome P450 enzymes, cytochrome P450 reductase, nitroglycerin, organic nitrates, pentaerythritol tetranitrate, POR

1 | INTRODUCTION

Organic nitrates (Table 1) are among the oldest drugs used in the treatment of angina and ischaemic heart disease (Thadani, 2014). It is accepted that organic nitrates are prodrugs, and release of **NO** or similar reactive nitrogen species requires specific enzymes (Daiber et al., 2004). The metabolite(s) of organic nitrates stimulate **soluble GC** (sGC) and, through the subsequent production of **cGMP**, vascular smooth muscle cell (VSMC) relaxation is achieved (Bennett et al., 1992; Mulsch et al., 1995). **Nitroglycerin** (NTG) and pentaerythritol tetranitrate (PETN) are the most potent organic nitrates as they contain more activated nitrate groups than, for example, isosorbide mononitrate (ISMN) (Wenzel et al., 2007). Although NTG is the most widely used agent, its clinical use is complicated by nitrate tolerance, an effect not observed for PETN (Munzel et al., 2005; Wenzel et al., 2007).

The mitochondrial enzyme, **aldehyde dehydrogenase** (ALDH2), is known to contribute to the biotransformation of NTG and PETN at clinical concentrations (Chen et al., 2002, 2005; Munzel et al., 2005). In contrast, at supra-pharmacological concentrations, **cytochromes P450** (CYP) can have a similar function (Divakaran & Loscalzo, 2017; Munzel et al., 2005), and reduction of CYP expression has been suggested to be one mechanism of nitrate tolerance development (Minamiyama et al., 2001, 2004). The liver (followed by the kidney) is rich in CYP enzymes, and hepatic biotransformation is certainly important for the activation and inactivation of a large panel of drugs (Minamiyama et al., 2001, 2004). Nevertheless, also in the heart and vessels, expression of CYP enzymes has been shown to be very important for the maintenance of vascular quiescence and for some anti-inflammatory functions, which are dependent on the CYP-mediated production of vasoactive lipids (Hu, Dziumbla, et al., 2017; Hu, Geyer, et al., 2017). Whether, however, the vascular cytochrome P450 reductase–CYP (POR/CYP) system contributes to NTG and PETN biotransformation is unclear.

CYP enzymes are membrane-bound and haem-containing terminal monooxygenases (Fleming, 2001). They are part of a multienzyme system comprising a flavin adenine dinucleotide (FAD)/flavin mononucleotide (FMN)-containing NADPH-POR (gene name) (Degtyarenko & Kulikova, 2001; Fleming, 2001). POR is a key enzyme in transferring electrons to CYP enzymes, and, therefore, POR is essential for all functions performed by every CYP, including metabolism of lipids, hormone and cholesterol synthesis, and drug detoxification. CYPs are coded by more than 60 genes, whereas POR is coded by a single gene only. This makes the knockout of POR a valuable tool to switch off all CYP enzymes in a cell (Riddick et al., 2013).

Hepatic CYPs were earlier suggested to biotransform NTG (Servent et al., 1989), and later, it was shown that NTG was an activator of sGC (Katsuki et al., 1977). Moreover, CYP enzymes are expressed not only in the liver but also in many extrahepatic tissues, including VSMCs (Serabjit-Singh et al., 1985). Thus, a local release of NO from prodrugs in vessels could be an important function of vascular POR/CYP. In fact, the biotransformation of NO prodrugs has not been studied in the blood vessels of a POR/CYP knockout mouse model.

In the current work, we generated an inducible, smooth muscle cell (SMC)-specific knockout mouse of POR to investigate the contribution of the POR/CYP system to local biotransformation of NO prodrugs in vessels.

What is already known

- Organic nitrates are prodrugs activated by mitochondrial aldehyde dehydrogenase in the liver and blood vessels.
- Organic nitrate prodrugs can also be metabolised by cytochromes P450 in the liver.

What this study adds

- Biotransformation of organic nitrates in blood vessels was investigated in a POR knockout mouse model.

What is the clinical significance

- The POR/CYP system in blood vessels does not contribute to local bioactivation of organic nitrates.

2 | METHODS

2.1 | smcPOR$^{-/-}$ mice and animal procedures

Smooth muscle cell-specific, **tamoxifen**-inducible, knockout mice of POR (smcPOR$^{-/-}$) were generated by crossing CPR$^{Flox/Flox}$ mice (CPR: POR) (Wu et al., 2003) with SMMHC-CreERT2 (Wirth et al., 2008) (Myh11, smooth muscle myosin heavy chain) mice (provided by Prof. Offermanns). In this mouse line, CreERT2 is under the control of the Myh11 promoter/enhancer region on the bacterial artificial chromosome (BAC) transgene. Only male mice could be utilized in this study, since the BAC transgene is carried by the Y chromosome.

TABLE 1 Summary of clinical organic nitrate drugs and other NO donors and their application

Nitrovasodilators	Clinical use	Still in use?	Side effects	References
Nitroglycerin (NTG)	Angina, chest pain, acute heart failure	Yes	Nitrate tolerance, headache, flushing, postural hypotension	DrugBank; Divakaran & Loscalzo, 2017
Pentaerythritol tetranitrate (PETN)	Angina	Yes	Headache, nausea, skin irritation, postural hypotension	DrugBank; Divakaran & Loscalzo, 2017
Isosorbide dinitrate (ISDN)	Angina	Yes	Nitrate tolerance, headache, postural hypotension	DrugBank; Divakaran & Loscalzo, 2017
Isosorbide mononitrate (ISMN)	Angina	Yes	Nitrate tolerance, headache, postural hypotension	DrugBank; Divakaran & Loscalzo, 2017
Molsidomine	Ischaemic heart disease, angina, chronic heart failure, and pulmonary hypertension	Yes	Headache, nausea, postural hypotension, hypersensitivity	DrugBank

Global knockout mice of POR ($POR^{-/-}$) were generated by crossing $CPR^{Flox/Flox}$ mice with CMV-GT-Rosa-*CreERT2*$^{TG/0}$. All knockout animals were generated on the C57BL/6 background. The knockout of POR was induced by feeding mice with tamoxifen (400 mg·kg^{-1}) for 10 days followed by a washout time of another 10 days. "CTL" denotes animals carrying the Cre-recombinase as well as the floxed POR gene without tamoxifen application.

All animal care and experimental procedures were performed in accordance with the German animal protection law and were carried out after approval by the local authorities (FU1188). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). Animals were housed in groups with free access to chow and water in a specified pathogen-free facility with a 12-h day/12-h night cycle.

2.2 | Immunoblotting analysis for smcPOR$^{-/-}$

The antibody-based procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018). Western blots were carried out on extracts from from freshly isolated thoracic aorta or other organs. Tissue samples were homogenised with a Tissue Lyser (Qiagen) in a 1-min cycle at 50 s^{-1} in 150 µl of Triton X-100 lysis buffer (pH 7.4, concentration in mmol·L^{-1}: 50 Tris–HCl, 150 NaCl, 10 sodium pyrophosphate, 20 sodium fluoride, Triton X-100 (1%), proteinase inhibitor mix, 1 phenylmethylsulfonyl fluoride, 2 orthovanadate, 1 × 10^{-5} okadaic acid). The homogenate was centrifuged at 17,000 *g* for 10 min at 4°C. The protein concentration was estimated by Bradford assays, and samples (30 µg protein) were fractionated by SDS/PAGE followed by Western blotting for POR and β-actin (AC-15, #A1978, mouse, dilution 1:10,000).

Thoracic aorta and liver were freshly isolated, weighed, and homogenised as described in a volume in order to get 10 µg·µl^{-1}. The homogenate was centrifuged at 17,000 *g* for 10 min at 4°C, and 5× Blue Buffer was added. Samples of the extract (10, 30, 100, and 300 µg) were fractionated by SDS/PAGE followed by Western blotting for POR, CYP51A1 or ERK.

2.3 | Reductase activity in aortic rings

Aortas from smcPOR$^{-/-}$ mice and control littermates were freshly isolated and cleaned of connective tissue. To remove the endothelium, aortic rings were washed for 15 s with Krebs–Henseleit buffer (composition in g·L^{-1}: 2 D-glucose, 0.14 magnesium sulphate, 0.16 potassium phosphate monobasic, 0.35 potassium chloride, 6.9 sodium chloride) supplemented with 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 5% glucose. Next, rings were washed three times with Krebs–Henseleit without additives. Aortas were embedded in OCT™ (Tissue-Tek®) and frozen on dry ice. Frozen tissues were sectioned (10 µm) over Superfrost® plus microscope slides. Rings were incubated 30 min at 37°C with 0.02% nitro blue tetrazolium (NBT) and NADPH 100 µM in HEPES Tyrode buffer (HT, containing in mmol·L^{-1}: 137 NaCl, 2.7 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 5 glucose, 0.36 NaH_2PO_4, 10 HEPES). Tissue sections were then washed once with HT buffer and cover slipped, and formazan crystals were viewed under a light microscope.

2.4 | Histology

The experimental details of the immunofluorescence and immunohistochemistry assays conform with the *BJP* guidelines (Alexander et al., 2018). Aortas from smcPOR$^{-/-}$ mice and control littermates were freshly isolated and cleaned of connective tissue. Aortas were embedded in OCT™ (Tissue-Tek®) and frozen on dry ice. Frozen tissues were cut (10 µm) over Superfrost® plus microscope slides.

For caldesmon-1 and α-smooth muscle actin detection by immunofluorescence, aortic rings were fixed (4% paraformaldehyde [PFA]) for 10 min at room temperature, permeabilized (0.3% Tween in PBS) for 5 min three times, and blocked with 3% BSA in PBS for 30 min. Sections were stained overnight with an antibody for caldesmon-1 or

α-smooth muscle actin (Cy3 conjugate, 1A4, #C6198, mouse, dilution 1:300). Sections stained with caldesmon were incubated for 30 min with donkey anti-rabbit Alexa Fluor 546 (#A10040, Thermo Fisher, dilution 1:500 in 3% BSA). Nuclei were counter-stained by DAPI (#D9542, Sigma, dilution 1:200). Sections were mounted using Dako Fluorescence Mounting Medium (#S3023, Agilent) and analysed using a Zeiss LSM 800 confocal microscope. Blinded image analysis was performed using ImageJ software.

For vessel morphology, frozen tissues were fixed for 10 min at room temperature with 4% PFA. Nuclei were stained with Mayer's haematoxylin (#T865.2, Roth, Karlsruhe, Germany) for 6 min followed by cytoplasm staining with eosin 2% (#1092040500, Merck) for 1.5 min. Tissues were then dehydrated as follows: 70% ethanol (EtOH), 100% EtOH, 100% isopropanol, and xylene. Tissue sections were cover slipped and vascular morphology was viewed under a light microscope.

2.5 | Nitrite measurements

Nitrite was measured as a footprint of NO with an NO analyser (NOA, Sievers NOA-280) apparatus using acetic acid and sodium iodide (AppliChem #A1887) in a nitrogen atmosphere. A reference curve with sodium nitrite (100 nM to 10 μM) was used for calibration in every experiment.

For measurements with microsomes, 10 μg of total protein per condition were incubated in HEPES buffer in the presence of NADPH (100 μM). For time course analysis, samples were measured 3, 10, 30, and 60 min after adding NADPH.

For plasma nitrite measurements, heparinized blood was centrifuged for 10 min at 2,000 *g* at 4°C. Plasma was further cleared by centrifugal filter units (Microcon®-10, Merck #MRCPRT010) for 70 min at 17,000 *g* at 4°C, and 50 μl were loaded into the NOA. Importantly, animals received bottled water with low nitrite levels for 5 days before the measurements.

Aortic nitrite production was measured from the supernatant of rings (1 mm) in 96-well plates incubated with either NTG or PETN (100 μM) for 30 min at 37°C. In selected experiments, aortic rings were pretreated for 5 min with SKF525 (100 μM) or benomyl (10 and 100 μM) to inactivate CYPs and block ALDH2, respectively, followed by incubation with NTG or PETN (25 min).

2.6 | Organ chamber experiments

Experiments were performed with isolated murine aortic rings in carbogen-aerated Krebs–Henseleit buffer. The phenylephrine concentration was cumulatively increased (0.03–0.3 $\mu mol \cdot L^{-1}$) to obtain a constriction level of ~80% of the initial KCl (80 mM) constriction. Subsequently, ACh, NTG, or PETN was cumulatively added to aortic rings. In selected experiments, aortic vessels were pre-exposed for 5 min to benomyl (100 μM). Dilator dose response curves were constructed relative to the constrictor response to phenylephrine.

2.7 | Blinding and randomization

Every mouse received an identification number for each experiment, and the operator was blind for the genotype. Animal group sizes differ due to number of littermates. One control and one knockout animal were analysed per experiment, and the order was alternated daily.

2.8 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). Statistical analysis was performed only for studies where each group size was at least five and no data points were excluded from statistical analysis in any test.

Unless otherwise indicated, data are shown as means ± SEM. Calculations were performed with Prism 8.0. In case of multiple testing, Bonferroni correction was applied. For multiple group comparisons, ANOVA followed by post hoc testing was performed. Individual statistics of unpaired samples was performed by *t* test and if not normally distributed by Mann–Whitney test. Vasodilator studies were analysed with ANOVA for repeated measurements. A P value of <.05 was considered as significant. n indicates the number of individual experiments or animals.

2.9 | Materials

NTG, PETN, and isosorbide dinitrate (ISDN) were kindly provided by Prof. Andreas Daiber (Universitätklinik Mainz, Germany). Tamoxifen (#DT400.R1) was from Genobios (Laval, France); SKF525 (proadifen hydrochloride, CYP suicide inhibitor, #P1061) and benomyl (an irreversible inhibitor of ALDH2, #45339) were purchased from Sigma Aldrich (Darmstadt, Germany). Sodium nitrite (#1065441000), isosorbide mononitrate (ISMN, #I0775010) and molsidomine (# M2901) were from Merck (Darmstadt, Germany) , NADPH (#A1395) from AppliChem (Darmstadt, Germany), and DETA NONOate (#ALX-430-014) from Enzo (Lörrrach, Germany); all other chemicals, if not specified, were purchased from Sigma Aldrich. Anti-POR (F-10, #sc-25270, mouse, dilution 1:500) antibody was purchased from Santa Cruz (Dallas, USA). Anti-CYP51A1 (#13431-1-AP, rabbit, dilution 1:1,000) antibody was purchased from Proteintech (St. Leon-Rot, Germany). Anti-ERK1/2 (L34F12, #4696, mouse, dilution 1:2,000) antibody was purchased from Cell Signaling (Danvers, USA). IRDye 800CW anti-mouse (#926-32212, dilution 1:10,000), anti-rabbit (#926-32213, dilution 1:10,000), and IRDye 680CW anti-mouse (#926-68072, dilution 1:10,000) secondary antibodies used for Western blotting were purchased from LI-COR (Bad Homburg, Germany). Microsomes (Supersomes™) produced in insect cells overexpressing different combinations of the human CYP system were obtained from Corning® (New York, USA). The following combinations were used: P450 reductase + cytochrome b_5 as a negative control which lacks CYP450 (POR + $Cytb_5$ #456244) and P450 reductase + cytochrome P450

(POR + CYP) with the following CYPs: CYP2C8 (#456212), CYP2C9 (#456218), CYP3A4 (#456207), and CYP1B1 (#456220).

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | Nitrovasodilators release nitrite in vitro when incubated with recombinant CYP preparations

To investigate whether POR/CYP can biotransform NO prodrugs, we selected CYPs which are expressed in murine and human aorta using published sequencing data as reference (Fleming, 2001; Kalluri et al., 2019; Tabula Muris Consortium, 2018).

CYP2C9, 2C8, 3A4, and 1B1 were selected as being typically expressed in vessels and commercially available in the form of recombinant Supersomes™. Release of NO was measured as nitrite (by NOA) in time course experiments with each POR/CYP combination incubated individually with NTG, PETN, ISDN, ISMN, and molsidomine (Figure 1). The negative control POR + Cyt_{b5} showed no signal with any nitrite donor tested, confirming that the catalytic CYP unit is required for the reaction. Importantly, POR/CYP without NADPH gave no signal in the assay. The nitrite production differed for the NO donors and the individual supersomes. At equimolar concentrations, nitrite production from NTG was higher than for any other compound. CYP2C8 and CYP2C9 were much more effective in producing nitrite of NTG and PETN than CYP3A4 and CPY1B1 (Figure 1a,b). In contrast, ISDN was effectively cleaved by CYP2C9, whereas CYP2C8, CYP3A4, and CYP1B1 did not metabolize it (Figure 1c). CYP1B1 was the only CYP oxidase able to generate nitrite from molsidomine (Figure 1e). Interestingly, none of the CYPs generated significant amounts of nitrite from ISMN (Figure 1d). NONOate

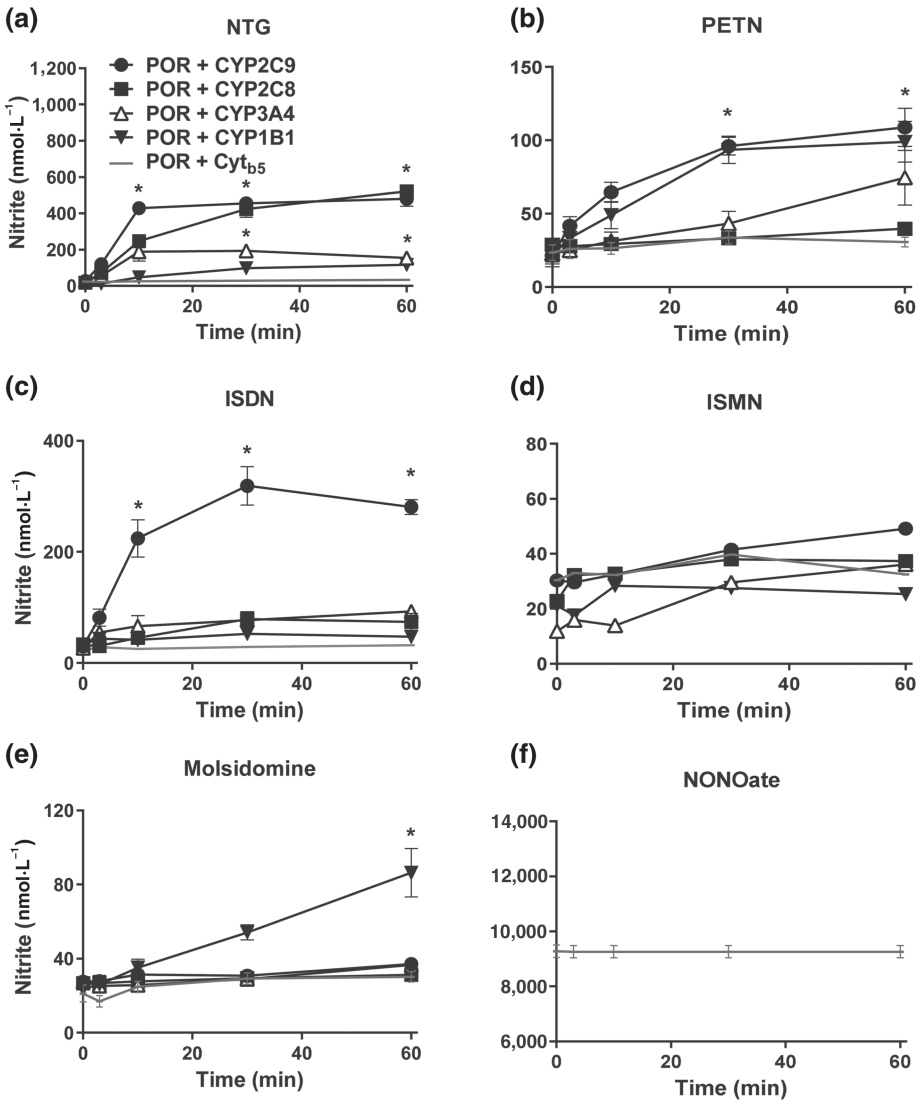

FIGURE 1 Production of nitrite from organic nitrates by CYP-containing microsomes. Nitrite (NO_2^-) levels, measured with the NOA, in microsomes overexpressing combinations of cytochrome P450 components (10 μg) incubated with the drugs indicated (100 μM) at 3, 10, 30, and 60 min. Samples were supplemented with NADPH (100 μM). (a–e) Nitrite production from microsomes incubated with NTG, PETN, ISDN, ISMN, or molsidomine is CYPs specific. (f) As positive control, NONOate which spontaneously releases NO in contact with water. Data shown are means ± SEM from $n = 5$ independent experiments. *$P < .05$, significantly different from POR + Cyt_{b5}

was used as a positive control for the nitrite measurements, as it spontaneously releases NO with zero-order kinetics (Figure 1f). These results show that POR/CYP in the form of recombinant microsomes can release nitrite from NO prodrugs in a CYP- and concentration-dependent manner. For the next experiments, we focused on two nitrovasodilators: NTG which is the most commonly used organic nitrate and PETN which does not induce nitrate tolerance.

3.2 | SKF525 inhibits nitrite release from NTG and PETN in recombinant POR/CYP microsomes

Next, we wanted to ensure that the biotransformation of NTG and PETN from CYP enzyme was truly dependent on enzymic activity. For that, CYP2C8 and CYP2C9 were preincubated for 5 min with SKF525, a non-selective CYP suicide inhibitor (Schenkman et al., 1972), before adding the prodrugs. The nitrite production from NTG by CYP2C9 and CYP2C8 was abolished by SKF525 (Figure 2a). Likewise, nitrite release from PETN was also abolished by SKF525 (Figure 2b).

Thus, POR/CYP enzymes were essential for the biotransformation of NTG and PETN in our recombinant overexpression system.

3.3 | Mice lacking POR in SMCs do not show a cardiovascular phenotype

As POR is broadly expressed in vascular cells, our first attempt was to study global, tamoxifen-inducible knockout mice of POR, as the constitutive knockout is embryonically lethal. However, global induction of POR deletion resulted in early onset of fatty liver (Figure S1A), and the knockout efficiency in aorta was variable and insufficient (Figure S1B). Potentially, this is a consequence of the insufficient conversion of tamoxifen to its active form hydroxytamoxifen by hepatic CYPs as the Cre-mediated POR deletion progressed in the liver. Knockout in the liver is usually faster and more efficient than in vessels, and potentially, hydroxytamoxifen levels decreased before a sufficient vascular knockout of the POR gene was achieved. As SMCs are the predominant vascular cell type and the effectors of relaxation, smooth muscle-specific, tamoxifen-inducible knockout mice (smcPOR$^{-/-}$) were generated and subsequently studied.

Reduction of POR protein in aortic rings of smcPOR$^{-/-}$ was more than 70% as shown by Western blotting (Figure 3a). The knockout of the protein was accompanied by a decrease in the reductase activity in vessels as shown by diminished NBT to formazan conversion, as a marker for the vascular reductase activity (Figure 3b left panel). This difference was particularly pronounced after removal of the endothelium, which should still express POR and other sources of NBT reducing enzymes, such as endothelial NOS (eNOS) in these animals (Figure 3b right panel).

smcPOR$^{-/-}$ mice showed no obvious vascular phenotype. In fact, there was no difference in the endothelium-dependent relaxation of aortic rings between CTL and smcPOR$^{-/-}$ mice (Figure 3c). Moreover, the vessel morphology was not altered in smcPOR$^{-/-}$ mice. The α-smooth muscle actin and caldesmon-1 expression, VSMC markers, was determined by immunofluorescence and was similar between CTL and smcPOR$^{-/-}$ mice (Figure S2A). Haematoxylin and eosin (H&E) staining of the vessels confirmed no morphological difference between CTL and smcPOR$^{-/-}$ mice (Figure S2B). As judged by the heart to body weight ratio, smcPOR$^{-/-}$ did not develop cardiac

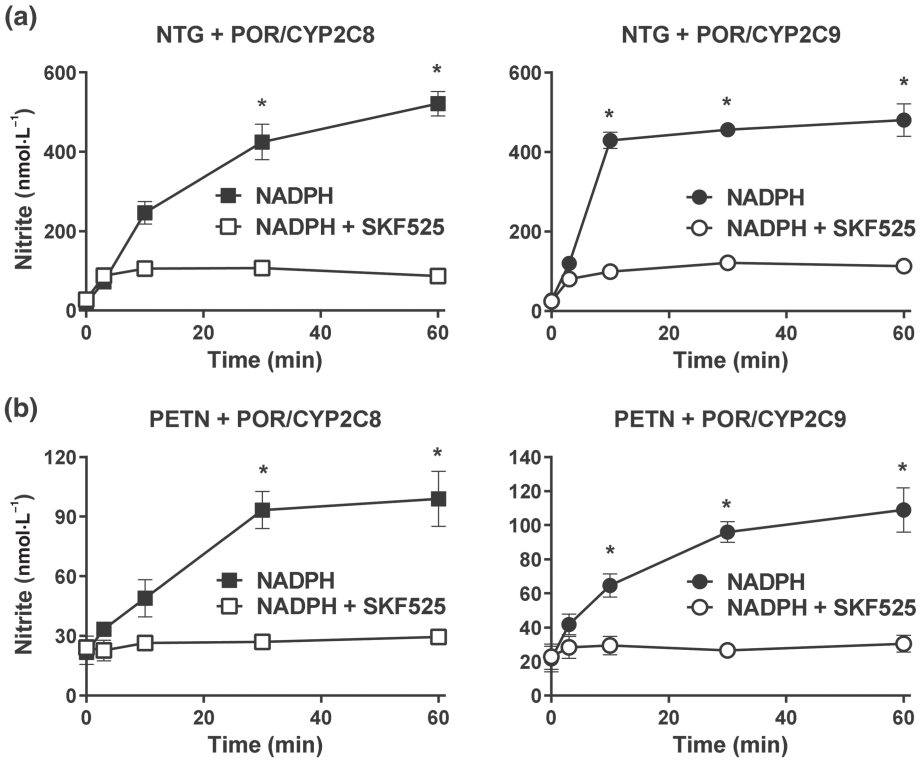

FIGURE 2 Effect of CYP inhibition on nitrite production of microsomal from organic nitrates is blocked by CYP inhibition. Nitrite (NO_2^-) measurements in microsomes overexpressing combinations of cytochrome P450 components (10 μg) as indicated, incubated with NTG or PETN (100 μM) at 3, 10, 30, and 60 min. Samples were supplemented with NADPH (100 μM). Microsomes were preincubated with or without SKF525, a suicide CYP inhibitor, for 5 min followed by incubation with drugs. Data shown are means ± SEM from n = 5 independent experiments. *P < .05, significantly different from NADPH

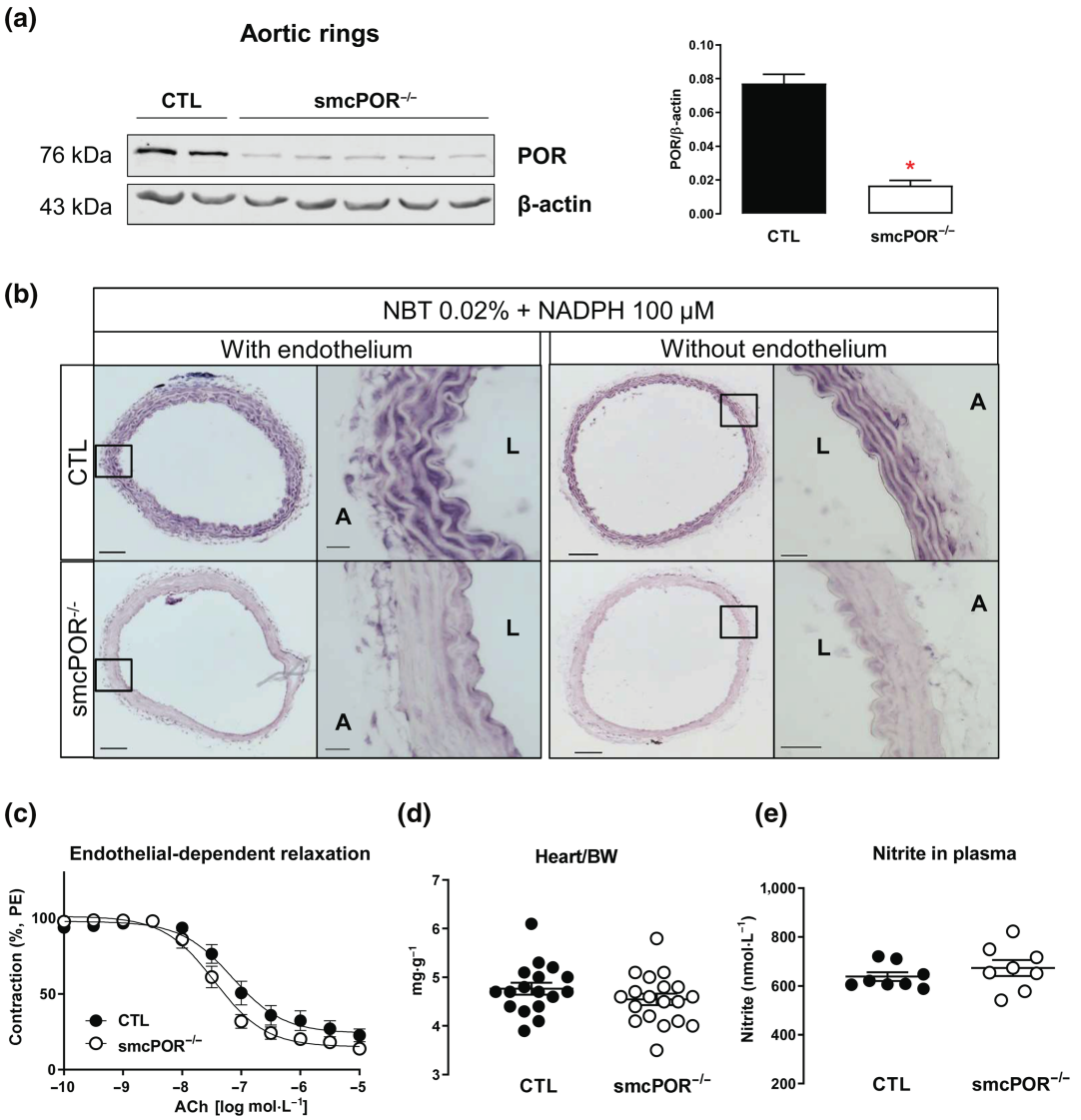

FIGURE 3 Characterization and efficiency of smcPOR knockout in mice. (a) POR expression in aortic rings as analysed by Western blot (representative image). (b) POR reductase activity in SMC was visualized by NADPH oxidation resulting in the deposition of formazan crystals in the presence of NBT (0.02%). Left panels: scale bars 100 µm and 5× magnification; right panels: scale bars 20 µm and 40× magnification. A, adventitial face; L, lumen. (c) Organ bath experiments of freshly isolated aortic artery in response to ACh; $n \geq 9$. (d) Ratio of heart to body weight; $n \geq 17$. Body weight CTL: 26.8 ± 1.79g; body weight smcPOR$^{-/-}$: 27.0 ± 2.29g. (e) Nitrite (measured as NO_2^-) from plasma of CTL and smcPOR$^{-/-}$ animals ($n = 8$) using NOA. Data shown are means ± SEM from n independent experiments, as shown. *$P < .05$, significantly different from CTL. CTL = CPR$^{Flox/Flox}$SMMHC-CreERT2$^{+/y}$ without tamoxifen; smcPOR$^{-/-}$ = CPR$^{Flox/Flox}$SMMHC-CreERT2$^{+/y}$ after tamoxifen

hypertrophy (Figure 3d), and also, plasma nitrite levels were similar between the two strains (Figure 3e). Thus, we obtained a substantial knockout of POR in SMCs and thereby could indirectly knockout the function of all CYPs. However, the knockout of POR/CYP in SMC does not lead to an obvious cardiovascular phenotype under basal conditions.

3.4 | smcPOR$^{-/-}$ does not alter relaxation of aortic arteries to organic nitrates

To test if the dilator response induced by organic nitrates involves POR/CYP, the aortic response from CTL and smcPOR$^{-/-}$ aortic rings was compared with and without removal of the endothelium. Successful removal of the endothelium was confirmed by the absence of endothelium-dependent relaxation (Figure S3).

The dilator curves to NTG (Figure 4a) as well as to PETN (Figure 4b) were identical between aortic rings of CTL and smcPOR$^{-/-}$ mice. Moreover, also the responses to the spontaneous donor DETA-NONOate were not affected by the knockout (Figure 4c). Although removal of the endothelium changed the sensitivity to organic nitrates per se (Figure S4), the response to nitrates was not different between endothelium-free aortic rings of CTL and smcPOR$^{-/-}$ (Figure S4A–C). Hence, although nitrite was produced from organic nitrates by POR/CYP in recombinant overexpression system (microsomes),

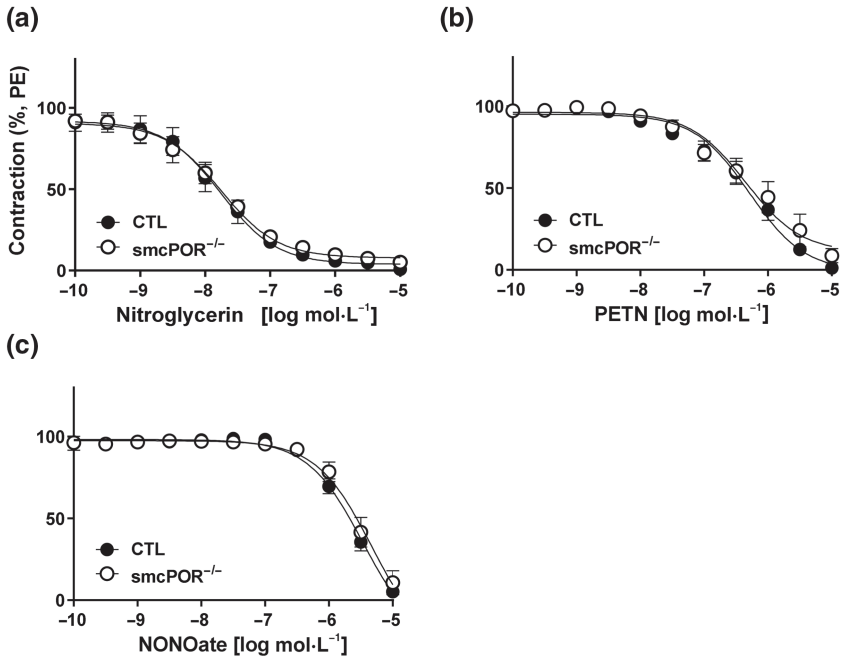

FIGURE 4 Contribution of smooth muscle POR to the aortic dilator responses to organic nitrates. Endothelium intact (a–c) aortic segments preconstricted with phenylephrine (PE) from CTL and smcPOR$^{-/-}$ mice were exposed for increasing concentrations of the dilators indicated. Data shown are means ± SEM from $n \geq 9$ animals. No significant differences from CTL were found. CTL = CPR$^{Flox/Flox}$SMMHC-CreERT2$^{+/y}$ without tamoxifen; smcPOR$^{-/-}$ = CPR$^{Flox/Flox}$SMMHC-CreERT2$^{+/y}$ after tamoxifen

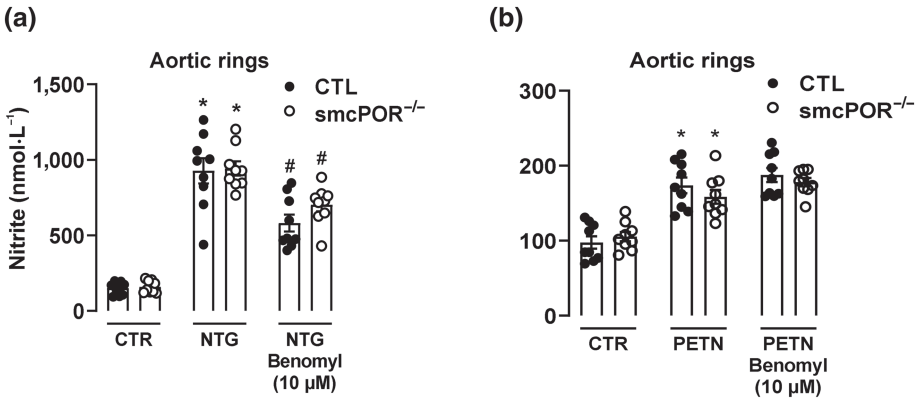

FIGURE 5 Nitrite (NO_2^-) measurements in aortic rings. Aortic segments from CTL and smcPOR$^{-/-}$ mice were incubated (37°C, 30 min) with NTG or PETN (100 μM) in the presence or absence of benomyl. Data shown are means ± SEM from $n = 9$ animals. $^*P < .05$, significantly different from CTR. $^\#P < .05$, significantly different from NTG. CTL = CPR$^{Flox/Flox}$SMMHC-CreERT2$^{+/y}$ without tamoxifen; smcPOR$^{-/-}$ = CPR$^{Flox/Flox}$SMMHC-CreERT2$^{+/y}$ after tamoxifen

inactivation of this system in vessels had no effects on the dilator response to organic nitrates.

3.5 | Inhibition of ALDH2 but not deletion of POR attenuates aortic nitrite production from organic nitrates

In addition to CYPs, the enzyme ALDH2 contributes to the activation of organic nitrates (Chen et al., 2002; Daiber et al., 2004). Therefore, the aortic nitrite production in response to organic nitrates was compared between CTL and smcPOR$^{-/-}$ aortic rings in the presence and absence of the ALDH2 inhibitor benomyl.

NTG (100 μM, 30 min) increased aortic nitrite production fivefold, whereas inhibition of ALDH2 with benomyl dose-dependently decreased this effect (Figure 5a). Deletion of POR or inhibition of CYPs with SKF525 (100 μM, Figure S5A) had no effect on nitrite production. The nitrite production from PETN was lower than that from NTG, but deletion of POR (Figure 5b) or inhibition (Figure S5B) of CYPs also had no effect on this response. Interestingly, benomyl was much less effective in blocking nitrite production by PETN, compared with NTG, suggesting that an unknown third enzyme contributes to PETN activation.

3.6 | Inhibition of ALDH2 does not unmask a role of CYP for organic nitrate activation

Given the important role of ALDH2 for organic nitrate activation, it was tested whether its inhibition by benomyl (10 μM) may unmask a role for POR/CYP. Higher concentrations of benomyl could not be studied as they interfered with the constrictor response. Benomyl had no effect on the dilator response to the direct NO donor DETA-NONOate or the endothelium-dependent vasodilator ACh (Figure 6a,b). In contrast to this, the response to NTG was shifted to the right (EC_{50} 21.3 nM without benomyl; 0.4 μM with benomyl,

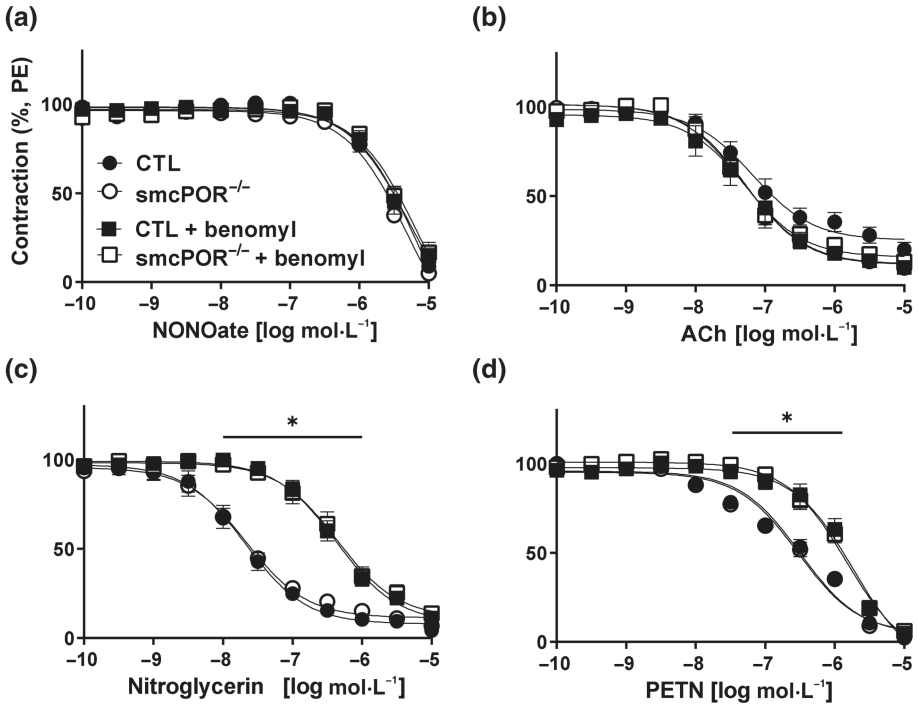

FIGURE 6 Effect of ALDH2 inhibition on the aortic dilator response to organic nitrates. Organ bath experiments in isolated aortic segments of CTL and smcPOR$^{-/-}$ mice in response to the nitrovasodilators indicated in the presence and absence of benomyl (10 μM). Data shown are means ± SEM from n = 10 animals. *P < .05, significantly different from WT and smcPOR$^{-/-}$ without benomyl. CTL = CPR$^{Flox/Flox}$SMMHC-CreERT2$^{+/y}$ without tamoxifen; smcPOR$^{-/-}$ = CPR$^{Flox/Flox}$SMMHC-CreERT2$^{+/y}$ after tamoxifen

Figure 6c). There was also a significant, although small, effect of the PETN-induced relaxation (EC_{50} 0.29 μM without benomyl; 1.40 μM with benomyl, Figure 6d). Responses between aortic rings from CTL and smcPOR$^{-/-}$ mice in the presence of benomyl were identical (Figure 6a–d). Furthermore, removal of the endothelium combined with benomyl (10 μM) also showed no difference in vessel relaxation between CTL and smcPOR$^{-/-}$ (Figure S6A–D). Thus, inhibition of ALDH2 did not unmask a contribution of POR to the dilator response of organic nitrates.

4 | DISCUSSION

In this study, we investigated the involvement of the vascular POR–CYP system in the biotransformation of organic nitrates. By using insect cell microsomes overexpressing the POR/CYP system, we demonstrated that the vascular CYPs could produce nitrite from organic nitrates. Moreover, this production was inhibited by SKF525, a non-selective CYP suicide inhibitor. However, using smcPOR$^{-/-}$ mice and NTG or PETN as organic nitrates, our results suggested that POR/CYP did not importantly contribute to the biotransformation of organic nitrates in vascular tissue.

Several studies in hepatic microsomal preparations have demonstrated the importance of CYP in the biotransformation of NTG into an activator of sGC (Bennett et al., 1992; Katsuki et al., 1977; McDonald & Bennett, 1990; Servent et al., 1989). In the present work, we could confirm that POR/CYP-containing microsomes indeed did generate nitrite from organic nitrates. It is possible that the liver, which is rich in POR/CYP, is the predominant site of transformation of organic nitrates in vivo. The resulting reactive nitrogen species would subsequently bind to haemoglobin (as S-nitroso-haemoglobin) in red blood cells or to plasma proteins to be transported to distant vessels.

POR/CYP are expressed in virtually every cell, although the CYP repertoire is unique to each cell type. Deletion of POR in SMCs of arteries did not affect the vascular response to organic nitrates, even if the endothelium was removed (Figure 7). This excludes not only smooth muscle CYPs but also endothelial CYPs as physiologically important sites of biotransformation. Indeed, since the report of the Stamler group (Chen et al., 2002), ALDH2 has been considered as the main enzyme responsible for the activation of organic nitrates in blood vessels. Subsequent studies suggested that ALDH2 indeed promotes NO release from NTG (Beretta et al., 2012; Opelt et al., 2016) and PETN (Griesberger et al., 2011; Wenzel et al., 2007).

In the present work, we could confirm the importance of vascular ALDH2 for the response to NTG. Interestingly, PETN-induced relaxation was only slightly altered by benomyl, and the effect of benomyl on PETN-induced nitrite production was minor. This is surprising, as genetic deletion of ALDH2 strongly attenuated the effects of PETN and suggests that specifics of the interaction of PETN with benomyl but not ALDH2 are responsible for this observation. Indeed, the ALDH2 inhibitor daidzin was equally potent in inhibiting the dilator response to PETN in the rat aorta as genetic deletion of ALDH2 was in the mouse aorta (Griesberger et al., 2011).

The fact that POR/CYP-containing liver microsomes generate nitrite from organic nitrates but that vascular POR/CYP does not contribute to the dilator response to organic nitrates allows for two interpretations. Either the CYP-dependent biotransformation of organic nitrates does not generate a dilatory active reactive nitrogen

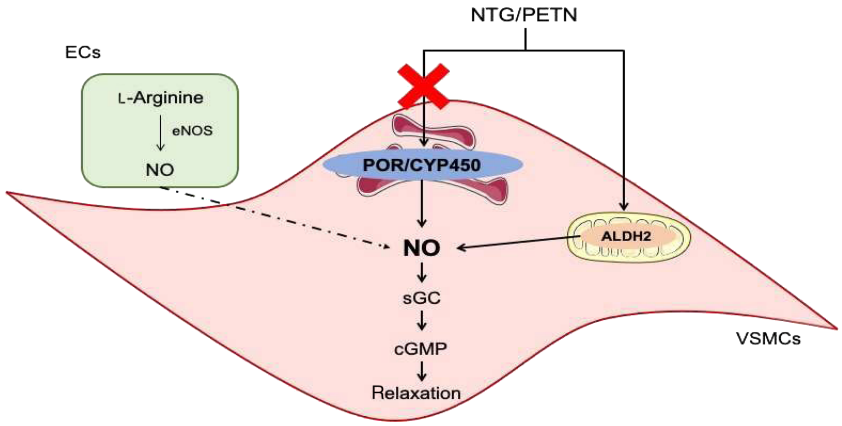

FIGURE 7 Pathway of the biotransformation of organic nitrates in vascular smooth muscle cells. Under basal condition, endothelial cells produce NO by eNOS. NO diffuses to smooth muscle cells resulting in sGC stimulation, cGMP production, and relaxation. ALDH2 contributes to the biotransformation of NTG and PETN. The metabolite(s) of NTG and PETN also stimulate sGC. POR/CYP450 are involved in the biotransformation of NTG and PETN in the liver. However, in vessels, this pathway turns out to be physiologically unimportant. ALDH2, mitochondrial aldehyde dehydrogenase; CYP450, cytochrome P450 enzymes; ECs, endothelial cells; eNOS, endothelial NOS; NTG, nitroglycerin; PETN, pentaerythritol tetranitrate; POR, cytochrome P450 reductase; sGC, soluble GC; VSMCs, vascular smooth muscle cells

intermediate, or the vascular CYP expression is so low, that the activity of the enzyme is physiologically unimportant. Whereas the first alternative is rather unlikely and not supported by the current literature, the present study suggests that indeed the vascular CYP expression is insufficient: Deletion of POR or inhibition of CYP had no effect on vascular nitrite production whereas CYP inhibition blocked nitrite production from microsomes. Thus, our data suggest that the CYP/POR axis can activate organic nitrates, but that in the vascular context, this system is insufficiently expressed (Figure S7). Such a concept supports the current paradigms in the field: ALDH2 and CYP are established as the two pathways in the biotransformation of NTG and PETN depending on the concentration applied (Divakaran & Loscalzo, 2017; Munzel et al., 2005). ALDH2 was identified as the main enzyme transforming NTG at clinical concentrations (Chen et al., 2002), and our results are in line with these conclusions (Daiber et al., 2004; Wenzel et al., 2007).

In conclusion, the present study supports the conclusion that ALDH2 locally releases nitrite from NTG and PETN in vessels, whereas the POR/CYP system does not importantly contribute to local bioactivation of organic nitrates.

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AUTHOR CONTRIBUTIONS

M.L. performed experiments, analysed data, and wrote the manuscript; P.F.M. performed experiments and analysed data; A.G.D. performed experiments and analysed data; X.D. generated and provided the CPR^{flox} mice; A.D. provided drugs and conceptual ideas; J.O.L. provided drugs and conceptual ideas; S.O. generated and provided the SMMHC-CreERT2 mouse; R.P.B. provided conceptual ideas and funding and wrote the manuscript; F.R. provided conceptual ideas and funding and wrote the manuscript. All authors read, revised, and approved the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no relevant financial, personal, or professional relationships to disclose which could be perceived as a conflict of interest or as potentially influencing or biasing the authors' work.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers, and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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SUPPORTING INFORMATION

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