Pentaerythritol Tetranitrate Targeting Myocardial Reactive Oxygen Species Production Improves Left Ventricular Remodeling and Function in Rats With Ischemic Heart Failure

Daniela Fraccarollo,* Paolo Galuppo,* Jonas Neuser,* Johann Bauersachs, Julian D. Widder

Abstract—Reduced nitric oxide bioavailability contributes to progression of cardiac dysfunction and remodeling in ischemic heart failure. Clinical use of organic nitrates as nitric oxide donors is limited by development of nitrate tolerance and reactive oxygen species formation. We investigated the effects of long-term therapy with pentaerythritol tetranitrate (PETN), an organic nitrate devoid of tolerance, in rats with congestive heart failure after extensive myocardial infarction. Seven days after coronary artery ligation, rats were randomly allocated to treatment with PETN (80 mg/kg BID) or placebo for 9 weeks. Long-term PETN therapy prevented the progressive left ventricular dilatation and improved left ventricular contractile function and relaxation in rats with congestive heart failure. Mitochondrial superoxide anion production was markedly increased in the failing left ventricular myocardium and nearly normalized by PETN treatment. Gene set enrichment analysis revealed that PETN beneficially modulated the dysregulation of mitochondrial genes involved in energy metabolism, paralleled by prevention of uncoupling protein-3, thioredoxin-2, and superoxide dismutase-2 downregulation. Moreover, PETN provided a remarkable protective effect against reactive fibrosis in chronically failing hearts. Mechanistically, induction of heme oxygenase-1 by PETN prevented mitochondrial superoxide generation, NOX4 upregulation, and ensuing formation of extracellular matrix proteins in fibroblasts from failing hearts. In summary, PETN targeting reactive oxygen species generation prevented the changes of mitochondrial antioxidant enzymes and progressive fibrotic remodeling, leading to amelioration of cardiac functional performance. Therefore, PETN might be a promising therapeutic option in the treatment of ischemic heart diseases involving oxidative stress and impairment in nitric oxide bioactivity. (Hypertension. 2015;66:00-00. DOI: 10.1161/HYPERTENSIONAHA.115.05931.)

Key Words: fibrosis ■ heart failure ■ pentaerythritol tetranitrate ■ rats ■ reactive oxygen species

Nitric oxide (NO) is one of the most important cardiovascular signaling molecules regulating vascular and myocardial function. NO, formed by NO synthases (NOS), modulates numerous physiological functions via soluble guanylate cyclase activation and subsequent increase of the second messenger cGMP.1,2 NO is rapidly oxidized by superoxide anions (O2−) to peroxynitrite. In ischemic heart disease and heart failure, endothelial dysfunction and vascular oxidative stress.12–15 Impaired NO bioavailability plays a crucial role in the pathophysiology of postinfarction ventricular remodeling triggering hypertrophy, chamber dilatation, and interstitial fibrosis.1,3,6 Various cardioprotective therapies act in part through an increase in NO formation. Organic nitrates, liberating NO or a related molecule, are used in the treatments of angina pectoris in ischemic heart diseases and in acute heart failure.7 The combination of isosorbide dinitrate and hydralazine was shown to improve survival among black patients having advanced heart failure already taking neurohormonal antagonists.8 Likewise, transdermal nitroglycerin application was shown to improve systolic function and exercise tolerance and to reduce left ventricular (LV) size.9 The potential of most organic nitrates is limited by the development of tolerance and induction of reactive oxygen species (ROS) leading to reduced NO bioavailability and hence endothelial dysfunction.10 Pentaerythritol tetranitrate (PETN) is an organic nitrate devoid of nitrate tolerance and oxidative stress because of induction of antioxidant pathways.10,11 PETN has been shown to protect against endothelial dysfunction and vascular oxidative stress.12–15

Potential effects of PETN treatment in congestive heart failure (CHF) have not been studied yet. Therefore, we investigated the effects of long-term treatment with PETN on progressive cardiac remodeling (dilation and cellular and molecular dysregulations) in CHF rats after extensive myocardial infarction (MI).

Methods

All procedures were approved by the institutional animal research committee and conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.
MI and Study Design

Left coronary artery ligations (MI) were performed in adult male Wistar rats (age, 8–12 weeks; weighing 200–250 g from Harlan Winkelmann, Germany). 6, 7 Starting on the seventh postoperative day, sham-operated animals received placebo treatment (n=12), and MI rats were randomly allocated to placebo (n=18) or PETN (80 mg/kg; n=14) treatment, administered twice daily by gavage. Two rats died in the placebo group and 1 in the PETN group during the 9 weeks of treatment. Hemodynamic and volume measurements were performed 10 weeks after left coronary ligation, when animals are in a chronic stable phase of heart failure. Only rats with CHF (LV filling pressure, >15 mm Hg) were included in the study (placebo, n=12; PETN, n=8).

Infarct Size, Capillary Density, and Cardiomyocyte Hypertrophy

The heart was arrested in diastole by potassium chloride. The right ventricle and LV, including septum, were separated in ice-cold saline and weighed. Infarct size was quantified histologically by planimetry. The LV was cut into 3 transverse sections: apex, middle ring (∼3 mm), and base. From the middle ring, 5-µm sections were cut at 100-µm intervals and stained with picrosirius red. Infarct size (fraction of the infarcted LV) was calculated as the average of all slices and expressed as a percentage of length. For analysis of capillary density, LV sections were stained using primary antibodies against CD31 (550300; BD Biosciences Pharmingen) followed by incubation with biotinylated antiamouse antibody, rat adsorbed (BA-2001, Vector Laboratories) and fluorescein avidin DCS (A-2011, Vector Laboratories). For cardiomyocyte cross-sectional area, LV frozen 5-µm sections were incubated with Alexa Fluor594 wheat germ agglutinin and Hoechst 33342 for staining of the plasma membrane and nucleus (I34406, Invitrogen). For mean myocyte size, the cross-sectional area of at least 100 cells, in which the nucleus and a clear myocyte outline could be visualized, was averaged. The crosses were used to identify myocyte and cell outlines were measured using lasso tool in Adobe Photoshop. 6

An expanded Methods section is available in the online-only Data Supplement.

Statistical Analysis

Values are presented as mean±SEM. Comparisons between groups of animals or treatments were made by 1-way ANOVA followed by Student–Newman–Keuls post hoc test. Statistical analysis was performed with the software StatView version 5.0.1 (SAS Institute Corp.). Values of P<0.05 were considered statistically significant.

Results

Global Parameters and Hemodynamics

MI size was not different between PETN- and placebo-treated animals (Table). Rats with CHF displayed right ventricular hypertrophy and pulmonary fluid accumulation. LV systolic pressure was reduced in CHF rats regardless of treatment, and mean arterial pressure was lower in CHF-PETN rats (Figure 1; Table). Long-term therapy with the organic nitrate PETN prevented the progressive LV dilatation and improved LV contractile function and relaxation: LV end-systolic and end-diastolic volumes were significantly reduced, associated with significantly improved dP/dt max and LV ejection fraction in CHF-PETN rats compared with placebo-treated animals (Figure 1; Table). Moreover, LV pressure isovolumic decay, a load-independent index of LV relaxation, was prolonged in CHF-placebo rats and significantly shortened by PETN (Table). LV dP/dt min, divided by instantaneous pressure, a load-independent measure of contractile function, was reduced in CHF-placebo rats and improved by PETN (Figure 1; Table). Lower mean arterial pressure and increased forward output significantly decreased vascular resistance in PETN-treated rats compared with untreated rats with CHF (Table).

Capillary Density and Cardiomyocyte Hypertrophy

Several factors involved in the process of angiogenesis have been identified to use NO as a second messenger. 1 Hence, we observed, by immunohistological analysis, a significantly decreased capillary network represented by CD31-positive cells in CHF rats on placebo. Treatment with PETN normalized capillary density to levels found in sham-operated animals (Figure 2A). Attenuation of LV dilation by PETN

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Statistics was performed by 1-way ANOVA followed by Newman–Keuls post hoc test. Mean±SEM (n=8–12). τ indicates LV pressure isovolumic decay; BW, body weight; CHF, congestive heart failure; dP/dt max, maximal rate of pressure decline; LV, left ventricle; LVEDP, left ventricular end-diastolic pressure; MAP, mean arterial pressure; MI, myocardial infarction; PETN, pentaerythritol tetranitrate; PLA, placebo; RV, right ventricle; and TPR, total peripheral resistance. 6

*P<0.05 vs sham. †P<0.05 vs CHF-PLA.
treatment in CHF rats was also associated with significantly reduced cardiomyocyte size (Figure 2A).

**Mitochondrial Superoxide Production**

Mitochondrial oxidative stress is an important driver of progressive cardiac remodeling and molecular alterations. Therefore, we assessed LV mitochondrial superoxide production using a highly sensitive isocratic ion-pair high-pressure liquid chromatography-electrochemical method (Figure 2B). Mitochondria from failing LV myocardium displayed significantly increased \( \mathrm{O}_2^{\cdot-} \) production. Long-term treatment with PETN normalized the formation of mitochondrial \( \mathrm{O}_2^{\cdot-} \) nearly to the basal level found in sham-operated animals.

\( \mathrm{O}_2^{\cdot-} \) rapidly reacts with NO, resulting in the formation of peroxynitrite anion, a powerful cytotoxic oxidant. Enhanced immunoreactivity for nitrotyrosine, a specific nitration product of peroxynitrite, was detected in the surviving LV myocardium of CHF-placebo rats, localized mainly in the vasculature and cardiomyocytes, compared with sham-operated and PETN-CHF animals (Figure S1 in the online-only Data Supplement). There was no difference in myocardial endothelial NOS expression among the groups studied (data not shown).

**Mitochondrial Metabolism**

To uncover the molecular connection between improved cardiac remodeling and mitochondrial superoxide production, we performed gene expression profile in the failing heart using gene set enrichment analysis. Gene set enrichment analysis showed that the deregulation of genes involved in mitochondrial energy metabolism was beneficially modulated by long-term PETN treatment (Figure 3A–3D). Noteworthy, among genes of the top subset contributing to the most enrichment score (Figure 3A), uncoupling protein-3 (UCP3), thioredoxin-2 (Txn2), superoxide dismutase-2 (SOD2), and mitochondrial proteins involved in maintaining cellular redox balance were significantly upregulated in CHF-PETN versus CHF-placebo hearts (Figure 3D).

**LV Fibrosis**

NO is involved in the regulation of a wide array of physiological processes. We performed microarray analysis to explore long-term effects of the NO-donor PETN on gene expression patterns in the failing LV myocardium (Figure 4A and 4B). The expression of several genes associated with extracellular matrix remodeling was regulated by PETN. We confirmed significantly reduced expression of collagen, connective tissue growth factor, fibrillin-1, transforming growth factor-\( \beta \) (TGF-\( \beta \)), and peristatin by quantitative real-time reverse transcription polymerase chain reaction analysis (Figure 4C). Histological and immunohistochemical analysis showed prevention of fibronectin and collagen accumulation (Figure 5A) and a striking reduction in interstitial fibrosis in the remote LV myocardium in CHF rats by long-term treatment with PETN (Figure 5B). Monocytes/macrophages regulating the differentiation of extracellular matrix–producing (myo)fibroblasts may be critically important in promoting fibrotic remodeling in the failing heart. Of note, immunohistochemical staining clearly showed more CD68\* monocytes/macrophages in areas remote from the ischemic injury in CHF-placebo versus CHF-PETN rats and sham-operated rats (Figure 5C).
Mechanism of Action of the NO-Donor PETN in Fibroblasts From Failing Heart

Our in vivo results suggest that mechanisms involving mitochondrial oxidative stress and extracellular matrix remodeling likely contributed to PETN-mediated protective effects against adverse cardiac remodeling and dysfunction. Therefore, to identify cell-specific mechanisms, we isolated cardiac fibroblasts, master producers of extracellular matrix proteins, from mice with heart failure. We investigated the effects of PETN on mitochondrial $\text{O}_2^-\text{•}^{-}$ production in response to TGF-$\beta_1$, playing a pivotal role in fibrotic remodeling of the noninfarcted myocardium. Stimulation with TGF-$\beta_1$ for 3 hours substantially increased mitochondrial $\text{O}_2^-\text{•}^{-}$ (measured by high-pressure liquid chromatography-electrochemical analysis of Mito-2-hydroxy-E+) in fibroblasts from failing myocardium (Figure 6A), which was almost prevented by preincubation with PETN for 2 hours. As shown in Figure 6B, in failing fibroblasts, PETN caused a significant increase in heme oxygenase-1 (HO-1) protein expression, which was further enhanced during exposure to TGF-$\beta_1$. Of note, coincubation with Sn-protoporphyrin IX, an inhibitor of heme oxygenase activity, completely reversed the protective effect against TGF-$\beta_1$-induced oxidative stress (Figure 6A).

The results suggest a major role of the heme oxygenase pathway in PETN-mediated prevention of early mitochondrial $\text{O}_2^-\text{•}^{-}$ generation.

We also tested the hypothesis that PETN may act as an antioxidant. Treatment of cardiac fibroblasts with PETN after stimulation with TGF-$\beta_1$ did not abolish the generation of Mito-2-hydroxy-E$^+$ (Figure S2A). In addition, direct superoxide scavenging activity of PETN was examined in a cell-free system using xanthine/xanthine oxidase as $\text{O}_2^-\text{•}^{-}$-generating system (Figure S2B). PETN did not abolish the $\text{O}_2^-\text{•}^{-}$ generation detected by lucigenin-enhanced chemiluminescence, indicating that the NO-donor PETN had no free radical scavenging and antioxidant activities.

Recent data showed that TGF-$\beta_1$–induced mitochondrial oxidative stress is essential for NOX4 expression. On the other hand, NOX4-derived superoxide production is critical for TGF-$\beta_1$–driven phenotypic conversion of fibroblasts to extracellular matrix–producing myofibroblasts. Accordingly, we studied whether PETN may modulate NOX protein expression and prevent the production of extracellular matrix proteins. In fibroblasts from failing myocardium, PETN prevented the induction of NOX4 in response to TGF-$\beta_1$ stimulation (Figure 6C) and significantly inhibited the expression of connective tissue...
growth factor and collagen. These effects were attenuated by Sn-protoporphyrin IX (Figure 6C). NOX4 protein expression significantly correlated with connective tissue growth factor (r=0.7, P<0.0001) and collagen (r=0.9, P<0.0001) expression, suggesting a mechanistic interdependence. Overall, HO-1 induction by PETN prevents mitochondrial superoxide generation and NOX4 upregulation, beneficially modulating extracellular matrix protein production.

**Aortic Mitochondrial Superoxide Production, HO-1, SOD2, and Endothelial NOS Expression**

Increased vascular oxidative stress is a key driver of heart failure progression and pathogenesis. Hence, we examined vascular mitochondrial superoxide production determining MitoSOX Red oxidation products by ion-pair high-pressure liquid chromatography-electrochemical method. As shown in Figure S3A, long-term PETN treatment prevented the increase in mitochondrial superoxide anion production in rats with CHF. In parallel to the beneficial effects on oxidative stress, PETN treatment enhanced aortic HO-1, SOD2, and endothelial NOS protein expression (Figure S3B).

**Discussion**

Long-term treatment with PETN, an organic nitrate devoid of tolerance and superoxide anion induction, prevented progressive LV remodeling, improved contractile dysfunction, and provided striking protection against reactive fibrosis in CHF.

Reduced NO bioavailability is a key feature of CHF, contributing to several pathological processes, including differentiation of extracellular matrix–producing fibroblasts and adverse fibrotic remodeling. Excessive accumulation of extracellular matrix components, mainly collagen, in the remote noninfarcted myocardium increases cardiac stiffness leading to abnormal relaxation and progressive dysfunction. In this study, the NO-donor PETN preserving extracellular matrix homeostasis likely restrained maladaptive remodeling. Contractile function and relaxation were improved associated with a significant reduction in LV chamber dilatation.

Although the cascade of molecular events/mechanisms underlying the beneficial effects of PETN cannot be easily elucidated in vivo in the experimental model of CHF, reduced myocardial superoxide radical formation seems to be a primary mechanism by which the NO donor conferred significant
protection against posts ischemic progressive cardiac dysfunction and pathological remodeling. In the setting of heart failure, ROS are increased. Thereby, NO is rapidly oxidized by superoxide anions to peroxynitrite, contributing to nitrosative stress ultimately leading to myocardial and vascular dysfunction.1,25,26 Common organic nitrates release NO, but a pharmacological approach solely built on the concept of releasing NO bares the risk of being ineffective. Furthermore, development of nitrate tolerance and the induction of superoxide anion production limits their long-term use in heart failure.10,27 PETN, in contrast to other organic nitrates, is not only free of superoxide anion induction but also actively lowers ROS production in vascular tissue.11,28,29 We now demonstrate that long-term treatment with PETN reduced superoxide radical formation in mitochondria in the failing heart, which could have beneficially affected not only fibrotic remodeling but also the dysregulation of mitochondrial genes involved in energy metabolism and antioxidant defenses, providing stress-specific cardioprotection.

Gene set enrichment analysis, able to individuate although small but cumulatively significant changes on sets of functionally related genes, showed that the deregulation of UCP3, SOD2, and Txn2, mitochondrial antioxidant enzymes, and critical regulators of cellular redox balance, was beneficially modulated by long-term PETN treatment in the failing myocardium. Inappropriate production of oxygen-free radicals in mitochondria, overwhelming the antioxidant defense systems, and the resulting oxidative damage to mitochondrial DNA and mitochondrial proteins have long been recognized as playing a causative role in the development and progression of cardiac remodeling and dysfunction.16–18 However, we cannot exclude that regulation of nicotinamide adenine dinucleotide phosphate oxidase–generated superoxide, as a nonmitochondrial

Figure 4. Long-term treatment with pentaerythritol tetranitrate (PETN) regulated the expression of genes associated with extracellular matrix remodeling in the failing heart. A, Heatmap of significantly downregulated, hierarchical clustered genes with fold change (FC) >1.5; \( P<0.05 \) \((n=3)\). B, MA plot of the contrast PETN vs placebo (PLA) treatment (M, log ratio; A, average); significantly upregulated (red) downregulated (green) genes (FC>1.3; \( P<0.05 \)). C, Quantitative real-time reverse transcription polymerase chain reaction analysis of actinin 1 (Actn1), collagen type I-\( \alpha1/2 \) (Col1\( \alpha1/2 \)), collagen type III-\( \alpha1 \) (Col3\( \alpha1 \)), connective tissue growth factor (CTGF), fibrillin-1 (Fbn1), transforming growth factor-\( \beta3 \) (TGF-\( \beta3 \)), and periostin (Postn). Statistical analysis was performed by 1-way ANOVA followed by Newman–Keuls post hoc test. Mean±SEM \((n=5–6)\); \*\( P<0.05 \) vs Sham; †\( P<0.05 \) vs CHF-PLA.
source of ROS, may also be involved in the cardioprotection of PETN against maladaptive remodeling.

Several studies reported reduced UCP3 expression in the failing heart and an inverse correlation between mitochondrial ROS formation and UCP3 expression. Altered mitochondrial redox balance may decrease the expression of UCP3, whereas mechanical unloading restored UCP3 in the failing human heart. Genetic deletion of UCP3 promoted mitochondrial dysfunction and ROS production, leading to adverse cardiac remodeling after MI. Likewise, conditional SOD2 gene deficiency led to hypertrophic and fibrotic remodeling and impaired contractile function. Recent data support an essential role for the mitochondrial antioxidant enzyme Txn2 in preserving cardiac function by suppressing mitochondrial ROS production. Mice with cardiac-specific deletion of Txn2 develop progressive dilated cardiomyopathy with impairment of contractile function, heart chamber dilation, and marked interstitial fibrosis. This extends our previous observations of a major role for Txn2 and ROS production in angiotensin II-induced myocardial hypertrophy and cardiovascular alterations. Interestingly, evidence that therapeutic targeting of impaired NO signaling by soluble guanylyl cyclase activation or angiotensin-converting enzyme inhibition restores mitochondrial expression of UCP3 and SOD2 in the failing heart after MI implies a critical role for NO in the regulation of the mitochondrial ROS detoxification system in chronic heart disease. In an elegant study, Wu et al recently showed that maternal treatment of spontaneously hypertensive rats with PETN reduces blood pressure in female offspring, linked to epigenetic changes and transcriptional activation, in turn inducing antioxidant enzymes. Beneficial modulation of cardioprotective/cardiotoxic genes by PETN treatment has also been reported in the myocardium of healthy rats. Therefore, we cannot exclude cardioprotective effects of PETN therapy in rats without coronary ligation.

Noteworthy are our findings concerning the link between induction of HO-1 by PETN and the inhibition of TGF-β1–induced profibrotic responses in fibroblasts from failing myocardium. PETN protects against endothelial dysfunction and vascular oxidative stress, largely via HO-1–dependent mechanism. In this study, we provided novel evidence that upregulation of the antioxidant enzyme HO-1 contributed to PETN-mediated cytoprotective effect by inhibiting mitochondrial ROS generation and NOX4 expression. Previous studies have reported that NOX4-dependent \( \mathrm{O}_2^- \) production by TGF-β1 is required for fibroblast differentiation into a profibrotic
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myofibroblast phenotype. More recent evidence emphasizes that ROS generated from mitochondrial electron transport chain complex III are required for TGF-β-mediated transcription of NOX4 and establish that the initial activation of NOX4 amplifies and sustains TGF-β1–induced oxidative stress. Therefore, prevention of early mitochondrial O2•− formation and ensuing NOX4 upregulation might be a key mechanism involved in the antifibrotic effect of PETN. The pharmacological effects of PETN, including NO release and induction of HO-1, could provide synergistic protection against cardiovascular diseases associated with oxidative stress and progressive fibrotic remodeling.

Hypertrophic remodeling after MI is characterized by an insufficient adaptation of the capillary network to the cardiomyocyte hypertrophy. Remarkably, long-term PETN treatment preserved capillary density in the failing heart. NO holds a crucial role in endothelial progenitor cell mobilization, and we previously demonstrated that PETN therapy increased circulating levels of endothelial progenitor cells and improved their functionality by diminishing intracellular ROS levels. NO has also been shown to mediate proliferation and migration contributing to an increase in angiogenesis. Proangiogenic properties of PETN might therefore be mediated by increased NO bioavailability.

Coronary artery ligation induces a wide spectrum of cardiac dysfunction, ranging from mild impairment to overt CHF, depending on MI size. We included in our study only rats with CHF, defined by LVEDP of >15 mm Hg. Therefore, additional favorable effects of PETN on prevention of heart failure could have been missed/overlooked. Of note, long-term PETN therapy provided cardioprotection even in rats with extensive MI and CHF, where the effects of current therapeutic strategies, such as angiotensin-converting enzyme inhibition, are minor.

In conclusion, long-term PETN treatment targeting superoxide generation and NO bioavailability most likely prevented the changes of mitochondrial scavenging pathways and progressive fibrotic remodeling, leading to improved cardiac functional performance in CHF. Given our promising results, PETN therapy might be a novel therapeutic approach in ischemic heart disease.

Perspectives

PETN, an organic nitrate devoid of nitrate tolerance and induction oxidative stress, is currently used in the treatment of angina pectoris. We investigated the effects of long-term treatment with PETN in CHF. The NO donor provided protection against postischemic progressive cardiac dysfunction and pathological remodeling. Prevention of mitochondrial superoxide generation and induction of HO-1 contributed to
PETN-mediated cardioprotective effects. Therefore, PETN seems to be a promising therapeutic option in the treatment of chronic heart failure. However, clinical studies are needed to widen clinical evidence.

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Disclosures
Dr Bauersachs received honoraria and research support from Actavis. The other authors report no conflicts.

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26. Loch T, Vakhрушева О, Piotrowska I, Ziółkowski W, Ebelt H, Braun T, Bober E. Different extent of cardiac malformation and resistance to


**Novelty and Significance**

**What Is New?**
Our data demonstrate that long-term treatment with pentaerythritol tetranitrate, an organic nitrate devoid of tolerance, improves left ventricular remodeling and function in rats with congestive heart failure most likely by reduction of mitochondrial reactive oxygen species formation.

**What Is Relevant?**
The use of most organic nitrates in heart failure is limited by development of nitrate tolerance and stimulation of reactive oxygen species formation. Our data suggest that pentaerythritol tetranitrate, a drug with known safety profile as used in Europe over decades for patients with stable angina, might be a promising therapeutic option in the treatment of heart failure.

**Summary**
Pentaerythritol tetranitrate improves cardiac remodeling and function in rats with heart failure by targeting mitochondrial reactive oxygen species formation.
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SUPPLEMENTAL MATERIAL

Pentaerythritol Tetranitrate Targeting Myocardial ROS-Production Improves Left Ventricular Remodeling and Function in Rats With Ischemic Heart Failure

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Fraccarollo et al.: PETN targeting ROS improves LV function

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D.F., P.G. and J.N. contributed equally to this work.
Hemodynamic and Volume Measurements

Left ventricular systolic (LVSP) and end-diastolic pressures (LVEDP), mean arterial pressure (MAP) and dP/dt were measured under under light isoflurane anesthesia and spontaneous respiration. Saline-filled catheters (PE50) were advanced from the right carotid artery into the left ventricle and connected via a three-way stopcock to a Millar micromanometer and Statham transducer. The time constant of LV pressure isovolumic decay [\( \tau \), regression of log(pressure) versus time] was calculated by Weiss method. In vivo LV pressure-volume relationship was analyzed using conductance catheter (SPR-774, Millar Instruments). The 1.4 Fr catheter was advanced from the right carotid artery into the left ventricle through the PE 50 saline-filled catheter after hemodynamic measurements. Pressure-volume signals were acquired by BioBench software (National Instruments). Pvan software (Millar Instruments) was used to analyze all pressure-volume loop data recorded at steady state and during injection of hypertonic saline for the calibration of parallel conductance volume (\( V_p \)). LV volume was calculated for each rat from the conductance volume corrected by the relative \( V_p \).\(^{1,2}\) LV ejection fraction was calculated with the formula: \( \text{EF} \% = \frac{(EDV - ESV)}{EDV} \times 100 \). Total peripheral resistance was calculated as the ratio of the difference between mean systemic and right atrial pressures and cardiac output. Pulmonary edema was assessed as net fluid weight (difference between the wet and dry weights).\(^{2,3}\)

Left ventricular fibrosis

Paraffin sections (5µm) of the interventricular septum were stained with 0.1% sirius red F3B in saturated picric acid. Sections were examined using a Nikon ECLIPSE 50i microscope equipped with filters to provide circularly polarized illumination. Tissue images were recorded with a cooled digital camera (DS-5Mc, Nikon) with a magnification 200X, and analyzed using SigmaScan Pro 5.0 image analysis software (Systat Software Inc.). Collagen content was expressed as a percentage of the area of each image.\(^{2,3}\)

Immunohistochemical analysis

LV paraffin 5µm sections were stained using primary antibody against collagen (ab34710, Abcam), fibronectin (610077, BD Biosciences), CD68 (MCA341, AbD Serotec) and nitrotyrosine (Ab7048, Abcam). Staining was performed using VECTASTAIN® Elite AB kit (PK-6100, Vector Laboratories) and DAB (550880, BD Biosciences).\(^{3,4}\)

Gene expression

Total RNA from LV samples (surviving or sham-operated LV myocardium) was extracted using TRIzol (Invitrogen,) and purified using the RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturers’ instructions. RNA quality was assessed with Bioanalyzer 2100 (Agilent). RNA samples were converted to biotinylated cRNA and hybridized to Affymetrix GeneChip Rat Expression Array 230 2.0 according to the manufacturer’s directions. Microarray data analysis was performed using R packages from the Bioconductor project (www.bioconductor.org) and the computational method Gene Set Enrichment Analysis (GSEA)\(^{3}\) version 2.2.0 and MSigDB collection C2 (curated gene set) version 5.0 (www.broadinstitute.org/gsea/). GSEA was used to detect coordinate expression in the three main groups (Sham, Placebo, PETN) and was run according to default parameters: probes for the same gene were collapsed into a single gene symbol, permutation number and type were set, 1000, to gene set respectively. By convention gene sets with p <0.05 and FDR < 25% were considered statistically significant.
Real-Time-quantitative-PCR
Reverse transcriptase of 1µg total RNA was performed using iScript™ Select cDNA Synthesis Kit with Oligo(dT)20 primers (Bio-Rad). Gene expression, normalized to glyceraldehyde-3-phosphat-dehydrogenase (GAPDH), was determined by customized Bio-Rad PrimePCR Assays™ following the manufacturer's instructions and the 2^{ΔΔCt} method.

Isolation of fibroblast
Fibroblasts were isolated from hearts of mice with heart failure 10 days after coronary ligation, using the Langendorff perfusion method.6 Isolation were performed according to AfCS Procedure Protocol PP00000125 (http://afcs.lbl.gov/reports/v1/CM0005/CM0005.htm). The non-cardiomyocytes cell suspension (obtained after centrifugation of the single cell suspension at 30g for 4 minutes) was centrifuged at 300g for 5 minutes. The pelleted cells were suspended in DMEM (Lonza), plating for 90 minutes in 35-mm tissue culture dishes, washed 3x with culture medium and then cultured in DMEM supplemented with 10% FBS. After 24 hours cells were passaged using Accutase (Sigma-Aldrich). Using this method, we routinely obtained cultures that were almost exclusively fibroblasts by the first passage.6 Fibroblasts were placed in serum-free media for 12 hours before experiments. Passage 1 was used in all experiments. Pentærythritol tetranitrate and Sn-protoporphyrin IX (Sn749-9, Frontier Scientific) were added 2 hours before TGF-β1 (7666-MB, R&D System) stimulation.

Western Blot analysis
For Western Blot analysis fibroblasts and aortic rings were lysed/homogenized in ice-cold RIPA buffer (9806, Cell Signaling Technology). Equal amounts of protein were mixed with sample loading buffer and separated on SDS-polyacrylamide gel. Proteins were electrophoresed on PVDF membranes (Immun-Blot 0.2 µm, Bio-Rad). The bands were detected using a chemiluminescence assay (ECL Plus, ECL, Amersham). Primary antibodies used recognize heme oxygenase-1 (ab13243, Abcam), Nox4 (NB110-58849, Novus Biologicals), collagen (Ab116223, Abcam), CTGF (sc-14939, Santa Cruz Biotechnology), eNOS (610296, BD Biosciences) and SOD2 (611580, BD Biosciences). GAPDH (Ab8245, Abcam) and α-tubulin (2144, Cell Signaling Technology) were used as internal loading control.

Determination of mitochondrial superoxide formation
Mitochondria were isolated from LV tissue (surviving or sham-operated LV myocardium) using the Oprime Mitochondria Isolation Kit (QIAGEN). Isolated mitochondria were incubated in Krebs-HEPES buffer containing 2µmol/L of the mitochondria targeted hydroethidine Mito-HE (MitoSOX™ Red, Invitrogen) at 37°C for 30 minutes, followed by centrifugation at 6000g for 10 minutes. Subsequently, mitochondria were washed with Krebs HEPES buffer, homogenized in cold methanol and centrifuged at 12000g.3,6 Cardiac fibroblasts were exposed to Mito-HE (1µmol/L) at 37°C for 20 minutes. Subsequently, cells were washed, harvested in cold methanol and centrifuged at 12000g. The descending thoracic aorta was dissected after removal of the heart and cleaned of connective tissue. Aortic rings (~10mm) were exposed to Mito-HE (5µmol/L) at 37°C for 30 minutes, washed, homogenized in cold methanol and centrifuged at 14000g. Mito-HE oxidation products were assessed using a highly sensitive HPLC-electrochemical (EC) method. Our isocratic HPLC-EC method, incorporating an ion-pair reagent in the mobile phase, allowed more distinct separation of Mito-HE, mito-2-hydroxy-E⁺ and mito-E⁺. Moreover, an internal standard (IS, 3,4 Dihydroxycinnamic acid) was used to correct the errors due to extraction of Mito-HE oxidation products from mitochondria/cells/aorta and variations of
injection volume and of HPLC-EC system. Data acquisition and analysis were performed using the Chromeleon®7 Software (Dionex). Results were normalized for protein concentration. Direct superoxide scavenging activity of PETN was examined in a cell-free system using xanthine/xanthine oxidase as O$_2$•$^-$ generating system. Lucigenin (5µM) in Krebs-HEPES buffer (50mM) was incubated with xanthine oxidase (5mU/ml) and xanthine (0.5mM).

References


Figure S1 Prevention of nitrotyrosine formation in the failing myocardium by long-term treatment with pentaerythritol tetranitrate (PETN). Positive immunohistochemical staining for nitrotyrosine (brown) was observed in placebo CHF hearts, localized mainly in the vasculature and cardiomyocytes. Nitrotyrosine immunoreactivity was relatively weaker in the LV myocardium of PETN-CHF rats and almost undetectable in sham-operated hearts.
**Figure S2** A. Treatment of cardiac fibroblasts with PETN after stimulation with TGF-β₁ (10ng/mL) for 3 hours did not abolish the generation of Mito-2-hydroxy-E⁺. B. Superoxide anion production detected by lucigenin enhanced chemiluminescence generated in a cell-free xanthine/xanthine oxidase system (X/XO). PETN (15μmol/L); SOD, Polyethylene glycol-conjugated superoxide dismutase (135U/ml); Tiron (30μmol/L).

Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls post hoc test. Mean ± SEM (n=4-8). *p<0.05 vs. unstimulated.
Figure S3 Long-term treatment with pentaerythritol tetranitrate (PETN) prevented the increase in vascular mitochondrial superoxide anion production and enhanced HO-1, eNOS and SOD2 protein expression. A, Mitochondrial superoxide anion formation measured by HPLC-EC analysis of Mito-2-hydroxy-E\(^{\bullet}\), and (B), HO-1, eNOS and SOD2 protein expression in aortic rings. Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls post hoc test. Mean ± SEM (n=6-10). *p<0.05 vs. Sham; †p<0.05 vs. CHF-PLA.