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Effects of nonintermittent treatment of rabbits with pentaerythritol tetranitrate on vascular reactivity and superoxide production

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Abstract

Pentaerythritol tetranitrate is an organic nitrate ester that undergoes metabolization to pentaerythritol, pentaerythritol trinitrate, pentaerythritol dinitrate and pentaerythritol mononitrate. Recent data suggested that pentaerythritol tetranitrate is endowed with vasoprotective activities in experimental atherosclerosis. This study was undertaken to gain insight into the underlying mechanism. The basic mechanism of action of all pentaerythritol nitrates was evaluated by measuring liberation of nitric oxide (NO), stimulation of human soluble guanylate cyclase and vasorelaxation in rabbit aorta. A subsequent in vivo study in New Zealand White rabbits was performed to investigate the effects of a 4 months lasting nonintermittent oral treatment with 6 mg pentaerythritol tetranitrate kg^{-1} day^{-1} on vascular superoxide production, endothelium dependent vasorelaxation and vasorelaxation to pentaerythritol tetranitrate itself. The formation rates of NO from the pentaerythritol nitrates (100 μM, $n = 5$) in presence of 5 mM cystein were (in nM min^{-1}): 62.1 ± 3.2 (pentaerythritol tetranitrate), 21.3 ± 0.9 (pentaerythritol trinitrate), 6.4 ± 0.6 (pentaerythritol dinitrate) and 3.2 ± 0.4 (pentaerythritol mononitrate). Similarly, the pD_2 values ($-\log M$) for half-maximal activation of soluble guanylate cyclase decreased from pentaerythritol tetranitrate (3.391 ± 0.09, $n = 4$) to pentaerythritol mononitrate (2.655 ± 0.04, $n = 3$) as did the pD_2 values (in $-\log M$) for half-maximal relaxation of rabbit aortic rings ($n = 7$) from pentaerythritol tetranitrate (8.3 ± 0.17) to pentaerythritol mononitrate (5.0 ± 0.11). Significant correlations were found between the NO formation rates and the pD_2 values for enzyme stimulation ($r = 0.98$, $P = 0.002$) and vasorelaxation ($r = 0.90$, $P = 0.049$) suggesting that these effects of the pentaerythritol nitrates were mediated by NO. The results of the in vivo study showed that aging induces a significant increase of aortic superoxide production (median values, $n = 10$) from 2.45 nM mg^{-1} min^{-1} (age 7 months) to 3.39 nM mg^{-1} min^{-1} (age 11 months, $P < 0.01$) that was prevented by concurrent treatment with pentaerythritol tetranitrate (2.76 nM mg^{-1} min^{-1}). In vitro vasorelaxation to pentaerythritol tetranitrate was identical in all groups indicating absence of nitrate tolerance. Endothelium-dependent vasorelaxation was also identical in all groups. These data suggest that oral treatment with pentaerythritol tetranitrate reduces vascular oxidant stress by an NO-dependent pathway, which may contribute to the vasoprotective activity of pentaerythritol tetranitrate in experimental atherosclerosis.

Keywords: Superoxide; Nitric oxide (NO); Atherosclerosis; Organic nitrate; Pentaerythritol tetranitrate; Guanylate cyclase, soluble

1. Introduction

Pentaerythritol tetranitrate is an organic nitrate sharing structural similarities with other typical organic nitrates, such as glyceryl trinitrate, isosorbide-5-nitrate or isosorbide-2,5-dinitrate (Ahlner et al., 1991). In the past, pentaerythritol tetranitrate was rarely used in Western Germany but was the main nitrovasodilator for treatment of angina and heart failure in former Eastern Germany. Although it is generally assumed that the mechanism of action of pentaerythritol tetranitrate is identical to that of other organic nitrates, there is only little information available about the basic pharmacological properties of this drug (Ahlner et al., 1991). In particular, no experimental evidence was provided to demonstrate liberation of nitric oxide (NO) and stimulation of soluble guanylate cyclase, which is the common mechanism of action of nitrovasodilators (Feelisch and Noack, 1987). In vivo, pentaerythritol tetranitrate is degraded to 4 metabolites: pentaerythritol trinitrate, pentaerythritol dinitrate, pentaerythritol mononitrate and pentaerythritol as indicated by detection of these metabolites in plasma of man after oral application (Davidson et al., 1970; Weber et al., 1995). The

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nitrated metabolites are most likely pharmacologically active intermediates (Parker et al., 1975) but the basic pharmacologic properties of these metabolites have never been investigated. Interestingly, pentaerythritol tetranitrate is believed to have only a low tendency to produce typical nitrate tolerance as indicated by animal experiments and clinical experience (Dück and Richard, 1990; Kojda et al., 1995; Fink et al., 1996).

The results of a recent study suggested that pentaerythritol tetranitrate is endowed with vasoprotective activities. Long-term treatment with pentaerythritol tetranitrate reduced the extent of intimal atherosclerotic lesions and prevented the development of impaired endothelium-dependent vasorelaxation (Kojda et al., 1995). In this study, we sought to determine some basic pharmacological properties of the pentaerythritol nitrates that are known to be similar among organic nitrates. These investigations were extended by an in vivo study to gain further insight in the mechanism of the vasoprotective activity of pentaerythritol tetranitrate. In detail, three groups of New Zealand White rabbits were treated with either lactose or a lactose/pentaerythritol tetranitrate trituration for 4 months and vascular superoxide production, endothelium-dependent vasorelaxation and vasorelaxation to a spontaneous NO donor and pentaerythritol tetranitrate itself was measured. Our results suggest that a reduction of vascular superoxide production contributes to the vasoprotective effects of pentaerythritol tetranitrate.

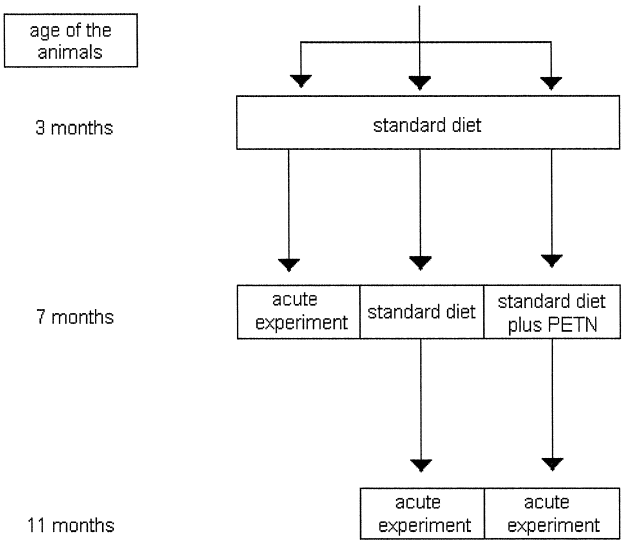

Fig. 1. Design of the in vivo study. Thirty New Zealand White Rabbits were randomly divided in three groups of 10 animals each. The control group was fed for 4 months and the aging group and the pentaerythritol tetranitrate (PETN) group were fed for another 4 months. Acute experiments at the end of the feeding periods included organ bath studies with isolated aortic rings and determination of vascular superoxide production.

2. Experimental procedures

2.1. Animal preparation of the in vivo study

For the in vivo study, we investigated a total of 30 New Zealand White rabbits. The mean body weight was 2252 ± 47.5 g, the age was 10–12 weeks. The animals were housed individually in stainless steel cages at a temperature of 18–20°C, a humidity of 50–60% and a day–night rhythm of 12 h and received water ad libitum. The study design is shown in Fig. 1. Rabbits were randomly divided in three groups of 10 animals each and fed 40 g (kg b.wt.)$^{-1}$ day^{-1} of a standard rabbit chow supplemented with either lactose or a pentaerythritol tetranitrate–lactose trituration, adjusting the daily pentaerythritol tetranitrate dose to 6 mg (kg b.wt.)$^{-1}$. The control group received the standard chow for 16 weeks. The aging group received the standard chow for 32 weeks. The pentaerythritol tetranitrate group received the standard chow for 16 weeks and the pentaerythritol tetranitrate chow for another 16 weeks. During this time the body weight of every animal was determined weekly and the concentration of plasma lipids (see below) was also monitored. After the feeding period the acute experiments were performed.

Permission for this study was provided by the regional government (No.: 23.05-230-3-54/95) and the experiments were performed according to the guidelines for the use of experimental animals as given by 'Deutsches Tierschutzgesetz' and to the 'Guide for the care and use of laboratory animals' of the US National Institutes of Health.

2.2. Vasorelaxation studies following the in vivo study

Aortas from the rabbits of the in vivo study were rapidly excised and immersed in cold oxygenated (95% O_2 + 5% CO_2) Krebs–Henseleit buffer (pH 7.4, composition see above). The aorta was divided and used for vasorelaxation studies and measurements of vascular superoxide production. Four ring segments (5-mm width) of thoracic aorta were mounted between stainless steel triangles in a water-jacketed organ bath (37°C) for measurement of tension development as recently described (Kojda et al., 1991). Previous experiments with KCl (10–60 mM) revealed an optimal resting tension of 2 g for development of contractile function in the vessels. After equilibration (1 h), contractile function of aortic segments was tested by application of KCl (60 mM). This was followed by a repeated application of phenylephrine (3 μM), which resulted in a maximal tension of approximately 130 mN. Function of the endothelium was then examined by cumulative addition of acetylcholine (0.001–10 μM) following precontraction with phenylephrine (0.1 μM). Thereafter the aortic rings were divided in subgroups and the vasorelaxations to different type of NO donors, such as *S*-nitroso-*N*-acetyl-DL-penicillamine (0.1 nM–10 μM) and pentaerythritol tetranitrate (0.1 nM–10 μM) were studied following precontraction with phenylephrine (0.2 μM).

Preliminary experiments revealed that the maximal achieved concentration of dimethylsulfoxide (0.01%), which was necessary to dissolve *S*-nitroso-*N*-acetyl-DL-penicillamine and pentaerythritol tetranitrate, exhibited no influence on aortic contractile function. In another set of preliminary experiments using rabbit aorta we confirmed that *S*-nitroso-*N*-acetyl-DL-penicillamine released NO largely extracellularly as the pD_2 value of the concentration-dependent relaxation caused by *S*-nitroso-*N*-acetyl-DL-penicillamine showed a 10-fold decrease in the presence of 1 μM oxyhemoglobin, a NO scavenger. In contrast, this concentration of oxyhemoglobin had no effect on vasorelaxations caused by the organic nitrate pentaerythritol tetranitrate.

2.3. Other vasorelaxation studies

In these studies, we used aortic ring segments from 3- to 4-month old normal New Zealand White rabbits. Overall the experimental procedure was similar to that described above. Equilibrated and precontracted ring segments were exposed to increasing concentrations of pentaerythritol tetranitrate, pentaerythritol trinitrate, pentaerythritol dinitrate and pentaerythritol mononitrate (0.1 nM–100 μM). The highest concentration of 100 μM was applied for pentaerythritol dinitrate and pentaerythritol mononitrate only.

2.4. Determination of NO release

NO release was quantified using the oxyhemoglobin assay as described previously (Feelisch and Noack, 1987; Noack et al., 1992). Briefly, 100 μM of pentaerythritol tetranitrate, pentaerythritol trinitrate, pentaerythritol dinitrate and pentaerythritol mononitrate were incubated in 50 mM phosphate buffer (pH 7.7) in the presence of cysteine (5 mM) and freshly prepared oxyhemoglobin (3–5 μM). The total volume of 3 ml was filled in a cuvette and NO-induced generation of methemoglobin was measured spectrophotometrically for 20 min. Control experiments were performed in the absence of the nitrate esters.

2.5. Quantification of vascular superoxide production

Generation of superoxide radicals by rabbit thoracic aorta was measured by use of the lucigenin assay described previously (Ohara et al., 1993; Allen, 1986). Briefly, aortic rings (5-mm width) were equilibrated in Krebs–Henseleit buffer (pH 7.4, 37°C) gassed with carbogen for 30 min and then transferred into vials (4°C) filled with precooled Krebs–Henseleit buffer and incubated for 60 min. The measurements of superoxide radicals were performed at 37°C in albumin buffer (pH 7.4) of the following composition (in mM): Na^+ 144.93, K^+ 7.23, Cl^- 138.77, $H_2PO_4^-$ 4.55, HPO_4^{2-} 8.03, glucose 5.55 and bovine serum albumine (0.1%, weight/volume). This buffer was enriched with lucigenin (0.5 mM). Aortic segments were transferred to vials containing this buffer, which were preheated to 37°C by placing them into the luminometer (Packard Luminometer Analyzer, Picolite A6112, Packard, Downers Grove, IL, USA) for a 40-min period. During the last 20 min of this period background radiation was measured. Preliminary experiments in both, rat and rabbit aorta showed that the superoxide production in nM mg^{-1} min^{-1} was fairly stable after the temperature of the vessels segments reached 37°C (after 2–3 min). Superoxide production in the vessel segment was estimated for 20 min and corrected for background radiation. The results shown were calculated from chemiluminescence measurements between 5 and 20 min.

The system was calibrated by the use of xanthin/xanthin oxidase to generate superoxide. Production of superoxide was quantified by reduction of cytochrome C. Horse heart cytochrome *C* (808 μM), xanthin (0.4 μM–3.2 μM) and buttermilk xanthin oxidase (grade III) were dissolved in albumin buffer and the increase in absorption was detected at λ_{max} (550 nM) for 10 min. Calibration of the luminometer was performed by adding xanthin oxidase (0.4 U ml^{-1}) to increasing concentrations of xanthin (0.1 μM–3.2 μM) dissolved in albumin buffer. A correlation between the concentration of superoxide and the chemiluminescence showed a coefficient of 0.968.

2.6. Determination of guanylate cyclase activity

Preparation of human platelet guanylate cyclase was performed as reported previously (Kojda and Noack, 1993). Briefly, 1000 ml human platelet rich plasma was mixed with 50 ml EDTA (0.1 M) and platelets were concentrated by centrifugation ($1000 \times g$ for 10 min). The platelets were washed twice with Tris buffer (50 mM, pH 7.6) containing 154 mM NaCl by repeated resuspension and centrifugation ($500 \times g$). Washed platelets were resuspended in the Tris buffer (16 ml) and slowly cooled to 4°C, which was the temperature for all following steps. Lysis of platelets was achieved by addition of 100 ml hypotonic Tris buffer (5 mM, pH 7.6) containing leupeptin (0.05%), phenylmethylsufonyl fluoride (0.2 mM) and dithiothreitol (1 mM). During lysis, platelets were sonicated (50 W, 30 s). The supernatant from the following centrifugation ($10\,000 \times g$ for 10 min) was collected and centrifuged again with $105\,000 \times g$ for 1 h. The obtained cytosolic fraction was loaded on a diethylaminoethanol (DEAE)–Sepharose column (HiLoad™ 26/10 Q Sepharose HP, Pharmacia, Freiburg, Germany) after preequilibration with Tris buffer containing 1 mM dithiothreitol. A linear sodium chloride (0–0.4 M) gradient in the same buffer was started. Active fractions (cGMP accumulation $> 30\%$ of the maximal value) were identified after stimulation with 500 μM *S*-nitroso-*N*-acetyl-DL-penicillamine in the presence of 1 mg ml^{-1} bovine serum albumin and were pooled

and stored in aliquots at −80°C. Protein content was measured by the method of Bradford (1976) using bovine serum albumin as a standard. The maximal *S*-nitroso-*N*-acetyl-DL-penicillamine-stimulated specific activity of the enzyme preparation used in the present investigation was 2.23 nM cGMP mg^{-1} min^{-1}.

Specific activity of soluble guanylate cyclase was measured by the formation of [^{32}P]cGMP from [α-^{32}P]GTP as described previously (Schultz and Böhme, 1984). Briefly, 20–40 μg of protein was incubated in a total volume of 100 μl of a triethanolamine–HCl buffer (50 mM, pH 7.4, 37°C) containing [α-^{32}P]GTP (5 nM, 0.4 μCi), GTP (100 μM), cGMP (1 mM), 3-isobutyl-1-methylxanthine (IBMX, 1 mM), $MgCl_2$ (1 mM), dithiothreitol (1 mM) and cysteine 5 mM in presence of increasing concentrations of pentaerythritol tetranitrate, pentaerythritol trinitrate, pentaerythritol dinitrate and pentaerythritol mononitrate (10 μM–10 mM) or vehicle (0.25% dimethylsulfoxide). None of the nitrates showed any activation of the enzyme in the absence of cystein.

2.7. Substances and solutions

S-Nitroso-*N*-acetyl-DL-penicillamine was synthesized in our laboratory according to Field et al. (1978) as described previously (Kojda et al., 1996). Pentaerythritol tetranitrate, pentaerythritol trinitrate, pentaerythritol dinitrate and pentaerythritol mononitrate were provided by ISIS-Pharma, Zwickau, Germany; all other chemicals were obtained from Merck, Darmstadt, Germany or from Sigma, Deisenhofen, Germany, in analytical grade.

The stock solutions of acetylcholine (10 mM), phenylephrine (10 mM), prostaglandine F_2 (10 mM), substance P (1 μM) and pentaerythritol mononitrate were prepared in distilled water. The stock solutions of *S*-nitroso-*N*-acetyl-DL-penicillamine, pentaerythritol tetranitrate, pentaerythritol trinitrate and pentaerythritol dinitrate (100 mmol l^{-1}) were prepared in dimethylsulfoxide. All stock solutions were prepared daily, diluted with buffer as required, kept on ice and protected from daylight until use. All concentrations indicated in the text and figures are expressed as final bath concentrations.

2.8. Statistics

The concentrations of the half-maximal vasorelaxant effect of the nitrates (pD_2 values) were calculated from the individual concentration–effect curves as proposed by Hafner et al. (1977). Vasorelaxation due to treatment with the compounds is expressed as percentage of the contractile response achieved with phenylephrine or prostaglandine $F_{2\alpha}$ at the beginning of the experiments.

All data were analysed by a standard computer programs (SAS PC Software 6.04, PROC Analysis of Variance, ANOVA) and are expressed as mean values and standard error of the mean (S.E.M.) or as median values.

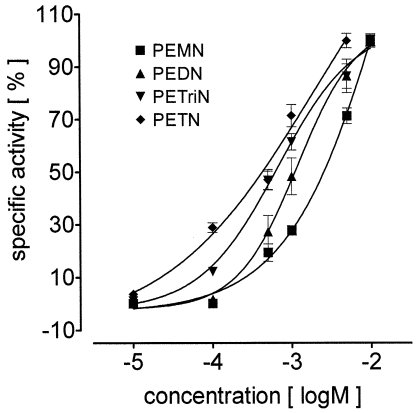

Fig. 2. Activation of human platelet soluble guanylate cyclase induced by the pentaerythritol nitrates in the presence of 5 mM cysteine. Plotted are the mean values (±S.E.M.) as related to the maximal activity achieved with the same compound (set to 100%). The greatest increase of specific activity to 542±82 pmol cGMP mg^{-1} min^{-1} was induced by 10 mM pentaerythritol tetranitrate (PETN = pentaerythritol tetranitrate, PETriN = pentaerythritol trinitrate, PEDN = pentaerythritol dinitrate, PEMN = pentaerythritol mononitrate).

Significant differences were evaluated using either unpaired two-tailed Student's *t*-test or unpaired two-tailed Mann–Whitney test (Graph Pad Prism®, also used to create the graphs) and a *P*-value below 0.05 was considered as significant.

3. Results

3.1. NO release and activation of soluble guanylate cyclase

All pentaerythritol nitrates concentration-dependently activated soluble guanylate cyclase (Fig. 2). Our results

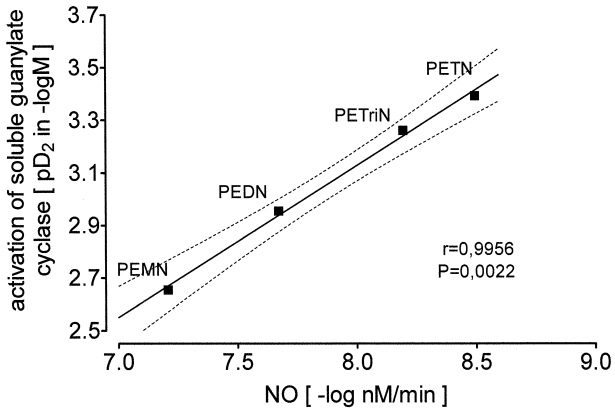

Fig. 3. Correlation between the concentration inducing half-maximal activation of human soluble guanylate cyclase (pD_2 value) and the cysteine-dependent release of NO from the pentaerythritol nitrates. The correlation coefficient (*r*) and the level of significance (*P*) are given in the insert. The graph shows that activation by the pentaerythritol nitrates of isolated soluble guanylate cyclase is most likely dependent on the release of NO.

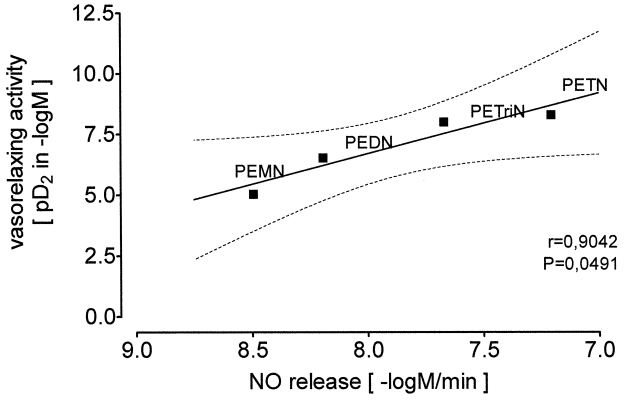

Fig. 4. Correlation between the concentration inducing half-maximal vasorelaxation (pD_2 values) of the pentaerythritol nitrates and the cysteine-dependent release of NO from these drugs. The correlation coefficient (r) and the level of significance (P) are given in the insert. The graph shows that vasorelaxation by the pentaerythritol nitrates is most likely dependent on the release of NO.

indicate that the number of nitrate ester groups is important for this activity. Hydrolysis of the nitrate esters to the corresponding alcohols resulted in a stepwise reduction of the enzyme stimulating potency. The magnitude of NO release shows the same pattern. There was an excellent correlation between the NO release and the soluble guanylate cyclase stimulating potency (Fig. 3) indicating that stimulation of soluble guanylate cyclase induced by all pentaerythritol nitrates is mediated by NO.

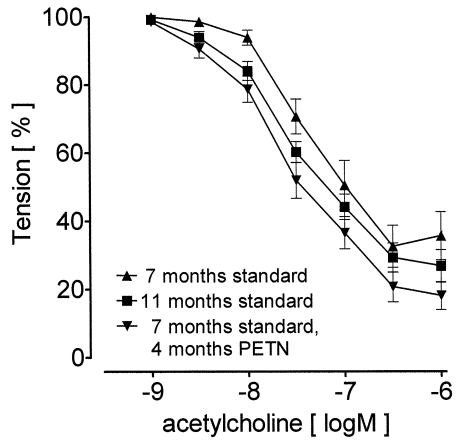

Fig. 5. The vasorelaxant activity of acetylcholine in isolated thoracic aorta segments of New Zealand White rabbits fed either a standard chow for 4 months (age: 7 months) or a standard chow for 8 months (age: 11 months) or a standard chow for 4 months followed by a standard chow supplemented with pentaerythritol tetranitrate (6 mg (kg b.wt.)$^{-1}$ day^{-1}) for another 4 months (age: 11 months). The vasodilatory response is expressed as percentage of precontraction induced by phenylephrine. Each concentration–response curve was plotted by taking the respective mean values of 10 separate experiments (S.E.M. indicated by bars). Neither aging nor pentaerythritol tetranitrate treatment had an influence on endothelium dependent vasorelaxation.

3.2. Vasorelaxant activity

The vasorelaxant activity of all pentaerythritol nitrates was determined in rabbit aorta. We found that the number of nitrate ester groups is important for the vasorelaxant activity. The pD_2 values (in $-\log M$) were as follows: 5.0 ± 0.11 (pentaerythritol mononitrate), 6.52 ± 0.12 (pentaerythritol dinitrate), 8.0 ± 0.06 (pentaerythritol trinitrate), 8.3 ± 0.17 (pentaerythritol tetranitrate). In the same experimental setting, the pD_2 value of glyceryl trinitrate was 7.96 ± 0.11 showing a vasodilatory potency comparable to pentaerythritol trinitrate. The pD_2 values of the pentaerythritol nitrates excellently correlated with the NO release

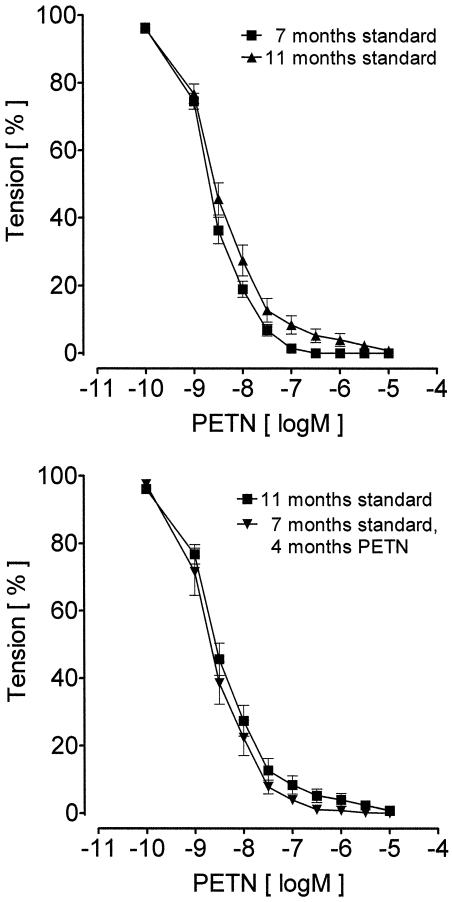

Fig. 6. The vasorelaxant activity of pentaerythritol tetranitrate (PETN) in isolated thoracic aorta segments of New Zealand White rabbits fed either a standard chow for 4 months (age: 7 months) or a standard chow for 8 months (age: 11 months) or a standard chow for 4 months followed by a standard chow supplemented with pentaerythritol tetranitrate (6 mg (kg b.wt.)$^{-1}$ day^{-1}) for another 4 months (age: 11 months). The vasodilatory response is expressed as percentage of precontraction induced by phenylephrine. Each concentration–response curve was plotted by taking the respective mean values of 10 separate experiments (S.E.M. indicated by bars). Neither aging (upper panel) nor pentaerythritol tetranitrate treatment (lower panel) had an influence on vasorelaxation induced by pentaerythritol tetranitrate indicating the absence of typical nitrate tolerance after long-term nonintermittent treatment with pentaerythritol tetranitrate.

(Fig. 4) indicating that vasorelaxation induced by these nitrates is induced by NO.

3.3. Effects of oral pentaerythritol tetranitrate treatment on vasorelaxation

None of the treatment regimens had an influence on endothelium dependent vasorelaxation as indicated by the unchanged vasodilator response to acetylcholine (Fig. 5). Aortic segments of each study group were also subjected to increasing concentrations of pentaerythritol tetranitrate. We found that the vasodilator potency of pentaerythritol tetranitrate was identical in each group indicating that neither age nor long-term oral treatment with pentaerythritol tetranitrate had an effect on the vasodilator potency of this drug (Fig. 6). Examination of the vasorelaxation induced by *S*-nitroso-*N*-acetyl-DL-penicillamine, a nitrosothiol which releases NO directly into the organ bath, showed no significant differences between the groups (Fig. 7). These results suggests that the NO-sensitivity of rabbit aortic rings remained unchanged.

3.4. Effects of oral pentaerythritol tetranitrate treatment on vascular superoxide production

There was a low basal superoxide production in aortic rings of the control rabbits. This superoxide production was slightly but significantly enhanced by aging (Fig. 8, upper panel). In contrast, the superoxide production of aortic rings from pentaerythritol tetranitrate-treated rabbits was almost identical to that observed in the control rabbits (Fig. 8, lower panel) suggesting that pentaerythritol tetranitrate prevented the increase in vascular superoxide production induced by aging.

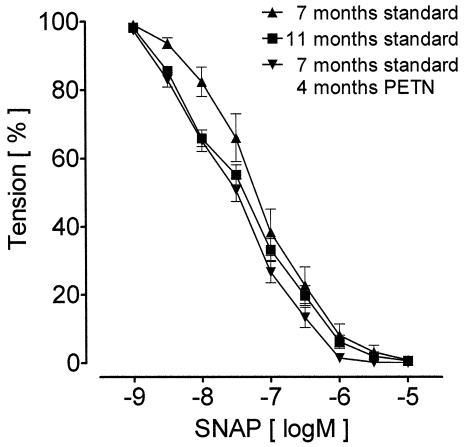

Fig. 7. The vasorelaxant activity of the NO-donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) in isolated thoracic aorta segments of New Zealand White rabbits fed either a standard chow for 4 months (age: 7 months) or a standard chow for 8 months (age: 11 months) or a standard chow for 4 months followed by a standard chow supplemented with pentaerythritol tetranitrate (PETN, 6 mg (kg b.wt.)$^{-1}$ day^{-1}) for another 4 months (age: 11 months). The vasodilatory response is expressed as percentage of precontraction induced by phenylephrine. Each concentration–response curve was plotted by taking the respective mean values of 10 separate experiments (S.E.M. indicated by bars). Neither aging nor pentaerythritol tetranitrate treatment had an influence on vasorelaxation induced by *S*-nitroso-*N*-acetyl-DL-penicillamine indicating that the vascular NO-sensitivity remained unchanged.

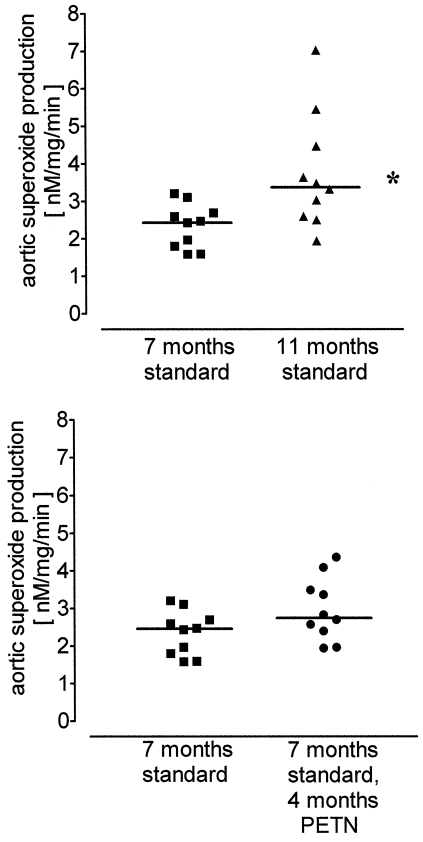

Fig. 8. Generation of superoxide anions as detected by use of the lucigenin assay in thoracic aorta segments of New Zealand White rabbits fed either a standard chow for 4 months (age: 7 months) or a standard chow for 8 months (age: 11 months) or a standard chow for 4 months followed by a standard chow supplemented with pentaerythritol tetranitrate (PETN, 6 mg (kg b.wt.)$^{-1}$ day^{-1}) for another 4 months (age: 11 months). Aging slightly increased vascular superoxide production (upper panel), while concurrent treatment with pentaerythritol tetranitrate appears to prevent this increase in vascular oxidant stress (lower panel). The lines denote the median values which are 2.453 nM mg^{-1} min^{-1} (control group), 3.399 nM mg^{-1} min^{-1} (aging group) and 2.762 nM mg^{-1} min^{-1} (pentaerythritol tetranitrate group, $* = P < 0.01$).

4. Discussion

The results of the present study showed that pentaerythritol tetranitrate is a typical organic nitrate as evidenced by a cysteine-dependent generation of NO, which excellently correlates with both, activation of soluble guanylate cyclase and vasorelaxation. The new finding is that long-term oral treatment of rabbits with a therapeutic dose of pentaerythritol tetranitrate prevented the small increase of vascular superoxide production induced by aging and did

not induce typical nitrate tolerance. These results suggest that pentaerythritol tetranitrate treatment may reduce vascular oxidant stress.

The new finding of the present study is that oral nonintermittent treatment with the organic nitrate pentaerythritol tetranitrate prevents the vascular of vascular superoxide production induced by aging. This result seems to be in contrast to previous findings showing that nonintermittent treatment of rabbits with the organic nitrate glyceryl trinitrate substantially increased aortic superoxide production (Münzel et al., 1995). In this study, increased superoxide production was associated with a marked attenuation of glyceryl trinitrate induced vasodilation indicating development of typical nitrate tolerance. In contrast, nitrate tolerance did not occur in our study as evidenced by unchanged vasodilation to pentaerythritol tetranitrate even after 4 months of nonintermittent oral treatment. Therefore, the lack of development of typical nitrate tolerance might serve as an explanation for the different observations in terms of vascular superoxide production.

Attenuation of the therapeutic activity of organic nitrate esters after nonintermittent treatment is not restricted to glyceryl trinitrate. This typical nitrate tolerance has also been reported for other organic nitrates, such as isosorbide-5-nitrate and isosorbide-2,5-dinitrate (Ahlner et al., 1991). In contrast, there is no clinical investigation demonstrating the occurrence of nitrate tolerance after nonintermittent therapy with pentaerythritol tetranitrate and recent experimental studies failed to prove its development. Continuous intravenous infusion of pentaerythritol tetranitrate in awake dogs for 5 days did not change the dilatory action of this drug on left coronary artery, while the coronary vasodilation induced by glyceryl trinitrate and isosorbide-2,5-dinitrate were markedly reduced (Fink et al., 1996). A previous study in our lab also failed to detect typical nitrate tolerance after 4 months of nonintermittent oral treatment of rabbits with pentaerythritol tetranitrate as evidenced by unchanged aortic vasodilation induced by pentaerythritol tetranitrate (Kojda et al., 1995) and a similar result was obtained in the present study (Fig. 6).

The reasons for the important difference between pentaerythritol tetranitrate and other organic nitrates in terms of tolerance development remain to be elucidated. Different mechanisms, such as reduced bioactivation of organic nitrates to NO and reduced sensitivity of vascular soluble guanylate cyclase have been proposed to explain nitrate tolerance (Ahlner et al., 1991). In addition, a neurohumoral counterregulation blunting the vasodilator effects of organic nitrates also occurs. Recent studies provided evidence for an activation of the renin angiotensin system and an involvement of endothelin-1 as key mechanisms inducing nitrate tolerance in vivo by neurohumoral counterregulation (Münzel et al., 1996). In these studies, nitrate tolerance was induced by transdermal application of glyceryl trinitrate for 3 days at a dosage of approximately 0.1 mg h^{-1} (kg b.wt.)$^{-1}$. This dosage is much higher than the therapeutic dose of glyceryl trinitrate released by the routinely used 10-mg patch (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico, 1994). In man, a 10-mg patch releases approximately 0.005 mg glyceryl trinitrate h^{-1} (kg b.wt.)$^{-1}$ and induces typical nitrate tolerance after continuous 24 h application for 3 days (Ahlner et al., 1991).

In our study, pentaerythritol tetranitrate was administered orally. The daily dosage of 6 mg pentaerythritol tetranitrate (kg b.wt.)$^{-1}$ closely corresponds to the clinically effective daily dosage of 150 mg to 300 mg, which was chosen according to the appearance of antianginal effects as measured by ST-segment depression or the onset of pectanginal pain after controlled physical strain (Dück and Richard, 1990). We cannot rule out that in rabbits the clinically effective dosage is simply to low to induce sustained hemodynamic changes and typical nitrate tolerance. Thus, the absence of nitrate tolerance in our experimental setting might be the result of the comparably low daily dosage. However, the results obtained with pentaerythritol tetranitrate in awake dogs showing absence of hemodynamic tolerance even at hemodynamically active doses (Fink et al., 1996) argue against this possibility. It is of note that the low daily dosage of pentaerythritol tetranitrate used in our study was effective. Given to rabbits, it induces beneficial effects, such as prevention of aging-induced vascular superoxide production (Fig. 8) and prevention of the impairment of endothelium-dependent vasorelaxation induced by long term feeding with cholesterol (Kojda et al., 1995).

Another explanation for the lack of tolerance development would be that pentaerythritol tetranitrate, although sharing structural similarities with other typical organic nitrates, act via a different mechanism of action. Organic nitrates undergo bioactivation to form NO, which is the pharmacologically active compound and stimulates soluble guanylate cyclase to produce cGMP (Ahlner et al., 1991). The bioactivation to NO occurs in aqueous solutions in the presence of cysteine (Feelisch and Noack, 1987) or in the intact cell by means of an enzymatic process (Chung and Fung, 1990). We demonstrate here that pentaerythritol tetranitrate and all nitrated metabolites are bioactivated in a similar manner. Pentaerythritol tetranitrate, pentaerythritol trinitrate, pentaerythritol dinitrate and pentaerythritol mononitrate show a cysteine dependent generation of NO, which closely correlates with both, activation of soluble guanylate cyclase and vasorelaxation. We found that the number of nitrate esters groups strongly determines the efficacy: the higher the number of nitrate ester groups the lower is the concentration for half-maximal activation of soluble guanylate cyclase or for half-maximal vasorelaxation. This finding confirms previous results obtained with other organic nitrates (Schröder et al., 1985). It is also consistent with an earlier study showing a similar loss of vasodilator activity from pentaerythritol tetranitrate to pentaerythritol mononitrate in the dog in vivo (Parker et al.,

1975). Taken together, our data strongly suggest that the mechanism of action of pentaerythritol tetranitrate is not different from that of other organic nitrates.

A major difference between pentaerythritol tetranitrate and other organic nitrates is its complex pharmacokinetic pattern. After ingestion neither pentaerythritol tetranitrate nor pentaerythritol trinitrate is detectable in plasma suggesting a biotransformation in the intestine (Posadas del Rio et al., 1988) and/or the liver (Weber et al., 1995). The other two nitrated metabolites peak 4 h (pentaerythritol dinitrate) and 7 h (pentaerythritol mononitrate) after application of a commercially available tablet in the plasma and have plasma half-lives of 4–5 h and 10–11 h, respectively. It is likely that these metabolites are involved in the therapeutic efficacy of pentaerythritol tetranitrate. The duration of action of pentaerythritol tetranitrate is greater than 5 h (Shellock et al., 1980; Giles et al., 1981) and both metabolites have been shown to be active in vitro and in vivo (Parker et al., 1975). In rabbits, both pentaerythritol dinitrate and pentaerythritol mononitrate have been detected in plasma samples taken after a 24-h washout period from animals treated with 6 mg pentaerythritol tetranitrate kg^{-1} day^{-1} (Kojda et al., 1995). These data give rise to the assumption that nonintermittent treatment with pentaerythritol tetranitrate might be associated with alternating peak concentrations of pharmacologically active metabolites, such as pentaerythritol dinitrate and pentaerythritol mononitrate. This might mimic the intermittent treatment regimen that is used to prevent nitrate tolerance induced by glyceryl trinitrate or isosorbide mononitrate (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico, 1994; ISIS-4 Collaborative Group, 1995) and might contribute to the lack of development of nitrate tolerance in rabbits (Fig. 6; Kojda et al., 1995), dogs (Fink et al., 1996) and possibly also in man (Dück and Richard, 1990).

Our results suggest that long-term nonintermittent treatment with pentaerythritol tetranitrate prevents the increase in vascular superoxide production induced by aging. This observation is new and might help to explain why pentaerythritol tetranitrate has beneficial effects in experimental atherosclerosis. In cholesterol-fed rabbits, treatment with pentaerythritol tetranitrate results in a reduction of the area of aortic lesions and in prevention of the impairment of endothelium-dependent vasorelaxation (Kojda et al., 1995). It was shown previously that feeding rabbits with a cholesterol-enriched chow increases aortic superoxide production (Ohara et al., 1993), which itself is believed to be a key event in atherogenesis (Ross, 1993; Steinberg, 1995; Cox and Cohen, 1996). The mechanism of action underlying the reduction of vascular superoxide production induced by pentaerythritol tetranitrate is unclear. It is assumed that NO as the pharmacologically active compound is involved. The detection of pentaerythritol mononitrate in the plasma of rabbits after a 24-h fasting period shows that in these animals pentaerythritol tetranitrate is absorbed and most likely metabolized to NO (Kojda et al., 1995). Possible mechanisms of an antiatherogenic action of NO might include its antimitogenic effects (Nakaki et al., 1990; De Mey et al., 1991) or its capacity to act as an antioxidant, which scavenges superoxide radicals generated in the vasculature, reduces the oxidation of low density lipoproteins and prevents the induction of membrane damage (Bruckdorfer et al., 1990).

In summary, our results show that long-term nonintermittent treatment of rabbits with a low daily dosage of pentaerythritol tetranitrate prevents the small increase of vascular superoxide production induced by aging. This vasoprotective activity is most likely related to intravascular production of NO from pentaerythritol tetranitrate. In vitro investigations showed excellent correlations between the NO release from pentaerythritol tetranitrate, pentaerythritol trinitrate, pentaerythritol dinitrate and pentaerythritol mononitrate and both, activation of soluble guanylate cyclase and vasorelaxant activity. Despite long-term nonintermittent application of pentaerythritol tetranitrate, a development of typical nitrate tolerance was not observed. Possible explanations for the absence of tolerance are the comparably low daily dosage and the alternating peak concentrations of the pharmacologically active metabolites pentaerythritol dinitrate and pentaerythritol mononitrate. It is concluded that pentaerythritol tetranitrate might reduce vascular oxidant stress.

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