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The Antioxidant Defense Protein Ferritin Is a Novel and Specific Target for Pentaerithrityl Tetranitrate in Endothelial Cells

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The organic nitrate pentaerithrityl tetranitrate (PETN) is known to exert long-term antioxidant and antiatherogenic effects by as yet unidentified mechanisms. In porcine aortic endothelial cells, a 24 h incubation with PETN (1–100 μM) or its metabolite pentaerithrityl trinitrate (PETriN) increased levels of the antioxidant protein ferritin up to three-fold over basal, whereas isosorbide dinitrate and isosorbide-5-mononitrate were without significant effect under these conditions. PETriN-induced ferritin expression was blocked by the NO scavenger PTIO but remained unaltered in the presence of ODQ, an inhibitor of soluble guanylyl cyclase. 8-Bromo cyclic GMP and dibutyryl cyclic GMP did not influence basal ferritin synthesis. The iron chelator desferrioxamine abolished ferritin induction by PETriN. Our results show that PETN or its active metabolite PETriN induce ferritin synthesis through NO- and iron-dependent but cyclic GMP-independent pathways. Increased activity of ferritin may contribute to, and at least in part explain, the specific antiatherogenic and antioxidant action of PETN.

Nitric acid esters such as glyceryl trinitrate (GTN) were introduced into therapy more than a century ago and are still widely used for the treatment of myocardial ischemia and its main symptom angina pectoris. The basic mechanisms responsible for the vasodilatory and anti-ischemic action of organic nitrates involve bioactivation of, and nitric oxide (NO) release from, these compounds which have therefore been termed NO donors. The intracellular target of NO (donors) is the soluble isozyme of guanylyl cyclase which upon activation by NO increases its synthesis rate of the second messenger cyclic GMP (1–3).

Sustained treatment of cardiovascular diseases with organic nitrates has long been known to induce tolerance to the hemodynamic and anti-ischemic effects of these drugs in humans and animals (4, 5). Despite its first description early this century, nitrate tolerance still poses an unsolved clinical problem and is one of the main limitations to the use of nitrates. Newer studies show that prolonged exposure of rabbits to GTN is associated with enhanced superoxide production in the blood vessel wall (6, 7) suggesting that oxidative stress may cause vascular desensitization to nitrates.

Pentaerithrityl tetranitrate (PETN) is a long-acting NO donor and has recently been described as an organic nitrate ester that in contrast to other nitric acid esters does not induce oxidative stress and is therefore free of tolerance (8, 9). Moreover, animal experiments revealed that PETN actively reduces oxygen radical formation in vivo (10) and specifically prevents atherogenesis and endothelial dysfunction in cholesterol-fed rabbits, possibly as a consequence of its radical scavenging properties (11). On the basis of these findings, clinical interest in this drug has substantially renewed, however the basic mechanisms underlying its particular antioxidant profile have remained unclear. Direct quenching or neutralization of superoxide radicals by NO (12) as sole underlying cause appears unlikely since the antioxidant activity of PETN has a delayed onset and persists even after PETN treatment has ended (10, 11). Therefore a plausible mode of action might be the induction of genes which protect cells from damage by reactive oxygen species. In recent studies, the induction of ferritin has been shown to provide marked antioxidant cellular protection by rapidly sequestering free cytosolic iron, the crucial catalyst of oxygen-centered radical formation via the Fenton reaction in biological systems (13, 14). Thus,

Abbreviations used: GTN, glyceryl trinitrate; ISDN, isosorbide dinitrate; ISMN, isosorbide-5-mononitrate; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one; PETN, pentaerithrityl tetranitrate; PETriN, pentaerithrityl trinitrate; PTIO, phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide.

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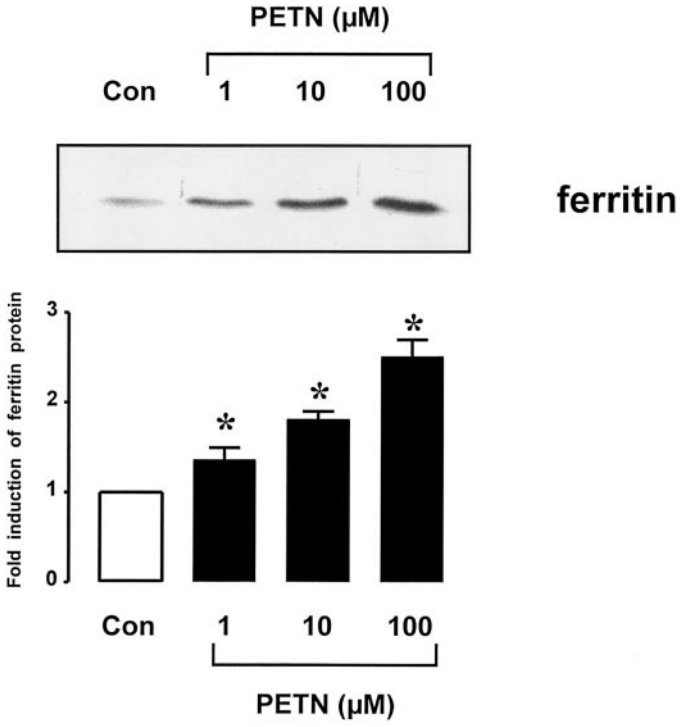

FIG. 1. Effect of PETN on ferritin protein expression in endothelial cells. Incubations, protein isolation and Western blot analysis were performed as described under Materials and Methods. The densitometric data (lower panel) are means ± SEM of $n = 3$ separate experiments. (*) $P < 0.05$, treatment vs control (Con), one-way ANOVA, and Bonferroni's multiple comparison test. A representative Western blot analysis is shown in the upper panel.

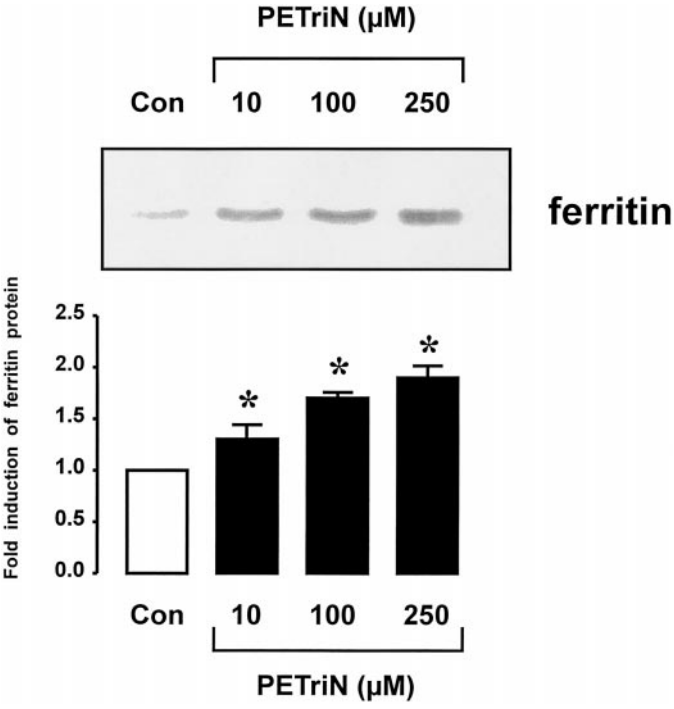

FIG. 2. Effect of PETriN on ferritin protein expression in endothelial cells. Incubations, protein isolation, and Western blot analysis were performed as described under Materials and Methods. The densitometric data (lower panel) are means ± SEM of $n = 3$ separate experiments. (*) $P < 0.05$, treatment vs control (Con), one-way ANOVA, and Bonferroni's multiple comparison test. A representative Western blot analysis is shown in the upper panel.

ferritin, which until then was thought to merely function as a "housekeeper" iron storage protein, has emerged as a critical and fast acting endogenous cytoprotectant that plays an important role in cellular antioxidant defense mechanisms (15, 16). Our aim, therefore, was to investigate whether ferritin is a potential site of action for PETN and to characterize the effect of PETN and its main active metabolite pentaerithrityl trinitrate (PETriN) on the expression of ferritin in endothelial cells.

MATERIALS AND METHODS

Materials. Fetal bovine serum, Dulbecco's modified Eagle medium and penicillin-streptomycin were obtained from Gibco (Eggenstein, FRG). The Chemiluminescence Western Blotting Kit and anti-rabbit peroxidase-conjugated secondary antibody were from Boehringer Mannheim (Mannheim, FRG). PETN and PETriN were provided by ISIS Pharma (Monheim, FRG). Spermine NONOate, phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) and 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) were purchased from Alexis Deutschland GmbH (Grünberg, FRG). Isosorbide dinitrate (ISDN) and isosorbide-5-mononitrate (ISMN) were from Schwarz Pharma (Monheim, FRG). The polyclonal antibody against human ferritin and all other chemicals were bought from Sigma (Deisenhofen, FRG).

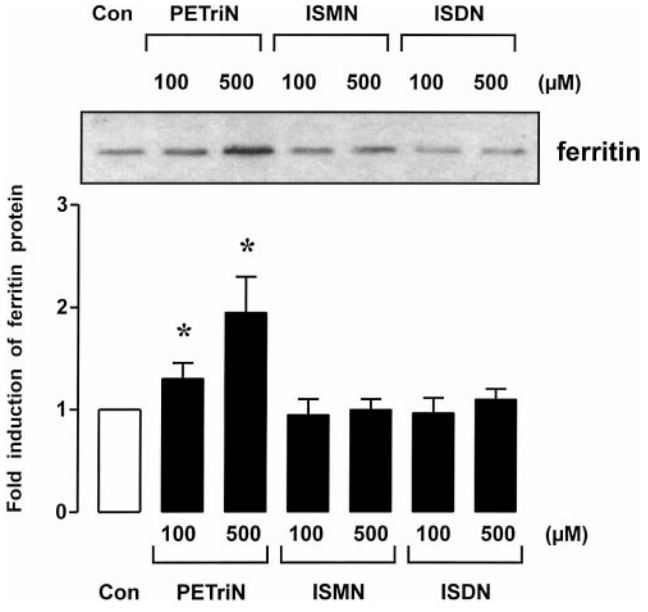

FIG. 3. Effect of PETriN, ISMN, and ISDN on ferritin protein expression in endothelial cells. Incubations, protein isolation, and Western blot analysis were performed as described under Materials and Methods. The densitometric data (lower panel) are means ± SEM of $n = 3$ separate experiments. (*) $P < 0.05$, treatment vs control (Con), one-way ANOVA, and Bonferroni's multiple comparison test. A representative Western blot analysis is shown in the upper panel.

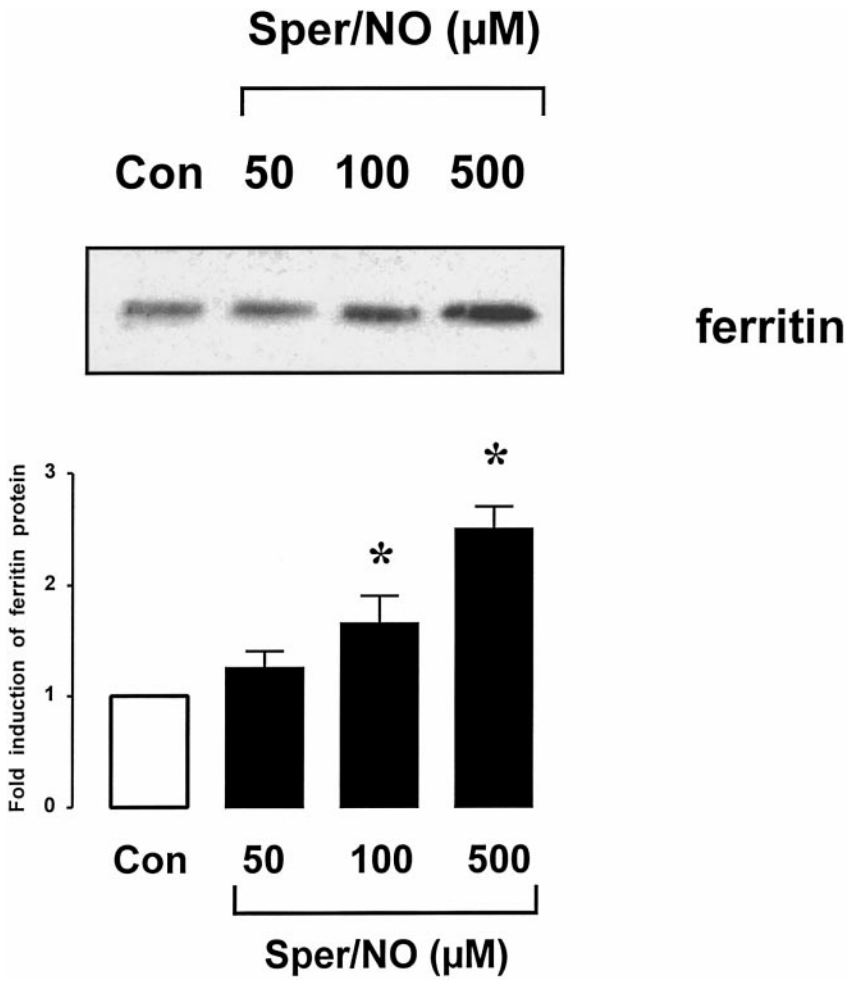

FIG. 4. Effect of spermine NONOate (Sper/NO) on ferritin protein expression in endothelial cells. Incubations, protein isolation, and Western blot analysis were performed as described under Materials and Methods. The densitometric data (lower panel) are means ± SEM of $n = 3$ separate experiments. (*) $P < 0.05$, treatment vs control (Con), one-way ANOVA, and Bonferroni's multiple comparison test. A representative Western blot analysis is shown in the upper panel.

Cell culture. Pig aortic endothelial cells were isolated and characterized as described previously (17). Endothelial cells were maintained and subcultured in Dulbecco's modified Eagle medium supplemented with 15% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Endothelial cells at passages 3 were used for experiments. The cells were grown in a humidified incubator at 37°C and 5% CO_2.

Incubation procedure and ferritin protein analysis. Endothelial cells were cultured in 100-mm culture dishes. Upon reaching confluence, cells were incubated with control media, PETN, PETriN, ISDN, ISMN, spermine NONOate, 8-bromo cyclic GMP, or dibutyryl cyclic GMP for 24 hours. PTIO, ODQ or desferrioxamine were added 15 minutes prior to PETriN. After the incubation, cells were washed with phosphate buffered saline and extracted as described previously (17, 18). Desferrioxamine was added to the cells 30 min prior to aspirin. Protein (50 μg/lane) was applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, protein was transferred to a nitrocellulose membrane, and a polyclonal antibody to human ferritin (Sigma) was used to identify ferritin protein content. Antigen antibody complexes were visualized with the horseradish peroxidase chemiluminescence system according to the manufacturer's instructions (Boehringer Mannheim). Quantitation of ferritin induction was performed using computer-assisted videodensitometry (Eagle Eye II-system, Stratagene).

RESULTS

In porcine aortic endothelial cells, a 24-h incubation with PETN (1-100 μM) increased ferritin protein levels up to 3-fold over basal (Fig. 1). The phase I PETN metabolite PETriN was also found to stimulate ferritin synthesis (Fig. 2) whereas ISDN and ISMN, two other long-acting nitrates, were without significant effect under these conditions (Fig. 3). PETriN-induced ferritin expression was mimicked by the spontaneous NO donor NONOate (Fig. 4) and blocked by the NO scavenger PTIO (Fig. 5A). The membrane permeable cyclic GMP analogues 8-bromo cyclic GMP and dibutyryl cyclic GMP did not influence basal ferritin synthesis (Fig. 6). Moreover, PETriN-induced ferritin synthesis remained unaltered in the presence of ODQ, an inhibitor

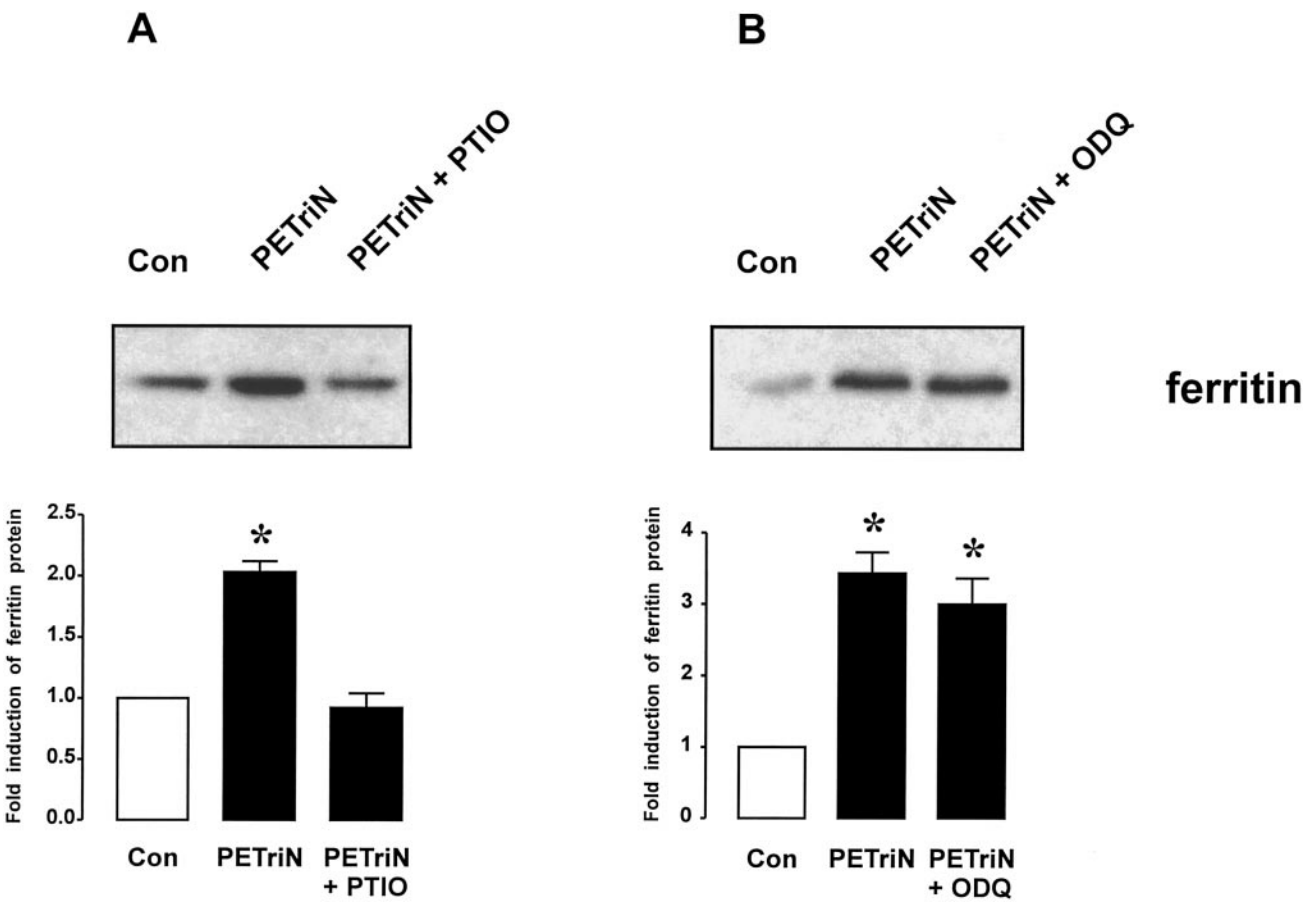

FIG. 5. Effect of the NO scavenger PTIO (100 μM) (A) and the inhibitor of soluble guanylyl cyclase ODQ (10 μM) (B) on ferritin protein induction by PETriN (500 μM) in endothelial cells. Incubations, protein isolation, and Western blot analysis were performed as described under Materials and Methods. The densitometric data (lower panels) are means ± SEM of $n = 3$ separate experiments. (*) $P < 0.05$, treatment vs control (Con), one-way ANOVA, and Bonferroni's multiple comparison test. Representative Western blot analyses are shown in the upper panels.

of soluble guanylyl cyclase (Fig. 5B). The iron chelator desferrioxamine abolished ferritin induction by PETriN (Fig. 7). PTIO, ODQ, or desferrioxamine alone were without significant influence on ferritin synthesis under these conditions (not shown).

DISCUSSION

The present study demonstrates, for the first time, that PETN and its active metabolite PETriN induce the synthesis of the antioxidant defense protein ferritin in endothelial cells. Ferritin, in addition to serving as an iron reserve for the maintenance of vital cellular functions, sequesters iron that might otherwise catalyze damaging oxidative reactions. The cytoprotective role of ferritin has remained largely hypothetical until it was shown that cells overexpressing this protein are more resistant to injury by oxygen radicals (13, 16, 17). In contrast, organisms lacking a functional ferritin gene or cells treated with ferritin antisense oligonucleotides are more sensitive to oxidative stress (19, 20). That ferritin may be a therapeutically relevant drug target in cardiovascular disease was also highlighted by recent work showing that endothelium-protective effects of aspirin are related to ferritin induction and iron sequestration (21–23). Moreover, it is known that iron-free apoferritin, exogenously added to tissues, enhances tolerance to oxidant stress after being internalized (13, 17, 22).

In our study, increased ferritin synthesis occurred at concentrations as low as 1 μM PETN which is within the range of plasma or tissue concentrations that can be expected during oral therapy (24). A possible clinical relevance of this pathway is also suggested by our finding that PETriN, a denitrated phase I metabolite of PETN, still lead to several-fold elevations of endothelial ferritin. PETriN undergoes hepatic circulation, tends to accumulate and is therefore held responsible for sustained vasodilatory and anti-ischemic effects of PETN (25). Therefore, most experiments in this investigation were carried out with the active PETN metabolite PEtriN. From the results obtained in this and in

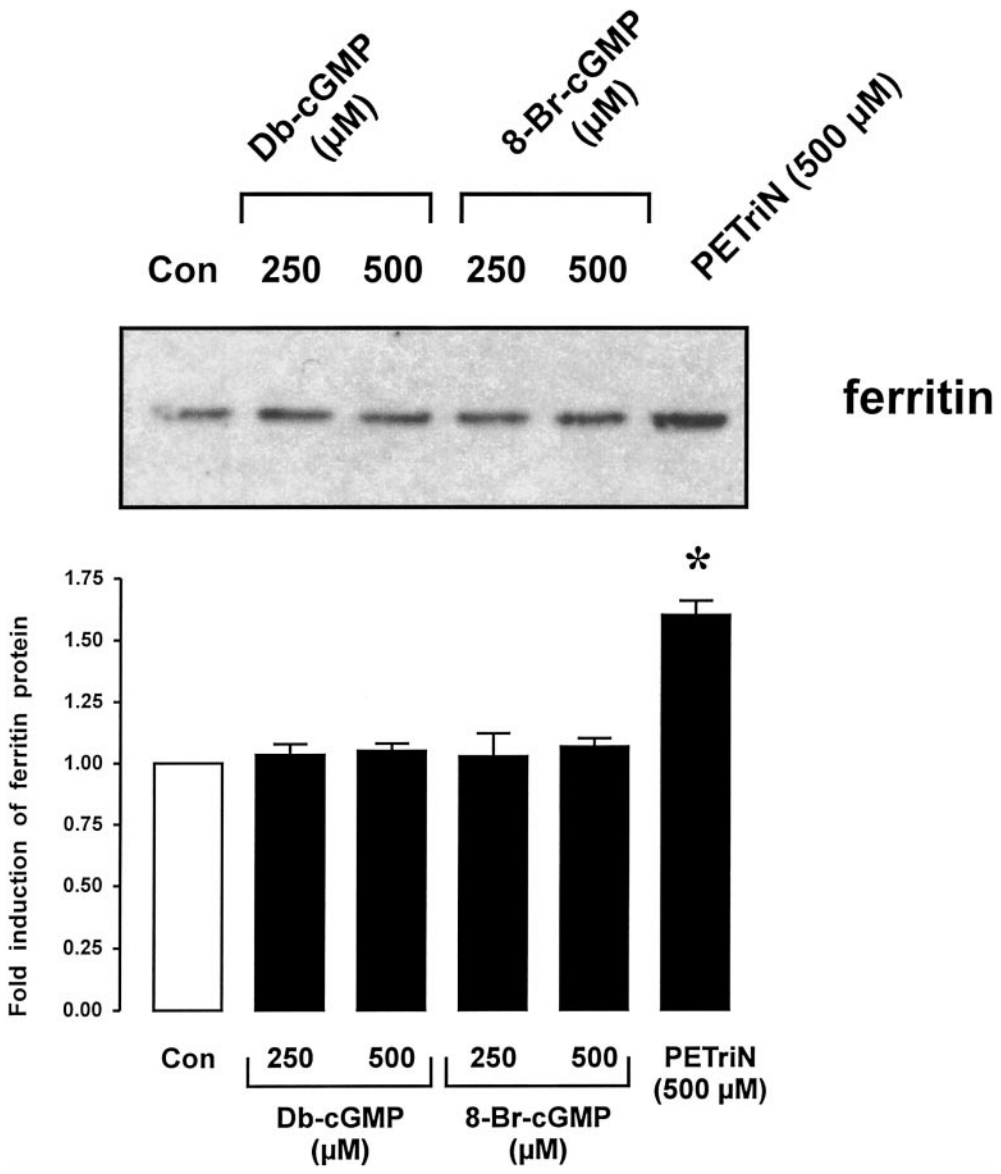

FIG. 6. Effect of dibutyryl cyclic GMP (Db-cGMP) and 8-bromo cyclic GMP (8-Br-cGMP) on ferritin protein expression in endothelial cells. Incubations, protein isolation, and Western blot analysis were performed as described under Materials and Methods. The densitometric data (lower panel) are means ± SEM of $n = 3$ separate experiments. (*) $P < 0.05$, treatment vs control (Con), one-way ANOVA, and Bonferroni's multiple comparison test. A representative Western blot analysis is shown in the upper panel.

previous studies (17, 22), it seems plausible that by increasing the synthesis of iron-scavenging ferritin, PETN may effectively interrupt the reaction cascade leading to oxidant stress and related events such as atherogenesis or development of nitrate tolerance.

Ferritin induction by PETriN was completely blocked in the presence of the NO scavenger PTIO (26) indicating a crucial role for NO in mediating increased ferritin synthesis. This is further supported by our finding that an increase in ferritin comparable to that of PETN was caused by spermine NONOate, an experimental drug that delivers NO spontaneously in aqueous solution and at a controlled release rate (27). Although earlier reports of increased repressor protein binding to ferritin mRNA in the presence of NO seemed to point to an inhibitory function of NO in ferritin expression (28, 29), the data from this study and other recent observations clearly document that NO stimulates ferritin synthesis in an iron-dependent but cyclic GMP-independent fashion (16, 17, 30). NO, possibly by increasing intracellular iron bioavailability (31), causes dissociation of iron regulatory protein-2 (IRP-2) from ferritin mRNA thus allowing ribosomal access and subsequent translation (32). That PETN may act through this pathway can be derived from two findings of our study. First, PETN-dependent ferritin induction

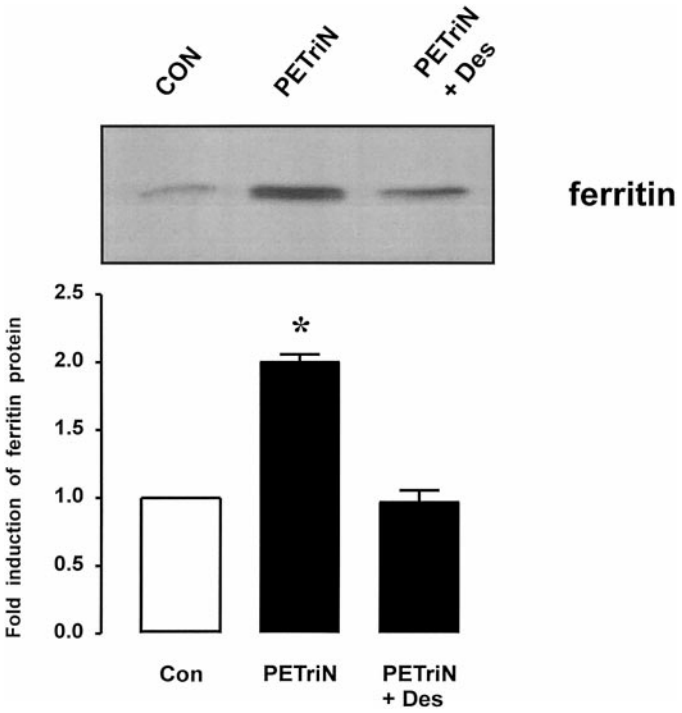

FIG. 7. Effect of desferrioxamine (Des, 100 μM) on ferritin protein induction by PETriN (500 μM) in endothelial cells. Incubations, protein isolation, and Western blot analysis were performed as described under Materials and Methods. The densitometric data (lower panel) are means ± SEM of $n = 3$ separate experiments. (*) $P < 0.05$, treatment vs control (Con), one-way ANOVA, and Bonferroni's multiple comparison test. A representative Western blot analysis is shown in the upper panel.

is also cyclic GMP-independent, which is evident from the lack of effect of membrane permeable cyclic GMP analogues and the inhibitor of soluble guanylyl cyclase ODQ (33). Second, PETN-induced ferritin synthesis requires the presence of free cytosolic iron since ferritin induction by PETN was abolished by of the iron chelator desferrioxamine. The latter observation indicates that PETN interacts with and activates the physiological iron-dependent translation of ferritin via IRP-2 described above (34).

Ferritin induction has a particular explanatory power for the long-term in vivo effects of PETN since (i) PETN reduces both oxygen radical formation and atherogenesis (9–11) and (ii) increased expression of ferritin not only leads to diminished oxygen radical formation but has also been observed in atherosclerotic lesions of patients with coronary heart disesase, which underlines the clinical relevance of this antioxidant pathway (15, 35). Two other long acting nitrates ISDN and ISMN had no significant effect on endothelial ferritin expression in this study which is in agreement with previous reports demonstrating only marginal or non-detectable amounts of NO released from these drugs in various cell types (36, 37). Interestingly and in contrast to PETN, both isosorbide nitrates are known to induce tolerance to their cardiovascualr effects, presumably via oxidant stress (7, 38). Moreover, in an earlier investigation aimed at comparing the antiatherogenic potential of nitrates, PETN but not ISMN prevented plaque formation and endothelial dysfunction in cholesterol-fed animals (11). Thus, the capacity to activate ferritin translation apparently distinguishes PETN from other long-acting nitrates and may explain their different patterns of action in vivo. Based on the outcome of recent clinical studies, an increase in cellular iron levels has been suggested as a coronary risk factor which via enhancing oxidative stress promotes atherosclerosis and raises the incidence of myocardial infarction (39–41). In the light of our investigations, activation of endogenous iron sequestration could be an important mechanism by which PETN, in addition to its vasodilatory action, protects against heart attacks and myocardial infarction.

Together, we have shown that PETN induces ferritin synthesis through NO-dependent but cyclic GMP-independent mechanisms. Increased activity of ferritin may contribute to, and at least in part explain, the specific antiatherogenic and antioxidant action of PETN.

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