Combined Superoxide Dismutase Mimetic and Peroxynitrite Scavenger Protects Against Neointima Formation After Endarterectomy in Association with Decreased Proliferation and Nitro-oxidative Stress

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Abstract  Objective: Reactive oxygen and nitrogen species (e.g., peroxynitrite) may trigger neointima formation leading to restenosis. In a rat carotid endarterectomy (CEA) model, we investigated the effects of the manganese(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP), a superoxide dismutase (SOD) mimetic and peroxynitrite scavenger on neointima formation.

Methods: CEA was performed in male Sprague–Dawley rats. Animals received either vehicle (control group; n = 15) or 15 mg kg⁻¹ day⁻¹ MnTBAP intraperitoneally for 3 weeks (treatment group; n = 13). Four groups of carotids were analysed: the left, uninjured carotids (sham) and the right, injured carotids (control CEA) from the control group, the right, injured carotids from the treatment group (CEA + MnTBAP) and an additional group of carotids that were harvested 1 h following endarterectomy. The analysis of carotid arteries was performed by histology, immunohistochemistry and real-time polymerase chain reaction (PCR). Plasma malondialdehyde (MDA) levels were measured by lipid hydroperoxidase assay.

Results: Stenosis rate (10.5 ± 8.1% vs. 45.4 ± 28.3%), the percentage of proliferating cell nuclear antigen-positive cells (13.4 ± 7.1% vs. 23.3 ± 11.0%) and nitrotyrosine immunoreactivity (5.8 ± 1.9 vs. 8.0 ± 2.0) were significantly reduced in the vascular wall of the CEA + MnTBAP group compared with control CEA group. Ratio of Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL)-positive nuclei was significantly lower after antioxidant therapy (41.7 ± 26.7% vs. 64.9 ± 18.5%). Plasma MDA levels increased after endarterectomy (11.7 ± 4.8 vs. 4.1 ± 2.0 μmol l⁻¹) and reduced in the treatment group (3.2 ± 2.1 μmol l⁻¹). No significant gene regulation after MnTBAP treatment could be noted.

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the expression of redox-sensitive genes. The vascular pathways involved in neointima formation or by regulating course of restenosis development by activation of signalling logic conditions in association with oxidative stress. Suggested to exert protective effects in several pathophysiological conditions. Two of the latter two are finally responsible for neointimal hyperplasia leading to recurrent stenosis of the treated vessels. The main factors that trigger the above-mentioned processes are endothelial damage and subsequent generation of reactive oxygen and nitrogen species (ROS and RNS), such as superoxide anion and peroxynitrite. ROS and RNS participate during the whole process of restenosis development by activation of signalling pathways involved in neointima formation or by regulating the expression of redox-sensitive genes. The vascular reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidases are the primary producers of ROS, while superoxide dismutase-1 (SOD-1 or CuZnSOD) is primarily responsible for ROS removal. ROS can induce the expression of pro-inflammatory genes and superoxide anions enhance the catabolism of nitric oxide (NO) via the formation of peroxynitrite.

Antioxidant drugs that reduce ROS/RNS levels have been represented for several years as important therapeutic targets for the treatment of restenosis. However, the clinical efficacy of free radical scavengers has been limited due to their short half-life and low penetration in vascular tissues. Manganese(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP), a cell-permeable SOD mimetic and peroxynitrite scavenger, has been suggested to exert protective effects in several pathophysiological conditions in association with oxidative stress.

The present study was designed to evaluate the association between neointimal hyperplasia and local as well as systemic signs of oxidative and nitrosative stress after vascular injury in a rat carotid endarterectomy (CEA) model established in our laboratory. In addition, we aimed to test the effects of the antioxidant MnTBAP on neointimal hyperplasia and gene regulation during stenosis formation.

Methods

Rat CEA

The protocol was approved by the Regional Ethical Committee for Laboratory Animal Use and conformed with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85–23, revised 1996). Thirty-two male Sprague–Dawley rats (250–300 g body weight) underwent right CEA as described previously. Briefly, after adequate anaesthesia with an intraperitoneal injection of ketamine hydrochloride (100 mg kg⁻¹) and xylazine hydrochloride (5 mg kg⁻¹), the right common carotid artery was exposed under a dissecting microscope via a midline cervical incision. After clamping of the right common carotid artery, it was punctured by a 27-G needle and the arteriotomy was extended to 6 mm with microscissors. To denude the endothelium, a sterile cotton-tipped applicator immersed in saponin (0.1%) was rubbed on the inner vessel surface. The arteriotomy was closed with a running 9/0 Ethilon monofilament nylon suture (Ethicon, Inc, Somerville, NJ, USA). The superficial cervical muscles and skin were closed with running 4/0 absorbable sutures. Animals were placed postoperatively on a standard rat diet and provided with water ad libitum.

Experimental groups

Animals were randomly assigned into three groups: (1) control CEA group (n = 15) treated with placebo and (2) the treatment group (n = 13) that underwent CEA and received MnTBAP (21 days, 15 mg kg⁻¹ day⁻¹, administered intraperitoneally) dissolved in 0.1 M Tris–HCl, 1 mM ethylenediamine tetraacetic acid (EDTA), pH 9.00. This dose was drawn from recent literature reports showing the efficacy of MnTBAP in rat models. In addition (3), four rats underwent CEA and the carotid arteries were harvested 1 h following surgery. Carotid arteries from the above-mentioned three experimental groups were divided into four groups: left, uninjured carotids from the control endarterectomy group (sham); carotid arteries harvested 1 h after endarterectomy (acute injury group); right, injured carotids from the control endarterectomy group (control CEA) and right, injured carotids from the treatment group (CEA + MnTBAP).

Histologic analysis

After 21 days, 10 carotids from both the sham and control CEA groups and nine carotids from the CEA + MnTBAP group were perfusion-fixed and harvested. First, EDTA-anticoagulated blood was taken from the inferior caval vein, then the vein was transected and the heart was cannulated. Normal saline solution was infused at 100 mmHg until the vena cava effluent ran clear, and then a solution of 4% formaldehyde was infused at a constant pressure of 100 mmHg in an equal volume to the saline infusion (200 ml) to complete the perfusion–fixation process. Morphometry was performed in

Conclusions: MnTBAP decreased neointima formation, which was associated with reduced vascular smooth muscle cell proliferation and attenuated local and systemic nitro-oxidative stress.

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three haematoxylin–eosin stained cross-sections of each animal (from the mid region of the operated vessel segment) by using computer-aided planimetry (BX51 microscope and Cell^A imaging software, Olympus, Hamburg, Germany). For a detailed description, see the online-only Data Supplement.

**Immunohistochemical staining**

Immunohistochemical analysis was done in one representative cross-section of each animal using the avidin–biotin method. Proliferating cell nuclear antigen (PCNA), nitrotyrosine (NT) and transforming growth factor β1 (TGFβ1) were determined. Immunohistochemical stainings were evaluated by a semiquantitative scoring system with the Cell^A imaging software (Olympus, Hamburg, Germany). For a detailed description, see the online-only Data Supplement.

**TUNEL assay**

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) was used to detect DNA fragmentation that results from oxidative DNA damage. For a detailed description of the TUNEL assay, see the online-only Data Supplement.

**Quantitative real-time reverse transcription–polymerase chain reaction (RT-qPCR)**

Five carotids from both the sham and the control CEA groups and four carotids from both the acute injury and CEA + MnTBAP groups were perfused with ice-cold saline solution, harvested and immediately snap-frozen in liquid nitrogen. Operated segments of the carotids were ground under liquid nitrogen in a pestle and mortar, and total RNA was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA concentration and purity were determined photometrically (at 260, 280 and 230 nm). RNA (1.4 μg from each group) was reverse-transcribed with QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Real-time PCR reactions were performed on the Light Cycler 480 Real-time PCR detection system using the Light Cycler 480 Probes Master and Universal Probe Library (UPL) probes (Roche, Mannheim, Germany). Expression of genes involved in inflammation, proliferation and extracellular matrix metabolism during neointima formation was determined. For more details, see the online-only Data Supplement and Online Table 1.

**Lipid hydroperoxidase assay**

EDTA–anticoagulated blood samples were collected from each animal in the control CEA and CEA + MnTBAP groups. In addition, blood samples were taken from five animals that did not undergo an operation or treatment (sham). Plasma was separated and placed immediately in liquid nitrogen. Malondialdehyde (MDA) level was measured by a lipid hydroperoxidase (LPO) assay kit following the protocol provided by the manufacturer (Calbiochem, Merck, Germany).

**Statistical analysis**

Values are expressed as mean ± standard deviation. Statistical analysis was performed by using the Origin 7 statistical software product. Two group comparisons were performed by Student’s t-test. A value of p < 0.05 was considered statistically significant.

**Results**

**Neointimal thickening is suppressed by MnTBAP**

Three weeks after endarterectomy, neointimal thickening was found in the carotids of the control endarterectomy group, resulting in a 45.41% stenosis of the original luminal area (Fig. 1A, D). In the MnTBAP treated group, significant reduction of neointima formation was observed. Stenosis was 10.48% in this group (p < 0.05, CEA + MnTBAP group compared with control CEA group) (Fig. 1A, E). Further parameters measured in the carotid arteries are shown in the Online Table 2. Vessel area and media area showed no significant differences between sham, control CEA and CEA + MnTBAP groups. Total neointima area and neointimal thickness were higher in the control CEA group as compared with sham, and significantly lower in the CEA + MnTBAP group as compared with control CEA group (Online Table 2). In addition, the neointimal/medial area ratio was calculated and was found to be significantly reduced after MnTBAP treatment (0.27 vs. 0.76 in the CEA + MnTBAP group vs. the control CEA group, p < 0.05) (Fig. 1B). In the sham group, normal morphologic structure was found with a single endothelial cell layer as shown in Fig. 1C.

**Proliferation rate and nitro-oxidative stress is decreased after MnTBAP treatment**

PCNA immunohistochemical staining is a common method to confirm cell proliferation activity after arterial injury. As shown in Fig. 2, the proportion of PCNA-positive cell nuclei in the vascular media was significantly higher in the control CEA group as compared with sham. Both in the medial and neointimal layer, PCNA-positivity significantly decreased in the CEA + MnTBAP group compared with control CEA group (Fig. 2A–E). On the other hand, TGFβ1 immunoreactivity was similar in all the three experimental groups (Fig. 2F–J). In the media of the control CEA group, there is a tendency for increased nitrosative stress as shown by higher nitrotyrosine immunoreactivity. Peroxynitrite formation is significantly decreased in the media of the CEA + MnTBAP group compared with the control CEA group. When analysing the neointimal layer only, the difference was not significant (Fig. 2K–O). In this study, the nonspecific TUNEL assay was used to assess medial and neointimal cells with oxidative DNA injury during stenosis formation. Indeed, an approximately sixfold increase in the number of TUNEL-positive medial smooth muscle cells occurred in the control CEA group compared with sham, proving an
excessive oxidative stress caused by endarterectomy, that persists 3 weeks after arterial injury. MnTBAP markedly decreased the amount of TUNEL-positive cells in the vascular wall; however, the difference was only significant in the medial but not in the neointimal layer (Fig. 3).

Decreased oxidative stress in the CEA + MnTBAP group could be confirmed by lipid hydroperoxidase assay as well. Three weeks after CEA, plasma MDA level increased 2.86-fold compared with those of the sham animals (11.70 ± 4.77 μmol l⁻¹ vs. 4.09 ± 2.03 μmol l⁻¹). A highly...
significant decrease could be observed after MnTBAP treatment (3.15 ± 2.19 μmol l⁻¹ in the CEA + MnTBAP group vs. 11.70 ± 4.77 μmol l⁻¹ in the control CEA group) (Fig. 4).

**Gene expression is not significantly affected by MnTBAP during stenosis formation**

The relative expression of endothelial nitric oxide synthase (eNOS) in the carotid artery was decreased 10-fold 1 h after endarterectomy, confirming effective endothelial damage. Three weeks after endothelial denudation, eNOS expression reached the level of those in the sham group, confirming a re-endothelialisation process. MnTBAP slightly increased eNOS expression; however, the difference was not significant (Fig. 5A). TGFβ1 expression did not change 1 h after endarterectomy and was twofold up-regulated 3 weeks after injury. MnTBAP exerted no effects on TGFβ1 gene expression (Fig. 5B). Vascular NAD(P)H oxidase (NOX-4) was up-regulated in the control CEA group, while superoxide dismutase-1 was down-regulated. The expression of both markers of oxidative stress remained unchanged in the CEA + MnTBAP group (Fig. 5C, D). Proto-oncogenes c-JUN and c-FOS were overexpressed 1 h after endothelial injury and decreased to the level of the sham group in 3 weeks. MnTBAP further decreased c-FOS expression, while those of c-JUN remained unchanged (Fig. 5E, F). Matrix-metalloproteinase-2 (MMP-2) significantly decreased 1 h as well as 3 weeks after endarterectomy, while MMP-9 was up-regulated at both time intervals. MnTBAP had no effects on the expression of both MMP genes (Fig. 5G, H).

**Discussion**

Reactive oxygen and nitrogen species play important role in neointima formation that leads to restenosis, which is supported by several previous works. However, few studies have addressed the antioxidant therapeutic approaches to block neointima formation, especially in in vivo models of surgical endarterectomy. In recent years, a rat CEA model has been established by our group. In this model, neointima formation is triggered by surgical arteriotomy, besides the commonly performed endothelial denudation. Our findings confirm higher nitro-oxidative stress in association with increased proliferation of vascular smooth muscle cells after CEA, as assessed by NT and PCNA immunostainings, TUNEL assay, lipid hydroperoxidase assay and RT-qPCR of NOX-4 and SOD-1 genes. SOD mimetic and peroxynitrite scavenger MnTBAP reduced neointima formation in rat carotid arteries, accompanied by lower

**Figure 3** Ratio (%) of TUNEL-positive (brown) nuclei in the medial (A) and neointimal (B) layer of the carotids. Representative photomicrographs of the TUNEL assay in the sham (C, n = 10), control CEA (D, n = 10) and treatment (E, n = 9) groups. *p < 0.05 vs. sham; †p < 0.05 vs. control CEA.

**Figure 4** Malondialdehyde (MDA) plasma concentrations in animals of the sham (n = 5), control CEA (n = 15) and CEA + MnTBAP (n = 13) groups. *p < 0.05 vs. sham; †p < 0.05 vs. control CEA.
Three weeks after CEA, almost 50% luminal stenosis was developed by the growing neointima. This is in line with the results of our previous work, where a comparable amount of neointimal hyperplasia could be observed in the control CEA group. Neointima/media area ratio is the most accurate way for morphometric analysis that considers small changes in vessel diameters caused by the surgery but that is independent of neointima formation. In neointima/media area ratio, we observed a threefold decrease in the treatment group.

As described in previous works, NO and superoxide anions generate peroxynitrite, which finally leads to reduced bioavailability of NO. In addition, endothelial damage also results in reduced NO formation due to impaired eNOS activity. Reduced NO-cyclic guanosine monophosphate (cGMP) signalling contributes to neointima formation, while different agents that activate this pathway have been shown to attenuate this process. The mechanism, however, is not yet completely understood; it is assumed that reduced migration/proliferation mediates the beneficial effects of increased NO-cGMP signalling. On the other hand, peroxynitrite — that is produced some weeks after injury, when NO output is expected to rise from regrowing endothelium — stimulates cell signalling pathways involved in proliferation. Our results show that endarterectomy-induced peroxynitrite formation was effectively reduced by MnTBAP, leading to decreased stenosis formation 3 weeks after endothelial injury. Peroxynitrite is able to directly damage lipids, proteins and DNA, and activate MMPs. Zhang et al. suggested the pathophysiological role of oxidative DNA damage in neointima formation. In our study, TUNEL, as a nonspecific assay, has been used to test the nitro-oxidative DNA damage as predominantly shown in vascular media and in the plasma- and reduced VSMC proliferation rate.

Figure 5  Effect of carotid endarterectomy and manganese(III)tetrakis(4-benzoic acid) porphyrin (MnTBAP) treatment on the relative expression of eNOS (A, endothelial nitric oxide synthase), TGF-β (B, transforming growth factor β1), NOX-4 (C, nicotinamide adenine dinucleotide phosphate oxidase 4), SOD-1 (D, superoxide dismutase-1), c-JUN (E, c-JUN proto-oncogene), c-FOS (F, c-FOS protooncogene), MMP-2 (G, matrix-metalloproteinase-2) and MMP-9 (H, matrix-metalloproteinase-9) mRNA expression in the carotid arteries of the sham (n = 5), acute injury (n = 4), control CEA (n = 5) and CEA + MnTBAP (n = 4) groups. In any case, β-actin as reference gene was used. *p < 0.05 vs. sham; #p < 0.05 vs. control CEA.
damage during neointima formation. In the control CEA group, excessive increase of TUNEL-positive medial cell count was observed, compared with sham, which significantly decreased in the CEA + MnTBAP group. The same tendency was observed in the neointima (the sham group was not evaluated here because it lacked the neointimal layer). In addition, we assessed lipid peroxidation by measuring MDA — the degradation product of polyunsaturated fatty acids hydroperoxides — levels in the blood plasma. Since this marker was three times higher in the control CEA group than in the sham group, we assume that locally produced ROS/RNS react with plasma lipids, resulting in increased MDA plasma levels. After treatment with MnTBAP, MDA decreased to the same level as measured in the sham group, suggesting an effective elimination of nitro-oxidative agents.

Interestingly, the lower proliferation rate, and the decreased oxidative and nitrosative stress (as assessed by immunohistochemistry) after MnTBAP treatment were highly significant in the media of the injured carotids, but in the neointimal layer, only tendencies with lower differences between the control CEA and CEA + MnTBAP groups could be shown. These results suggest that medial and neointimal cells in the injured arteries are differentially affected by nitro-oxidative stimuli or by the antioxidant treatment and may use different signalling pathways as suggested by other authors as well.9

Gene expression seems not to be affected by the antioxidant MnTBAP, which may suggest a post-translational effect of this drug, predominantly due to the decreased level of nitrosative and oxidative agents (e.g., decreased NT immunoreactivity, oxidative DNA damage or lower lipid peroxidation). Despite this, RT-qPCR provided some interesting data, comparable to those of previously published works. We observed a highly significant reduction in eNOS expression in the acute injury group that indicates a successful endothelial damage during CEA. Three weeks after surgery, eNOS expression reached the level of that of the sham group, confirming an effective re-endothelialisation of the injured arteries.18 Growth factors such as TGFβ1 are important cofactors in proliferating tissues, such as neointima, which is confirmed by our observation of the twofold elevation in TGFβ1 gene expression 3 weeks after surgery.24 We found a 1.6-fold up-regulation in vascular NOX-4 that is the main source of superoxide generation in the vascular wall after injury. Superoxide can stimulate cell proliferation as suggested by several former studies.14,25,26 Simultaneously, SOD-1 — primarily responsible for superoxide elimination in the vessel wall — was down-regulated in the control CEA group as compared with sham.27 The overproduced superoxide anions react with NO, resulting in peroxynitrite formation. Furthermore, we observed a rapid up-regulation of the proto-oncogenes c-Jun and c-Fos in the carotid arteries 1 h after endarterectomy. As described by several authors, activation of proto-oncogenes contributes to the very early phase of neointima formation; then, their level drops to baseline in the following stages of restenosis development.28,29 Indeed, we found c-Jun and c-Fos expression in control CEA group to be identical to those of the sham group. MMPs play a pivotal role in neointima formation, by degrading extracellular matrix components to enable VSMCs to migrate through the internal elastic lamina. In line with the findings of Webb et al., we found MMP-2 to be constitutively produced by normal uninjured (sham) arteries; its level fell in the early postoperative time (1 h) and increased afterwards.30 MMP-9 expression was barely detectable in the sham carotids, was up-regulated 1 h after injury and was still highly overexpressed at 3-week follow-up.

Limitations

The CEA model used in this study does not resemble the complex situation of a severely diseased atherosclerotic artery in a patient. However, the native rat carotid artery is an established model of intimal hyperplasia.9,13 Knockout mice models of atherosclerosis also lack direct comparability with the human situation. Further studies with animal models of atherosclerosis are needed.

Conclusions

This study provides new data for an antioxidant therapeutic approach of preventing neointimal hyperplasia in a rat CEA model. The SOD mimetic and peroxynitrite scavenger MnTBAP attenuated the stenosis formation by decreasing VSMC proliferation and reducing nitro-oxidative stress.

Conflict of Interest

None.

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Appendix

Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejvs.2010.03.024.

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