Tempol therapy attenuates medial smooth muscle cell apoptosis and neointima formation after balloon catheter injury in carotid artery of diabetic rats

D. K. Jagadeesha,1,2 Timothy E. Lindley,1 Jason DeLeon,1 Ram V. Sharma,1,2 Francis Miller,2,3,4 and Ramesh C. Bhalla1,2

1Department of Anatomy and Cell Biology, 2The Cardiovascular Center, 3Department of Internal Medicine, and 4Free Radical and Radiation Biology Program, Roy J. and Lucille A. Carver College of Medicine, The University of Iowa, Iowa City, Iowa

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Jagadeesha, D. K., Timothy E. Lindley, Jason DeLeon, Ram V. Sharma, Francis Miller, and Ramesh C. Bhalla. Tempol therapy attenuates medial smooth muscle cell apoptosis and neointima formation after balloon catheter injury in carotid artery of diabetic rats. Am J Physiol Heart Circ Physiol 289: H1047–H1053, 2005. First published April 15, 2005; doi:10.1152/ajpheart.01071.2004.—Accumulating data support the hypothesis that reactive oxygen species (ROS) play a critical role in the vascular complications observed in diabetes. However, the mechanisms of ROS-mediated vascular complications in diabetes are not clear. We tested the hypothesis that ROS-mediated increase in proapoptotic factor Bax expression leads to medial smooth muscle cell (SMC) apoptosis that is associated with neointima formation. We used a fructose-rich diet for 4 wk to model Type 2 diabetes in rats. SOD mimetic membrane-permeable 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol, 1 mM) was administered in drinking water to scavenge superoxide starting 1 day before surgery and continued during the duration of the experiment. Vascular injury resulted in a significant increase in medial SMC apoptosis that was associated with neointima formation. The number of medial SMC positive for Bax immunostaining significantly increased in injured arteries compared with uninjured arteries. Superoxide scavenging by Tempol treatment inhibited both the Bax-positive index as well as the apoptotic index of medial SMC in response to vascular injury. Tempol treatment inhibited apoptotic loss of medial SMC, thus increasing their density in the injured arteries. These alterations in the media were associated with a marked decrease in neointima formation in injured arteries. We conclude that Bax expression may play an important role in vascular SMC apoptosis and, finally, that this regulatory mechanism is redox sensitive.

vascular smooth muscle; vascular injury; Type 2 diabetes; vascular smooth muscle apoptosis; superoxides; Bax protein

TYPE 2 DIABETES is the most critical risk factor for all patients with coronary artery disease. The pathophysiology is associated with increased vascular proliferative response in diabetic patients and is characterized by late morbidity and mortality (21). Functional derangement of vascular smooth muscle cells (SMCs) as a manifestation of cardiovascular complications of diabetes has been observed (1, 32, 38). During the past few years, convincing evidence has emerged indicating that increased oxidative stress plays a significant role in the pathogenesis of vascular complications associated with type 2 diabetes (37). Indeed, Type 2 diabetes is characterized by increased oxidative stress (39). It is postulated that glucose is prone to oxidation to generate reactive oxygen species (ROS), which may cause increased peroxidation (43).

The hyperglycemia and increased fatty acids in the bloodstream observed in Type 2 diabetes can cause oxidative stress and damage to the mitochondrial membrane (7). Systemic antioxidant therapy with probucol has been shown to reduce intima formation and restenosis after vascular injury (27). However, a limitation of most of the antioxidants used so far, including probucol, has been the need to administer the drug for 2 to 4 wk before angioplasty to demonstrate its beneficial effect in reducing neointima formation (9, 27). Therefore, we examined the effects of the SOD mimetic membrane-permeable 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol, Sigma) administered 1 day before surgery and continued during the entire experimental period. This low-molecular-weight cyclic nitroxide acts in a catalytic fashion and is regenerated in the process of quenching superoxide. It has been shown to reduce oxidative stress-mediated injury during ischemia-reperfusion (10) and inflammation (18).

Emerging evidence suggests that increased ROS production leads to vascular SMC apoptosis following injury (24). Apoptosis is a highly regulated cell death process characterized by cellular and nuclear fragmentation. SMC apoptosis associated with significant intimal hyperplasia has been observed in several models of vascular injury (13, 15, 25, 31, 36). In animal models of balloon vascular injury, marked superoxide production and massive apoptosis of medial SMCs were observed within the first hour, resulting in loss of cell density in the media layer (30). Apoptosis of medial SMCs precedes neointima formation, suggesting that cell death of medial SMCs may trigger cell migration and proliferation to form neointima (25).

Although signal transduction pathways involved in redox-linked vascular SMC apoptosis are poorly defined, recent evidence suggests that Bcl family genes may play a crucial role. Proapoptotic Bcl family members such as Bax and Bak cause induction of apoptosis, disrupting outer mitochondrial membrane integrity (19, 42). On the other hand, antiapoptotic members such as Bcl-2 and Bcl-xL prevent apoptosis by heterodimerization with Bax and Bak, thus preventing outer mitochondrial membrane disruption (3, 44). Given the link between increased ROS and medial SMC apoptosis, it is important to develop strategies to protect against ROS-mediated cell death in arteries.
apoptosis as well as emerging evidence that Bax may play a role in apoptosis, we hypothesized that antioxidant therapy with Tempol would inhibit medial SMC apoptosis and neointima formation in balloon-injured rat carotid arteries by inhibiting Bax induction. To test this hypothesis, we examined the effects of antioxidant Tempol in balloon injury-induced expression of Bax on medial SMC apoptosis and neointima formation in rats. These findings provide further evidence for the role of ROS in the pathogenesis of neointima formation after vascular injury. These data suggest that inhibition of redox-sensitive signaling pathways by Tempol may represent an important therapeutic approach in the treatment of vascular proliferative diseases in diabetes.

**MATERIALS AND METHODS**

*Animals.* All experiments were performed within the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Animal Care and Use Committee at The University of Iowa. Adult male Sprague-Dawley rats weighing 300–325 g were used for this study. Animals were housed in a room with a 12:12-h light-dark cycle and an ambient temperature of 22°C. Animals were fed a fructose-rich diet (60%; Harlan) for 30 days before the surgery to induce type 2 diabetes as has been described earlier (14, 16).

*Plasma blood glucose and insulin estimation.* Blood plasma was collected from nonfasting animals by eye puncture to measure plasma insulin using the ultrasensitive rat insulin ELISA kit (Crystal Chem). Blood glucose was measured with a standardized portable glucometer (ACCU-Chek; Roche Diagnostics).

*Surgical procedure and drug treatment protocol.* Catheter balloon injury of the left common carotid artery was performed essentially as described previously (8). In brief, male Sprague-Dawley rats weighing 300–325 g were preanesthetized with halothane (Halocarbon, River Edge, NJ) and then anesthetized by intraperitoneal injection of ketamine (10 mg/kg). To produce balloon injury, we exposed the bifurcation of the left common carotid artery through a midline incision and temporarily ligated the left common and internal carotid arteries. A 2-F embolectomy balloon catheter (Baxter Edwards Life-sciences, Irvine, CA) was introduced into the isolated common carotid artery through an arteriotomy site in the external carotid artery and advanced to the distal ligation of the common carotid artery. The balloon was inflated with air and drawn toward the arteriotomy site three times to produce a distending, deendothelializing injury. After the catheter was withdrawn, the proximal end of the external carotid artery was ligated, and blood flow was restored. The surgical incision was closed, and the rats were allowed to recover from anesthesia. The right uninjured artery was used as control tissue. Although investigators in our laboratory (8) and others (5, 23) have used uninjured contralateral artery as a control, it should be kept in mind that a sham procedure in which the control artery is stretched and handled without injury to the endothelium may represent a better control tissue. For one group of animals, Tempol (1 mM), freshly prepared, was started 1 day before surgery and was continued throughout the experiment. For the assessment of cellularity, paraffin-embedded arterial sections were stained with H&E, and the area was calculated using a computerized program. The cellularity in the medial layer was expressed as the Hoechst index, defined as the positive nuclear staining averaged for one fixed area (0.1 mm²) in the medial layer. Histological samples were randomly examined between treatment groups by an independent investigator who was blind to the treatment protocol.

*Superoxide measurement by enhanced chemiluminescence.* The carotid arteries of rats fed a normal diet were immediately frozen in 4% paraformaldehyde for 48 h before paraffin embedding.

*Histomorphometric measurement.* For morphometric analysis, paraffin-embedded sections were used for each of the arterial specimens. Cross sections (6 µm) were obtained from uninjured and injured carotid arteries from control and Tempol-treated groups and stained with hematoxylin and eosin. The images were digitized using an Olympus BX 51 microscope (Leeds Precision Instruments), and photographs were captured using a digital camera. Morphometric analysis of these arterial sections was performed as described previously (8). For each arterial cross section, the intimal and medial areas were measured and the ratio of intima to media area was calculated. Morphometric analysis was performed using NIH Image analysis software.

**In situ labeling of fragmented DNA.** Formalin-fixed and paraffin-embedded tissue sections were deparaffinized, rehydrated, and treated with proteinase K (20 µg/ml), and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. In situ end labeling of apoptotic cells was performed as described in the protocol provided by the manufacturers of the in situ apoptosis detection kit (Trevigen). In brief, after quenching, end labeling with digoxigenin-DUTP by TdT enzyme in buffer was carried out for 1 h at 37°C in a humidifying chamber. After treatment with stop/wash buffer, sections were incubated with anti-digoxigenin antibody-peroxidase conjugate, rinsed, and stained with diaminobenzidine tetrahydrochloride. Negative controls were incubated with PBS instead of TdT enzyme, and positive controls were treated with DNase 1. Sections were counterstained with 1% methyl green. Apoptotic cells were counted in the medial layer of a cross section of the artery, and apoptotic index was calculated as the number of labeled nuclei per the number of total nuclei × 100. After DNA nick-end labeling (TUNEL), tissue sections in which apoptosis was detected in the SMCs were identified by immunostaining with an alkaline phosphatase-conjugated monoclonal antibody to a-smooth muscle actin.

*Immunohistochemistry for activated caspase-3 and Bax.* To compare and extend the responses estimated by the TUNEL assay, we employed caspase-3 immunohistochemistry for the detection of apoptosis of vascular SMCs by using antibodies that specifically recognize only the large subunit of activated caspase-3, as described previously (5, 11). Tissue sections (6 µm) were mounted on poly-L-lysine-coated glass slides. After deparaffinization and rehydration, sections were blocked for endogenous peroxidase activity with 3% hydrogen peroxide in methanol. Sections were then incubated in an anti-caspase-3 primary antibody that specifically recognizes the large fragment (17 kDa) of activated caspase-3 (1:50; Calbiochem, San Diego, CA) and a rabbit polyclonal anti-mouse Bax-α antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) separately overnight at 4°C. After washing, the slides were incubated with biotinylated secondary antibody for 30 min at room temperature. After two washes in PBS, sections were incubated in streptavidin-horseradish peroxidase (Vector Laboratories), washed two more times in PBS, and then incubated for 5 min in the dark with diaminobenzidine. Sections were counterstained using 1% methyl green. Data are expressed as the number of Bax-positive and activated caspase-3-positive cells per the total number of nuclei × 100.

*Estimation of SMC density in media.* For the assessment of cellularity, paraffin-embedded arterial sections were stained with H&E, and the area was calculated using a computerized program. The cellularity in the medial layer was expressed as the Hoechst index, defined as the positive nuclear staining averaged for one fixed area (0.1 mm²) in the medial layer. Histological samples were randomly examined between treatment groups by an independent investigator who was blind to the treatment protocol.
OCT on dry ice. Frozen sections (40 μm) were incubated in 1 ml of PBS containing 5 μM lucigenin (bis-N-methylacridinium nitrate) for 2 min in the dark. The sample was then placed in a luminometer (Monolight 2010) for measurement of relative light units emitted every 30 s for 4 min. The values obtained between 2 and 4 min were averaged to determine basal emission levels. NADPH (50 μM; Sigma) was then added, and the relative light units emitted were again recorded every 30 s for 4 min. The values obtained between 2 and 4 min were averaged, and the basal emission level was subtracted.

Statistical analysis. Data are expressed as means ± SE or means (SD), as indicated. The statistical comparison was performed using one-way analysis of variance (ANOVA) followed by the Bonferroni test. A value of \( P < 0.05 \) was considered significant.

RESULTS

Fructose-rich diet increases plasma glucose and insulin levels. The effects of fructose-rich diet on plasma glucose and insulin concentration are shown in Fig. 1. The glucose levels were significantly higher in animals fed fructose-rich diet compared with animals fed normal chow [fructose, 177 (50) vs. normal, 136 (16) mg/dl, mean (SD); \( P < 0.05, n = 18 \)]. Although the insulin levels were higher in animals fed fructose-rich diet compared with animals fed normal chow diet, they did not reach statistical significance [fructose, 4.8 (1.4) vs. normal, 3.9 (1.8) ng/dl, mean (SD); \( n = 15 \)]. It is possible that the variability in glucose and insulin levels was increased because animals were not fasted before blood samples were collected.

Tempol inhibits vascular injury-induced increases in apoptosis of medial SMC. Balloon distension and endothelium denudation injury resulted in an increase in medial SMC apoptosis characterized by shrinkage and nuclear condensation visualized by the TUNEL assay that stains nuclei containing nicked DNA, a characteristic signifying the early stages of apoptotic cell death. Quantification of the apoptotic SMCs in the medial layer showed a significant increase in apoptotic index in the injured artery compared with the uninjured artery of the diabetic rats (injured, 12.9 ± 0.44 vs. uninjured, 2.5 ± 0.18%; \( P < 0.05, n = 5 \)) (Fig. 2A). Tempol treatment significantly inhibited apoptosis of the medial SMCs of injured arteries (injured, 12.9 ± 0.4 vs. injured + Tempol, 5.2 ± 0.3%; \( P < 0.05, n = 5 \)) (Fig. 2A).

In the initial experiments, we observed that Tempol treatment did not affect the TUNEL-positive medial SMC indexes in uninjured arteries (data not shown). In addition to TUNEL assay, we employed immunohistochemistry to detect apoptosis of SMCs by using antibodies that specifically recognize the large subunit of activated caspase-3. Activated caspase-3-positive apoptotic cells were quantified, and apoptotic indexes (apoptotic cells/total cells × 100) were calculated. The activated caspase-3-positive apoptotic index was significantly increased in the injured arteries compared with the uninjured control arteries (uninjured, 3.13 ± 0.42 vs. injured, 18.8 ± 0.45%; \( P < 0.05, n = 5 \)) (Fig. 2B). Tempol treatment significantly reduced the apoptotic indexes in the injured arteries (injured, 18.8 ± 0.45 vs. injured + Tempol, 7.51 ± 0.62%; \( P < 0.05, n = 5 \)). These data demonstrate that inhibition of oxidative stress by Tempol treatment attenuated medial SMC apoptosis and suggest that signaling pathways associated with ROS are involved in medial SMC apoptosis induced by vascular injury.
Antioxidant Tempol inhibits ROS generation in injured arteries. To verify the effectiveness of oral administration of Tempol to attenuate the elevation in superoxide levels in the injured vessel, we estimated superoxide levels by using the lucigenin-enhanced chemiluminescence method. Injury produced a significant increase in ROS levels in carotid arteries, and Tempol treatment prevented this increase (Fig. 5). These data show an increase in superoxide levels following vascular injury, and Tempol treatment can effectively inhibit superoxide levels.

Tempol treatment attenuates neointima formation in balloon-injured rat carotid artery. Two weeks after injury, a concentric neointimal lesion was found at the site of injury (Fig. 6). The neointima consisted mainly of SMCs as determined by immunostaining for α-SMC actin. Quantitative analysis of the intima-to-media ratio of pressure-fixed vessel cross sections showed that the ratio was significantly increased (*P < 0.05) in injured compared with uninjured arteries (uninjured, 0.12 ± 0.008 vs. injured, 0.73 ± 0.04), and Tempol treatment significantly reduced the neointima-to-media ratio in injured arteries of diabetic rats (injured, 0.73 ± 0.04 vs. injured + Tempol, 0.22 ± 0.04; *P < 0.05, n = 5).

Discussion

The possible targets of ROS in SMCs and their relation to neointima formation in diabetes mellitus were discussed in a recent review (37). However, the mechanism by which ROS controls neointima formation remains unclear. Our studies demonstrate that the membrane-permeable SOD mimetic Tempol is an effective pharmacological compound in inhibiting injury-induced superoxide levels, SMC apoptosis, and neointima formation at the site of injury. We propose that after vascular injury, ROS play a central role in the vascular remodeling process, and we further speculate that antioxidant therapy may retard injury-induced remodeling of the vessel wall in diabetes.

Vascular SMC apoptosis plays a critical role in the pathogenesis of a number of cardiovascular diseases (40). The occurrence of SMC apoptosis after balloon angioplasty has been demonstrated in both animal and human models (4, 6, 13, 15, 20, 25). Previous studies with the balloon catheter injury of
rat carotid artery have demonstrated extensive apoptosis of medial SMCs, and subsequent neointima formation reaches maximum by day 14 postsurgery (30). Therefore, we have examined in this study the effects of vascular injury at 14 days postinjury, by which time neointima formation is well developed. It has been postulated that loss of cellularity due to apoptosis of medial SMCs may play a critical role in triggering a signaling pathway that would allow the remaining SMCs to reenter the cell cycle for the repopulation of media and subsequent intima formation due to migration and proliferation of medial SMCs (31, 41). However, the mechanisms underlying vascular SMC apoptosis in response to vascular injury are not completely elucidated.

To begin to test the hypothesis that increased ROS play a role in medial SMC apoptosis, we used antioxidant Tempol and quantified apoptotic cells in the medial layer by TUNEL assay and by immunohistochemistry for activated caspase-3 (Fig. 2). We found that 2 wk after vascular injury, there was a significant increase in the apoptotic indexes of medial SMCs in the injured arteries compared with the uninjured arteries. Tempol treatment significantly inhibited \( P < 0.05 \) apoptosis of medial SMCs in the injured arteries. These findings are consistent with a number of reports showing that medial SMC apoptosis plays a significant role in intimal hyperplasia in several models of vascular injury and in atherosclerotic lesions (13, 15, 25, 31, 36). Although a relationship between medial SMC apoptosis and neointima formation has been postulated, a link between medial SMC apoptosis and intimal hyperplasia has been lacking. Next, we examined whether inhibition of medial SMC apoptosis would increase medial SMC density. Our results demonstrate that Tempol treatment prevents the loss of medial SMC density (Fig. 3), which is associated with the inhibition of neointima formation (Figs. 6 and 7). How Tempol increases medial SMC density is not clear at this time. It is possible that Tempol may inhibit the initial loss of medial SMC in response to injury. Alternatively, Tempol may regulate proliferation of the remaining medial SMCs. Further studies are needed to examine the effect of Tempol on the dynamics of medial SMC apoptosis and proliferation at different time intervals immediately following injury. Although there was an association between the extent of medial SMC apoptosis and the extent of neointima formation, we have not demonstrated a cause-and-effect relationship between these two events in this study. It is likely that Tempol may be inhibiting neointima formation by mechanisms independent of those inhibiting SMC apoptosis. ROS has been shown to increase SMC growth and migration (11, 26, 35). It is likely that Tempol may be inhibiting neointima formation by inhibiting SMC migration and proliferation. Future studies using alternate methods to inhibit medial SMC apoptosis and testing its effects on neointima formation are required to further elucidate the role of medial SMC apoptosis in neointima formation.

The loss of medial SMCs due to apoptosis coincides with intimal hyperplasia. Thus the intima-to-media ratio in the injured rats was \( 0.73 \pm 0.04 \) and was reduced to \( 0.22 \pm 0.04 \) in Tempol-treated animals. Morphometric examination 2 wk after vascular injury showed that inhibition of medial SMC apoptosis by Tempol treatment of diabetic rats correlated with reduced neointima formation compared with untreated rats (Fig. 7). These data confirm the previous observations showing that antioxidants inhibit neointima formation after injury (31). Furthermore, our data suggest that the medial SMC apoptotic index and a decreased medial SMC density both have a bearing on the extent of neointima formation. We hypothesize that apoptotic cells send a message to healthy cells to differentiate, leading to cell migration and proliferation. Thus antioxidant Tempol therapy, by maintaining medial SMC density after vascular injury, may minimize the stimulus for healthy cells to

![Image](http://http://ajpheart.physiology.org/10.1152/ajpheart.00589.2004)
migrate and proliferate. Further studies are needed to determine the long-term beneficial effects of antioxidant treatment on intimal hyperplasia.

Previous studies in other tissues have indicated that apoptosis is regulated by a balance between the expression of proapoptotic mediators such as Bax and the antiapoptotic mediators Bcl-x and Bcl-2 (34). However, studies in vascular lesions have demonstrated that Bcl-x is expressed primarily in neointima cells, and the downregulation of Bcl-x by antisense oligonucleotides does not affect the apoptosis of the medial SMCs or their cellularity in injured arteries (33). To elucidate potential mechanisms of ROS-mediated apoptosis, we studied expression of Bax, a proapoptotic protein, in medial SMCs by using immunohistochemistry. The proportion of Bax-positive SMCs in the medial layer was markedly increased at 2 wk postinjury, concomitant with an increase in medial SMC apoptosis, suggesting that modulation of this protein may play a crucial role in apoptotic response to injury. These findings are in agreement with the role of Bax in the initial phase of apoptosis (22) and are consistent with the observations that adenovirus-mediated overexpression of Bax induces apoptosis in many cells (28, 29).

We have observed that the proportion of Bax-positive cells in the medial layer is attenuated by Tempol treatment, with a concomitant inhibition of apoptotic index. These findings suggest that increased ROS generation mediates its effects, at least in part, by increasing Bax expression, resulting in medial SMC apoptosis. Our findings are consistent with previous studies showing that oxidative stress causes apoptosis in vascular SMCs due to an increase in the ratio of Bax to Bcl-2 in the blood vessel wall as estimated using biochemical procedures (2). However, the immunohistochemical techniques used in our study permitted us to estimate the number of Bax-positive cells in the media directly. A number of biochemical functions have been defined for Bax that support its suggested role as a proapoptotic protein, including induction of cytochrome c release from mitochondria (17). The present study has provided evidence that the inhibition of Bax by antioxidant therapy can inhibit medial SMC apoptosis, resulting in the inhibition of intima formation.

Investigators in our laboratory (12) have previously demonstrated that matrix metalloproteinase (MMP)-9 expression is regulated by the intracellular redox state of vascular SMCs. Therefore, it is conceivable that injury-induced ROS generation may increase MMP-9 induction, and perturbations in cell-matrix interactions may result in increased vascular SMC apoptosis. The inhibition of oxidative stress by Tempol treatment will attenuate this cellular pathway. Thus it will be of interest to determine the induction of MMP-9 following injury and the effect of ROS inhibition on this signaling pathway. We postulate that inhibition of MMP-9 induction at the time of injury may prevent early inhibition of medial SMC apoptosis, leading to subsequent decrease in neointimal hyperplasia.

In conclusion, in vivo treatment of diabetic rats with the antioxidant agent Tempol inhibits the induction in apoptosis of medial SMCs and prevents the loss of medial SMC density with its concomitant inhibition of neointima formation. These data suggest that antioxidant therapy with Tempol may prove beneficial to attenuate injury-induced vascular wall remodeling in diabetes.

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