Oxidative Stress Promotes Endothelial Cell Apoptosis and Loss of Microvessels in the Spontaneously Hypertensive Rats

Nobuhiko Kobayashi, Frank A. DeLano, Geert W. Schmid-Schönbein

Objective—Endothelial cell apoptosis caused by oxidative stress may lead to the loss of microvessels (rarefaction) in hypertension. We examine here the effects of antioxidants on cell apoptosis and rarefaction.

Methods and Results—The juvenile spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats were treated with superoxide scavengers, Tempol or Tiron, during growth. After the treatment, oxidative stress status, endothelial cell apoptosis rate, and microvessel length density in skeletal muscle and mesentery were evaluated in comparison with age-matched controls. Untreated 16-week-old SHR had higher oxidative stress ($P<0.01$) and cell apoptosis rate ($P<0.05$) and lower microvessel length density ($371 \pm 17 \text{ mm/mm}^3$ [$P<0.01$]) compared with age-matched WKY rats ($435 \pm 15 \text{ mm/mm}^3$). In the SHR, but not in WKY rats, systemically applied antioxidants attenuated oxidative stress and cell apoptosis rate ($P<0.05$ versus untreated controls) and prevented the loss of microvessels ($411 \pm 15 \text{ mm/mm}^3$ for Tempol [$P<0.01$ versus untreated control] and $399 \pm 17 \text{ mm/mm}^3$ for Tiron [$P<0.05$]).

Conclusions—Antioxidant treatment with cell-permeable superoxide scavengers inhibits endothelial cell apoptosis and prevents microvessel rarefaction in the SHR during growth. (Arterioscler Thromb Vasc Biol. 2005;25:0-0.)

Key Words: arterial hypertension □ capillary □ endothelial cell apoptosis □ microvessels □ oxidative stress □ rarefaction □ superoxide

A number of hypertensive animal models and patients with essential hypertension exhibit a reduction in microvessel length density in tissues such as skeletal muscle, skin, conjunctiva, and myocardium. This phenomenon, designated as structural or anatomic rarefaction, leads to an increase in peripheral vascular resistance and localized reduction in oxygen delivery to the tissue. Substantial evidence indicates that microvascular rarefaction is associated with the pathogenesis of hypertension and may be accompanied by parenchymal cell death. The disappearance of microvessels in hypertensive subjects may be a secondary event after blood pressure elevation. However, recent research in man demonstrates microvascular rarefaction in pre-established stage of hypertensive subjects who still maintain near normal blood pressure. In addition, the loss of microvessels is implicated in development of nonhypertensive pathologic conditions including diabetic organ failure. These findings seem to suggest that factors other than elevated arterial pressure may promote the disappearance of microvessels. Accumulating evidence indicates that the increase in endothelial cell apoptosis in microvessels may cause rarefaction in hypertensive subjects, although the mechanism of enhanced cell apoptosis is still undergoing investigation.

We hypothesize that oxidative stress promotes endothelial cell apoptosis in microvessels and induces rarefaction in the spontaneously hypertensive rats (SHR). In the SHR, microvascular endothelium is exposed to enhanced oxidative stress due to an increase in xanthine-oxidase and NADPH-oxidase activity and/or a reduction in superoxide-dismutase activity. Reactive oxygen species (ROS) modulate diverse functions and exert secondary effects on microvessels, eg, an inhibition of endothelium-dependent vasodilation and a promotion of leukocyte adhesion to endothelium. In vitro exposure to oxidative stress reportedly promotes apoptotic cell death in bovine and human aortic endothelial cells. However, it is still uncertain whether ROS enhance endothelial cell apoptosis in hypertensive animals. Thus, the present study was designed to investigate whether chronic antioxidant treatment reduces endothelial cell apoptosis in microvessels and prevents structural rarefaction in the SHR.

Materials and Methods

The animal protocols were approved by the Animal Subject Committee of the University of California San Diego and conformed to the Guide for the Care and Use of Laboratory Animals by the United States National Institutes of Health (NIH Publication No. 85-23, 1996). Four-week-old male SHR and their normotensive controls, the Wistar-Kyoto (WKY) rats, were purchased from Charles River Breeding Laboratories (Wilmington, Mass.). The animals underwent systemic antioxidant treatment with cell-permeable superoxide scavengers, Tempol (4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl; Sigma) or Tiron (1,2-dihydroxybenzene-

Original received January 11, 2005; final version accepted July 11, 2005.
From the Department of Bioengineering, Whitaker Institute of Biomedical Engineering, University of California, San Diego, La Jolla, Calif.
Correspondence to Dr Geert W. Schmid-Schönbein, Department of Bioengineering, Whitaker Institute of Biomedical Engineering, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0412. E-mail gwss@bioeng.ucsd.edu

Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

DOI: 10.1161/01.ATV.0000178993.13222.f2
3,5-disulphonic acid disodium salt (Sigma) dissolved in drinking water (1 or 10 mmol/L respectively), whereas age-matched controls were maintained with regular water. After the administration of Tempol (4 [n=6] or 12 [n=6] weeks from 4 weeks of age) or Tiron (8 weeks from 8 weeks of age [n=6]), we examined systemic arterial pressure, microvessel length density in skeletal muscle, and oxidative stress status and endothelial cell apoptosis rate in muscle and mesentery microvessels. In selected untreated SHRs (16-week-old), the effects of topical antioxidants on microvascular endothelial cells were also evaluated by superfusion of skeletal muscle. Microscopic images of microvessels in cremaster muscle and mesentery (20 to 30 randomly selected fields in each experiment) were digitally stored and evaluated by off-line analysis. Oxidative stress and endothelial cell apoptosis in microvessels were evaluated in arterioles, capillaries, and venules separately, because biological characters such as ROS-generating or scavenging activity seem to differ among different vessel types.20–22

**Systemic Arterial Pressure**

Subsequent to intramuscular injection of sedative agents (10 mg/kg body weight of Xylazine and Nembutal), we cannulated femoral artery and vein (PE-50; Clay-Adams, Parsippany, NJ). Twenty minutes later, arterial pressure was recorded over 15 minutes (MacLab system; ADInstruments Pty Ltd, Colorado Springs, Colo) and mean values were computed.

**Oxidative Stress and Endothelial Cell Apoptosis in Muscular Microvessels**

Measuring systemic arterial pressure, we prepared cremaster muscle for intravital microscopic observation under superfusion with Krebs-Henseleit (K-H) solution.23 Tempol (0.5 mmol/L) or Tiron (5 mmol/L) was dissolved in K-H solution only in the experiments that aimed to investigate local effects of antioxidants.

Oxidative stress status in microvascular endothelial cells was examined by microfluorography with hydroethidine (Molecular Probes, Inc, Eugene, Ore), in which the cells exposed to ROS were labeled with fluorescent ethidium bromide (EB) (Figure 1A; please see http://atvb.ahajournals.org).

The incidence of endothelial cell apoptosis in muscular microvessels was evaluated by co-labeling with DNA-binding fluorescent molecules, propidium iodide (PI) (Sigma) and YO-PRO-1 (YP) (Molecular Probes, Inc). Early-stage apoptotic cells are positive only for YP, whereas necrotic or end-stage apoptotic cells are labeled with both fluorescent dyes. Early-stage apoptotic cells were labeled with fluorescent ethidium bromide (EB) (Figure 1A; please see http://atvb.ahajournals.org).

**Oxidative Stress and Endothelial Cell Apoptosis in Mesentery Microvessels**

After storing microscopic images of muscular microvessels, we exteriorized rat mesentery and superfused it for 1.5 hours with tetraniotroblue tetrazolium chloride (TNBT) (Sigma) dissolved in phosphate-buffered saline (2 mg/mL). Because superoxide converts light yellow TNBT into dark blue formazan (Figure 2A), the logarithmic value of the ratio between the maximum optical density on microvessels and the minimum density in avascular parts of the mesentery (TNBT light absorption) is considered to indicate oxidative stress status in microvessels (please see http://atvb.ahajournals.org).25–28

Cell apoptosis levels in mesenteric microvessels was examined by TdT-mediated dUTP-biotin nick end labeling (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling [TUNEL]) according to instructions (VasoTACS; Trevigen, Inc, Gaithersburg, Md).22 The applicability of TUNEL labeling to whole-mount mesentery was confirmed by the staining of sample tissue pretreated with DNase, in which the TUNEL assay labeled most microvascular endothelial cells.
Microvessel Length Density

The cremaster muscle was excised and labeled with fluorescein isothiocyanate-conjugated Bandeiraea simplicifolia-I lectin dissolved in phosphate-buffered saline (200 μg/mL, Sigma), which selectively bonded to small vessels with a diameter <20 μm. Using a confocal microscope (Bio-Rad MRC-1024UV), we obtained stacked fluorescent images composed of 20 consecutive sections over 50-μm thickness (please see http://atvb.ahajournals.org) and examined microvessel length density, ie, the total vessel length per unit tissue volume, in the same manner as reported previously.21,30

Statistical Analysis

Blood pressure records and microscopic images were analyzed by an operator blinded in regard to the group of rat undergoing examination. Comparisons among age-matched rat groups were made by 1-way ANOVA followed by Fisher’s PLSD test. Two-way repeated measure ANOVA was applied to the time course analysis of apoptotic cell counts in muscular microvessels. Age-associated change in oxidative stress status and cell apoptosis rate was examined by Student t test between the younger and older rats. Differences were considered statistically significant for P<0.05. All measurements are presented as mean±SD.

Results

Systemic Arterial Pressure

Controls

Systemic hypertension developed in untreated SHR (n=6) as they matured, whereas WKY rats (n=6) presented constant arterial pressure during growth (Table). The difference between 2 types of animals was statistically significant at 16 weeks of age (P<0.01; Table).

Antioxidant Treatment

Oral intake of Tempol (n=6) or Tiron (n=6) partially prevented arterial pressure increase in the SHR during growth (P<0.05; Table) but did not elicit significant blood pressure shift in WKY rats (Table).

Muscle superfusion with Tempol or Tiron dissolved in K-H solution did not reduce systemic blood pressure in 16-week-old SHR during intravital microscopic observation. Mean arterial pressure before and after microfluorography was 138±19 and 142±24 mm Hg for pure K-H solution (n=5), 134±9 and 140±17 mm Hg for Tempol (n=5), and 132±11 and 138±13 mm Hg for Tiron solution (n=5).

Oxidative Stress Status in Microvessels: Hydroethidine Fluorography in Skeletal Muscle

Controls

In older WKY and SHR, a larger number of endothelial cells were labeled with EB compared with younger animals (P<0.05; Figure 1). EB-positive cell counts were higher in the SHR than in age-matched WKY rats (P<0.01; Figure 1).

Antioxidant Treatment

Orally applied Tempol or Tiron suppressed the appearance of EB-positive endothelial cells in the SHR (P<0.05; Figure 1B), whereas the antioxidants did not affect oxidative stress.
Apoptosis and to allow entrance of PI molecules. This may indicate that cells committed to apoptotic cell death process require longer time to reach end stage of apoptosis and to allow entrance of PI molecules.

Controls
The number of apoptotic endothelial cells per millimeter of vessel length was higher in untreated SHR than in WKY rats (P<0.01; Figure 3). Age-related difference in apoptotic cell counts was not significant both in WKY and in the SHR (Figure 3).

Antioxidant Treatment
Orally applied Tempol or Tiron partially prevented endothelial cell apoptosis in the SHR (P<0.01; Figure 3) but much less so in WKY rats (Figure 3).

Cell apoptosis in 16-week-old SHR was also suppressed by topical antioxidants. The 4-hour increase in apoptotic endothelial cell counts was: 0.20±0.12 in arterioles, 0.29±0.10 in capillaries, and 0.21±0.11 in venules (n=5, P<0.05 versus untreated control) or Tiron (0.11±0.15 cells/mm vessel length in arterioles, 0.13±0.09 in capillaries, and 0.19±0.17 in venules (n=5, P<0.05 versus untreated control)).

TNBT Labeling of Mesentery
Controls
The optical density in avascular mesentery sectors was equally low in all groups (Figure 2A). TNBT light absorption, an index of ROS activity in microvessels, was increased both in WKY and in the SHR as they matured (P<0.05; Figure 2B). Untreated SHR showed higher optical density compared with age-matched WKY rats (P<0.01; Figure 2).

Antioxidant Treatment
Systemically applied Tempol or Tiron reduced TNBT light absorption in the SHR (P<0.05; Figure 2), whereas the optical density in WKY rats was not affected by antioxidants (Figure 2).

Microvascular Endothelial Cell Apoptosis: Nuclear Labeling With YP and PI in Muscular Microvessels
Apoptotic endothelial cells, which emitted only YP fluorescence (please see http://atvb.ahajournals.org), were increased with longer exposure time (P<0.01; Figure 3), and most of them remained PI negative 4 hours after muscle exteriorization. This may indicate that cells committed to apoptotic cell death process require longer time to reach end stage of apoptosis and to allow entrance of PI molecules.

Controls
Untreated SHR had a larger number of TUNEL-positive microvessels compared with age-matched WKY rats (P<0.01; Figure 4B). The difference between younger and

### Table: Age, Body Weight, Arterial Blood Pressure, and Heart Rate

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Body Weight (g)</th>
<th>Arterial Blood Pressure (mm Hg)</th>
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<tr>
<td>n</td>
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<td>Mean</td>
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**Younger animals**

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<td>Wky</td>
<td>8.6±1.3</td>
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<td>117±18</td>
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<td>8.4±0.6</td>
<td>217±21</td>
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**Older animals**

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<td>SHR</td>
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The findings after systemic antioxidant treatment with Tempol (4 or 12 weeks from 4 weeks of age) or Tiron (8 weeks from 8 weeks of age). WKY indicates Wistar–Kyoto rats; SHR, spontaneously hypertensive rats.

*P<0.05, **P<0.01 vs same type of younger animals; †P<0.05, ‡P<0.01 vs other type of age-matched animals.

in WKY rats (Figure 1B). Higher oxidative stress in the SHR (16-week-old) was also attenuated by topical Tempol (0.72±0.12 cells/mm vessel length in arterioles, 0.62±0.10 in capillaries, and 0.84±0.11 in venules [n=5, P<0.05 versus untreated control]) or Tiron (0.81±0.15 cells/mm vessel length in arterioles, 0.57±0.09 in capillaries, and 0.91±0.17 in venules [n=5, P<0.05 versus untreated control]).

**TNBT Labeling of Mesentery**

**Controls**

The optical density in avascular mesentery sectors was equally low in all groups (Figure 2A). TNBT light absorption, an index of ROS activity in microvessels, was increased both in WKY and in the SHR as they matured (P<0.05; Figure 2B). Untreated SHR showed higher optical density compared with age-matched WKY rats (P<0.01; Figure 2).

**Antioxidant Treatment**

Systemically applied Tempol or Tiron reduced TNBT light absorption in the SHR (P<0.05; Figure 2), whereas the optical density in WKY rats was not affected by antioxidants (Figure 2).
older animals was significant only in arterioles in the SHR (P<0.05; Figure 4B).

**Antioxidant Treatment**

Systemically applied Tempol or Tiron reduced TUNEL-positive counts of all classes of microvessels in the SHR (P<0.05; Figure 4B) but not in WKY rats.

**Microvessel Length Density in Cremaster Muscle**

**Controls**

Untreated SHR showed lower vessel length density compared with age-matched WKY rats (411±14 versus 444±12 mm/mm³ at 8 weeks of age [n=6, P<0.01] and 371±17 versus 435±15 mm/mm³ at 16 weeks of age [n=6, P<0.01]; please see http://atvb.ahajournals.org).

**Antioxidant Treatment**

The loss of microvessels in the SHR during growth was partially prevented by systemic antioxidant treatment with Tempol (428±15 mm/mm³ at 8 weeks of age [n=6, P<0.05 versus untreated control] and 411±15 mm/mm³ at 16 weeks of age [n=6, P<0.01]) or Tiron (399±17 mm/mm³ at 16 weeks of age [n=6, P<0.05 versus untreated control]) (please see http://atvb.ahajournals.org).

**Discussion**

The present study demonstrates that cell-permeable antioxidants prevent microvascular endothelial cell apoptosis and loss of microvessels in the SHR during growth.

In line with recent reports,31–34 systemically applied Tempol or Tiron prevented arterial pressure increase in the growing SHR. The superoxide-dismutase mimetics may inhibit blood pressure elevation as a result of improved endothelium-dependent vasodilation, because superoxide anion reacts with nitric oxide and impairs nitric oxide-induced vasorelaxation.35,36 Our findings seem to raise another possibility that the antioxidants prevent loss of microvessels and consequently suppress the increase in systemic vascular resistance. Although it is still controversial whether mi-
crovessel rarefaction in hypertensive subjects is a cause or a result of blood pressure elevation, growing evidence indicates that the disappearance of microvessels precedes the development of systemic hypertension. The loss of microvessels observed in the younger SHR, in which arterial pressure has not been fully elevated yet, seems to support this notion.

We examined the incidence of microvascular endothelial cell apoptosis by the TUNEL method and by nuclear labeling in vivo with fluorescent life–death indicators. The dual staining with YP and PI has been repeatedly used in vitro, especially in flow cytometric analysis, but not in vivo. The PI molecule, a well-established cell death marker, enters necrotic or advanced-stage apoptotic cells, whereas YO-PRO-1 passes through cation channels in the plasma membrane, such as ATP-gated P2X7 receptor, which are activated in the early stage of cell apoptosis. These small molecules are suitable for detecting endothelial cell apoptosis in vivo, because they can easily penetrate connective tissue and diffuse into microvessels. The results of microfluorography and TUNEL labeling show that under conditions after surgical exposure, microvascular endothelial cells in the SHR are committed to an apoptotic process more easily than in WKY rats, and higher cell apoptosis rate in the SHR is attenuated by antioxidants. Recent research suggests that enhanced endothelial cell apoptosis promotes the disappearance of microvessels and leads to structural rarefaction in hypertensive subjects. It seems likely that the cell apoptosis inhibition by antioxidants has contributed to the prevention of microvessel rarefaction in the present study.

Treatment of the SHR with Tempol or Tiron leads to antiapoptotic effects on microvascular endothelial cells in addition to alleviation of the oxidative stress. The reaction was not observed in the WKY rats in which ROS activity was much lower than in age-matched SHR. These findings seem to suggest that inhibition of cell apoptosis by antioxidants could be the consequence of improved oxidative stress status in microvessels. The blood pressure reduction may be another possible mechanism by which Tempol or Tiron protects endothelial cells from apoptosis. However, several pieces of evidence point toward the ROS suppression as a mechanism of cell apoptosis inhibition for the following reasons. First, topical antioxidants prevented endothelial cell apoptosis in microvessels without affecting systemic arterial pressure. Second, in the younger SHR, systemic treatment with Tempol decreased endothelial cell apoptosis without a significant shift of blood pressure. Besides, cell apoptosis was suppressed not only in arterioles but also in capillaries and venules, vessels in which blood pressure is equal to normotensive animals. There is no evidence that the shift in arterial pressure has a significant effect on pressure in capillaries or venules. Although the precise mechanism by which oxidative stress promotes endothelial cell apoptosis in microvessels could not be elucidated in this study, evidence from in vitro systems suggest that several pathways, eg, an activation of JNK/p38 MAP kinase, changes in mitochondrial integrity, and an impairment of nitric oxide bioavailability, may mediate ROS-induced apoptosis in vascular
endothelial cells, each of which may act independent of arterial pressure.

In the SHR, overproduction and/or reduced dismutation of superoxide anion are considered to be a primary factor promoting oxidative stress in microvessels.\textsuperscript{13–15,47} The ROS activity examined by hydroethidine microfluorography and TNBT labeling increased with age. Considering that both methods applied in the present study are relatively specific for the detection of superoxide,\textsuperscript{25–28,48} the findings seem concordant with previous reports describing age-associated increase in superoxide activity in rodent vessels.\textsuperscript{49,50} However, despite the different levels in oxidative stress, the incidence of endothelial cell apoptosis in microvessels did not differ significantly between the younger and older animals. The discrepancy may be caused by age-related enhancement of cell resistance to oxidative insult. Cell aging reportedly renders human fibroblasts less susceptible to ROS-induced cell apoptosis,\textsuperscript{51} although the mechanism responsible for resistance to apoptosis is still uncertain. Further investigations should clarify the details of age-associated changes in cell vulnerability to oxidative stress.

Antiangiogenic effects of Tempol and Tiron have been demonstrated in several cell types cultured under prooxidant conditions.\textsuperscript{52–55} In contrast, these chemicals seem to promote apoptotic cell death in some cancer cell lines maintained in standard culture conditions.\textsuperscript{56–58} In a highly prooxidant intracellular milieu, a nitroxy radical Tempol and a semiquinone radical Tiron mainly react with excess ROS and presumably attenuate oxidative stress. Meanwhile, under relatively low oxidative conditions, their antioxidant effects are expected to be small and instead may be overshadowed by cytotoxic radical activity. Although the antioxidant treatment did not enhance cell apoptosis in WKY rats in the present study, the double-edged character of these chemicals should be taken into consideration when attempting clinical applications.

Acknowledgments

This research was supported by the National Institutes of Health, Heart, Lung, and Blood Institute (HL 10881).

References


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Microvessel length density (mm/mm$^3$)

- **WKY**
  - Control
  - Tempol
  - Tiron

- **SHR**
  - Control
  - Tempol
  - Tiron

Graph showing microvessel length density for 8-week and 16-week old WKY and SHR rats treated with Control, Tempol, and Tiron. NS indicates no significant difference. ** and †† indicate significant differences with p-values less than 0.05 and 0.01, respectively.

Sample size (n=6) for each group.