Endothelial Apoptosis Induced by Oxidative Stress Through Activation of NF-κB

Antiapoptotic Effect of Antioxidant Agents on Endothelial Cells

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Abstract—Injury of endothelial cells has been assumed to be an initial trigger of the development of atherosclerosis. In this study, we investigated the molecular mechanisms of endothelial cell death induced by hypoxia, which leads to oxidative stress. To study the relation between hypoxia-induced cell death and activation of nuclear factor-κB (NF-κB) in a hypoxic state, we evaluated the effect of 2 antioxidant drugs, probucol and pyrrolidine dithiocarbamate (PDTC), on human endothelial apoptosis. Although hypoxic treatment of human aortic endothelial cells resulted in a significant decrease in cell number and a significant increase in apoptotic cells compared with that of cells under normoxia (P < 0.01), treatment with probucol (50 μmol/L) or PDTC (100 μmol/L) significantly attenuated the decrease in cell number (P < 0.01) and was accompanied by inhibition of NF-κB activation. Furthermore, downregulation of bcl-2 caused by hypoxia was inhibited by these drugs. We further investigated the translocation of bax protein from the cytoplasm to the mitochondrial heavy fraction membrane, as translocation of bax protein is considered to be a determinant of apoptosis. Interestingly, we found that antioxidant treatment inhibited the translocation of bax protein caused by hypoxia. Moreover, upregulation of p53, a proapoptotic molecule, was observed in hypoxia, whereas treatment with probucol attenuated the expression of p53 accompanied by suppression of NF-κB activation. These data suggest functional links between p53 and endothelial apoptosis through the activation of NF-κB. Overall, the current study demonstrated that oxidative stress induced apoptosis in human aortic endothelial cells through the downregulation of bcl-2, translocation of bax, and upregulation of p53, probably through NF-κB activation. Oxidative stress may play an important role in endothelial apoptosis mediated by hypoxia, through the activation of NF-κB. (Hypertension. 2001; 38:48-55.)

Key Words: hypoxia ■ apoptosis ■ endothelium-derived factors ■ bax

Endothelial dysfunction is a trigger of the development of atherosclerosis, followed by vascular remodeling, because endothelial cells have an important role as a biological barrier in the suppression of growth of vascular smooth muscle cells (VSMCs), maintenance of vascular tonus, and protection from monocyte and platelet adhesion.1–5 Therefore, it would be worthwhile to develop a treatment to protect the function of endothelial cells for the treatment of vascular diseases. Indeed, re-endothelialization using endothelium-specific growth factors is becoming a useful application of gene therapy for atherosclerosis and restenosis after angioplasty.6–8

Oxidative stress has been reported to be a major factor in damage of endothelial cells.9–12 Oxidative modification has been implicated in the development of atherosclerosis through a variety of mechanisms, especially those leading to endothelial dysfunction.2–5 The ability of antioxidants to inhibit the progression of atherosclerosis has been tested in several experimental animal models. Antioxidant agents, including vitamin E and probucol, have been shown to inhibit LDL oxidation13,14 and retard the development of atherosclerosis in hyperlipidemic animal models15,16 or genetically hypercholesterolemic animal models.17 Indeed, several prospective trials have been reported in which an antioxidant agent, probucol, prevented the progression of atherosclerosis18 and restenosis after angioplasty.19,20 To evaluate the effect of oxidative stress on endothelial cells, we used a hypoxic condition because hypoxia is known to lead to oxidative stress.9,10 We focused on the nuclear factor (NF-κB), which has been shown to respond to a variety of metabolic stress signals, including hypoxia.21,22 This heterodimeric protein is constitutively expressed in cells and,
once activated, translocates from the cytoplasm to the nucleus to activate gene transcription. NF-κB can rapidly transduce hypoxic signals by increasing its DNA-binding activity and can lead to the transcription of various cytokines to cause inflammation and adhesion of monocytes, neutrophils, and macrophages, resulting in cell damage. Recent studies demonstrated more direct actions of NF-κB in the process of apoptosis in various cells.23–26 To understand the pathogenesis of vascular diseases, it is extremely important to elucidate the molecular mechanisms of endothelial cell death through the activation of NF-κB in response to hypoxia.

Methods

Cell Culture and Hypoxic Treatment

Human aortic endothelial cells were obtained from Clonetics (San Diego, Calif) and grown in modified MCDB 131 medium supplied with fetal calf serum 5%, penicillin 10 U/mL, streptomycin 100 mg/mL, epidermal growth factor 10 ng/mL, basic fibroblast growth factor 2 ng/mL, and dexamethasone 1 μmol/L in the standard fashion.27 All the cells were used within passage 5 to 6.

When the cells reached confluence, the medium was changed to fresh DSF (defined serum–free) medium. DSF medium was supplemented with insulin (5 × 10⁻⁷ M), transferrin (5 mg/mL), and ascorbate (0.2 mmol/L).28 The cells were incubated in DSF medium for 24 hours before they were subjected to a hypoxic condition. A hypoxic condition was achieved with BBL gas pack (Beckton Dickson), which releases hydrogen gas that reacts with oxygen in the container so that the level of oxygen drops to an undetectable level (<0.01%).

Treatment With Antioxidant Agents

A stock solution of probucol (Daichi Pharmaceutical Co) dissolved at 5 mmol/L in ethanol was prepared and stored at −20°C. For experiments, an initial solution containing probucol 100 μmol/L, ethanol 1% (v/v), and BSA 1% in DSF was prepared and then diluted to a final concentration of probucol 50 μmol/L, ethanol 0.2% (v/v), and BSA 0.2%. Pyrrolidine dithiocarbamate (PDTC) (Sigma) was dissolved in a 0.5% serum medium and used at 100 μmol/L. After incubation with DSF under normoxia, the dishes were incubated at 37°C for 60 minutes (pretreatment) with fresh DSF containing these compounds. An index of cell proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Counting of Apoptotic Cells

As an assay of cell death by apoptosis, we used fluorescent DNA-binding dyes to define nuclear chromatin morphological features as a quantitative index of apoptosis within the cell culture system.29,30 Cells to be analyzed for apoptosis were stained with Hoechst 33342 and viewed under fluorescence microscopy as previously described.31,32 After 48 hours of hypoxia, cells were incubated with Hoechst 33342 (5 μg/mL in PBS) for 20 minutes at 37°C after washing in the medium, to stain them for DNA. Individual nuclei were visualized at ×400 to distinguish the normal uniform nuclear pattern from the characteristic condensed coalesced chromatin pattern of apoptotic cells. To quantify apoptosis, 400 nuclei from random microscopic fields were analyzed by an observer blinded to the treatment groups. The total number of apoptotic cells in each section was summed and expressed as the percentage of the total cell number. At least 10 individual sections were evaluated per slide. Each observer was blinded to other data concerning the cells, as well as to the results of the other observer.

Also, we measured caspase-3 activity to quantify the apoptotic ratio. Endothelial cells on the culture dish were trypsinized with trypsin EDTA (GIBCO BRM) and centrifuged for several minutes. Then, the pellet was dissolved in PBS, and 1 mmol/L, digitonin (Wako Pure Chemical Industries) was added to the pellet. The mixture was incubated for 10 minutes at 37°C, then centrifuged again at 15 000 rpm for several minutes at 4°C. The supernatant contained caspase protein; 40 μg protein was used for the assay. Because caspase-3 cuts a specific amino acid sequence, the cutting-off activity of substrate (Peptide Institute) containing 20 μg protein and 50 μmol/L acetyl-Asp-Glu-Val-Asp-aminonitrofluoromethylcoumarin (DEVD-APC) dissolved in DMSO was measured with a microplate fluorescence reader (Hitachi F-3000), which emits a wavelength of 380 nm of wavelength. The absorbance at a wavelength of 460 nm was measured. The absorbance was measured twice, before the addition of substrate and 60 minutes after addition under incubation at 37°C. The difference between the first and second measurement was considered as the activity of caspase-3.

Western Blotting

Western blotting was used to examine whether caspase-3, bcl-2, and p53 were present in the cells. After 48 hours (bcl-2 and p53) or incubation under hypoxia, the proteins were extracted from cells for Western blotting analysis. The cells were collected by scraping and centrifugation, followed by extraction of total protein with lysis buffer (50 mmol/L Tris-HCl pH 8.0, 20 mmol/L EDTA, 1% SDS, 100 mmol/L NaCl). Proteins were separated by SDS/PAGE, transferred to nitrocellulose membrane (Hybond ECL, Amersham), and incubated with a monoclonal antibody to bcl-2 (1:50; Santa Cruz) or p53 (1:20, Calbiochem). The precipitated protein was collected at 3000 rpm for 15 minutes and resuspended in homogenization buffer containing 0.35 mol/L sucrose. NF-κB decoy ODN was labeled with [³²P]labeled probe (0.5 to 1 ng, 10 000 to 15 000 cpm) and 1 μg polydeoxyinosinic-deoxycytidic acid (Sigma Chemical Co) were incubated with nuclear extract for 30 minutes at room temperature and then loaded onto a 5% polyacrylamide gel.
mitochondrial membrane protein. Each sample was analyzed by the same method as described above using a polyclonal antibody to bax (Santa Cruz).

**Preparation of Cationic HVJ Liposomes**

The procedure of preparation of cationic HVJ liposomes has been previously reported.\(^3\) In brief, a lipid mixture containing 6 mg phosphatidylcholine, 3 mg cholesterol, and 0.75 mg 3\(\beta\)-[N-(N\(9\),N-dimethylaminoethane)carbamoyl]cholesterol was dissolved in chloroform and evaporated using a rotary evaporator. The dried mixture was hydrated with 200 \(\mu\)L balanced salt solution (BSS; NaCl 137 mmol/L, KCl 5.4 mmol/L, Tris-HCl 10 mmol/L, pH 7.6) containing decoy oligodeoxynucleotides (ODN). Liposomes were prepared by shaking and sonication. Purified HVJ (Z strain) was inactivated by UV irradiation. The liposome suspension (0.5 mL, containing 10 mg lipid) was mixed with HVJ (30 000 hemagglutinating units) in a total volume of 1 mL BSS. Free HVJ was removed from the HVJ liposomes by sucrose density gradient centrifugation. The final concentrations of decoy ODN were equivalent to 15 \(\mu\)mol/L. The preventive effect of NF-\(\kappa\)B decoy ODN on NF-\(\kappa\)B activation of endothelial cells has been already reported.\(^3\)

Sequences of the phosphorothioate ODN used were as follows: NF-\(\kappa\)B decoy ODN (consensus sequences are underlined), 5\' - CCTTGAAGGGATTTCCCTCC-3\', 3\'-GGAACTTCCCTAAAGG-GAGG-5\'; and Scrambled decoy ODN, 5\'-TTG CCGTAC-CTGACTTAGCC-3', 3'-AACGGCATGGACTGAATCGG-5'. NF-\(\kappa\)B decoy ODN have been shown to bind the NF-\(\kappa\)B transcriptional factor.\(^3\)

**In Vitro Transfection of NF-\(\kappa\)B Decoy ODN**

Endothelial cells were inoculated on day 0 and grown to confluence in 5% calf serum. After confluence was reached, the medium was changed to fresh DSF. Cationic HVJ liposome complex (100 \(\mu\)L) containing NF-\(\kappa\)B decoy ODN or scrambled decoy ODN was added to the dishes. The cells were incubated at 4\(^\circ\)C for 5 minutes and then at 37\(^\circ\)C for 30 minutes. After incubation, the medium was changed to fresh DSF medium, and cells were incubated under hypoxic condition. After 12 hours on hypoxia, we extracted 2 proteins (mitochondrial membrane protein and cytoplasmic protein) to study translocation of bax.
Figure 3. A, Gel-mobility shift assay for NF-κB binding site. 1 indicates nuclear extract (20 μg) from endothelial cells cultured under normoxic condition incubated with P32-labeled NF-κB probe for 30 minutes at room temperature without any competitor; 2, nuclear extract (20 μg) from endothelial cells cultured under hypoxic condition incubated with P32-labeled NF-κB probe without any competitor; 3, nuclear extract (20 μg) from endothelial cells cultured under hypoxic condition with probucol incubated with P32-labeled NF-κB probe without any competitor; 4, nuclear extract (20 μg) from endothelial cells cultured under hypoxic conditions with probucol incubated with P32-labeled NF-κB probe without any competitor; and 5, nuclear extract (20 μg) from endothelial cells cultured under hypoxic condition incubated with PDTC incubated with P32-labeled NF-κB probe without any competitor. B, NF-κB-binding activity assessed by densitometry. Values are expressed as percentage of NF-κB-binding activity under normoxia. Values are calculated from 4 independent experiments (n=4 for each group). ##P<0.01 versus normoxia; hypoxia: 100%, hypoxia: 64±9%, P<0.01 versus normoxia; hypoxia + PDTC: 76±6%, P<0.01 versus hypoxia; hypoxia + probucol: 59±6%, P<0.01 versus hypoxia.

**Statistical Analysis**

All values are expressed as mean ± SEM. ANOVA with subsequent Scheffe’s test was used to determine the significance of differences in multiple comparisons. P<0.05 was considered significant.

**Results**

**Effect of Probucol and PDTC on Hypoxia-Induced Cell Death**

Cell number under hypoxic condition was significantly decreased in a time-dependent manner (P<0.01), as shown in Figure 1. Accordingly, apoptotic cells were also significantly increased in response to hypoxia, as assessed by nuclear staining (Figure 2, P<0.01). Interestingly, the addition of probucol (an antioxidant agent) or PDTC (an inhibitor of NF-κB) attenuated the decrease in cell number and the increase in number of apoptotic cells (Figures 1 and 2). The suppression of apoptosis by probucol and PDTC was also confirmed by the measurement of caspase-3–like activity (Figure 2c), as caspase 3, an Interleukin-1β-converting enzyme (ICE) homolog, cleaves poly (ADP-ribose) polymerase (PARP) during early apoptosis. It is noteworthy that the binding affinity of NF-κB was markedly increased in endothelial cells after 12 hours of treatment under hypoxic condition compared with normoxic condition, as assessed by gel mobility shift assay (Figure 3), consistent with our previous report. Moreover, treatment with these drugs suppressed the activation of NF-κB caused by hypoxia as shown in Figure 3. These data, together with our previous report that inhibition of NF-κB activation by decoy oligonucleotides resulted in the prevention of endothelial apoptosis, suggest that activation of NF-κB caused by oxidative stress followed by hypoxia mediates apoptosis and cell death in endothelial cells.

**Upregulation of bcl-2 by Probucol or PDTC**

To study the molecular mechanisms of hypoxia-induced apoptosis, we performed Western blotting for 2 apoptotic-related proteins, bcl-2 and bax. Under hypoxic condition, the level of bcl-2 protein was significantly decreased (Figure 4). However, when the cells were treated with probucol or PDTC, the level of bcl-2 protein returned to the same level as that under normoxia. On the other hand, the total expression level of bax was not changed in any conditions (data not shown). These data suggest that the mechanisms of apoptosis mediated by the activation of NF-κB induced by hypoxia involve at least the bcl-2 pathway. In addition, antioxidants can act as a protector of endothelial cells through the upregulation of bcl-2 (Figure 4). Thus, the ratio of bcl-2 to bax was significantly increased in endothelial cells by treatment with antioxidants compared with that of control (normoxia: 100%, hypoxia: 64±9%, P<0.01 versus normoxia; hypoxia + PDTC: 76±6%, P<0.01 versus hypoxia; hypoxia + probucol: 59±6%, P<0.01 versus hypoxia).

Figure 4. A, Typical example of Western blot of bcl-2 (29 kDa) protein in endothelial cells. 1 indicates normoxia; 2, hypoxia; 3, hypoxia+PDTC; and 4, hypoxia+Probucol. B, Percent change in protein level of bcl-2 in endothelial cells assessed by densitometry. Values are expressed as percentages of protein level of bcl-2 under normoxia. Values are from 4 independent experiments (n=4 for each group). ##P<0.01 versus normoxia; hypoxia: 100%, hypoxia: 64±9%, P<0.01 versus normoxia; hypoxia + PDTC: 76±6%, P<0.01 versus hypoxia; hypoxia + probucol: 59±6%, P<0.01 versus hypoxia.
probucol: 80±10%, P<0.01 versus hypoxia; n=4 from 4 independent experiments).  

**Translocation of bax**

Translocation of bax protein from the cytoplasm to the mitochondrial heavy fraction membrane is an important factor in apoptosis. Therefore, we evaluated translocation of bax mediated by hypoxia to study whether the suppression of NF-κB by probucol or PDTC can inhibit its translocation. Under hypoxic condition, most of bax was translocated from the cytoplasm to the mitochondrial heavy fraction membrane (Figure 5a and 5b), accompanied by activation of NF-κB. Importantly, the addition of probucol or PDTC inhibited the translocation in response to hypoxia. To further confirm the role of NF-κB activation on translocation of bax, we used an NF-κB specific inhibitor, NF-κB decoy ODN. Interestingly, transfection of NF-κB decoy ODN significantly inhibited the translocation of bax (Figure 5c and 5d). As previously, we reported that inhibition of NF-κB activation by decoy approach attenuated endothelial cell death through bcl-2 downregulation induced by hypoxia. Together with the present study, NF-κB activation plays an important role in hypoxia-induced endothelial cell death through translocation of bax and downregulation of bcl-2.

**p53-Mediated Apoptosis in Hypoxia**

Recent studies demonstrated that NF-κB regulates p53 gene expression. We previously reported that p53 is overexpressed in VSMCs in response to hypoxia, resulting in apoptosis. In this study, we investigated whether p53 is related to apoptosis induced by the activation of NF-κB in hypoxia. The protein of p53 was upregulated under hypoxic condition as assessed by Western blotting (Figure 6a and 6b), whereas the addition of PDTC or probucol attenuated the induction of p53 (Figure 6a and 6b), accompanied by suppression of NF-κB. Also, transfection of NF-κB decoy ODN significantly attenuated the induction of p53, even under hypoxic condition. These data reveal that there may be an interaction between p53 and NF-κB, suggesting that the mechanisms of apoptosis mediated by hypoxia are caused by not only the level of bcl-2 and translocation of bax, but also the level of p53.

**Discussion**

Although cell death by hypoxia as a well-known oxidative stress has been generally believed to be manifested as necrosis, recent biochemical observations have suggested the possibility of hypoxia-induced apoptosis. We previ-
ously reported that apoptosis of VSMCs in hypoxia is mediated by an increase in the ratio of bax to bcl-2, accompanied by the upregulation of p53. Similarly, in the present study, we demonstrated that endothelial apoptosis caused by hypoxia was related to an increase in the ratio of bax to bcl-2 (Figure 4). More importantly, activation of NF-κB–binding activity was also clearly observed in endothelial cells under hypoxic condition. Numerous stimuli—including oxidative stress, tumor necrosis factor-α, and high glucose—are known to induce apoptosis in endothelial cells. Interestingly, these stimuli also increased NF-κB–binding activity in endothelial cells, as previously demonstrated. Therefore, we hypothesized that an increase in NF-κB–binding activity might be involved in hypoxia-induced endothelial cell death. Importantly, PDTC, an inhibitor of NF-κB, could attenuate the downregulation of bcl-2, resulting in the prevention of endothelial apoptosis. Moreover, probucol also could suppress the activation of NF-κB and inhibit endothelial apoptosis. The present study suggested a phenomenon that hypoxia-induced apoptosis might be mediated by the NF-κB–bcl-2 pathway (Figure 7).

Unexpectedly, the protein level of bax was not changed. However, our data showed a quite interesting phenomenon, translocation of bax. It has been reported that 1 of the crucial steps before bax can exert its proapoptotic activity is translocation from the cytoplasm to the mitochondria. In apoptotic cells, cytochrome c release from the mitochondria is induced mainly by translocation of bax. In the present study, translocation of bax was inhibited by PDTC, probucol, or NF-κB decoy ODN, accompanied by the suppression of NF-κB. Taken together, NF-κB activation plays an important role in hypoxia-induced endothelial cell death through translocation of bax and bcl-2 downregulation. Although the relation between NF-κB and bax is not fully understood, downregulation of bcl-2 might promote translocation of bax (Figure 7), because bcl-2 is reported to suppress translocation of bax in other cells. Our data also showed that p53 overexpression was induced by the activation of NF-κB. However, the relation between p53 and translocation is unclear. Indeed, it was reported that translocation of bax protein to the mitochondria was not observed in p53-induced apoptosis, although bax was upregulated.

In this study, we failed to answer how NF-κB decreased bcl-2 expression. However, our present study may answer this question. According to the present data, p53 was overexpressed in response to hypoxia, whereas the suppression of NF-κB by PDTC, probucol, or NF-κB decoy ODN attenuated the increase in the level of p53. One of the functions of p53 is to repress expression of bcl-2 gene, which exhibits an
antiapoptotic action through a cis-acting p53 negative-response element located in the 5′-untranslated region. Therefore, the downregulation of bcl-2 because of the activation of NF-κB might be mediated by the induction of p53 (Figure 7). Although the role of NF-κB in p53 promoter activation is not fully understood, several papers have reported the interaction of NF-κB to p53 activation of. Wu et al36 was the first to report that NF-κB regulates p53 gene expression. They also reported36 cytoplasmic regulation of p53 by NF-κB, p53 activation following the tyrosine phosphorylation of IκB. On the other hand, Sun et al57 reported that the region of the p53 promoter required to respond to genotoxic stress extends from −70 to −40, which overlaps the NF-κB site. In addition, they showed that the NF-κB element in the p53 promoter has a high affinity for NF-κB p50 homodimers, but it is not transactivated by p65 or inhibited by IκB, suggesting a nuclear rather than a cytoplasmic regulation of p53 by NF-κB.57 Other investigators58 demonstrated that activation of p53 transcription in response to hypoxia or reoxygenation by NF-κB may be mediated by upstream factors such as src, ras, and raf, suggesting a nuclear regulation of p53. However, the possibility that NF-κB may influence the activation of p53 indirectly, for example, via c-myc expression, should be considered. Thus, NF-κB may play a role in p53 regulation in response to various stresses, including oxidative stress. It can be supposed that oxidative stress induces p53 expression through the activation of NF-κB, resulting in G1 arrest and apoptosis, and that overexpressed p53 also regulates bcl-2 negatively. In the clinical field, prospective trials in which probucol prevented the progression of atherosclerosis may also be supported by our data.18–20

Here, we demonstrated that activation of NF-κB by oxidative stress induced human aortic endothelial cell death and apoptosis through the suppression of bcl-2, translocation of bax, and induction of p53 (Figure 7). Blockade of NF-κB activation by antioxidant agents is suggested to be an effective strategy in the treatment of atherosclerosis. However, the endothelial apoptosis induced by NF-κB is contradictory to the previous findings in cancer cells. It would be important to clarify the detailed mechanisms of NF-κB-dependent endothelial apoptosis.

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