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Hydrogen Peroxide Induces Up-Regulation of Fas in Human Endothelial Cells

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Hydrogen peroxide (H₂O₂), an oxidant generated by inflammatory cells, is an important mediator of injury of endothelial cells (ECs). Here we show that H₂O₂ induces up-regulation of the expression of Fas, a death signal, in human ECs in culture. Flow cytometric analysis with a mAb against human Fas showed that incubation for 24 h with H₂O₂ induced a dose-dependent increase in the level of Fas in ECs. Coincubation with catalase, which rapidly degrades H₂O₂, inhibited H₂O₂-induced up-regulation of Fas. H₂O₂ also induced a dose-dependent increase in Fas mRNA level. A significant increase in Fas mRNA levels was observed from 6 h after stimulation with H₂O₂. Vanadate, a protein phosphatase inhibitor, significantly enhanced Fas mRNA and protein levels in H₂O₂-treated ECs. On the other hand, genistein, a tyrosine kinase inhibitor, inhibited H₂O₂-induced Fas mRNA expression. Furthermore, a flow cytometric method with propidium iodide staining and electron microscopic analysis showed that incubation with an agonistic Ab against Fas (anti-Fas IgM) induced apoptosis in H₂O₂-treated cells. These findings suggest that H₂O₂ induces up-regulation of Fas in ECs and that activation of protein tyrosine kinase may be involved in the mechanism of H₂O₂-induced Fas expression. Therefore, Fas-mediated apoptosis may have a pathologic role in H₂O₂-induced EC injury and thereby provide a new therapeutic target. The Journal of Immunology, 1998, 160: 4042–4047.

Materials and Methods

Materials

mAbs against human Fas, clone UB2 (IgG) and clone CH11 (IgM) were from Medical Biologic Laboratories (Nagoya, Japan). FITC-labeled goat anti-mouse IgG was obtained from Seikagaku (Tokyo, Japan). Catalase and genistein were purchased from Sigma (St. Louis, MO). Sodium pervanadate and propidium iodide were from Wako Pure Chemical Industries (Osaka, Japan).

Cell culture

HUVEC were purchased from Kurabo (Osaka, Japan) and maintained in medium (HuMedia-EG; Kurabo) containing 2% FCS, 10 ng/ml recombinant acidic fibroblast growth factor, 10 ng/ml recombinant acidic endothelium growth factor, and antibiotics. Cultured cells were identified as ECs on the basis of typical morphology and factor VIII immunostaining.

Detection of Fas by flow cytometry

After treatment with various compounds, floating cells were removed by rinsing the cell layers with PBS containing 0.2 mM EDTA, and then adherent cells were harvested with trypsin to analyze Fas expression. ECs (10⁶) were incubated with PBS containing 5% FCS and 10 μg/ml murine Ab against Fas (UB2) for 1 h at 4°C, washed with PBS three times, and then incubated with 10 μg/ml FITC-conjugated goat anti-mouse IgG for 30 min at 4°C. Fas expression on the cell surface was analyzed by a method using a flow cytometer (FACS) on FL-1 channel.

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2 Abbreviations used in this paper: Fas-L, Fas ligand; ECs, endothelial cells; NO, nitric oxide; VSMCs, vascular smooth muscle cells; NF-κB, nuclear factor-κB; AP-1, activator protein 1; H₂O₂, hydrogen peroxide.
Analysis of Fas mRNA

Total RNA from ECs was extracted by a guanidine isothiocynate/acid phenol method (27). Poly(A)^+ RNA was prepared using Oligo(dT)-Latex (Takara Biomedicals, Japan). Northern blot analysis was performed as previously described (28). The probe DNA of Fas was a 2.5-kbp XhoI fragment containing human Fas cDNA (29). The cDNA probes for human Fas and human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were labeled with [32P]dCTP (111 TBq/mmol) by using the multiprime DNA labeling kit (Amersham International, Buckinghamshire, U.K.). Hybridization with a G3PDH cDNA probe was used to monitor uniform loading of RNA on Northern blots.

DNA analysis of apoptosis by flow cytometry

Apoptosis was monitored by measuring the population distribution of DNA content (30). After treatment with H2O2 and anti-Fas IgM, ECs (10^6) were suspended in 100 μl of PBS and fixed with 900 μl of cold ethanol and then resuspended in staining buffer (1 mg/ml RNaseA, 20 μg/ml propidium iodide, 0.01% Nonidet P-40). The DNA content of the cells was analyzed by flow cytometry on FL2 channel.

Electron microscopy

Cells were fixed with 1% glutaraldehyde, 1% formaldehyde (prepared fresh from paraformaldehyde), and 0.2 mM CaCl2 in 0.12 M phosphate buffer (pH 7.3) for 5 to 30 min, osmicated in phosphate-buffered 2% OsO4 for 5 min, dehydrated in a graded series of alcohol, and embedded in epoxy resin. After polymerization of the resin, the culture dish was removed from the epoxy resin block. The blocks were cut into thin sections with a diamond knife and examined after contrasting with uranyl acetate and lead citrate.

Results

Induction of Fas in H2O2-treated ECs

Figure 1 shows the effect of H2O2 on Fas expression in HUVEC determined by flow cytometric analysis. Incubation for 24 h with H2O2 at concentrations from 0.2 to 1.0 mM induced a dose-dependent increase in Fas expression in ECs. However, catalase (920 U/ml), an enzyme that hydrolyzes H2O2 to O2 and H2O (31), inhibited the up-regulation of Fas induced by 0.5 mM H2O2 (Fig. 1D).

Expression of Fas mRNA in H2O2-treated ECs

Figure 2A shows the time-dependent effect of H2O2 at a concentration of 0.5 mM on the expression of Fas mRNA in ECs. Fas mRNA was detected in control cells. Two bands of 2.7 and 1.9 kb were detected in Fas mRNA in ECs. A significant increase in Fas mRNA level was observed from 6 h after stimulation with H2O2. As shown in Figure 2B, incubation for 12 h with H2O2 at concentrations from 0.2 to 1.0 mM induced a dose-dependent increase in Fas mRNA level in ECs. Coincubation with catalase inhibited the up-regulation of Fas mRNA expression induced by 0.5 mM H2O2.

Vanadate enhances H2O2-induced Fas expression

Next, we examined whether activation of protein tyrosine kinase is involved in the mechanism of H2O2-induced up-regulation of Fas expression. As shown in Figure 3A, incubation with 100 μM vanadate, a phosphatase inhibitor, significantly enhanced H2O2-induced Fas expression. However, the basal level of Fas was not affected by vanadate alone (Fig. 3B). Vanadate also enhanced the increase in Fas mRNA level induced by H2O2 (Fig. 3A). On the other hand, incubation with genistein, an inhibitor of protein tyrosine kinase, significantly inhibited H2O2-induced up-regulation of Fas mRNA expression (Fig. 3B).

H2O2-induced Fas can mediate apoptosis

We next examined whether H2O2-induced Fas can mediate apoptosis. A flow cytometric method with propidium iodide staining was used for quantitating endonucleolytic cleavage of DNA in cells undergoing apoptosis. ECs in control culture demonstrated...
normal diploid DNA content with DNA peaks of G₁ and G₂-M. The apoptotic population was small (13.4%) (Fig. 5A). Incubation for 24 h with H₂O₂ (0.5 mM) alone did not induce a significant change in the apoptotic population (17.6%) compared with control cells (Fig. 5B). On the other hand, incubation for 8 h with anti-Fas IgM induced a large hypodiploid population (41.8%) undergoing apoptosis with less than 2N DNA in H₂O₂-pretreated cells (Fig. 5C). However, anti-Fas IgM did not induce a significant change in the apoptotic population in cells without pretreatment with H₂O₂ (12.1%) (Fig. 5D). Furthermore, electron microscopic analysis showed typical morphologic changes of apoptosis such as cellular shrinkage, membrane blebbing, and chromatin condensation in most cells treated with H₂O₂ and anti-Fas IgM (Fig. 6, A and B). In addition, pretreatment with H₂O₂ at concentrations from 0.2 to 1 mM induced a dose-dependent enhancement of anti-Fas IgM-induced apoptosis in ECs, although H₂O₂ at a high concentration of 1 mM alone induced apoptosis (Fig. 7). These findings indicate that H₂O₂-induced Fas is functional in mediating apoptosis in ECs.

Discussion

In this study, we demonstrated that H₂O₂ induces up-regulation of Fas in ECs. H₂O₂ is one of the most important antimicrobial and antitumor weapons of polymorphonuclear leukocytes (32). H₂O₂ has also been implicated in cellular and tissue injury in pathologic conditions, for example, ischemia-reperfusion injury, hyperoxia, and inflammation (33–38). In ischemia-reperfusion injury, interaction of stimulated polymorphonuclear leukocytes with ECs is involved in its pathogenesis. Thus, ECs lining blood vessels appear to be the initial target of activated polymorphonuclear cell-derived oxidants. In addition, stimulation of various cells with either cytokines, phorbol esters, or growth factors increases the secretion of H₂O₂ in the extracellular space in vitro (39–43). High concentrations of this diffusable reactive oxygen intermediate exert toxic effects on susceptible cells. However, low concentrations of H₂O₂ alter cellular functions by modulating signal transduction in certain

FIGURE 2. Fas mRNA expression in ECs treated with H₂O₂. A, Poly(A)⁺ RNA was prepared from ECs incubated with H₂O₂ (0.5 mM) for the indicated times before harvest. B, Poly(A)⁺ RNA was prepared from ECs treated for 12 h with H₂O₂ at the indicated concentrations in the presence or absence of catalase (920 U/ml).

FIGURE 3. Effect of vanadate on Fas expression. Panel A shows the relative expression of Fas on ECs coincubated with both H₂O₂ (0.5 mM) and vanadate (0.1 mM) for 24 h. Panel B shows Fas expression on ECs coincubated with vanadate alone. Histograms obtained with each treatment are shown in broad lines. Histograms of controls are shown in solid lines, and dashed lines represent histograms of ECs treated with 0.5 mM H₂O₂.

FIGURE 4. Effect of vanadate on H₂O₂-induced Fas mRNA in ECs. A, ECs were incubated with 0.5 mM H₂O₂ for 12 h in the presence or absence of 0.1 mM vanadate. B, ECs were incubated for 6 h with 0.5 mM H₂O₂ in the presence or absence of 0.1 mM genistein. Northern blot analysis was performed as described in the text.
cells, including ECs, in vitro (20–25). Although it has been shown that activated polymorphonuclear leukocytes can generate H2O2 concentrations of up to 0.2 mM in vitro, and cigarette smoke passed through saline yields H2O2 concentrations of 0.05 to 0.1 mM (9, 44–45), it is unknown how much H2O2 is actually produced in inflammatory lesions in vivo. In addition, inactivation of the antioxidative pathway in a pathologic state may enhance the effect of H2O2 on ECs. For example, recent evidence has shown that NO, another diffusable reactive oxygen intermediate, inhibits glutathione peroxidase, an antioxidative enzyme that scavenges various peroxides, and increases the levels of intracellular peroxide (46). Since inducible NO synthase, which produces high levels of NO, is up-regulated in cytokine-activated cells and hypoxia/reperfusion tissues (47, 48), it may be possible that the concentration of H2O2 is locally much higher in inflammatory lesions.

Since it has been reported that H2O2 is a potent inducer of many biologic factors, including platelet-activating factors (49) and vascular endothelial growth factor (50), we examined whether H2O2-induced Fas expression is mediated by a new protein synthesis. However, H2O2-induced up-regulation of Fas mRNA expression occurred in the presence of the protein synthesis inhibitor cycloheximide (data not shown), indicating that de novo protein synthesis was not required and suggesting a direct involvement of H2O2. Exogenously added H2O2 can freely diffuse across cell membranes (31) and induce tyrosine phosphorylation in several cell types (51, 52). We observed that the tyrosine phosphatase inhibitor vanadate enhanced H2O2-induced up-regulation of Fas expression. It should be noted that the combination of H2O2 and vanadate generates the compound pervanadate, biologic activity of which has been demonstrated to be far greater than that of vanadate, and which strongly enhances tyrosine phosphorylation (53). Furthermore, genistein, an inhibitor of tyrosine kinase activity, inhibited H2O2-induced up-regulation of Fas mRNA expression.

These findings suggest that H2O2 induces up-regulation of Fas expression by increasing tyrosine kinase activity in ECs. Many of the oxidant-sensitive genes have nuclear factor (NF)-κB or activator protein 1 (AP-1) regulatory elements in their promoter regions, and increased binding of endothelial proteins to both types of elements has been reported after oxidant stimuli (54–56). Furthermore, H2O2 increases nuclear levels of NF-κB and AP-1 through a tyrosine kinase-dependent mechanism in ECs (57) and lymphocytes (58). In this respect, it is of interest to find regulatory elements matching the reported consensus sequences for NF-κB and AP-1 within the 5′ flanking sequence of the human Fas gene (59–61).

Although EC apoptosis is important in normal tissue homeostasis and during development (62–64), it is also present during remodeling of damaged tissues (65–67). Sgonc et al. (68) recently reported that EC apoptosis is a primary pathogenic event underlying skin lesions in avian and human scleroderma. Furthermore, Laurence et al. (69) also reported that Fas-mediated apoptosis in ECs may be of pathophysiologic importance in thrombotic thrombocytopenic purpura. In our studies, pretreatment with H2O2 induced a dose-dependent enhancement of apoptosis induced by an agonistic Ab to Fas (anti-Fas IgM). These results suggest that H2O2-induced Fas is functional to induce apoptosis in EC. On the other hand, it should be noted that Richardson et al. reported that IFN-γ-treated ECs did not undergo apoptosis upon Fas ligation (70). We observed that anti-Fas IgM did not induce apoptosis in control ECs. The lack of anti-Fas IgM-induced apoptosis in control ECs that express some baseline Fas mRNA may relate to threshold levels of Fas protein required for cross-linking. However, it is also possible that H2O2 treatment may sensitize ECs against Fas-induced apoptosis. In this regard, it is noteworthy that c-myc-induced apoptosis was recently shown to require interaction of Fas and Fas-L by sensitizing cells to the Fas death signal in 3T3 fibroblasts.

FIGURE 5. Flow cytometric DNA analysis of the effects of H2O2 and anti-Fas IgM on ECs. After incubation with anti-Fas IgM, ECs were fixed with ethanol and stained with 20 μg/ml of propidium iodide. A, ECs were incubated in serum-free medium for 24 h. B, ECs were incubated for 24 h with 0.5 mM H2O2, rinsed with fresh medium, and then incubated for a further 8 h with vehicle without H2O2. C, ECs were preincubated for 24 h with 0.5 mM H2O2, rinsed with fresh medium, and then incubated for a further 8 h with 1.0 mg/ml of anti-Fas IgM without H2O2. D, ECs were incubated for 24 h with serum-free medium and then incubated for a further 8 h with 1.0 mg/ml of anti-Fas IgM.
In addition, we also observed that H₂O₂ at a high concentration of 1 mM alone induced apoptosis in ECs. Our preliminary experiments showed that apoptosis induced by 1 mM H₂O₂ was partially inhibited by coincubation with a neutralizing Ab to Fas-L (data not shown), suggesting that up-regulation of Fas-L may also participate in the mechanism of H₂O₂-induced apoptosis in ECs (71). Taken together, these findings suggest that Fas-mediated apoptosis in ECs may contribute to the mechanism of H₂O₂-induced tissue injury and the extravasation of inflammatory cells. Therefore, it will be of interest to examine whether inhibitors of Fas-mediated apoptosis (73) may be clinically useful in preventing inflammatory cell-mediated tissue injury.

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FIGURE 6. Electron microscopic appearance of ECs after treatment with H₂O₂ and anti-Fas IgM (magnification ×4,100 and ×10,500, respectively). A. ECs were incubated for 8 h with 1.0 mg/ml of anti-Fas IgM without pretreatment with H₂O₂. B. ECs were incubated for 24 h with H₂O₂ (0.5 mM) and then treated with anti-Fas IgM for 8 h.

FIGURE 7. Dose-dependent effects of H₂O₂ on anti-Fas IgM-induced apoptosis in ECs. After preincubation of ECs with H₂O₂ at the indicated concentrations, ECs were washed with serum-free medium and then incubated for a further 8 h with 1.0 mg/ml of anti-Fas IgM. DNA analysis of apoptosis by flow cytometry was performed as described in the text. Values are mean ± SD of three individual experiments, each containing four replicates. *p < 0.05, significantly different from control. **p < 0.05, significantly different from cells treated with H₂O₂ alone.
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