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

Toshimitsu Suhara, Keisuke Fukuo, Tomosada Sugimoto, Shigeto Morimoto, Takeshi Nakahashi, Shigeki Hata, Masumi Shimizu and Toshio Ogihara

*J Immunol* 1998; 160:4042-4047;
http://www.jimmunol.org/content/160/8/4042

---

**References**  
This article cites 63 articles, 28 of which you can access for free at:  
http://www.jimmunol.org/content/160/8/4042.full#ref-list-1

**Subscription**  
Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

**Permissions**  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
Hydrogen Peroxide Induces Up-Regulation of Fas in Human Endothelial Cells

Toshimitsu Suhara,* Keisuke Fukuo,† Tomosada Sugimoto,‡ Shigeto Morimoto,* Takeshi Nakahashi,* Shigeki Hata,* Masumi Shimizu,* and Toshio Ogihara*

Hydrogen peroxide (H$_2$O$_2$), an oxidant generated by inflammatory cells, is an important mediator of injury of endothelial cells (ECs). Here we show that H$_2$O$_2$ 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 H$_2$O$_2$ induced a dose-dependent increase in the level of Fas in ECs. Coincubation with catalase, which rapidly degrades H$_2$O$_2$, inhibited H$_2$O$_2$-induced up-regulation of Fas. H$_2$O$_2$ 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 H$_2$O$_2$. Vanadate, a protein phosphatase inhibitor, significantly enhanced Fas mRNA and protein levels in H$_2$O$_2$-treated ECs. On the other hand, genistein, a tyrosine kinase inhibitor, inhibited H$_2$O$_2$-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 H$_2$O$_2$-treated cells. These findings suggest that H$_2$O$_2$ induces up-regulation of Fas in ECs and that activation of protein tyrosine kinase may be involved in the mechanism of H$_2$O$_2$-induced Fas expression. Therefore, Fas-mediated apoptosis may have a pathologic role in H$_2$O$_2$-induced EC injury and thereby provide a new therapeutic target. The Journal of Immunology, 1998, 160: 4042–4047.

Received for publication May 28, 1997. Accepted for publication December 17, 1997.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Address correspondence and reprint requests to Dr. Keisuke Fukuo, Department of Geriatric Medicine, University Medical School, 2-2 Yamadaoka, Suita, Osaka 565, Japan. E-mail address: fukuo@geriat.med.osaka-u.ac.jp

2 Abbreviations used in this paper: Fas-L, Fas ligand; ECs, endothelial cells; NO, nitric oxide; VSMCs, vascular smooth muscle cells; NF-kB, nuclear factor-kB; AP-1, activator protein 1; H$_2$O$_2$, hydrogen peroxide.
Analysis of Fas mRNA

Total RNA from ECs was extracted by a guanidine isothiocyanate/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. 4A). On the other hand, incubation with genistein, an inhibitor of protein tyrosine kinase, significantly inhibited H2O2-induced up-regulation of Fas mRNA expression (Fig. 4B).

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, inter-
action 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 cy-
tokines, phorbol esters, or growth factors increases the secretion of
H2O2 in the extracellular space in vitro (39–43). High concentra-
tions 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 diffusible 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.
In addition, we also observed that H$_2$O$_2$ at a high concentration of 1 mM alone induced apoptosis in ECs. Our preliminary experiments showed that apoptosis induced by 1 mM H$_2$O$_2$ 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 H$_2$O$_2$-induced apoptosis in ECs (71). Taken together, these findings suggest that Fas-mediated apoptosis in ECs may contribute to the mechanism of H$_2$O$_2$-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.

Acknowledgments

We thank Dr. S. Nagata for providing human Fas cDNA, Taeko Kaimoto for technical assistance, and Tomoko Adachi for secretarial assistance.

References


