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*J Immunol* 2000; 165:2190-2197; doi: 10.4049/jimmunol.165.4.2190
http://www.jimmunol.org/content/165/4/2190

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Requirement of Hydrogen Peroxide Generation in TGF-β1 Signal Transduction in Human Lung Fibroblast Cells: Involvement of Hydrogen Peroxide and Ca²⁺ in TGF-β1-Induced IL-6 Expression

Eunsung Jun,† Kee Nyung Lee,† Hyang Ran Ju,*, Seung Hyun Han,*, Joo Young Im,*, Hyung Sik Kang,*, Tae Ho Lee,† Yun Soo Bae,† Kwon Soo Ha,‡ Kwon Soo Ha,§ Zee Won Lee,§ Sue Goo Rhee,¶ and Inpyo Choi‡*‡

Stimulation of human lung fibroblast cells with TGF-β1 resulted in a transient burst of reactive oxygen species with maximal increase at 5 min after treatment. This reactive oxygen species increase was inhibited by the antioxidant, N-acetyl-l-cysteine (NAC). TGF-β1 treatment stimulated IL-6 gene expression and protein synthesis in human lung fibroblast cells. Antioxidants including NAC, glutathione, and catalase reduced TGF-β1-induced IL-6 gene expression, and direct H₂O₂ treatment induced IL-6 expression in a dose-dependent manner. NAC also reduced TGF-β1-induced AP-1 binding activity, which is involved in IL-6 gene expression. It has been reported that Ca²⁺ influx is stimulated by TGF-β1 treatment. EGTA suppressed TGF-β1- or H₂O₂-induced IL-6 expression, and ionomycin increased IL-6 expression, with simultaneously modulating AP-1 activity in the same pattern. PD98059, an inhibitor of mitogen-activated protein kinase (MAPK) kinase/extracellular signal-related kinase kinase 1, suppressed TGF-β1- or H₂O₂-induced IL-6 and AP-1 activation. In addition, TGF-β1 or H₂O₂ increased MAPK activity which was reduced by EGTA and NAC, suggesting that MAPK is involved in TGF-β1-induced IL-6 expression. Taken together, these results indicate that TGF-β1 induces a transient increase of intracellular H₂O₂ production, which regulates downstream events such as Ca²⁺ influx, MAPK, and AP-1 activation and IL-6 gene expression. The Journal of Immunology, 2000, 165: 2190–2197.

Transforming growth factor-β1 is a multifunctional cytokine that modulates the expression of fibronectin and collagen in fibroblasts, epithelial cell differentiation, adipogenesis, myogenesis, and chondrogenesis (1–3). Some of the diverse biological effects exerted by TGF-β1 are mediated by its ability to regulate the production of other growth factors and cytokines. It has been reported that TGF-β1 delivers diverse intracellular signals through type I and type II receptors which have serine/threonine kinase activity (4).

Recent studies suggest that reactive oxygen species (ROS)³ such as hydrogen peroxide and superoxide anions (O₂⁻) function as intracellular messengers in receptor signaling. Production of ROS has been observed in a variety of cells stimulated with several growth factors such as platelet-derived growth factor (PDGF) (5, 6), fibroblast growth factor (7), epidermal growth factor (EGF) (8), transforming growth factor-β1 (9, 10), and IL-1 (9). This ROS production has been related to the activation of transcription factors such as NF-κB (11, 12), egr-1 (13), and AP-1 (14), mitogen-activated protein kinase (MAPK) (15, 16), phospholipases (17, 18); to the triggering of apoptosis (19, 20); and to the inhibition of protein tyrosine phosphatases (21).

Although the chemical nature of ROS produced in stimulated cells has not been clearly identified yet, H₂O₂ was shown to be a major species of ROS when the activation is due to PDGF and EGF. Externally added H₂O₂, at high concentration, is toxic to cells because it inflicts oxidative damage on cellular components. However, at low concentrations, H₂O₂ enhances protein tyrosine phosphorylation and induces cell proliferation and differentiation.

The H₂O₂ production in response to the growth factors is rapid and transient, reaching its peak within 5–10 min (5, 6, 8). Inhibition of this H₂O₂ production by N-acetyl-l-cysteine (NAC) or catalase completely blocks receptor-mediated events such as protein phosphorylation and induces cell proliferation (5, 8).

TGF-β1 also stimulates H₂O₂ production in bovine pulmonary artery endothelial cells (22), vascular endothelial cells (23), mouse osteoblastic cells (24), and human lung fibroblast (HLF) cells (25, 26). However, unlike the case of growth factors, the H₂O₂ generation in response to TGF-β1 is slow and prolonged. For example, the TGF-β1-induced H₂O₂ generation in HLF cells occurred within 4–8 h after treatment, peaked at 16 h, and returned to baseline at 48 h (25). Recently, it has been reported that Ca²⁺ is an intracellular messenger of the action of TGF-β1 (27, 28). TGF-β1 stimulates Ca²⁺ influx and mediates an increase in the intracellular...
Ca\(^{2+}\) concentration [Ca\(^{2+}\)], which modulates the induction of CTF-1 transcriptional activity in NIH3T3 cells (27). In MIN6 insulinoma cells, TGF-\(\beta\) elevates the level of [Ca\(^{2+}\)] which is totally dependent on Ca\(^{2+}\) entry (28). However, the roles of ROS and the relation between ROS and Ca\(^{2+}\) influx in TGF-\(\beta\) signaling are not clear yet.

In the present study, we demonstrate a new possible mechanism of the rapid and transient H\(_2\)O\(_2\) production in response to TGF-\(\beta\) in HLF cells. TGF-\(\beta\) induced intracellular H\(_2\)O\(_2\) burst with maximal increase at 5 min after treatment, which was reduced by NAC. In addition, TGF-\(\beta\) or direct addition of H\(_2\)O\(_2\) induced IL-6 gene expression in HLF cells, including the activation of MAPK and AP-1 activity. Also, the possible interaction between intracellular ROS and Ca\(^{2+}\) influx in TGF-\(\beta\)-mediated signaling was investigated.

**Materials and Methods**

**Materials**

Recombinant human TGF-\(\beta\) was purchased from R&D System (Minneapolis, MN). Catalase, superoxide dismutase, NAC, glutathione, hydrogen peroxide, and EGTA were obtained from Sigma (St. Louis, MO). Ionomycin was purchased from Calbiochem (La Jolla, CA). PD98059 was purchased from New England BioLabs (Beverly, MA). Anti-ACTIVE MAPK Ab was purchased from Promega (Madison, WI), and anti-pan extracellular signal-regulated kinase (ERK) Ab was from Transduction Laboratories (Lexington, KY). Moloney murine leukemia virus reverse transcriptase was purchased from Promega, and Taq DNA polymerase was from Takara (Shiga, Japan).

**Cell culture**

HLF cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin under 5% CO\(_2\). After reaching 80–90% confluency, the cells were placed into the quiescent state by reducing the serum concentration to 0.1% for 1 day before stimulation with TGF-\(\beta\), H\(_2\)O\(_2\), or ionomycin in serum-free medium. There were no adverse effects of serum starvation for 1 day on cell morphology and viability. Cell viability was determined by trypan blue exclusion.

**Measurement of ROS level**

The intracellular ROS level was measured using a fluorescent dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA, Eastman Kodak, Rochester, NY). DCFH-DA is a nonpolar compound that is readily diffusible into cells, where it is hydrolyzed to the nonfluorescent polar derivative DCFH and thereby trapped within cells (8). In the presence of an oxidant, DCFH is converted into the highly fluorescent 2',7'-dichlorofluorescein (DCF).

For assays, cells (1.0 \(\times\) 10\(^6\)/ml) were loaded with 50 \(\mu\)M DCFH-DA. After 5 min incubation in the dark, cells were analyzed by confocal microscopy (8). ROS release from HLF into the medium was assayed by fluorometric method using homovanillic acid as described (25).

**FIGURE 1.** Time course of TGF-\(\beta\)-induced H\(_2\)O\(_2\) production from HLF cells. From 80 to 90% confluent HLF cells cultured in DMEM supplemented with 10% FBS were placed in the reduced serum concentration of 0.5% FBS for 24 h. Quiescent HLF cells were incubated in the presence or absence of 1 ng/ml TGF-\(\beta\) for various times. After incubation, DCF fluorescence was measured with a confocal laser-scanning microscope (A) and mean DCF fluorescence was measured (B) as described in Materials and Methods. C, Quiescent HLF cells pretreated with 5 mM NAC for 10 min were stimulated with 1 ng/ml TGF-\(\beta\) for 10 min. Then, the intracellular ROS level was measured by labeling with DCFH-DA. D, The rate of H\(_2\)O\(_2\) release from quiescent HLF cells was measured after 1 ng/ml TGF-\(\beta\) treatment for various times by fluorometric method. Data represent mean \(\pm\) SD of three independent experiments.
FIGURE 2. Stimulation of IL-6 expression by TGF-β1 treatment in HLF cells. HLF cells were treated with the indicated amounts of TGF-β1 for the various times. Twenty micrograms of total cellular RNA extracted using RNAzol B were blotted with human IL-6 cDNA probe as described in Materials and Methods. A, Effects of different concentrations (ng/ml) of TGF-β1 on IL-6 expression at 12 h after TGF-β1 treatment. B, Time course (hours) of TGF-β1-induced IL-6 expression; C. The culture supernatants of TGF-β1-treated HLF cells were collected at the various times and assayed for IL-6 ELISA. Data represent mean ± SD of four independent experiments.

Northern blot and RT-PCR analysis

Total cellular RNA was extracted by using RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturer’s instruction. Twenty micrograms of RNA were separated in agarose gel containing 2.2 M formaldehyde and then transferred overnight onto nylon membrane and UV cross-linked before prehybridization. Radiolabeling of the 460-bp fragment of human IL-6 cDNA cleaved by Taq I and BamHI was performed using random hexamer priming with [α-32P]CTP and a Klenow fragment of DNA polymerase I (Boeringer Mannheim, Mannheim, Germany). The membrane and probe were hybridized overnight at 42°C in buffer containing 50% formamide. The filters were washed and autoradiographed. Aliquots (3 μg) of total RNA were transcribed into cDNA at 37°C for 1 h in a total volume of 25 μl with 2.5 U of Moloney murine leukemia virus reverse transcriptase. PCR were then performed with 0.05 volume of the reverse transcription reaction for amplification of IL-6 and β-actin. Amplifications were performed in a total volume of 30 μl containing 0.5 U Taq DNA polymerase and 10 pmol primers specific for IL-6 (5′-TCAGGCTT GAGGAGAGATTGCCTGACGTCAGAGAGCTAG-3′ and 5′-GGGA GGGAAGAAGGAGA-3′) and for β-actin (5′-GGGAGGCCACCACTGAGAATGACG-3′ and 5′-GGGAACCCCTACCCATCACTGTA-3′). Amplifications were performed with 25 cycles for β-actin and 30 cycles for IL-6. The amplification profile included denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. After PCR, reaction mixtures were analyzed by agarose gel electrophoresis.

EMSA

After overnight culture with 0.1% serum, the cultures were stimulated for 2 h. The nuclear extracts were prepared according to the procedure previously described (29). DNA mobility shift assays were performed using double-stranded oligonucleotides comprising the consensus sequences for AP-1 (5′-GGCCGCCTTGGTGAAGACTCAGCAGCCGAA-3′), NF-κB (5′-GGGA GTTGAGGGGGACCTTTCACCGGC-3′), and cAMP response element binding protein (5′-GGGAGGATGCTGCGTCGACGAGCTAG-3′). Oligonucleotides were terminal-labeled with [α-32P]dCTP using a Klenow fragment of DNA polymerase I. Aliquots of nuclear extracts (5 μg) were incubated with labeled oligonucleotides in a total volume of 20 μl under the following conditions: 4% glycerol; 1 mM MgCl2; 0.5 mM EDTA; 0.5 mM DTT; 50 mM NaCl; 10 mM Tris-HCl (pH 7.5); and 2 μg poly(dI-dC). Incubations were conducted at room temperature for 30 min, and DNA-protein complexes were analyzed on a 6% polyacrylamide gel.

IL-6 ELISA

Human IL-6 concentrations were determined by IL-6 ELISA. In brief, polystyrene 96-well microtiter plates were coated overnight at 4°C with 100 μl diluted monoclonal anti-IL-6 Ab (100 μg/ml). After washing with PBS containing 0.05% Tween 20 (PBST), plates were blocked with 1% BSA in PBS at room temperature for 2 h, and rinsed with PBST. The culture supernatants (100 μl) were added and incubated for 2 h at room temperature and then washed with PBST. After a washing, 100 μl biotinylated polyclonal anti-IL-6 Ab (250 ng/ml) was added and incubated at room temperature for 2 h. Subsequent to the washing steps, 100 μl of streptavidin-HRP (1:5000) was added and incubated at room temperature for 2 h and proceeded to signal generation by using o-phenylenediamine and hydrogen peroxide. OD were measured at 495 nm, and IL-6 concentrations in test samples were determined from the standard curve of human rIL-6. Data shown are representative of at least three separate experiments.

Western blot analysis

Serum-deprived cells were treated with the agonists in the presence and absence of appropriate inhibitors for the indicated time periods. Cells were lysed in lysis buffer (20 mM HEPES (pH 7.9), 100 mM KCl, 300 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 1 mM Na3VO4, 1 mM PMSF, 100 μg/ml aprotinin, and 1 μg/ml leupeptin). The protein concentrations were determined using Bradford reagent (Bio-Rad, Hercules, CA). Cell lysates containing equal amounts of protein were resolved by 10% PAGE and transferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad). The blot was treated with anti-ACTIVE MAPK Ab or anti-pan ERK Ab, followed by incubation with appropriate peroxidase-conjugated secondary Abs. The Ag-Ab complexes were detected using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ).

Measurement of intracellular Ca2+ concentration ([Ca2+]i)

[Ca2+]i was measured by the use of a laser scanning confocal microscope. HLF cells, grown on coverslips and serum-starved for 1 day, were incubated with 4 μM fluo-3-acetoxymethyl ester (fluo-3-AM) in serum-free medium for 40 min and washed three times with serum-free medium. Each coverslip containing stained cells was mounted on a perfusion chamber (self-designed), subjected to a confocal laser scanning microscope (Carl Zeiss LSM 410), and then scanned every 5 s with a 488 nm excitation argon laser and a 515 nm long pass emission filter. H2O2 was added to the
cells by using an automatic pumping system (self-designed). All images (~130 images) from the scanning were processed to analyze changes of [Ca²⁺] in a single cell level. The results were expressed as the relative fluorescence intensity.

Results

Intracellular generation of transient ROS by TGF-β1 treatment

TGF-β1 (1 ng/ml)-induced intracellular ROS levels in HLF cells were measured with DCFH-DA and laser-scanning confocal microscopy at various time points. As shown in Fig. 1, A and B, DCF fluorescence displayed a rapid increase with maximal intensity at 5 min after treatment and was followed by a decline in fluorescence to the basal level by 20 min. Similar results were observed in three other experiments, and the maximal level of intracellular relative DCF fluorescence appeared to be about a 6.5-fold increase above the baseline value. The increase in DCF fluorescence was sensitive to the treatment of radical scavenger, NAC (Fig. 1C). We also observed the delayed ROS production that was maximal at 16 h as reported previously (Fig. 1D). It appears, therefore, that TGF-β1 induces the rapid and transient ROS change in addition to the sustained, slow increase in HLF cells.

Role of ROS in TGF-β1-induced IL-6 expression

To further elucidate the role of ROS in TGF-β1 signal transduction, the regulation of IL-6 expression by TGF-β1 was investigated. The amount of 1 ng/ml TGF-β1 was sufficient to induce the IL-6 mRNA (Fig. 2A). TGF-β1 (1 ng/ml) treatment produced a progressive increase in IL-6 mRNA concentrations, beginning as early as 30 min, peaking at 12 h, and decreasing after 24 h (Fig. 2B). Also, stimulation with TGF-β1 (1 ng/ml) caused a time-dependent increase in IL-6 protein accumulation in the culture supernatants (Fig. 2C). Next, the effect of various antioxidants on TGF-β1-induced IL-6 expression was analyzed (Fig. 3). Cells were stimulated with TGF-β1 (1 ng/ml) in the presence of catalase (100 U/ml), superoxide dismutase (100 U/ml), NAC (10 mM), or glutathione (GSH, 10 mM) for 6 h. The treatment of these reagents showed no adverse effects on cells, as judged by cell morphology and viability. Catalase, NAC, and GSH reduced the TGF-β1-stimulated IL-6 mRNA expression significantly, whereas superoxide dismutase had a stimulatory effect, probably due to the generation of H₂O₂ from O₂⁻ (Fig. 3A). These results suggest that ROS responsible for the TGF-β1-mediated increase in IL-6 mRNA expression is likely H₂O₂. Furthermore, direct addition of exogenous H₂O₂ also increased IL-6 mRNA expression in a dose-dependent manner (Fig. 3B). Comparing the kinetics of ROS generation and IL-6 gene expression in Figs. 1 and 2, it seems that first transient ROS is involved in IL-6 gene expression. At 30 min after TGF-β1 treatment, the first transient ROS production has been declined to the basal level at least until 60 min (Fig. 1B), whereas IL-6 gene expression was detectable from 30 min after treatment (Fig. 2B). To analyze this observation further, NAC was added to the culture at different times before and after TGF-β1 treatment (Fig. 3C). IL-6 expression at 6 h after TGF-β1 treatment was abolished when NAC was added at ~30 or 5 min after TGF-β1 treatment. However, the addition of NAC at 30 min or later after TGF-β1 treatment had little effect on IL-6 gene expression, suggesting that the first transient ROS production is mainly responsible for IL-6 gene expression at 6 h after TGF-β1 treatment.

FIGURE 3. Effects of antioxidants and H₂O₂ on IL-6 gene expression. A, HLF cells were treated with 1 ng/ml TGF-β1 for 6 h in the presence or absence of antioxidants: c, 100 U/ml catalase; s, 100 U/ml superoxide dismutase; g, 10 mM GSH; n, 10 mM NAC. The antioxidants were pretreated for 30 min before addition of TGF-β1. Northern blot was performed as described in Materials and Methods. B, HLF cells were treated with the indicated concentrations (mM) of H₂O₂ for 6 h. Then, Northern blot was performed. C, Quiescent HLF cells treated with 5 mM NAC for various times were stimulated with 1 ng/ml TGF-β1 for 6 h. IL-6 Northern blot was performed as described in Materials and Methods. Data are representative of three similar experiments.

FIGURE 4. Effects of NAC on TGF-β1-induced AP-1 activity. Serum-starved HLF cells were treated with TGF-β1 (1 ng/ml) or H₂O₂ (0.5 mM) for 2 h with or without the pretreatment of NAC (10 mM) for 30 min. Nuclear extracts (5 μg) were incubated for 30 min with 0.5 ng 32P-labeled double-stranded AP-1 probe. Right, competition analysis with a 50-fold excess of unlabeled AP-1 probe. Data are representative of five experiments performed.
Roles of ROS in TGF-β1-activated AP-1 binding activity

It has been reported that TGF-β1 activates the expression of several genes such as type I collagen (30) and retinoid X receptor (31) via AP-1 activation. AP-1 element in IL-6 promoter (nucleotide 2284 to 2276) is involved in IL-6 gene expression. When HLF cells were treated with TGF-β1, AP-1 activation was observed (Fig. 4). However, NAC treatment abolished TGF-β1-induced AP-1 activation. In the same line, H2O2 treatment also activated AP-1, suggesting that AP-1 is an essential transcription factor for ROS-mediated TGF-β1-induced IL-6. Other transcription factors including NF-κB in IL-6 promoter were not activated by TGF-β1 treatment (data not shown).

Effects of EGTA on TGF-β1-induced IL-6 expression and AP-1 binding activity

It has been reported that TGF-β1 stimulates Ca2+ influx and mediates an elevation of [Ca2+] (27, 28), which suggests the possible roles of Ca2+-dependent events in TGF-β1-mediated signaling. To study the roles of Ca2+ in TGF-β1-induced IL-6 expression, the extracellular Ca2+ chelator, EGTA, was used in combined with TGF-β1. The TGF-β1-induced IL-6 mRNA expression was significantly reduced in EGTA-pretreated cells (Fig. 5A). EGTA itself had no apparent effects on cell viability and morphology. In addition, treatment of cells with Ca2+ ionophore resulted in IL-6 mRNA expression (Fig. 5B). In addition, we investigated the effect of EGTA on TGF-β1-induced AP-1 binding activity (Fig. 5C). The binding activity was reduced in EGTA-pretreated cells, consistent with the observation in IL-6 mRNA expression. Ionomycin treatment also increased the AP-1 complex significantly (Fig. 5C), whereas other cis elements in IL-6 promoter including cAMP response element binding protein were not activated by ionomycin treatment (data not shown). These results suggest that Ca2+ is responsible for TGF-β1-induced IL-6 mRNA expression in HLF cells. Fig. 5D showed that TGF-β1 treatment increased [Ca2+] level rapidly with similar time kinetics (maximal intensity at 3–5 min after treatment) as seen in H2O2 treatment (Fig. 1). Antioxidants such as NAC and catalase abolished TGF-β1-induced [Ca2+] level successfully, indicating that ROS generation is required for TGF-β1-mediated [Ca2+] increase in HLF cells.

Requirement of Ca2+ for H2O2 stimulation of IL-6 mRNA expression

Thus far, the above results indicate that the increase of ROS and Ca2+ is required for TGF-β1-induced IL-6 gene expression. Next, we examined further the order of signaling cascade mediated by H2O2 and Ca2+ in IL-6 gene expression. The EGTA treatment reduced the H2O2-induced IL-6 gene expression, whereas NAC did not inhibit the ionomycin-induced IL-6 gene expression (Fig. 5D). Therefore, ROS and Ca2+ have a dual role in the TGF-β1-induced IL-6 expression via the AP-1 complex.

FIGURE 5. Effects of EGTA on TGF-β1-induced IL-6 gene expression. A, Serum-starved cells were treated with TGF-β1 (1 ng/ml) for 2 h in the absence or presence of EGTA (4 mM). EGTA was pretreated for 30 min before addition of TGF-β1. Total RNAs were isolated and analyzed by RT-PCR. RT-PCR was performed with specific primers for IL-6 as well as for β-actin as an internal control. The gels shown are representative of three independent experiments. B, Serum-starved cells were incubated with indicated concentration of ionomycin for 2 h. RT-PCR was performed as described. C, Nucleic extracts were isolated and EMSA was performed as described in Materials and Methods. Right, Competition analysis with a 50-fold excess of unlabeled AP-1 probe. F, The lane containing only the free probe. D, Serum-starved cells pretreated without or with inhibitors (500 U/ml catalase or 10 mM NAC) were incubated with 4 μM fluo-3-AM in serum-free medium for 40 min and washed three times with serum-free medium. TGF-β1 (1 ng/ml) was added to the cells by using an automatic pumping system (self-designed). All images from the scanning were processed to analyze changes of [Ca2+] in a single cell level. The results were expressed as the relative fluorescence intensity (RFI). A representative experiment of four is presented.
Also, EGTA and NAC regulated H$_2$O$_2$- or ionomycin-induced AP-1 binding activity in the same pattern (Fig. 6B). In addition, when HLF cells were treated with H$_2$O$_2$, [Ca$^{2+}$] was elevated ~3-fold (Fig. 6C), further confirming that Ca$^{2+}$ mobilization event is the downstream of ROS in signaling cascade for IL-6 gene expression.

**Requirement of MAPK activation in TGF-β1-induced IL-6 expression**

Evidences that TGF-β1 is capable of activating MAPK pathways have been reported (32, 33). We investigated the roles of MAPK/ERK activation in TGF-β1-induced IL-6 expression using an ERK kinase 1-specific inhibitor, PD98059. The PD98059 treatment significantly reduced the TGF-β1-induced IL-6 gene expression as well as H$_2$O$_2$- and ionomycin-induced IL-6 gene expression (Fig. 7A). Similar results were also observed in AP-1 EMSA (Fig. 7B). When the activation of ERK was analyzed by immunoblotting using phosphospecific ERK Ab (32), ERK phosphorylation was greatly increased by TGF-β1 treatment, whereas either EGTA or NAC treatment abolished the TGF-β1-induced ERK activation (Fig. 8). Furthermore, EGTA treatment inhibited the H$_2$O$_2$-induced ERK activation. Taken together, these results suggest that ROS and Ca$^{2+}$ are required for the TGF-β1-induced ERK activation and are in turn essential for the TGF-β1-induced IL-6 expression. In addition, these results confirmed the evidence that ROS increase is positioned as an upstream event of Ca$^{2+}$ increase in the pathway of TGF-β1-induced IL-6 expression.

**Discussion**

There is growing evidence that ROS play important roles as a second messenger in signal transduction. Transient elevations in
intracellular ROS occur in response to growth factors and cytokines. As a prooxidant cytokine, TGF-β1 is known to induce a slow and sustained H₂O₂ production in several types of cells. In mouse osteoblastic cells, TGF-β1 induced H₂O₂ production, which peaked at 80 min after stimulation and declined to the basal level at 120 min (24). In HLF and pulmonary artery endothelial cells (22, 25, 26), TGF-β1 treatment resulted in a sustained increase of H₂O₂ with the maximal level at 16 h after treatment. H₂O₂ generated by TGF-β1 treatment was shown to be involved in the induction of egr-1 expression (24) and macrophage CSF via NF-κB activation (23).

In the present study, we observed that TGF-β1 induced a rapid burst of H₂O₂ production which peaked at 5 min. The mode of generation and biological function of the TGF-β1-induced H₂O₂ we observed here are distinct from those of the previously reported, sustained H₂O₂ production. Rather, it is similar to classical ROS generation by growth factors. TGF-β1 induced rapid and transient H₂O₂ generation as shown in the case of PDGF and EGF. In addition, we observed a slow, sustained H₂O₂ production after the initial burst. The physiological relationship between two different H₂O₂ generation induced by TGF-β1 treatment is not clear yet, but the first transient H₂O₂ generation seems to have a role in the TGF-β1 signaling cascade in several aspects: 1) H₂O₂ as a signaling molecule for TGF-β1-induced IL-6 expression. Several studies reported that ROS is involved in cytokine production via activation of transcription factors such as NF-κB and AP-1. The regulation of IL-8 gene expression was mediated by DMSO-sensitive oxidant stress in human whole blood (34). Hypoxia increases TNF-α and IFN-γ expression in mouse pulmonary lymphocytes (35). IL-6, which is involved in inflammation, was also induced by CpG DNA (36), asbestos exposure (37), or TNF-α (10) treatment via antioxidant-sensitive pathways. TGF-β1, as a proinflammatory cytokine, induces IL-6 production in some cell lines including human monocytes, keratinocytes (38), and bone marrow stromal cells (39). However, it is not determined whether the ROS production by TGF-β1 is correlated with IL-6 production. Our results (Fig. 3) showed that TGF-β1-mediated IL-6 expression was inhibited by antioxidants and that direct addition of H₂O₂ increased IL-6 expression, demonstrating that ROS is a mediator of TGF-β1-induced IL-6 expression. 2) H₂O₂ as a signaling molecule for TGF-β1-mediated AP-1 activation. Recently, Eickelberg et al. (40) reported that TGF-β1 induces IL-6 expression via activating AP-1 consisting of JunD homodimers in HLF. Consistent with these results, Fig. 4 showed that TGF-β1 activated AP-1 binding activity, which was also mediated by H₂O₂ production. 3) H₂O₂ as a signaling molecule for Ca²⁺ dynamics. Alevizopoulos et al. (27) demonstrated that TGF-β1 increases Ca²⁺ influx, which activates Ca²⁺-dependent signaling enzymes such as calcineurin and Ca²⁺/calmodulin-dependent kinase. Our results showed that TGF-β1 increased [Ca²⁺] in HLF cells with similar time kinetics with ROS generation and TGF-β1-induced [Ca²⁺] was abolished with antioxidants such as NAC and catalase. Also, EGTA inhibited TGF-β1-induced IL-6 expression and AP-1 activity, and ionomycin increased it. In addition, EGTA inhibited H₂O₂-induced IL-6 expression and AP-1 activity, but NAC did not inhibit ionomycin-induced IL-6 expression and AP-1 activity, suggesting Ca²⁺ generation is required for H₂O₂ downstream signaling. Recently, Lee et al. (41) reported that H₂O₂ treatment induces the increase in [Ca²⁺] in rat fibroblasts. Our results (Fig. 6) demonstrated that H₂O₂ treatment increased [Ca²⁺], which was sustained until ~10 min as observed in other types of cells (our unpublished observations). These results confirmed the H₂O₂ modulates the level of [Ca²⁺] in HLF cells. Finally, H₂O₂ as a mediator for TGF-β1-induced MAPK activation. It has been reported that TGF-β1 activates MAPK in macrophages (32) and rat lung fibroblasts (33) and that MAPK is required for AP-1 activation (42). In HLF cells,
PD98059, an inhibitor of MAPK, inhibited TGF-β1- or ionomycin-induced IL-6 expression and AP-1 activation. In addition, EGTA and NAC inhibited TGF-β1-induced MAPK activation.

To our understanding, this is the first report that the rapid burst of H2O2 by TGF-β1, which turns on the downstream signaling events including MAPK activation and IL-6 gene expression. To reconcile our observations with previous reports, we propose the following scheme (Fig. 9). TGF-β1 elicits the first rapid and transient H2O2 production, which increases [Ca2+]i. This [Ca2+]i increase subsequently activates MAPK and AP-1 activation/IL-6 generation. Recently, it has been reported that Ca2+ activates MAPK via G protein, protein kinase C, or calmodulin-dependent kinase II (43, 44). In this scheme, the relationship between the second phase of H2O2 generation and other signaling events is not known yet. Further detailed studies are required to define the roles of each event in TGF-β1-mediated signaling. These observations can provide a clue to explaining how TGF-β1 regulates proinflammatory cytokine production and inflammatory events as a key inflammatory cytokine by modulating intracellular redox status.

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