Production of Hydrogen Peroxide by Transforming Growth Factor-β1 and Its Involvement in Induction of egr-1 in Mouse Osteoblastic Cells

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Abstract. TGF-β1 controls the expression of numerous genes, including early response and cellular matrix genes. However, the signal-transducing mechanism underlying this regulation of gene expression is not fully understood. In this study, we investigated whether redox regulation plays a role in the TGF-β1 signal transduction in the mouse osteoblastic cell line (MC3T3-E1). The overall intracellular oxidized state of the cells, when measured using 2′,7′-dichlorofluorescin diacetate by laser-scanning confocal microscopy, was increased transiently after the addition of TGF-β1. This increase was abolished by the addition of oxygen radical scavengers such as catalase and *N*-acetylcysteine. In a variant cell line lacking the TGF-β1 receptor, the intracellular oxidized state was not modulated by treatment with TGF-β1. We then examined the expression of early growth response-1 (egr-1) gene, which is inducible by TGF-β1 and H_2O_2. Radical scavengers inhibited the induction of egr-1 by TGF-β1, but not that by 12-*O*-tetradecanoylphorbol-13 acetate. A nuclear run-on assay indicated that this inhibition was at the transcriptional level. From transient expression experiments using chloramphenicol acetyltransferase gene linked to serially deleted egr-1 gene 5′-upstream region, the CArG element in the 5′ flanking region of egr-1 was identified as an essential sequence in the transcriptional activation for both TGF-β1 and H_2O_2 stimulation. These findings suggest that H_2O_2 acts as a mediator for the TGF-β1–induced transcription of egr-1 gene.

TRANSFORMING growth factor-β1 is a multifunctional cytokine regulating cell proliferation, differentiation, and other biological responses (Roberts and Sporn, 1988; Barnard et al., 1990). The diverse biological effects exerted by TGF-β1 depend on cell types, growth status of cells including culture conditions, and the presence of other growth factors. This factor was originally recognized as a stimulator for the anchorage-independent growth of normal cells. However, it is now widely accepted as a potent growth inhibitor of epithelial cells in vitro (Massagué, 1987; Masui et al., 1986), while it acts as a bifunctional regulator for mesenchymal-derived cells (Robey et al., 1987; Centrella et al., 1987; Shibanuma et al., 1991).

TGF-β1 modulates the expression of a variety of genes: it increases the expression of extracellular matrix proteins and protease inhibitor genes (Ignotz and Massagué, 1986; Roberts, et al., 1988; Lund et al., 1986; Thompson et al., 1988) and it decreases the expression of genes for proteases that degrade matrix proteins (Edwards et al., 1987; Kerr et al., 1988). TGF-β1 also induced the expression of immediate-early response genes such as c-*jun*, junB, early growth response-1 (egr-1)[1], and TSC-22 (Pertovaara et al., 1989; Koskinen et al., 1991; Shibanuma et al., 1992). Pietenpol et al. (1990*a*; 1990*b*) reported that the growth inhibition caused by TGF-β1 correlated with a decrease in c-*myc* gene transcription. The products of the retinoblastoma gene and the p53 gene seem to be also involved in this growth inhibition and result in cell cycle arrest (Pietenpol et al., 1990*b*, 1991; Laiho et al., 1990). We have recently cloned a new TGF-β1–inducible gene (TSC-36) whose expresion reached a peak at 6–8 h after stimulation with TGF-β1, and this gene seems to encode an extracellular matrix protein (Shibanuma et al., 1993).

Although the multifaceted gene control actions of TGF-β1 have been explored, the exact mechanisms by which TGF-β1 exerts its effects is still only partly understood. Lin et al. (1992) recently cloned TGF-β1 type II receptor with a serine/threonine kinase domain. The receptor acts as a key component in the transduction of TGF-β1–signaling by form-

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1. *Abbreviations used in this paper*: CAT, chloramphenicol acetyltransferase; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DCFH, nonfluorescent polar derivative of DCFH-DA; egr-1, early growth response-1 (gene); GSH, intracellular glutathione; NAC, *N*-acetyl-L-cysteine; PDTC, pyrrolidine carbodithioic acid ammonium; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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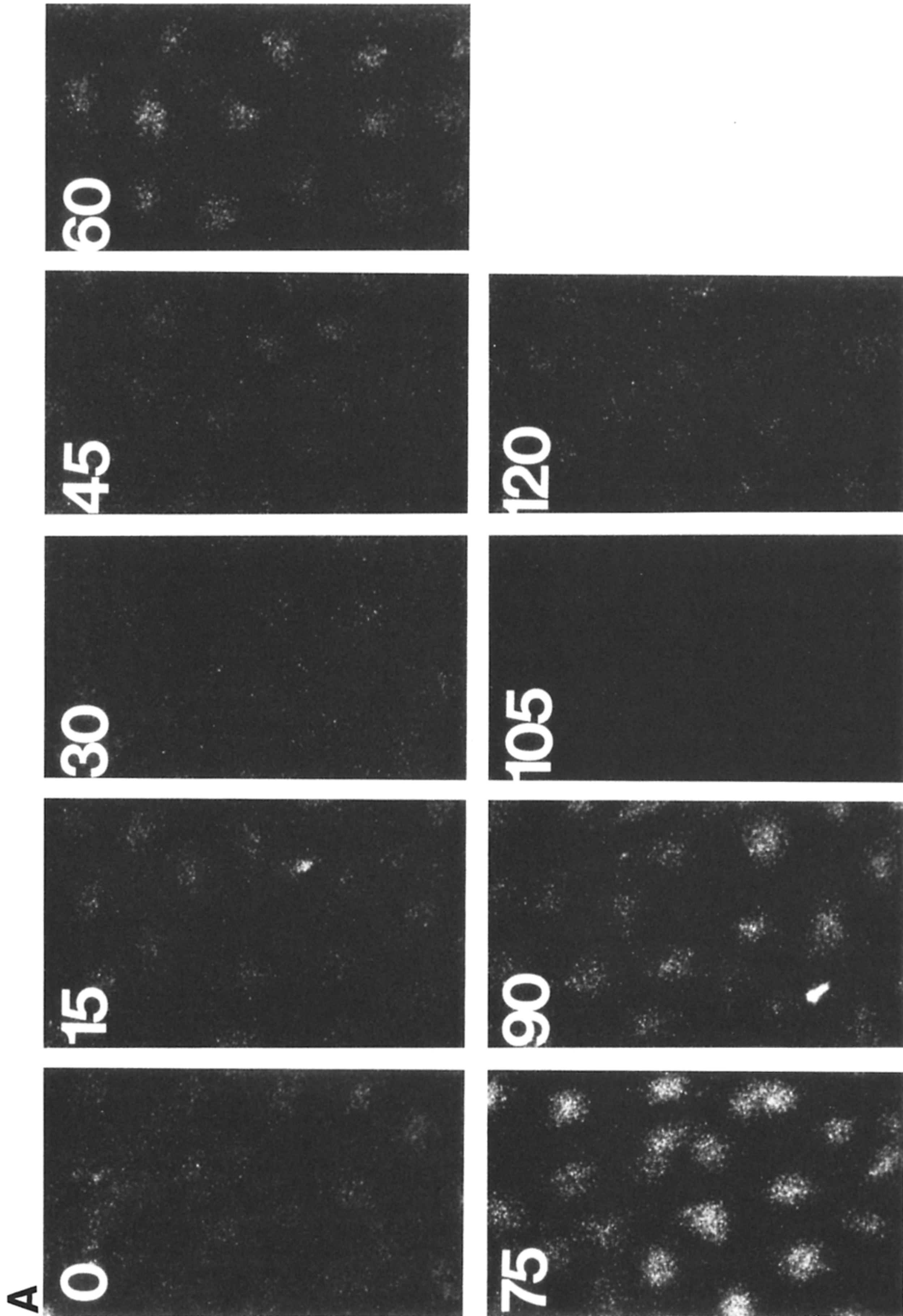
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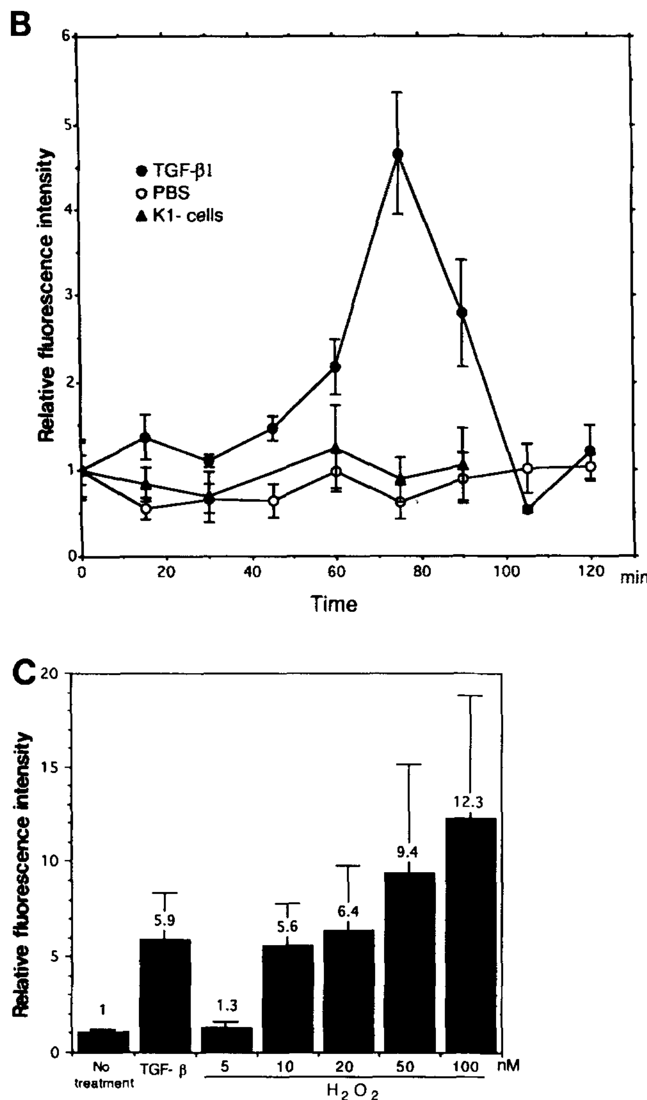

Figure 1. Changes in intracellular redox state of MC3T3-E1 cells after treatment with TGF-β1. (*a*) Cells in preconfluent state were transferred to 0.1% BSA medium, and were treated with TGF-β1 (5 ng/ml) for the indicated times (minutes). After treatment, medium was replaced with Hanks' solution containing 5 μM DCFH-DA, and fluorescence intensity was measured 5 min later with a confocal laser scanning microscope. (*B*) Relative fluorescence intensity of each cell was calculated relative to untreated control cells. (○) Untreated MC3T3 cells, (●) MC3T3 cells treated with TGF-β1, (▲) v-Ki-ras-transformed K1 cells treated with TGF-β1. (*C*) Cells were either treated with TGF-β1 (5 ng/ml) (column *2*) for 75 min or with H_2O_2 (columns *3-7*) for 10 min. About 50 cells were used for determination of each point. The results show the means ± standard deviation. The experiment was repeated three times with reproducible results.

ing a complex with type I receptor (Wrana et al., 1992; Ebner et al., 1993). The involvement of G-protein (Howe et al., 1990) and protein phosphatases as mediators of TGF-β1 action has also been proposed (Gruppuso et al., 1991). In spite of these findings, the question of the signaling pathways from the receptor to nuclei and the nature of the second messengers still remains to be elucidated.

Our previous findings suggest that the actions of TGF-β1 could be mediated by hydrogen peroxide, H_2O_2. Both TGF-β1 and H_2O_2 reversibly blocked cells at the late G1 phase (Shibanuma et al., 1991). The cell cycle arrest by TGF-β1 was partly abolished by catalase. Furthermore, TGF-β1 stimulated the release of H_2O_2 from the cells into the culture medium in MC3T3-E1 cells. These observations suggest that H_2O_2 is a mediator in the action of TGF-β1.

Active oxygens are, in general, considered to be cytotoxic, damaging cells by inactivating and modifying the cellular components (Cerutti, 1985). However, accumulating evidence suggests that oxygen radicals at low doses may function as physiological mediators of cellular responses. For example, H_2O_2 was shown to mimic the activity of insulin in glucose transport and in lipid synthesis in rat adipocytes (Mukhwejee et al., 1978; May and De Haen, 1979). Schreck et al. (1991) and Schreck and Baeuerle (1991) reported that radical scavengers blocked the increase in NFκB-binding activity caused by tumor necrosis factor-α, and that H_2O_2 increased NFκB-binding activity. H_2O_2 also stimulated the expression of immediate early response genes (Nose et al., 1991).

In the present study, we examined the effect of TGF-β1 on the intracellular oxidation state in MC3T3-E1 cells. In addition, we explored whether alterations in oxidation states are involved in modulating egr-1 gene expression.

Materials and Methods

Cell Culture

The mouse osteoblastic cell line, MC3T3-E1 (Kurihara et al., 1986), and its v-Ki-ras-transformant, MC3T3-K1 (Nose et al., 1989), were cultured in Dulbecco's modified medium supplemented with 10% fetal bovine serum and 50 μg/ml kanamycin under a humidified atmosphere of 5% CO_2 in air. Cells were kept in continuous culture for ≤3 mo after thawing.

Chemicals

Recombinant human TGF-β1 was obtained from King Brewing Co. Ltd. (Kakogawa, Japan), and 12-*O*-tetradecanoylphorbol-13-acetate, *N*-acetyl L-cysteine (NAC) and catalase derived from *Aspergillus niger* were products of Sigma Chemical Co. (St. Louis, MO). Pyrrolidine carbodithioic acid ammonium (PDTC) was purchased from Nakarai Tesque, Inc. (Kyoto). Potassium ferricyanide and ascorbic acid were obtained from Wako Pure Chemicals (Tokyo). NAC and ascorbic acid solutions were adjusted to pH 7.4 by the addition of NaOH. 2',7'-dichlorofluorescin diacetate (DCFH-DA) was obtained from Eastman Kodak Corp. (Rochester, NY). The kinase inhibitors H-7, H-9, and W-7 were obtained from Seikagaku Kogyo (Tokyo). Staurosporine was purchased from Kyowa Medix Co. Ltd. (Tokyo).

Assay of Intracellular Redox State

Intracellular redox state levels were measured using a fluorescent dye, DCFH-DA. DCFH-DA is a nonpolar compound that is converted into a nonfluorescent polar derivative (DCFH) by cellular esterases after incorporation into cells. DCFH is membrane-impermeable and rapidly oxidized to the highly fluorescent 2',7'-dichlorofluorescein in the presence of intracellular hydrogen peroxide and peroxidases (Szejda et al., 1984; Bass et al., 1983). For assays, medium was replaced with Hanks' solution containing 5 μM DCFH-DA at appropriate times after stimulation. After 5 min of incubation at room temperature, the fluorescent intensity of ~50 cells for each point was measured by confocal laser scanning microscopy (MRC500; Bio Rad Laboratories, Richmond, CA). The excitation wave length at 513 nm was used. Relative fluorescence intensity was calculated using untreated control cells as standard.

RNA Extraction and Analysis

At ~80% confluence, the cells were starved in serum-free DME containing

0.1% BSA (fraction V; Sigma Chemical Co.) for 48-60 h. The quiescent cells were treated with the agents for the times indicated in the figure legends. Total RNA was extracted using the guanidium/hot phenol method (Maniatis et al., 1982). 20 μg/lane of RNA was electrophoresed on a 1.5% agarose gel containing 2.2 M formaldehyde, blotted onto a nylon membrane (Hybond N; Amersham International, Amersham, UK), and hybridized with probes labeled by nick translation, as described previously (Shibanuma et al., 1992). The probes used were mouse α-tubulin cDNA (Mα1; Lewis et al., 1985) and TGF-β1-inducible TSC-36 cDNA (Shibanuma et al., 1993). Mouse egr-1 cDNA (Tsai-Morris et al., 1988; Cao et al., 1990) was kindly provided by Dr. Sakhatme (University of Chicago, Chicago, IL), and a 1.1-kb fragment corresponding to nucleotide 1958-3068 was used as a probe.

Nuclear Run-on Transcription

Nuclei were isolated from cells treated with TGF-β1 in the presence or absence of 2.5 mM NAC, and they were transcribed in vitro as described previously (Nose et al., 1991). The ^{32}P-labeled transcripts were isolated and hybridized with plasmid DNA immobilized on nylon membranes. The membranes were washed and autoradiographed.

Plasmid Construction

A phage clone containing the complete egr-1 gene, including the 5'- and 3'-flanking sequences (Tsai-Morris et al., 1988), was isolated from mouse liver genomic EMBL3 library (Clontech Laboratories, Inc., Palo Alto, CA) after screening. A mouse egr-1 cDNa fragment was used as a probe for the screening process. A 6.1-kb EcoRI fragment was subcloned into pUC118. An EcoRI/SacII fragment from −1394 to +123 basepairs, containing the egr-1 transcriptional promoter and start site, was subcloned upstream of the chloramphenicol acetyltransferase (CAT) gene in the plasmid pCAT basic vector (Promega Corp., Madison, WI) to construct pEgr1394. 5'-deletion mutants were prepared by cutting with exonuclease III and S1 nuclease, as previously described (Maniatis et al., 1982). pEgr1394, 875, 417, 393, 356, 111, 94, and 72 represent the CAT constructs that contain the egr-1 gene 5'-upstream fragments of indicated numbers of bases counting from the cap site down to +123 basepairs. The deletion end points were determined by dideoxynucleotide sequencing.

Transfection and CAT Assay

Transfection of the CAT constructs (7.5 μg/100-mm dish) was performed by the conventional calcium phosphate precipitation method (Graham and van der Eb, 1973; Gorman et al., 1982). 4 h later, the cells were subjected to 15% glycerol shock and placed in serum-free DME containing 0.1% BSA. 38 h after the transfection, TGF-β1 (5 ng/ml) or H_2O_2 (0.2 mM) was added for additional 8 h. Cell extracts were prepared as described in Gorman et al. (1982). CAT activity was measured by a liquid scintillation counting method (Neumann et al., 1987) using cell lysates in 250 μl of a mixture of 10 mM Tris-HCl (pH 7.8), 1 mM chloramphenicol, and 0.5 μCi [^{3}H]acetyl CoA. CAT activity was evaluated by calculating increase in dpm within a linear range of response. The plasmid pCH110 (5 μg/dish) containing the β-galactosidase gene (Pharmacia, Uppsala, Sweden) was cotransfected to correct for differences in transfection efficiency. The activity of β-galactosidase was determined as described by Herbomel et al. (1984).

Results

Increase of Intracellular Oxidized State in MC3T3-E1 Cells by TGFβ1

Our previous study showed that there was small increase in the H_2O_2 concentrations in the culture medium after treatment with TGF-β1, as detected by 3-(*p*-hydroxyphenyl)-propionic acid and horseradish peroxidase assays (Shibanuma et al., 1991). In the present study, intracellular oxidized states were measured using a more sensitive fluorescent assay method with DCFH-DA and laser-scanning confocal microscopy. The dye is incorporated into cells and is converted to a fluorescent compound by oxidation. The intensity of fluorescence reflects the intracellular oxidized state (Bass et al., 1983; Robertson et al., 1990).

Quiescent cultures of MC3T3 cells were treated with TGF-β1 (5 ng/ml) for various times. As shown in Figs. 1 *A* and *B*, the intensity of DCFH fluorescence began to increase 60 min after treatment, reaching a maximal level of fivefold higher than that in unstimulated cells at 75 min after treatment. This was followed by a decline in fluorescence to the basal level by 120 min. Some cells showed high fluorescence in nuclei, possibly because of accumulation of the dye in nuclear fraction. The maximal level of intracellular fluorescence was comparable to that treated with 10-20 nM H_2O_2 (Fig. 1 *C*). In a *ras*-transformed variant of MC3T3 cells (K1) that were resistant to TGF-β1 through loss of its binding sites (Nose et al., 1989), no change in fluorescence intensity was observed after treatment with TGF-β1 (Fig. 1 *B*). These results suggested that an increase in the intracellular oxidized state was induced specifically by TGF-β1 through the binding to its receptors.

DCFH can be oxidized by any peroxidase and hydroperoxide, including H_2O_2 (Bass et al., 1983; Keston and Brandt, 1965). To specify the active oxygen species that were responsible for the oxidation of DCFH, we examined whether catalase, an enzyme that specifically hydrolizes H_2O_2, prevented the oxidation of DCFH. MC3T3 cells were treated with TGF-β1 in the absence (Fig. 2, *top* and *bottom*, *column B*) or presence (Fig. 2, *top* and *bottom*, *column C*) of catalase (350 U/ml). The addition of catalase completely inhibited the increase in DCFH oxidation as measured 75 min after TGF-β1 treatment. Heat-inactivated catalase had no effect on DCFH oxidation (Fig. 2, *top* and *bottom*, *column E*). These findings suggest that H_2O_2 participated in DCFH oxidation after the TGF-β1 treatment. Nonspecific thiol radical scavengers, NAC and PDTC, also inhibited the increase in the intracellular oxidized state induced by TGF-β1; NAC at 2.5 mM had a 25% inhibitory effect on the increase in DCFH oxidation, and PDTC at 0.1 mM inhibited this increase by 50% (data not shown).

Inhibitory Effect of Radical Scavengers on the Expression of egr-1 Gene by TGF-β1

We then investigated whether H_2O_2 generated by TGF-β1 plays a role in gene expression. Our previous study demonstrated that active oxygens stimulated the expression of the immediate early response genes such as c-*fos*, c-*jun*, c-*myc*, and egr-1 (Nose et al., 1991). Of these genes, egr-1 was found to respond to both TGF-β1 and H_2O_2 in MC3T3 cells.

Fig. 3 shows Northern blotting of egr-1 gene at various times after treatment with either TGF-β1 (2 ng/ml) or H_2O_2 (0.15 mM). H_2O_2, at concentrations $\leq$0.2 mM, did not affect cellular viability as determined by dye exclusion test and plating efficiency assay (data not shown). As seen in Fig. 3, egr-1 gene expression increased within 30 min after stimulation with TGF-β1, reaching the maximal level 2 h after treatment. H_2O_2-induced egr-1 mRNA more rapidly and transiently, reaching a peak within 30 min after the treatment. Slower induction of egr-1 by TGF-β1 compared to that by H_2O_2 showed the delay of ~90 min. This delay roughly coincided with the time course of changes in the intracellular oxidized state determined by the confocal laser scanning microscopy (Fig. 1).

We examined the effects of radical scavengers, i.e. catalase, NAC, PDTC, potassium ferricyanide (Chaudhri et al.,

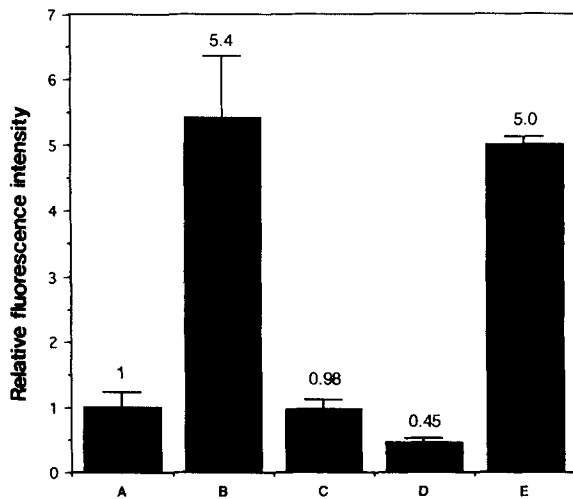

Figure 2. Effects of catalase on the increase in intracellular oxidized state induced by TGF-β1. Cells were treated for 75 min with either TGF-β1 (5 ng/ml) (*B*), TGF-β1 + catalase (350 U/ml) (*C*), catalase (*D*), or TGF-β1 + catalase (heat-inactivated) (*E*). Fluorescence intensity was measured and calculated as described in the legends to Fig. 1 *B*. The experiments were repeated four times, and the results were reproducible.

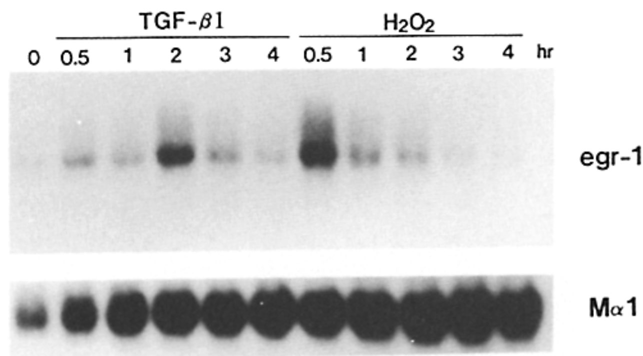

Figure 3. Time course of induction of egr-1 mRNA by TGF β1 and H_2O_2. MC3T3 cells were either untreated (lane *1*), treated with TGF-β1 (2 ng/ml) (lanes *2–6*), or H_2O_2 (0.15 mM) (lanes *7–11*) for indicated times. Total RNA was extracted, run on a gel, and transferred to membrane filters. The filters were hybridized with ^{32}P-labeled probes, washed, and autoradiographed. Mouse α-tubulin probe (M α 1) was used to monitor the amount of RNA in each lane.

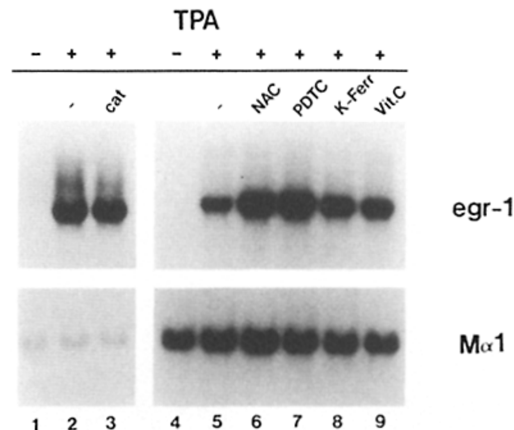

Figure 5. Effects of radical scavengers on the induction of egr-1 by TPA. Cells were untreated (lanes *1* and *4*), or they were treated for 2 h with 50 ng/ml TPA in the presence (lane *2* and *5*) or presence of 170 U/ml catalase (lane *3*), 2.5 mM NAC (lane *6*), 50 μM PDTC (lane *7*), 5 mM potassium ferricyanide (lane *8*), or 5 mM ascorbic acid (lane *9*). RNA was analyzed as described in the legends to Fig. 3.

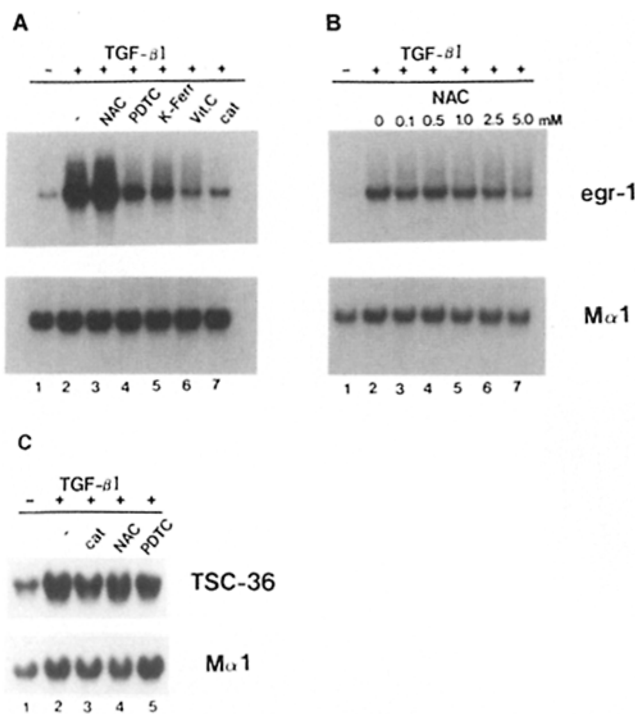

Figure 4. Effects of radical scavengers on the induction of egr-1 and TSC-36 mRNA by TGF-β1. (*A*) MC3T3 cells were either untreated (lane *1*), or they were treated with TGF-β1 (2 ng/ml) in the absence (lane *2*) or presence of 0.5 mM NAC (lane *3*), 50 μM PDTC (lane *4*), 5 mM potassium ferricyanide (*K-Ferr*; lane *5*), 5 mM ascorbic acid (*Vit.C*; lane *6*), or 170 U/ml catalase (*cat*; lane *7*) for 2 h. (*B*) Cells were untreated (lane *1*), or they were treated with TGF-β1 (2 ng/ml) in the absence (lane *2*) or presence of increasing concentrations (lane *3–7*, 0.1, 0.5, 1.0, 2.5, and 5.0 mM, respectively) of NAC. (*C*) Cells were untreated (lane *1*), or they were treated with 2 ng/ml TGF-β1 in the absence (lane *2*) or presence of 170 U/ml catalase (lane *3*), 2.5 mM NAC (lane *4*), or 50 μM PDTC (lane *5*) for 8 h. RNA was analyzed using ^{32}P-labeled probes as described in the legend to Fig. 3.

1988), and vitamin C, on the induction of egr-1 by TGF-β1. As shown in Fig. 4 *A*, the addition of these antioxidants abrogated the induction of egr-1 by TGF-β1, except for NAC. The inhibitory effect of NAC on egr-1 induction was found to be dependent on its concentrations (Fig. 4 *B*). Catalase was very effective in the inhibition of egr-1 induction (Fig. 4 *A*, lane *7*), consistent with oxidized state caused by TGF-β1 treatment (Fig. 2).

These radical scavengers did not inhibit the induction of another TGF-β1–inducible gene, TSC-36 (Fig. 4*C*). Furthermore, egr-1 induction by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was not affected by these scavengers (Fig. 5). These results indicate that the inhibitory effect of scavengers was specific for egr-1 gene expression induced by TGF-β1. A nuclear run-on assay was carried out to test whether the induction of egr-1 by TGF-β1, and its inhibition by NAC were regulated at the transcriptional level. As shown in Fig. 6, TGF-β1–activated transcription of egr-1 gene, and NAC at 2.5 mM led to the reduction of this TGF-β1–induced transcription. Shorter exposure of the film showed that transcrip-

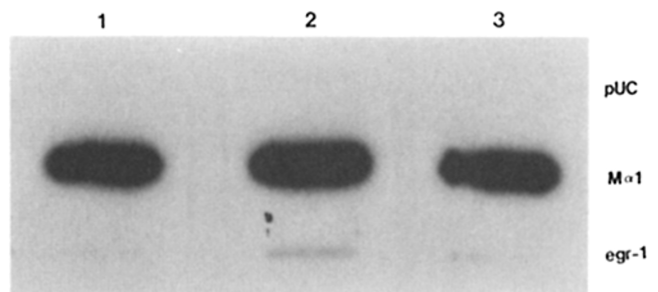

Figure 6. Nuclear run-on assay. Nuclei were isolated from untreated cells (lane *1*), and from cells treated with TGF-β1 (2 ng/ml) for 90 min in the absence (lane 2) or presence of 2.5 mM NAC (lane *3*), and were transcribed in the presence of [^{32}P]UTP. The transcripts were hybridized with the probes immobilized on membranes. The membranes were washed and autoradiographed.

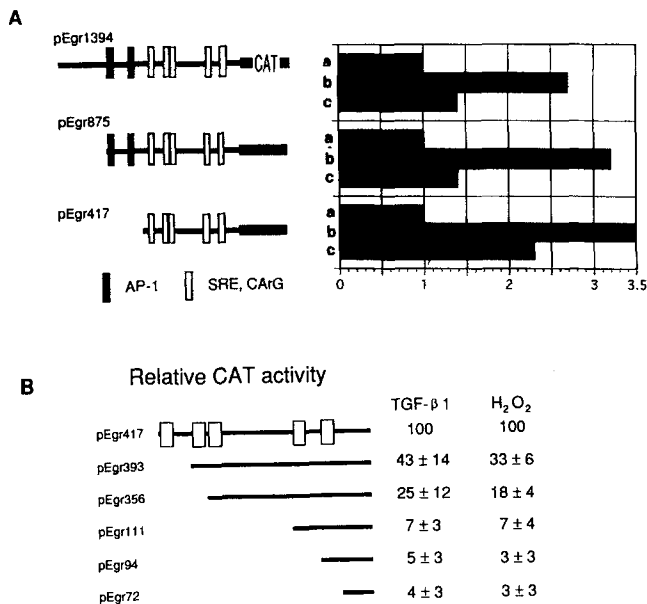

Figure 7. Schematic representation of egr-1/CAT constructs and stimulation of egr-1 enhancer by TGF-β1 and H_2O_2. MC3T3 cells were transfected with each plasmid by the calcium phosphate precipitation method, and they were cultured in DME containing 1 mg/ml BSA for 38 h. The cells were either untreated, or they were treated with TGF-β1 (5 ng/ml) or 0.2 mM H_2O_2. Cells were harvested 8 h later, and CAT activity was measured. (*A*) *a*, Untreated; *b*, TGF-β1-treated; *c*, H_2O_2-treated. Values indicate relative CAT activity. (*B*) Relative CAT activity of serial deletion constructs in TGF-β1-treated and H_2O_2-treated cells. Open and closed boxes indicate sites containing CArG and AP-1 elements, respectively. The experiments were carried out three times, and the results show the means ± standard deviation.

tion of Mαl stayed constant during 90 min after treatment period (data not shown).

A Common Target Sequence for TGF-β1- and H_2O_2 Stimulation of egr-1 Gene Transcription

To elucidate the transcriptional regulatory mechanism responsible for redox-mediated TGF-β1 signal transduction, we attempted to identify a common *cis*-acting DNA element for both TGF-β1 and H_2O_2. For this purpose, the deletion mutants upstream of the transcriptional start site of the egr-1 gene were fused to a CAT reporter gene. The mouse egr-1 upstream region contains five $CC(A/T)_6GG$ (CArG) boxes plus the incomplete CCAATATGGC sequence (position −340 to −331 bp, Tsai-Morris et al., 1988). These CArG elements were progressively deleted from pEgr1394, a plasmid containing the mouse egr-1 upstream region between −1394 to +123 bp with five CArG elements and two AP-1-like elements (Fig. 7).

The highest CAT activity was observed with pEgr417, which contained all five CArG elements, in cells treated either with TGF-β1 or H_2O_2 (Fig. 7 *B*). Treatment with TGF-β1 or H_2O_2 led to a 2.3- and 3.5-fold increase in CAT activity, respectively, compared to that of unstimulated cells. A progressive decrease in response was observed as each CArG element was deleted. Removal of the three CArG elements from the 5′-end (pEgr111) resulted in very weak induction by both agents, indicating that TGF-β1 and H_2O_2 require at least three CArG elements from the cap site for the induction. The above findings identify the CArG elements as a common target sequence for both TGF-β1 and H_2O_2.

Effects of Protein Kinase Inhibitors on the Expression of egr-1

Protein kinases participate in signal transductions elicited by diverse stimuli. Possible involvement of protein kinases in the redox-mediated signaling pathways was examined by protein kinase inhibitors. The results of Northern blots show that 50 μM of H-7, an inhibitor of protein kinases C, A, and G, repressed almost completely the egr-1 induction by TGF-β1 and H_2O_2 (Fig. 8). However, neither staurosporine, a potent inhibitor of protein kinase C, nor H-9, an inhibitor of cAMP-dependent kinase, affected the egr-1 induction by TGF-β1 and H_2O_2. Moreover, the induction of egr-1 by TGF-β1 was retained in the protein kinase C–downregulated cells after prolonged treatment with TPA (24 h) (data not shown). Based on these observations, we postulate that an H-7–sensitive kinase, but not protein kinase C, might be involved in the gene expression of egr-1 by TGF-β1 and H_2O_2.

Discussion

In the present study, we demonstrated that TGF-β1 increased the intracellular oxidized state in mouse osteoblastic cells, but not in a TGF-β1 receptor–deficient variant. This increase was almost completely abolished by the addition of catalase, indicating that H_2O_2 was a major cause of increased oxidized state. The production of H_2O_2 was also blocked, albeit to a lesser extent, by the nonspecific thiol agents, NAC and PDTC. NAC raises the levels of intracellular glutathione (GSH), which controls the cellular redox state as a substrate of GSH peroxidase. In addition, NAC and PDTC both directly quench intracellular free radical (Staal et al., 1990). Catalase, on the other hand, cannot be incorporated into cells, thereby acting on the outside of the cells. These results suggest that H_2O_2 might be first released from the cells after treatment with TGF-β1, and then diffused into the cells.

In the previous study (Shibanuma et al., 1991), we measured levels of H_2O_2 in the medium by using 3-(*p*-hydroxyphenyl)-propionic acid and horseradish peroxidase, and we detected H_2O_2 in the medium of cells stimulated by TGF-β1 only in cells at G_1 phase and not in G_0 phase cells. In the present study, however, the method using DCFH-DA and confocal laser microscopy made it possible to detect the increase of the intracellular oxidative state. Consistent with the previous work, the increase in the intracellular oxidative state was more pronounced and sustained in cells in late G1 phase (data not shown).

The pathways for generation of free radicals in MC3T3 cells are not fully understood. NAD(P)H oxidase, which produces superoxide anion from O_2, is known to be localized in the outer cell membrane (Ramasarma, 1982). In neutrophils and macrophages, burst of oxygen radical occurs within seconds after stimulation (Sagone et al., 1976; Fridovich, 1983). In contrast, the change in redox state in response to TGF-β1 was slow and transient. In addition, radical scavengers did not inhibit egr-1 induction by TPA, which is known to activate NAD(P)H oxidase (Maridonneau-Parini,

1986). Thus, the generating system of H_2O_2 activated by TGF-β1 in MC3T3 cells seems to be distinct from the NAD(P)H oxidase system, probably because of a specific, still-unknown generating system.

Changes in the redox state of cells are thought to induce modifications of cellular signaling molecules, including protein kinases, protein phosphatases, and transcription factors. Regulation by redox of a portion tyrosine kinase (Bauskin et al., 1991; Devary et al., 1992) and a protein phosphatase (Guy et al., 1993) has also been reported. In the present study, possible biological roles of oxidized state in gene expression was studied with the egr-1 gene, which was transcriptionally activated by both TGF-β1 and H_2O_2. The results of Fig. 4, *A* and *B*, show that scavengers for active oxygens specifically inhibited the induction of egr-1 by TGF-β1, indicating that the action of TGF-β1 is regulated by redox, at least in term of egr-1 gene transcription.

CAT assay using the egr-1 promoter region indicated that the CArG element is a common target for the transcriptional activation of egr-1 by both TGF-β1 and H_2O_2. A recent finding (Datta et al., 1993) demonstrated that the transcription induction of egr-1 caused by ionizing radiation also required CArG elements. The involvement of active oxygens in this transcriptional activation was implied from the experiment with NAC. The CArG element is known to bind to serum response factor, whose binding activity is enhanced by its phosphorylation (Treisman, 1993).

Recent observations provide evidence that redox regulation is a general mechanism for the posttranslational control of transcription factor function. Changes in reduction-oxidation potential have been shown to influence the DNA binding activity of several transcription factors, such as Oxy R (Storz et al., 1990), FOS/JUN (Abate et al., 1990) and NFκB (Schreck et al., 1991). Oxy R is a best example of this redox regulation in the direct activation of a transcription factor in bacterial systems. DNA-binding activity of mammalian FOS/JUN is reported to be modified by the oxidation of their cysteine residues (Abate et al., 1990). Most of these works are, however, limited to in vitro experiments, and our present results indicate that redox regulation of transcription exists in vivo as well.

Active oxygens are produced by various types of cultured cells under the influence of numerous stimuli (Meier et al., 1989; Robertson et al., 1990). They are generally regarded as harmful to cells, because they modify cellular macromolecules, including lipids, proteins, carbohydrates, and nucleic acids. Cells have several different types of systems to protect them against such free radical-induced damages; systems involving intracellular glutathione, catalase, superoxide dismutases, and glutathione peroxidase (Fridovich, 1978; Meister, 1983). Most active oxygens can be deleterious to cells, but cytokines, especially suppressors of growth such as tumor necrosis factor-α and interleukin-1, are known to stimulate cells to produce active oxygens (Beutler and Cerami, 1986; Meier et al., 1989).

We previously proposed that active oxygens can work as a competence factor for growth in quiescent fibroblasts (Shibanuma et al., 1990). It now seems reasonable to believe that active oxygens at certain levels could work as biosignals in physiological conditions. The molecular mechanisms responsible for such signals may include direct oxidation of protein factors by sulfenylation (Storz et al., 1990) and S-thiolation (Gilbert, 1984). Our results with the inhibitors of protein kinases indicate that the activation of H-7–sensitive protein kinase(s) is necessary for the induction of egr-1 by either TGF-β1 or H_2O_2, and such kinase(s) could be a direct target of redox regulation. Protein kinases that are stimulated by interleukin-1 and tumor necrosis factor (Guesdon et al., 1993) could be regulated by redox.

The best example of the second messenger actions of active oxygen species is nitric oxide. Nitric oxide is one member of active oxygen species, and is well known as a major messenger molecule regulating immune function and blood vessel dilatation. It also serves as a neurotransmitter in the brain and peripheral nervous system (Lowenstein and Snyder, 1992). We propose that hydrogen peroxide could be another member of active oxygens that operates as a second messenger in growth factor signalings.

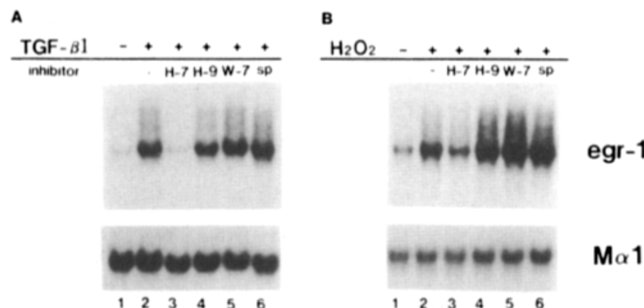

Figure 8. Effects of protein kinase inhibitors on egr-1 induction by TGF-β1 or H_2O_2. Cells were untreated (lane *1*), or they were treated with 2 ng/ml TGF-β1 (*A*) or 0.15 mM H_2O_2 (*B*) for 2 h in the absence (lane *2*) or presence of 50 μM H-7 (lane *3*), H-9 (lane *4*), W-7 (lane *5*), or 0.5 μM staurosporine (*sp*, lane *6*). RNA was extracted and analyzed as described in the legend to Fig. 3.

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