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Inhibition by *N*-acetyl-L-cysteine of interleukin-6 mRNA induction and activation of NFκB by tumor necrosis factor α in a mouse fibroblastic cell line, Balb/3T3

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Abstract Redox-based modulation plays a role in transcriptional control of gene expression. In the present study, we investigated the possible role of reactive oxygen species in the induction of interleukin-6 (IL-6) mRNA and in increases in NFκB binding activity by tumor necrosis factor (TNF) α using a mouse fibroblastic cell line, Balb/3T3. Expression of IL-6 mRNA is known to be dependent upon NFκB that binds to the 5′-flanking region of the IL-6 gene. We found that: (i) TNFα increased IL-6 mRNA levels and this increase was inhibited by *N*-acetyl-L-cysteine (NAC), a scavenger of reactive oxygen species. (ii) NFκB binding activity in this cell line was also increased by TNFα, and the increase was inhibited in the presence of NAC. (iii) The treatment of cells with low doses of hydrogen peroxide increased the NFκB binding activity. (iv) Expression of a reporter gene in which the chloramphenicol acetyltransferase (CAT) gene was under the control of NFκB binding sites was induced by hydrogen peroxide. These results suggest that the induction of IL-6 mRNA is regulated by a mechanism involving reactive oxygen species and that NFκB, whose activity is sensitive to the cellular redox state, plays an important role in this induction in a fibroblastic cell line, Balb/3T3, stimulated with TNFα.

Key words: Interleukin-6; *N*-acetyl-L-cysteine; NFκB

1. Introduction

A growing body of evidence suggests that reactive oxygen species play a physiological role as second messengers in a variety of cellular processes, including growth regulation [1–9]. These species are produced by various types of cells in response to a variety of stimuli, including growth factors [10–15]. Our earlier studies indicated that superoxide anions were implicated in the pH_i increase elicited by growth stimuli in U937 cells and mouse thymocytes [16] and that hydrogen peroxide operated as a competent factor in quiescent Balb/3T3 cells [5]. In a more recent study, we found that the inhibitory effect of tumor growth factor β1 (TGFβ1) on the growth of a mouse osteoblastic cell line, MC3T3, was mediated, at least in part, by hydrogen peroxide [7].

Although the precise mechanisms underlying these effects of reactive oxygen species on cellular growth remain largely unknown, an increasing number of reports has shown that a variety of genes that are thought to be associated with growth control are induced by reactive oxygen species. We and others have reported that immediate-early genes such as the c-*fos*, c-*myc*, c-*jun*, *egr*-1, *KC*, and *JE* genes were induced by hydrogen peroxide [47–6,17–19] and that the induction of at least some of them was controlled at a transcriptional level [17–19]. Recently we demonstrated that induction of *egr*-1 gene by TGFβ1 was mediated by hydrogen peroxide [17]. With regard to the induction of these genes, the critical problem is to identify the transcription factors that are responsible for the induction and are activated by reactive oxygen species. Some transcriptional regulatory elements on DNA sequences and transcription factors responsible for the induction of these genes by hydrogen peroxide have already been identified. We have previously shown that the serum responsive element (SRE) of c-*fos* was activated by hydrogen peroxide; however, the most prominent example is NFκB binding activity. Schreck et al.[20], in experiments with human T cells, demonstrated that NFκB was activated by hydrogen peroxide and that its activation by various agents was inhibited by thiol compounds. Redox regulation of NFκB binding activity was also reported by others [21–24]. However, there have been few reports investigating whether the levels of mRNAs whose expression were dependent on NFκB binding activity were actually modulated by the change in cellular redox state.

In this study, we examined the role played by reactive oxygen species in the activation of NFκB and in the expression of IL-6 mRNA, one of the best studied examples among the genes whose expression are transcriptionally regulated by NFκB [25–27], using a fibroblastic cell line Balb/3T3 treated with TNFα.

2. Materials and methods

2.1. Chemicals

Hydrogen peroxide was obtained from Mitsubishi Gas Co. (Tokyo, Japan), mouse recombinant TNFα from Boehringer Mannheim Co. (Indianapolis, IN). *N*-Acetyl-L-cysteine (NAC), cycloheximide (CHX), and phorbol 12-myristate 13-acetate (TPA) were purchased from Sigma Chemicals (St. Louis, MO).

2.2. Cell culture

The Balb/3T3 A31–1 clone was obtained from the Japan Cancer Research Resources Bank. Cells were grown in Eagle's MEM supplemented with 10% fetal bovine serum under 5% CO_2 in air. We used quiescent (contact-inhibited and serum-depleted) cells cultured without renewal of medium for 7 days after inoculation.

2.3. Gel mobility shift assay

Nuclear extracts were prepared following the procedure of Dignam et al. [28]. The cells were collected with a rubber policeman, suspended in hypotonic buffer A (10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM phenylmethyl-sulfonyl fluoride; PMSF, and 1 mM dithiothreitol;

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Abbreviations: IL-6, interleukin-6; TNFα, tumor necrosis factor α; NFκB, nuclear factor κB; NAC, *N*-acetyl-L-cysteine

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DTT), and centrifuged and disrupted with a Dounce homogenizer. The nuclear fraction obtained by centrifugation was extracted with buffer C (20 mM HEPES (pH 7.9), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 1 mM PMSF, and 1 mM DTT) at 4°C for 30 min. The extract purified by centrifugation was dialyzed against 50 × vol of buffer D (20 mM HEPES (pH 7.9), 0.1 M KCl, 0.2 mM EDTA, 20% glycerol, 1 mM PMSF, and 1 mM DTT) at 4°C for 5 h. The dialysate was cleared by centrifugation, and aliquots were kept frozen at −80°C. Sodium molybdate (10 mM) was added to all buffers to prevent phosphatase activity.

The probes and competitors used were: (i) a HIV enhancer DNA fragment from −92 to −79 [29] termed NFκB (wild type)(AGCTTGGGGACTTTCCAGCCG and its complementary strand GATCCGGCTGGAAAGTCCCCA), or termed NFκB (mutant type) (AGCTTGCTCACTTTCCAGCCG and GATCCGGCTGGAAAGTGAGCA) subcloned into the *Hin*dIII/ *Bam*HI sites of pUC18, (ii) TPA-responsive element (TRE), a human metallothionein IIA (hMTIIA) DNA fragment (GATCGCAAGTGACTCAGCGCG and GATCCGCGCTGAGTCACTTGC) subcloned into the *Xba*I/*Bam*HI site of pUC18 [30] and (iii) cyclic AMP responsive element (CRE), a rat somatostatin DNA fragment (GATCCCTGGGGGCGCCTCCTTGGCTGACGTCAGAGAGAGAGG and GATCCCTCTCTCTCTGACGTCAGCCAAGGAGGCGCCCCCAGG) subcloned into the *Bam*HI site of pUC18 [31]. The franking sequence GATC served as a linker for subcloning. The NFκB, TRE, and CRE elements are underlined. The inserts were cut out from the plasmids at each site for the restriction endonucleases and terminally labeled with [^{32}P]dCTP and Klenow fragments.

For the binding assay, nuclear extracts (10 μg of protein) were incubated in 24 ml of binding buffer (20 mM HEPES (pH 7.9), 50 mM KCl, 4 mM $MgCl_2$, 4 mM spermidine, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.05% (v/v) NP-40, 20% (v/v) glycerol, and 1 mg/ml of bovine serum albumin) containing 1 μg of poly(dI-dC), and various competitor DNA (100 ng) as indicated for 1 h at 4°C. The ^{32}P-labeled probe (0.2 ng, 5,000 cpm) was added last, and the mixture was incubated for 30 min at room temperature. Samples were analyzed on native 4% polyacrylamide gel containing 6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA for 2 h at 11 V/cm. The gels were dried and autoradiographed with a intensifying screen at −70°C.

2.4. Transient expression assay

Vectors expressing chloramphenicol acetyltransferase (CAT) were constructed, using $pA_{10}CAT_2$[32] modified by Dr. Satake (Kyoto University). The part of the 72 bp repeat of the SV40 enhancer contained in $pA_{10}CAT_2$ plasmid was deleted by treatment with *Bal*31 and then ligated to a *Bgl*II linker. The resulting plasmid was cut with *Bgl*II, and the HIV enhancer fragment (−105/−79) was inserted into this site. For transfection, cells were plated at 3×10^6 cells/100-mm plastic dish. Twenty four h later, they were transfected with plasmid DNA by the conventional calcium phosphate precipitation technique [33]. The resulting precipitates were removed 6 h later and the cells were treated with 20% dimethylsulfoxide. The cells were cultured for 24 h after transfection and then treated with H_2O_2 or TPA for 48 h. The cell lysate was prepared as described by Gorman et al. [34]. The lysates were incubated in 0.15 M Tris-HCl (pH 7.6), containing 0.5 μCi [^{14}C]chloramphenicol and 1 mM acetyl- CoA for 1 h at 37°C. Chloramphenicol acetyltransferase activity was determined by thin-layer chromatography.

2.5. RNA extraction and analysis

Total RNA was extracted by the guanidium/hot phenol method [35]; 20 μg samples of RNA were separated in agarose gel containing 2.2 M formaldehyde. The RNA was then transferred to a nylon membrane (Hybond N, Amersham) and hybridized with ^{32}P-labeled probes in 50% formamide at 42°C for 24 h. The probes used were mouse IL-6 cDNA, a fragment cut out by digestion with *Hpa*II and *Bgl*II from pSRKmIL-6 (a kind gift from Dr. N. Arai, DNAX Research Institute, CA) and mouse α-tubulin cDNA (Mα1) [36]. The filters were washed, as described before, and autoradiographed [35].

3. Results

TNFα is a cytokine that stimulates cells to produce reactive oxygen species [37] and increases intracellular oxidized state to the level corresponding to that in cells treated with 0.1–0.2 mM H_2O_2 (data not shown). We treated quiescent Balb/3T3 cells with TNFα and first examined the effect of NAC, that is a scavenger of reactive oxygen species, on the inducibility of IL-6 mRNA. IL-6 mRNA was accumulated 2–4 h after the treatment with TNFα, but in the presence of NAC, this accumulation was diminished (Fig. 1).

Because the induction of IL-6 mRNA is thought to be dependent on NFκB activation [25–27], we next investigated (i) whether the activation of NFκB binding was increased by TNFα in Balb/3T3 cells, and (ii) whether this activation was also sensitive to NAC. As shown in human T cells [20], TNFα increased the binding activity of NFκB in quiescent Balb/3T3 cells (Fig. 2, lane 2), and this increase was decreased in the presence of NAC (Fig. 2, lane 4). That the effect of NAC was specific to NFκB binding activity was shown by our finding that the formation of complexes to other elements, i.e. cAMP-responsive element (CRE), and TPA- responsive element (TRE), was virtually unaffected (Fig. 2, lane 6, 8, 10 and 12). The increase of NFκB binding activity was not sensitive to cycloheximide (CHX) (Fig. 2, lane 3), implying that this activation was controlled post-transcriptionally.

The above result suggested that reactive oxygen species were involved in the activation of NFκB binding. To investigate further whether activity of NFκB binding could be modulated by reactive oxygen species in the fibroblastic cells, we treated the cells with 0.15 mM hydrogen peroxide. At this dose, hydrogen peroxide was not toxic and stimulated cells to proliferate in the presence of insulin [5]. The concentrations of H_2O_2 used in the present experiment, thus, seems to be relevant and physiological ones. The gel mobility shift assay of the nuclear extract prepared from these cells showed a remarkable increase of binding activity to the NFκB site derived from human immunodeficiency virus (HIV) enhancer, while no such increase of binding occurred to the mutant NFκB site (Fig. 3A, lanes 2,5). The activity reached maximum level 2 h after hydrogen peroxide treatment and thereafter rapidly returned to a basal level (data not shown). The specificity of this binding was assured by competition analysis with the wild and mutant type

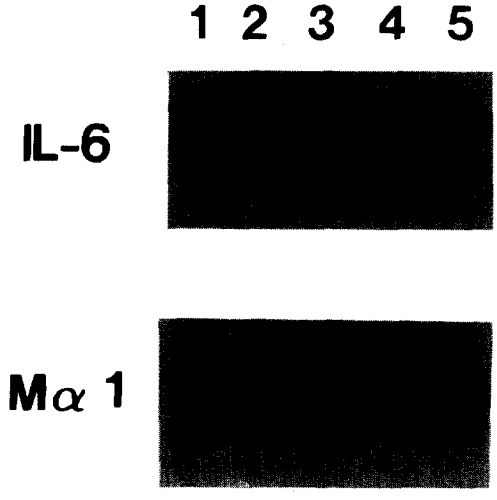

Fig. 1. Induction of IL-6 mRNA in Balb/3T3 cells by TNFα and effects of NAC on its induction. Cells were untreated (lane 1), or treated with TNFα (100 U/ml) for 2 h (lanes 2,3) or 4 h (lanes 4,5). NAC (20 mM) was added simultaneously with TNFα (lanes 3,5).

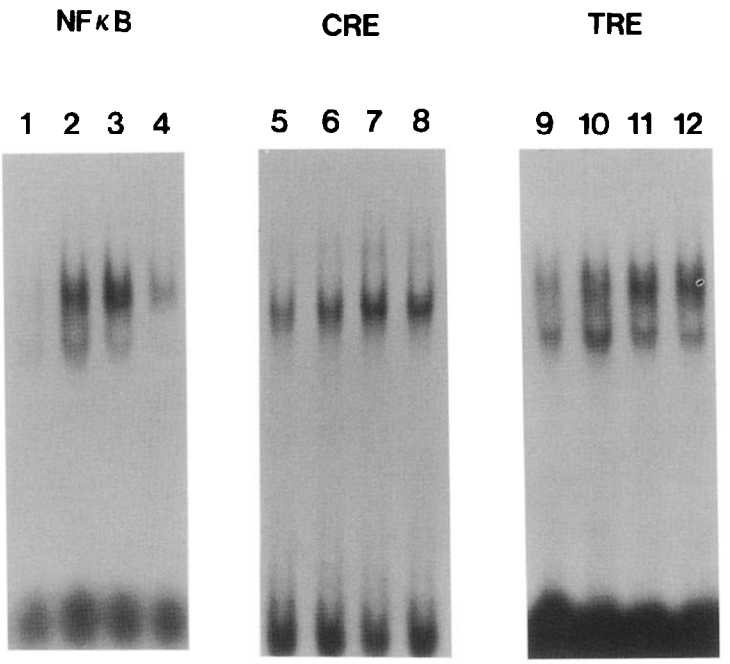

Fig. 2. NFκB, CRE, and TRE binding activity induced by TNFα. The nuclear extracts were prepared from Balb/3T3 cells untreated (lanes 1,5,9) and treated with TNFα (100 U/ml, 4 h) (lanes 2,3,4,6,7,8,10,11,12) in the presence of either CHX (20 mg/ml) (lanes 3,7,11) or NAC (20 mM) (lanes 4,8,12). Probes used were: lanes 1–4, wild-type of NFκB; lanes 5–8, CRE; lanes 9–12, TRE.

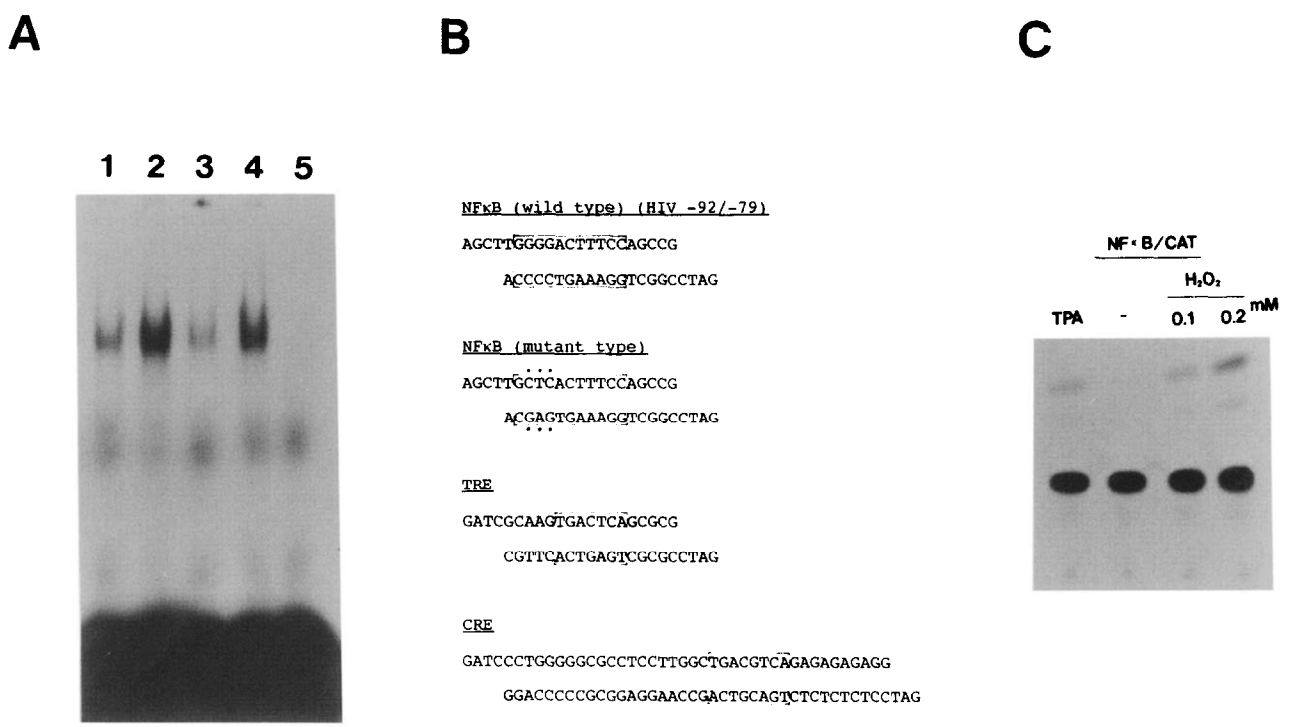

Fig. 3. (A) Gel mobility shift assay of wild-type NFκB binding site. The nuclear extracts were prepared from Balb/3T3 cells: lane 1, no treatment; lanes 2–5, treated with hydrogen peroxide (0.15 mM, 2 h). Probes used were: lanes 1–4, wild-type NFκB; lane 5, mutant-type NFκB. Unlabeled wild (lane 3) and mutant (lane 4)-types of competitors were present in the binding assay. The assay was performed as described in section 2. (B) Sequences of probes and competitors used in this study. NFκB, TRE, and CRE elements are boxed. (C) Transient activation of NFκB binding site by hydrogen peroxide and TPA. Cells were transfected with 10 μg/dish of NFκB/CAT DNA. One day after transfection, the cells were either left untreated (lane 2) or were treated with 50 ng/ml TPA (lane 1) or hydrogen peroxide 0.1 mM (lane 3) or 0.2 mM (lane 4) for 48 h. CAT activity was measured by thin-layer chromatography.

sequences of the κB site. Only the wild type sequence competed with the binding (Fig. 3A, lanes 3,4). This result demonstrated that reactive oxygen species were not only necessary but also sufficient for the activation of NFκB binding.

To examine whether NFκB binding activation by hydrogen peroxide could lead to gene expression, we transfected into cells a reporter gene construct in which the chloramphenicol acetyltransferase (CAT) gene was under the control of NFκB binding sites derived from HIV enhancer. The cells were treated with hydrogen peroxide, following which CAT activity in the cell lysates was measured. As shown in Fig. 3C, increased CAT activity was clearly detected after the hydrogen peroxide treatment; 0.2 mM of hydrogen peroxide was more effective than 50 ng/ml TPA, one of potent activators of NFκB. Although NFκB binding and transcriptional activation from the promoter linked to NFκB binding sites were induced by hydrogen peroxide, hydrogen peroxide alone could not increase IL-6 mRNA level (data not shown).

4. Discussion

We have been studying about the mechanisms of growth control and gene expression by reactive oxygen species in fibroblasts. We selected Balb/3T3 cell line, since hydrogen peroxide was found to operate as a competent factor in quiescent state of this line [5]. Recent findings that reactive oxygen species play an important role in the activation of NFκB, a transcription factor in human T cells [20], prompted us to investigate the involvement of such species in the activation of NFκB and gene expression in a fibroblastic cell line, Balb/3T3.

In this study, we found that treatment of Balb/3T3 cells with TNFα induced the expression of IL-6 mRNA and this induction was inhibited by NAC, a scavenger of reactive oxygen species. These changes of IL-6 mRNA level seem to reflect those of NFκB binding activity within the cells. Actually TNFα activated NFκB binding and this activation was inhibited by NAC in Balb/3T3 cells. Although it has been shown that NFκB binding activity was modulated by reactive oxygen species, biological relevance of such a modulation to gene expression in vivo has been unclear. Our results raised the possibility that a certain kind of gene expression was regulated by reactive oxygen species through the modulation of NFκB binding activity in vivo. Besides IL-6 gene, genes of immunomodulatory cytokines such as β-interferon and TNFα [25–27,38] are the target genes of NFκB. These cytokines affect cellular growth in many types of cells, including fibroblasts. Consequently, it is likely that reactive oxygen species play an important role in the activation of NFκB binding, thereby affecting gene expression and cellular growth.

In a fibroblastic cell line, both TRE and SRE binding activities have been shown to be increased by hydrogen peroxide [18]. In this study, NFκB binding was also shown to be activated by hydrogen peroxide. These binding activities induced by hydrogen peroxide are thought to be functional as indicated by the results of a CAT assay [18]. Taken toghether these results, the action of hydrogen peroxide as a competent factor in quiescent Balb/3T3 cells could result from these concomitant activation of several transcription factors.

Some growth factors producing reactive oxygen species, such as TNFα, might make use of such functions of these species in their signaling pathway. TNFα is secreted by activated macrophages and influences a variety of biological processes, including mitogenesis, differentiation, angiogenesis, and immunological regulation [39]. One particular aspect of this cytokine is its cytotoxic/cytostatic action against certain tumor cells both in vitro and in vivo. However, it has recently been found that TNFα affects cellular growth both positively and negatively, depending on cell type and growth conditions [40]. Interestingly, in quiescent Balb/3T3 cells, Palombella et al. [41] demonstrated that TNFα exhibited a stimulatory effect on cell growth just like hydrogen peroxide did [5]. This effect might be mediated at least in part by reactive oxygen species working as a competent factor.

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