Nuclear factor κB-dependent mechanisms coordinate the synergistic effect of PMA and cytokines on the induction of superoxide dismutase 2

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Manganese superoxide dismutase (MnSOD) serves a protective role under conditions of oxidative stress mediated by such diverse agents as adriamycin, radiation, chemical hypoxia and ischaemia and might act as a newly recognized type of tumour-suppressor. MnSOD is an inducible enzyme; however, the signalling molecules and pathways involved in its induction have not been fully elucidated. Recently we reported the identification of a 342 bp enhancer within the second intron (I2E) of the human gene encoding MnSOD (SOD2), which contains sites for binding nuclear factor κB (NF-κB), CCAAT-enhancer-binding protein (C/EBP) and nuclear factor 1 (NF-1). Using a human fibroblast cell line transformed by simian virus 40, we have identified the I2E fragment as being responsive to PMA. Furthermore, simultaneous treatment with PMA and cytokines (tumour necrosis factor α and interleukin 1β) synergistically increases MnSOD induction. The use of mutant constructs identified the NF-κB element within the enhancer fragment as being essential for the PMA and PMA/cytokine effect. Mutations in the C/EBP- and NF-1-binding sites revealed a potential co-operation between proteins that bind to these sites and the NF-κB element. Evaluation of inhibitory κB (IκB)-α and IκB-β proteins reveals agent-specific differences in their turnover kinetics. Both C/EBP and NF-κB DNA-binding activities were increased in cells receiving a combination of cytokine and PMA. Supershift and immunoprecipitation studies suggest a physical interaction between C/EBP and NF-κB proteins. Taken together, these studies suggest the activation of multiple transcription factors as well as pathways leading to increased NF-κB activity as being the mechanisms responsible for the synergistic induction of MnSOD by PMA and cytokines.

Key words: CCAAT-enhancer-binding protein, gene regulation, interleukin 1β, oxidative stress, tumour necrosis factor α.

INTRODUCTION

Manganese superoxide dismutase (MnSOD) is an essential antioxidant that catalyses the dismutation of superoxide radicals, generating H_2O_2 and molecular oxygen [1]. Reactive oxygen species (ROS), including the superoxide (O_2^-) radical, are normal by-products of aerobic metabolism. There is accumulating evidence to suggest a role for ROS in a variety of pathological conditions, including aging, vascular disease and neurodegenerative diseases such as amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease and Alzheimer's disease (reviewed in [2]). The contribution of ROS to these diseases might result from either an increase in their production and/or an ineffective system for their removal. Within the superoxide dismutase family of isoenzymes, MnSOD has been shown to be essential for normal development. In animal models in which the *SOD2* gene has been knocked out, neonatal lethality occurs as a result of cardiomyopathy [3] and neurodegeneration [4]. This finding distinguishes MnSOD from both the cytosolic (Cu,Zn SOD) and extracellular (ECSOD) copper- and zinc-containing superoxide dismutase isoenzymes. Knock-out of either the *CuZnSOD* or the *ECSOD* gene did not result in neonatal lethality [5,6].

With the use of both transgenic cell and animal models, MnSOD has been shown to attenuate injury or death under conditions of enhanced oxidative stress. In cell models, over-expression of MnSOD protects against tumour necrosis factor α (TNF-α) [7], exposure to iron [8], nitric-oxide-generating agents [8,9], alkalosis [10] and chemical hypoxia [11]. In animals that have been generated to overexpress MnSOD, attenuation of oxygen-induced lung injury [12], doxorubicin-induced cardiac injury [13] as well as ischaemia and trauma-induced brain injuries [8,14] have been observed.

In addition, numerous studies suggest a role in tumour suppression by MnSOD. Studies have shown that tumour cells, which normally express low levels of MnSOD, undergo phenotypic changes as a result of transfection and subsequent over-expression of MnSOD [15–17]. Furthermore, MnSOD has been shown to suppress tumour growth in nude mice [15,16] and to inhibit the metastatic potential of transplanted tumours [18].

Alterations in the cellular redox status by agents such as TNF-α [19], interleukin 1β (IL-1β) [20], dinitrophenol [21], paraquat [22] and PMA [23] can up-regulate *SOD2* gene transcription. In addition, studies both *in vitro* and *in vivo* have shown that pretreatment with agents that enhance MnSOD expression can protect against subsequent oxidative stress mediated by hyperoxia [24], paraquat [25], radiation [26] and reperfusion injury [27]. Therefore the identification of specific signalling molecules involved in MnSOD induction could lead to the development of novel therapeutic interventions for many of the aforementioned conditions.

Recently we identified a 342 bp enhancer, I2E, within the second intron of the human *SOD2* gene, that is responsive to TNF-α and IL-1β [28]. This enhancer element contains a binding

Abbreviations used: BME, Eagle's basal medium; C/EBP, CCAAT-enhancer-binding protein; I2E, enhancer element within second intron of *SOD2* gene; IκB, inhibitory κB; IL-1β, interleukin 1β; MnSOD, manganese superoxide dismutase; NF-1, nuclear factor 1; NF-κB, nuclear factor κB; PKC, protein kinase C; ROS, reactive oxygen species; TNF-α, tumour necrosis factor α.

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site for nuclear factor κB (NF-κB) that is essential for induction by TNF-α and IL-1β. In addition there are binding sites for CCAAT-enhancer-binding protein (C/EBP) and nuclear factor 1 (NF-1), which might also contribute to the regulation of MnSOD expression. In this study we evaluated the potential role of this enhancer in the inducibility of SOD2 by PMA. Unlike cytokines, phorbol esters modulate gene expression predominantly by activation of protein kinase C (PKC). Furthermore, owing to the potential of both PKC-dependent (PMA) and PKC-independent (TNF-α and IL-1β) pathways involved in SOD2 induction, we used a combination treatment of cytokines and PMA to determine whether MnSOD expression could be further enhanced over that caused by the application of each agent individually.

EXPERIMENTAL

Cell culture

The simian-virus-40-transformed human lung fibroblast cell line (VA-13), which was purchased from the American Type Culture Collection, was maintained in Eagle's basal medium (BME) supplemented with 10% (v/v) fetal bovine serum, 1% (w/v) glutamine and 1% antibiotics. Cells were grown at 37 °C in a humidified air/CO_2 (19:1) atmosphere.

Plasmids and site-directed mutagenesis

A *Bam*HI fragment containing a 3.4 kb 5' flanking region was used to generate the minimal promoter fragment (P7). To create the P7 fragment, PCR primers with recognition sequences for the *Kpn*I and *Bgl*II restriction enzymes were added to allow subcloning upstream of the luciferase reporter gene. The following primer set was used (the underlined 9 bp sequence indicates the *Kpn*I recognition site in the upper-strand primer and the *Bgl*II recognition site in the lower-strand primer starting at +24): upper strand (−210), 5'-CGGGGTACCGCCTCCTTTCTCCCGTGCCCTGGG-3'; lower strand (+24), 5'-GGAAGATCTGCCGAAGCCACCACAGCCACGGAGT-3'.

The P7 fragment was subcloned into a PGL3 luciferase vector (Promega). Digestion with *Bam*HI of a 39 b λ phage clone containing the entire human *MnSOD* gene resulted in a 8074 bp fragment that was subsequently used as a template for generating a 342 bp fragment within the second intron (I2E). The primers were designed to amplify the I2E region with *Kpn*I sites. PCR was performed in a 100 μl master mixture containing 10 μl of 10×PCR buffer [200 mM Tris/HCl (pH 8.8)/20 mM $MgSO_4$/100 mM KCl/100 mM $(NH_4)_2SO_4$/1% (v/v) Triton-X-100/1 mg/ml nuclease-free BSA], 4 μl of 10 mM dNTP mix (2.5 mM each of dATP, dCTP, dGTP and dTTP), 1 μl of each primer (upper strand, 5'-CGGGGTACCGGGGTTATGAAATTTGTTGAGTA-3'; lower strand, 5'-CGGGGTACCCCACAAGTAAAGGACTGAAATTAA-3') at 10 pM, 100 ng of plasmid DNA and 1 μl of a high-fidelity *Pfu* DNA polymerase (2.5 units/μl; Stratagene). The thermal cycling settings were: after initial denaturation at 95 °C for 5 min, 30 amplification cycles were performed with denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min.

Site-specific mutations within the C/EBP-1, C/EBPX, C/EBP-2, NF-κB and NF-1 elements of the I2E construct were prepared by a Chameleon™ double-stranded site-directed mutagenesis kit (Stratagene). After the target plasmid DNA (I2E/P7/PGL3) had been denatured by heat, two oligonucleotide primers were annealed to one of the strands. One primer contained a specific mutation in a defined sequence of the target DNA and the other primer was used as a selection primer to change one non-essential unique restriction site to *Xba* I. Native T7 polymerase and T4 DNA ligase were used for primer extension around the plasmid. The heterozygous plasmid DNA was digested with *Xba*I and then transformed into a repair-deficient *mutS Escherichia coli* strain. Plasmid DNA was isolated, digested with *Xba*I and then transformed into *E. coli* XLI-Blue competent cells to yield the selected mutant plasmid DNA. The selection primer corresponding to a change in the *Xba*I digestion site was 5'-GATCGCCGTGTAATTCTGGAGTCGGGGCGGCCGGCCGCTTC-3'. The mutated primer for each transcription-factor-binding site in the I2E fragment was designed as follows (target regions are indicated by underlines): C/EBP-1, 5'-GATTAAAAGAGGAGGAAGTTATTACATTCTGGAAGATTTAC-3'; C/EBPX, 5'-GAGGAGGAAGTTACCACAT-TCTTTAAGATTTACTTGAGAC-3'; C/EBP-2, 5'-AACAAATTTGGGAAACATTTCCTGGGGAGAGACTGGGGAA-3'; NF-κB, 5'-TGGGGAGAGACTGTTAATACTTCAGTTGTGAAAGTACTT; NF-1, 5'-GGGAATACCCCAGTTATGACCGTACTTCCTGTAAGGCAAC-3'.

After amplification, the I2E region and mutants were subcloned into the modified PGL3 vector (Promega) containing the luciferase reporter gene driven by the minimal human MnSOD promoter (P7/PGL3) [29]. The nucleotide composition of all constructs was verified by DNA sequencing methods.

Transient transfection

Cells were plated at a density of 3×10^6 cells per 100 cm^2 plate. After 24 h the cells were transfected with a modified calcium phosphate method, as described by Graham and Van Der Eb [30]. Cells were co-transfected with 1 μM of plasmid construct containing either the I2E fragment or mutants of the I2E construct and 0.1 μM of pRL-TK (Promega) containing the *Renilla* cDNA. After 8 h the cells were washed twice with PBS and incubated in fresh BME. After 24 h, cells were trypsin-treated and replated in a 24-well plate at a density of 10^5 cells per well. After 24 h cells were treated with either one of 200 i.u./ml recombinant TNF-α (R & D Systems), 100 nM PMA (Sigma) or 2 ng/ml IL-1β (Endogen), or combinations thereof. In preliminary experiments, concentrations of PMA, TNF-α and IL-1β were optimized to maximize luciferase expression in the VA-13 cells without causing cytotoxicity. At 12 h after treatment, the cells were washed with PBS and lysed in reporter passive lysis buffer (Promega). Samples were analysed for luciferase activity with the Dual-Luc Reporter Assay System (Promega) in accordance with the manufacturer's instructions, in a TD-20/20 luminometer (Turner Designs).

Stable transfection

The plasmid DNA (I2E-P7/PGL3) was linearized with *Bam*HI and co-transfected with the pSV2-NEO plasmid by using lipofectin (Gibco). After 48 h the cells were exposed to 400 μg/ml geneticin (G418 sulphate). Clones were obtained and propagated from single transfected cells plated on 60 mm dishes. Cell lines obtained from individual clones were maintained in BME with 10% (v/v) fetal bovine serum, 1% (w/v) glutamine, 1% (w/v) penicillin/streptomycin plus 400 μg/ml G418. For experiments with the stable transfectants, 10^5 cells were plated in a 24-well plate and treated as described above. Luciferase activity was measured with the Luciferase Assay System (Promega) in ac-

cordance with the manufacturer's instructions. All luciferase assays were normalized to protein concentration.

Northern blot analysis

Total RNA was isolated by the method of Chirgwin et al. [31]. RNA was estimated spectrophotometrically at 260 nm. Total RNA (30 μg) was loaded on a 1 % (w/v) agarose/formaldehyde gel for electrophoresis and then transferred to a nylon membrane (Nytran Plus; Schleicher & Schuell, Keene, NH, U.S.A.). The membrane was baked at 80 °C for 2 h, prehybridized at 42 °C in prehybridization solution [50 % formamide/5 × saline/sodium phosphate/EDTA (150 mM NaCl/10 mM NaH_2PO_4/1 mM EDTA)/5 × Denhardt's solution/0.1 % SDS/100 μg/ml sonicated sperm DNA], and then hybridized for 72 h at 42 °C. Probes were ^{32}P-labelled with the random priming method. The filters were washed at room temperature twice for 15 min with 2 × SSC (where 1 × SSC = 150 mM NaCl/15 mM sodium citrate)/0.1 % SDS/0.1 % sodium pyrophosphate, and twice for 20 min at 65 °C with 0.1 × SSC/0.1 % SDS/0.1 % sodium pyrophosphate. The blots were then exposed to X-ray film at −70 °C. The probes used were a human MnSOD cDNA and a chicken β-actin cDNA (OnCor, Gaithersburg, MD, U.S.A.).

Western blot analysis for inhibitory κB (IκB)-α and IκB-β

Cytoplasmic extracts (50 μg) of control and treated cells were analysed for levels of IκB-α and IκB-β. Samples were separated by SDS/PAGE [12.5 % (w/v) gel] and transferred to nitrocellulose. Transfer efficiency was assessed by incubation with 0.1 % Ponceau. The membrane was washed with distilled water to remove the excess stain and blocked in Blotto [5 % (v/v) milk/10 mM Tris/HCl (pH 8.0)/150 mM NaCl/0.05 % (v/v) Tween 20] for 1 h at room temperature. For analysis, either an affinity-purified rabbit anti-(IκB-α) or anti-(IκB-β) antibody (1:1000 dilution) purchased from Santa Cruz Biotechnology was used. After two washings in TBST [10 mM Tris/HCl/150 mM NaCl/0.05 % (v/v) Tween-20], the blot was incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology) at a 1:5000 dilution in Blotto for 1.5 h at room temperature. The blot was washed three times with TBST and once with TBS. Protein bands were detected with the enhanced chemiluminescence detection system (ECL®; Amersham, Little Chalfont, Bucks., U.K.). Densitometric analysis was performed with ImageQuant for Windows (version 5.1).

Electrophoretic mobility-shift assay

Cells were plated at a density of 6×10^5 cells per 100 mm dish, then treated as described above. Nuclear extracts, as well as the cytoplasmic fraction, were isolated as described by Dignam et al. [32] with the inclusion of 35 % (v/v) glycerol and protease inhibitors (pepstatin, aprotinin and leupeptin) at 1 μg/ml in the extraction buffer. The phosphatase inhibitors NaF (5 mM) and Na_3VO_4 (1 mM) were included. Protein concentration was determined by a colorimetric assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Oligonucleotides corresponding to C/EBP, NF-κB and NF-1 *cis*-acting elements within the I2E fragment were either purchased from Santa Cruz (C/EBP) or synthesized by Life Technologies as follows: C/EBP, 5'-TGCAGATTGCGCAATCTGCA-3'; C/EBP mutant, 5'-TGCAGAGACTAGTCTCTGCA-3'; NF-κB, 5'-GAGACTGGGGAATACCCCAGT-3'; NF-κB mutant, 5'-GAGACTGGTTAATACTTCAGT-3'; NF-1, 5'-CAGTTGTGAAAGTACTTCC-3'.

After annealing, each oligonucleotide was radioactively end-labelled with [γ-^{32}P]ATP (3000 Ci/mmol at 10 mCi/ml) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA, U.S.A.). The probes were purified on a 20 % (w/v) native PAGE gel, which was then exposed to Kodak film and the band corresponding to the double strand was excised. The DNA was eluted overnight at 37 °C in 300 μl of 10 mM Tris/HCl/1 mM EDTA buffer (pH 7.4). The activity of labelled probe was counted and stored at −70 °C. Nuclear extract protein (2.5–10 μg) was used; the final volume of each reaction was 20 μl. Each reaction contained 4 μl of 5 × binding buffer [20 % (v/v) glycerol/5 mM $MgCl_2$/2.5 mM EDTA/5 mM dithiothreitol/50 mM Tris/HCl (pH 7.5)/0.25 mg/ml poly(dI-dC)] and 30000 c.p.m. of labelled probe. Samples were incubated at room temperature for 20 min. Supershift experiments to determine the components of the complex bound to the I2E NF-κB element after combination treatment were performed by the addition of 2 μl of the primary antibody [p50 (sc-1190x), p65 (sc-372x), C/EBP-α (sc-61X) or C/EBP-β (sc-150X)] to the binding reaction and extending the incubation to 1 h at room temperature. The reaction was stopped by the addition of 2 μl of 10 × DNA loading buffer [25 mM Tris/HCl (pH 7.5)/0.02 % Bromophenol Blue/4 % (v/v) glycerol]. DNA–protein complexes were separated from unbound probe on a native 6 % (w/v) PAGE gel in 0.5 × Tris/borate/EDTA buffer. Gels were dried under vacuum and exposed to Kodak film at −70 °C for 24–48 h. All antibodies were purchased from Santa Cruz Biotechnology.

Immunoprecipitation

Immunoprecipitation studies were performed with nuclear extracts from VA-13 cells in RIPA buffer [9.1 mM Na_2HPO_4/1.7 mM NaH_2PO_4/150 mM NaCl (pH 7.4)/1 % (v/v) Nonidet P40/0.5 % sodium deoxycholate/0.1 % SDS/10 mg/ml PMSF/1 μg/ml aprotinin]. The antibodies used included a rabbit anti-(C/EBP-β) antibody (Santa Cruz) and an agarose-conjugated p50 antibody (Santa Cruz). Nuclear extracts were incubated at 4 °C overnight with the corresponding antibodies. After a 2 h incubation, Protein A/G (20 μl; Santa Cruz Biotechnology) was added to the reaction with the C/EBP-β antibody. Immunoprecipitates were collected by centrifuging the sample at 2500 rev./min (605 ***g***) for 5 min, then washing four times with RIPA buffer. Samples were resuspended in sample loading buffer, subjected to SDS/PAGE [12.5 % (w/v) gel] and then transferred to nitrocellulose. After transfer samples were analysed by Western blotting.

Statistical analysis

All experiments were replicated in triplicate and representative findings are shown. Analysis of variance was performed for multiple samples and statistical significance was determined by using Bonferroni's test.

RESULTS

Combination treatment of VA-13 cells enhances SOD2 mRNA levels

To establish that the endogenous human *MnSOD* gene was responsive to treatments, we determined the effect of PMA alone and in combination with cytokines (TNF-α and IL-1β) on endogenous MnSOD mRNA levels in the VA-13 cell line. Cells were treated with either one of TNF-α (200 i.u./ml), IL-1β (2 ng/ml) or PMA (100 nM) or combinations thereof; RNA was isolated after 12 h. Hybridization with a human MnSOD cDNA probe revealed that treatment of VA-13 cells with either TNF-α or IL-1β resulted in similar MnSOD mRNA levels after 12 h

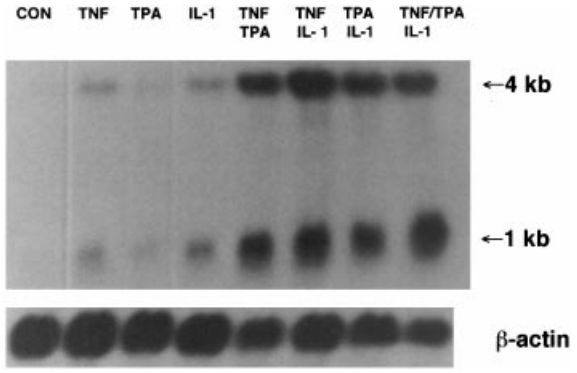

Figure 1 MnSOD mRNA levels increase synergistically with combination treatment

Northern analysis of MnSOD mRNA in VA-13 cells as a result of cytokine and/or phorbol ester treatment [TNF-α (200 i.u./ml), PMA (TPA) (100 nM), IL-1β (2 ng/ml)]. The 1 kb and 4 kb human MnSOD transcripts are indicated. The blots were stripped and reprobed with a β-actin probe (bottom panel).

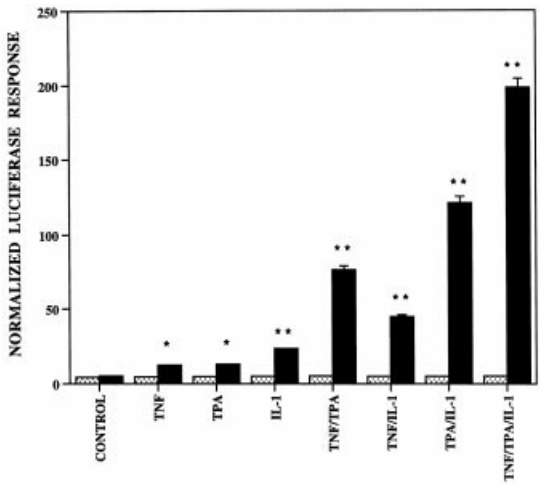

Figure 2 Intronic enhancer is responsive to PMA

The effect of TNF-α (200 i.u./ml), IL-1β (2 ng/ml) or PMA (TPA) (100 nM) and combinations thereof on the I2E/P7-dependent luciferase reporter gene. Results are means $\pm$ S.E.M. for three independent experiments. $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.0001$ [significant differences between construct (I2E/P7/PGL3) and vector control (P7/PGL3) receiving the same treatment]. Stippled-shaded columns, P7; black columns, 12E/P7.

(Figure 1). Furthermore, MnSOD mRNA levels were higher after cytokine treatment than with PMA (Figure 1). After all treatments an increase in both the 4 kb and 1 kb transcripts was seen. Blots were stripped and reprobed with a chicken β-actin cDNA probe as a control for RNA integrity and loading.

NF-κB element within the second intron is essential for induction by PMA

Previously we have identified an enhancer element within the second intron of the *SOD2* gene that is responsive to TNF-α and IL-1β in a manner independent of orientation or position [28]. To identify whether the intronic enhancer could respond to PMA, a known inducing agent of MnSOD, VA-13 cells were transiently transfected with the I2E/P7/PGL3 construct, which contains several binding sites for C/EBP, NF-κB and NF-1 within a 342 bp intronic fragment. Figure 2 indicates that elements within the 342 bp fragment confer responsiveness to PMA. Furthermore, we found that simultaneous treatment of cells with PMA and one or more cytokines (TNF-α or IL-1β) synergistically enhanced the luciferase response, whereas a combined treatment of TNF-α and IL-1β had an 'additive' effect on luciferase activity (Figure 2). With combination treatment the reporter assay results paralleled the increase in MnSOD mRNA.

To examine the relative importance of transcriptional elements in the intronic region after treatment with PMA alone or in combination (TNF-α/PMA, TNF-α/IL-1β, PMA/IL-1β, TNF-α/PMA/IL-1β), specific mutations for each binding site (C/EBP-1, C/EBP-X, C/EBP-2, NF-κB and NF-1) within the second intron were generated by site-directed mutagenesis. Mutation of the NF-κB site completely destroyed the TNF-α (Figure 3A), PMA (Figure 3B), IL-1β (Figure 3C), TNF-α/IL-1β (Figure 3E) and TPA/cytokine (Figures 3D, 3F and 3G) responsiveness of the I2E fragment, whereas the responsiveness was only decreased with the other mutation constructs. With PMA treatment alone, the predominant effect on the C/EBP sites occurred at C/EBP-1 (Figure 3B), where a significant ($P < 0.0001$) decrease in luciferase expression was observed compared with the I2E/P7/PGL3 construct. However, when treating simultaneously with PMA and either cytokine, all three C/EBP sites, as well as the NF-1 site, showed significant ($P < 0.0001$) decreases in luciferase expression compared with the I2E/P7/PGL3 construct (Figures 3D, 3F and 3G). These findings suggest a potential interaction between proteins that can bind the C/EBP, NF-1 and NF-κB sites, which might contribute to the synergistic response observed with the combination treatment.

Generation of VA-13 stable clones expressing the luciferase reporter gene under control of the intronic enhancer

To further our understanding of the intronic enhancer's role in the inducibility of the *SOD2* gene, we stably transfected the I2E/P7/PGL3 construct into the VA-13 cell line. Co-transfection with the pSV2-NEO plasmid permitted selection in medium containing 400 μg/ml geneticin. Three clones (VA13-I2E4, VA13-I2E7 and VA13-I2E8) were isolated and characterized by luciferase assay and by Southern blot analysis. Digestion with restriction enzyme (*Eco*RI or *Kpn*I) showed integration of the I2E construct into all three clones; the highest copy number was in VA13-I2E7 (results not shown).

To ensure that the combination effect of PMA and cytokines observed in the transient transfection was reproducible when the DNA was stably integrated into the genome, we treated each of the VA-13 stable clones for 12 h with either one of TNF-α (200 i.u./ml), IL-1β (2 ng/ml) or PMA (100 nM), or combinations thereof, and measured luciferase activity as described previously. When using the stable clones, luciferase response was normalized to protein concentration. Figure 4 shows the fold increase for each treatment in each of the three clones. The VA13-I2E7 clone, which was shown by Southern analysis to have a higher copy number of the I2E construct, had a larger degree of induction than either clone VA13-I2E4 or clone VA13-I2E8. The pattern of induction in the stable clones was the same as that found with the transient transfection experiments as a result of treatment with PMA, TNF-α or IL-1β, either alone or in combination. A combination of PMA and cytokines gave a synergistic response, whereas simultaneous treatment with TNF-α and IL-1β resulted in an additive response.

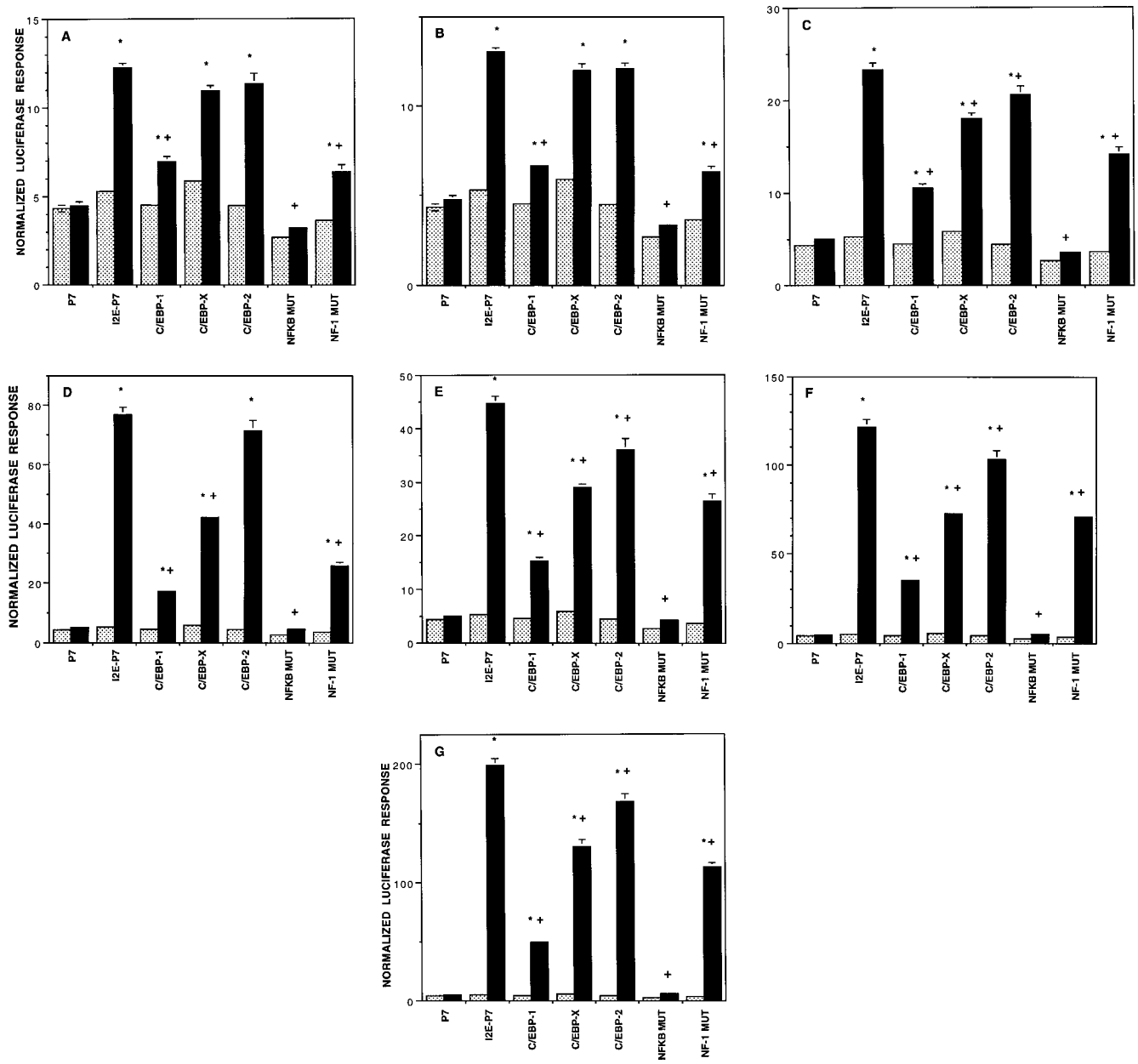

Figure 3 Intronic enhancer responsiveness to PMA is NF-κB dependent

The role of various transcription elements in the intronic enhancer's responsiveness to TNF-α, IL-1β, PMA and combinations thereof. The treatments were TNF-α (**A**), PMA (**B**), IL-1β (**C**), TNF-α/PMA (**D**), TNF-α/IL-1β (**E**), PMA/IL-1β (**F**) and TNF-α/PMA/IL-1β (**G**). Results are means ± S.E.M. for three independent experiments. $*P \leqslant 0.0001$ (significant difference between treatment and non-treatment with same construct); $+P \leqslant 0.0001$ (significant difference between the mutant construct and the I2E/P7/PGL3 construct with the same treatment). Key to all panels: stippled-shaded columns, control; black columns, treatment.

Kinetic analysis of degradation of IκB-α and IκB-β proteins reveals distinct differences between cytokine and phorbol ester effect in VA-13 cells

IκB-α and IκB-β are endogenous inhibitors of NF-κB. Both inhibitors form heteromeric complexes with p50 and p65 proteins, resulting in their cytoplasmic sequestration. The inhibitors can be phosphorylated by specific kinases on stimulation, which can lead to their degradation and subsequent release of p50:p65 heterodimers; these can then translocate to the nucleus and modulate transcriptional events. Because different kinases are involved in the phosphorylation of IκB-α and IκB-β, the degradation of these inhibitors might occur at different rates. Therefore we chose to evaluate the kinetics of proteolytic degradation of both IκB-α and IκB-β as a result of treatment with cytokine or phorbol ester, alone or in combination, in the VA-13 cells.

IκB-α

In the VA-13 cells, cytokine treatment led to a rapid degradation of IκB-α. Within 10 min IκB-α began to decrease and by 30 min the protein level decreased on average by 70% in the IL-1β-

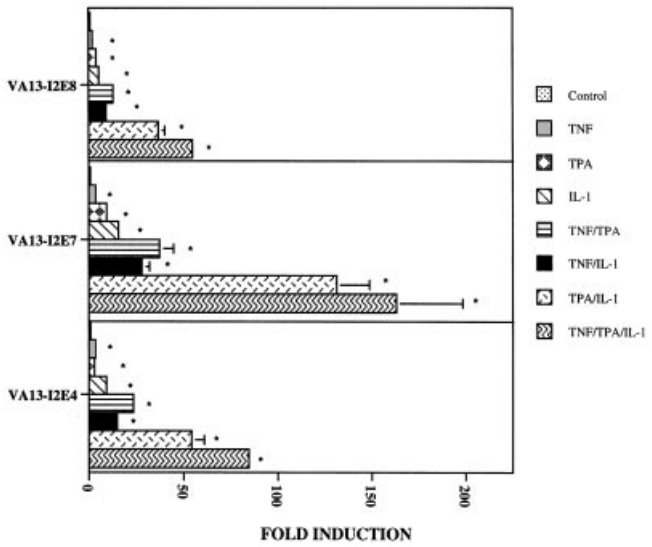

Figure 4 Combination analysis in I2E/P7/PGL3 stable clones

The effects of TNF-α (200 i.u./ml), PMA (TPA) (100 nM), IL-1β (2 ng/ml) and combinations thereof on the expression of the I2E/P7 dependent luciferase reporter gene are shown. Results are means $\pm$ S.E.M. for three independent experiments. $^{*}P \leqslant 0.01$ (significant differences between treatment and non-treatment with same construct).

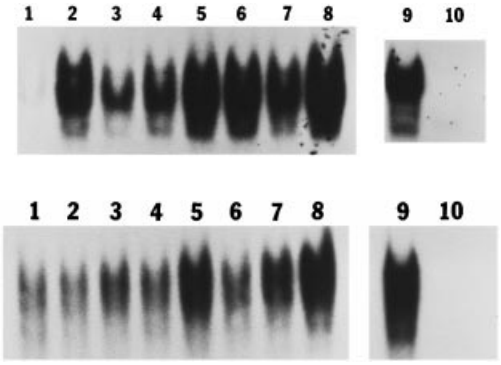

Figure 6 PMA enhances C/EBP DNA-binding activity

Electrophoretic mobility-shift assay of nuclear extracts (10 μg) with radiolabelled oligonucleotides. Lanes 1–9 used either the NF-κB wild-type (upper panel) or the C/EBP wild-type (lower panel) probe. Lane 10, mutated probes for DNA-binding activities of NF-κB (upper panel) and C/EBP (lower panel). Treatments were as follows: lane 1, control; lane 2, TNF-α; lane 3, PMA; lane 4, IL-1β; lane 5, TNF-α/PMA; lane 6, TNF-α/IL-1β; lane 7, PMA/IL-1β; lane 8, TNF-α/PMA/IL-1β. Lanes 9 and 10 show the specificity of binding between the wild-type (lane 9) and mutated (lane 10) probes on treatment with TNF-α/PMA/IL-1β.

treated cells and 94 % in the TNF-α-treated cells (Figure 5). By 90 min IκB-α levels rebounded to above control levels. The decrease in IκB-α on treatment with PMA did not occur until after 10 min, suggesting a slower proteolytic event than that observed with cytokines (Figure 5). Treatment of the VA-13 cells with PMA for 30 min led to only a minor decrease in IκB-α levels (21.9 ± 4.9 %; mean $\pm$ S.D.). A combination of cytokine and phorbol ester enhanced the degradation of IκB-α as seen at the 10 min time point in Figure 5.

IκB-β

Degradation of IκB-β occurred more slowly than that of IκB-α on treatment with TNF-α (Figure 5). No degradation was seen within 10 min; however, at 30 min IκB-β levels had decreased by 28 ± 6.3 % and remained low until 90 min (Figure 5). IL-1β treatment led to a greater decrease in IκB-β levels, namely 48.8 ± 14.3 % and 66 ± 10.2 % after 30 and 90 min of treatment respectively (Figure 5). The degradation pattern of IκB-β on treatment with PMA was similar to that of IκB-α (Figure 5). Only minor degradation was seen at 30 and 90 min (10.9 ± 3.0 %) after treatment with PMA (Figure 5). The combination of TNF-α/PMA resulted in enhanced degradation of IκB-β at 30 and 90 min; however, this was less than occurred with combinations with IL-1β (Figure 5).

PMA enhances C/EBP DNA-binding activity

The intronic enhancer contains multiple C/EBP elements, an NF-κB and an NF-1 element; we therefore examined the DNA-binding patterns of these proteins after treatment with PMA, TNF-α or IL-1β, either alone or in combination. Cells were treated for 1 h and harvested; nuclear extracts were then prepared as described previously.

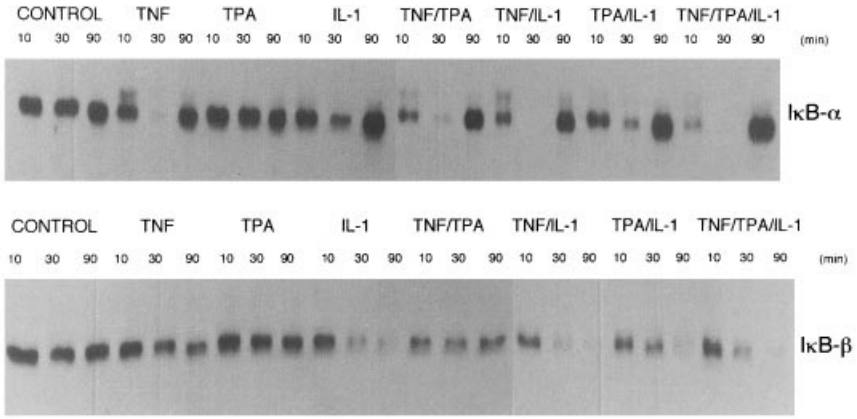

Figure 5 Kinetic analysis of IκB turnover reveals agent-specific differences

Western analysis of IκB-α and IκB-β levels in the cytoplasmic extracts of cells treated for 10, 30 or 90 min with cytokines and/or phorbol ester. Abbreviation: TPA, PMA.

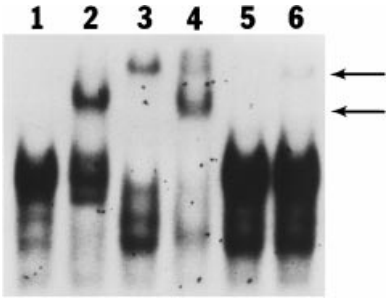

Figure 7 Supershift analysis of nuclear extracts (5 μg) of VA-13 cells with a radiolabelled NF-κB oligonucleotide and antibodies against p50, p65, C/EBP-α and C/EBP-β

Lane 1, nuclear extract without antibody; lane 2, anti-p50; lane 3, anti-p65; lane 4, anti-p50 and anti-p65; lane 5, anti-(C/EBP-α); lane 6, anti-(C/EBP-β). All samples represent a combined TNF-α/PMA/IL-1β treatment. Arrows indicate the NF-κB complex with bound antibodies.

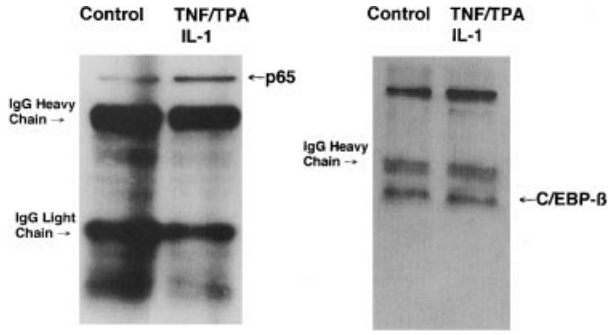

Figure 8 C/EBP and NF-κB form a heteromeric complex in VA-13 cells

By using a polyclonal antibody against either C/EBP-β (left panel) or p50 (right panel) and nuclear extracts from control and treated [TNF-α/PMA (TPA)/IL-1β] VA-13 cells, p65 or C/EBP-β respectively was immunoprecipitated.

NF-κB

An increase in DNA-binding activity of NF-κB was observed with all treatments after 1 h (Figure 6, upper panel, lanes 2–9). The increase on treatment with PMA (Figure 6, upper panel, lane 3) was less than that with either cytokine alone (lanes 2 and 4). Combinations including either TNF-α/PMA (Figure 6, upper panel, lane 5), PMA/IL-1β (lane 7) or TNF-α/PMA/IL-1β (lane 8) led to increases in the DNA-binding activity of NF-κB that were greater than with the individual agents (lanes 2–4). Addition of all three agents led to the greatest increase in NF-κB DNA-binding activity (Figure 6, upper panel, lane 8).

C/EBP

C/EBP DNA-binding activity increased in the presence of PMA treatment, either alone (Figure 6, lower panel, lane 3) or in combination with TNF-α (lane 5), IL-1β (lane 7) or both (lane 8), suggesting a possible role for this transcription factor in the synergistic response exerted by the PMA/cytokine combination.

NF-1

No apparent change in the DNA-binding activity of NF-1 occurred as a result of treatment with cytokine or PMA, alone or in combination (results not shown).

CEBP-β binds to the intronic NF-κB element on combination treatment

The combined treatment with TNF-α, PMA and IL-1β led to significant increases in the DNA-binding activity of both NF-κB and C/EBP in the VA-13 cell line (Figure 6, lanes 8). Therefore, to determine the specific components bound to the intronic NF-κB element, we used antibodies against p50, p65, C/EBP-α and C/EBP-β. Figure 7 shows the characteristic shift with the anti-p50 (lanes 2 and 4) and/or anti-p65 (lanes 3 and 4) antibodies. There was no shift with the anti-C/EBP-α antibody (Figure 7, lane 5). However, an antibody against C/EBP-β did result in a more slowly migrating band, suggesting an interaction of NF-κB and C/EBP proteins at the intronic NF-κB element as a result of phorbol ester/cytokine treatment (Figure 7, lane 6).

C/EBP and NF-κB proteins form a complex in VA-13 cells that might affect transcriptional regulation of the *SOD2* gene

To investigate further a potential co-operation between C/EBP and NF-κB proteins in the induction of MnSOD, we sought to determine whether a physical interaction occurred between these two transcription factors in the VA-13 cells. Using antibodies against either C/EBP-β or p50, we immunoprecipitated complexes from nuclear extracts of either control or TNF-α/PMA/IL-1β-treated cells. Using the anti-C/EBP-β antibody, we were able to immunoprecipitate p65 from both control and treated cells. The amount of immunoprecipitated p65 increased as a result of treatment (Figure 8). We were also able to immunoprecipitate C/EBP-β from both control and treated cells by using an antibody against p50 (Figure 8).

DISCUSSION

The basal expression of MnSOD is essential to all aerobic organisms and an enhanced activity is beneficial under conditions of oxidative stress. Many studies have shown that a variety of agents induce MnSOD, including TNF-α, IL-1β and PMA. However, we still have little knowledge about the sequence of events that mediate the induction of this important antioxidant enzyme. Alterations in the cellular redox status can lead to the modulation of numerous transcription factors, including activator protein 1 ('AP-1') and NF-κB (reviewed in [33]). Because both of these transcription factors have binding sites within the 5' flanking region of the *SOD2* gene, it has been speculated that they might have a role in the regulation of MnSOD expression. Recently we identified an NF-κB element within the second intron of the *SOD2* gene that is responsible for its induction by TNF-α and IL-1β [28]. By using reporter gene analysis, NF-κB sites in the 5' and 3' flanking regions of the *SOD2* gene were shown to have no effect on transcription upon cytokine treatment, unless they were transposed to the intronic region. This result suggests that contributions from other transcription factors in the intronic region are important in MnSOD regulation. Mutational analysis of the three C/EBP sites and the NF-1 site, which flank the I2E NF-κB element, suggests a potential contributory role for these proteins upon treatment with either cytokine. To extend the findings from previous studies on MnSOD induction, we evaluated the potential responsiveness of the intronic enhancer to PMA to determine whether MnSOD

induction by phorbol esters might be mediated through the I2E NF-κB element. In addition we treated VA-13 cells with a combination of PMA and cytokines to determine whether we could further enhance the expression of the human *SOD2* gene by the co-ordinated activation of multiple signalling pathways.

Using a variety of cell lines, Fujii et al. [23] first reported that PMA could induce MnSOD expression through the activation of transcription of the gene in TNF-resistant, but not in TNF-sensitive, cell lines. Studies by Whitsett et al. [34] in a pulmonary adenocarcinoma cell line (H441) showed an increase in steady-state mRNA levels as well as an increase in the rate of transcription of the *SOD2* gene after treatment with PMA. A recent study by Kim et al. [35] identified a cAMP-responsive-element-like sequence in the *SOD2* gene's 5' flanking region that was responsive to treatment with PMA in A549 human lung carcinoma cells. The level of MnSOD mRNA accumulation with PMA treatment in the A549 cells was much greater than the level of PMA-induced luciferase expression obtained with their promoter construct, leading the authors to suggest that other responsive elements might exist in gene sequences that were not examined in their work. PMA has been shown to activate NF-κB via the PKC pathway [36,37], and inhibitors of PKC can block the effect of PMA on MnSOD expression [35,38]. In addition, Warner et al. [39] reported that TNF-α up-regulated MnSOD expression independently of PKC in H441 cells, suggesting a role for multiple pathways in MnSOD regulation.

Our results provide new insight on MnSOD induction mediated by PMA and cytokines by identifying key molecules in the regulation of this essential antioxidant. Transfection analysis revealed that I2E was responsive to PMA in the VA-13 cells. Treatment of the VA-13 cells with a combination of phorbol ester and cytokines led to a higher level of transcriptional activity than when the cells were treated with the individual agents. These effects were also observed when the enhancer elements were integrated into the cellular genome. Furthermore, the highest levels of MnSOD mRNA accumulation were seen in the cells receiving the combination treatments, indicating that the results obtained from the I2E-dependent reporter assays simulated the effects observed with the endogenous *MnSOD* gene. Our results indicate that the synergistic effect of PMA and cytokines is mediated by at least two complementary mechanisms.

Mutational analysis within the I2E fragment reveals the relative contribution of specific transcription-factor-binding sites on treatment with PMA alone or in combination. Induction with PMA and PMA/cytokine combinations was completely eliminated when the I2E NF-κB element was mutated. Mutation of the C/EBP-1 site was the most effective in decreasing luciferase expression as a result of treatment with PMA; however, all three C/EBP sites seemed to contribute to the enhanced luciferase activity as a result of combination treatment. These findings suggest a potential interaction between proteins that bind the C/EBP and NF-κB sites, which might contribute to the synergism.

Activation of NF-κB is a multi-step process that can involve phosphorylation of the IκB family of inhibitors, followed by their ubiquitination and subsequent proteolytic degradation by the 26 S proteasome. Degradation of the IκB protein causes the release of the sequestered NF-κB, an unmasking of the nuclear localization signal and subsequent translocation to the nucleus, where gene expression can be modulated. Pretreatment of the stably transfected VA-13 cells for 2 h with the inhibitor benzyloxycarbonyl-leucinyl-leucinyl-leucinal (MG-132) led to a complete block of PMA- and PMA/cytokine-mediated I2E/P7-dependent luciferase activation, suggesting a proteosome-dependent process (results not shown).

Because IκB-α and IκB-β are the two prominent members of the IκB family members in VA-13 cells, we studied the kinetics of their degradation after treatment with phorbol ester and/or cytokines. We found agent-specific differences in the degradation of both IκB-α and IκB-β in VA-13 cells after stimulation. In this cell line both cytokines caused a significant decrease in IκB-α and IκB-β levels. IκB-α was more readily degraded in the TNF-α-treated cells, whereas IL-1β was a better inducer of IκB-β degradation. The MnSOD mRNA levels observed after 12 h of treatment with either TNF-α or IL-1β were similar and the combined treatment showed an additive effect of these two agents, suggesting a potential co-operative role for both of these inhibitors in the induction of the *SOD2* gene. PMA was a weak inducer of both IκB-α and IκB-β degradation when used alone; however, all combination treatments resulted in a more pronounced degradation. The degradation of IκB-β occurred more slowly but was more sustained than IκB-α degradation with all treatments. Unlike IκB-α, the transcription of the IκB-β gene is not increased by activators of NF-κB [40]. This might explain the sustained decrease in IκB-β level in the VA-13 cells. In contrast, a subclass of Ras proteins has been identified that associate predominantly with NF-κB–IκB-β complexes in cells [41]. This interaction decreases the rate of degradation of IκB proteins and might explain the differential kinetics of turnover of IκB-α and IκB-β. It is therefore also possible that TNF-α or IL-1β might alter the cellular status of the Ras proteins, leading to differential degradation of IκB-α and IκB-β. On the basis of the kinetic analysis of IκB proteolytic degradation, we determined I2E NF-κB DNA binding activity as a function of the different treatments. The pattern correlated with the changes in IκB degradation. Taken together, these studies suggest that both inhibitors contribute to NF-κB-mediated induction in the VA-13 cells.

The DNA-binding activity of C/EBP was sensitive to the administration of PMA. No effect on this DNA-binding activity was observed with TNF-α or IL-1β treatment alone. Treatment of the cells with PMA either alone or in combination led to an increase in DNA-binding activity of C/EBP. These results suggest that C/EBP might be important in PMA-mediated synergistic induction of the human *MnSOD* gene. Phorbol esters have been shown to cause increased C/EBP synthesis, phosphorylation and DNA binding to promoters of genes such as multidrug resistance and prostaglandin-endoperoxide synthase 2, resulting in modulation of expression [42–46].

Members of the C/EBP family have been reported to interact with several different transcription factors including NF-κB. Stein et al. [47] showed a heterodimerization between the bZIP region of the C/EBP protein that is characterized by a basic region involved in DNA binding and a leucine-zipper motif involved in dimerization with the Rel homology domain of NF-κB. These interactions have been shown to activate the expression of some genes synergistically (IL-6 and IL-8) while antagonizing the expression of others (angiotensinogen) [48–50]. Studies by Ray et al. [49] showed that a C/EBP–NF-κB complex, which could bind either a NF-κB or C/EBP consensus sequence, was better at activating the serum amyloid A gene than either of the two transcription factors alone.

Our results from the DNA binding and transcription studies suggest a positive interaction between NF-κB and C/EBP as a result of combination treatment. Using antibodies against either p50 or C/EBP-β, we were able to immunoprecipitate C/EBP or p65 respectively from both control and stimulated VA-13 cells. We were able to immunoprecipitate low levels of p65 from control cells by using the anti-C/EBP antibody and the level of immunoreactive p65 was increased in the combination-treated cells. Because PMA has a greater effect on the DNA-binding

activity of C/EBP in our model system, interactions between C/EBP and NF-κB might be a key modulator of the observed synergistic expression of the *SOD2* gene by PMA and TNF-α or IL-1β.

This paper is the first to identify an enhancer element within the second intron of the human *SOD2* gene as a potential site of gene regulation after treatment with phorbol ester. We show that, within the I2E fragment, the NF-κB element is essential for the PMA- and PMA/cytokine-mediated effect. Furthermore, our findings of enhanced MnSOD expression on treatment with PMA and either TNF-α or IL-1β identifies two inhibitors, IκB-α and IκB-β, and C/EBP as key molecules in these pathways. These findings might help to develop novel approaches to the modulation of the cellular antioxidant status.

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