Activation of the NF-κB transcription factor in a T-lymphocytic cell line by hypochlorous acid

Sonia SCHOONBROODT*, Sylvie LEGRAND-POELS*, Martin BEST-BELPOMME† and Jacques PIETTE*‡

*Laboratory of Virology, Institute of Pathology B23, University of Liège, B-4000 Liège, Belgium, and †Laboratory of Molecular and Cellular Genetics, URA CNRS 1135, Université Pierre et Marie Curie, F-75230 Paris Cedex 05, France

Reactive oxygen species (ROS) such as hydrogen peroxide serve as second messengers in the induction of the transcription factor NF-κB, and hence in the activation and replication of human immunodeficiency virus type 1 (HIV-1) in human cells. During inflammatory reactions, many oxidative species are produced, one of which is hypochlorous acid (HOCl), which is responsible for the microbicidal effects of activated human polymorphonuclear leukocytes. Treatment of a T-lymphocytic cell line with micromolar concentrations of HOCl promoted the appearance of transcription factor NF-κB (the heterodimer p50/p65) in the nucleus of the cells, even in the absence of *de novo* protein synthesis. Western blot analysis of the NF-κB inhibitory subunits (IκB) demonstrated that both IκB-α proteolysis and p105 processing were induced by the treatment. NF-κB activation was very effective when cells were subjected to hyperthermia before being treated with HOCl. Various antioxidants, such as pyrrolidine dithiocarbamate, *p*-bromophenacyl-bromide and nordihydroguaiaretic acid could strongly reduce NF-κB translocation, demonstrating the importance of oxidative species in the transduction mechanism. Moreover, ACH-2 cells treated with HOCl or H_2O_2 released tumour necrosis factor-α (TNF-α) in the supernatants. The importance of TNF-α release in NF-κB induction by HOCl or H_2O_2 was demonstrated by the fact that: (1) the nuclear appearance of NF-κB was promoted in untreated cells; and (2) synergism between TNF-α and HOCl was detected. Collectively, these results suggest that HOCl should be considered as an oxidative species capable of inducing NF-κB in a T-lymphocytic cell line through a transduction mechanism involving ROS, and having a long-distance effect through subsequent TNF-α release.

INTRODUCTION

One of the key experimental results concerning the role of reactive oxygen species (ROS) in gene expression is the elucidation of the mode of action of the NF-κB protein complex as a transcriptional factor [1] (for reviews see [2–4]). NF-κB is composed of a number of proteins related to the proto-oncogene c-*rel* (reviewed in [5] and [6]). These proteins are NF-κB1 (p50), NF-κB2 (p52), c-Rel, Rel A (p65) and Rel B [7–10]. The different NF-κB proteins associate to form a variety of homo- and hetero-dimers, which interact with a series of related DNA target sites, collectively called NF-κB sites [11]. Most of the dimeric NF-κB complexes are stored in the cytoplasm of non-stimulated cells as inactive complexes, as a result of interaction with a group of inhibitory proteins, collectively known as IκB [12]. NF-κB1 and NF-κB2 are initially synthesized as large precursors of 105 and 100 kDa in size respectively [13–15]. These precursors do not bind DNA, but they can interact with other Rel-related proteins and function in an IκB-like fashion by sequestering various Rel-related protein homo- and hetero-dimers in the cytoplasm [16,17]. Proteolytic degradation of the p105 and p100 precursors leads to the production of mature DNA-binding subunits and the release of the active Rel-related protein dimers containing p50 and p52 respectively. The rate of processing of both precursors appears to be regulated and can be increased in response to extracellular stimuli [16].

Submitting T-lymphocytes to oxidative stress, as mimicked by the addition of extracellular H_2O_2, induces the nuclear appearance and DNA-binding activity of NF-κB [1], followed by transcriptional activation of the proviral DNA in cells non-productively infected with human immunodeficiency virus type 1 (HIV-1) [18]. Presumably, the mechanism involves passive diffusion of H_2O_2 through the cell membrane. There, it indirectly triggers the controlled proteolytic degradation of IκB molecules [19,20]. The transduction mechanism leading to NF-κB translocation is still poorly understood, but seems to implicate a series of kinases, such as the T-cell tyrosine kinase ZAP-70 [21], which is responsive to H_2O_2 treatment and requires the expression of CD3 for the response. Moreover, ZAP-70 is known to associate with the ζ and ε chains of the T-cell receptor after treatment with H_2O_2 [22,23].

Phagocytic cells produce a number of ROS, all derived from the reduction of molecular oxygen to superoxide anion catalysed by an NADPH oxidase (see [24] for review). Neutrophils (polymorphonuclear cells) and eosinophils, but not mature macrophages, upon stimulation release myeloperoxidase (MPO) into phagolysosomes or into the extracellular medium [25]. MPO from neutrophils catalyses the reaction of H_2O with Cl^- ions to produce hypochlorous acid (HOCl), whereas the eosinophil MPO preferentially uses Br^-, ions even though the Cl^- ion concentration is 1000-fold higher in plasma [26]. HOCl can react with amines to produce chloramines and N-chlorinated derivatives which have long lifetimes in plasma [27]. Thus, MPO-generated HOCl and *N*-chloramines may represent the most

Abbreviations used: BPB, *p*-bromophenacyl-bromide; DTT, dithiothreitol; HIV-1, human immunodeficiency virus type 1; IL-1β, interleukin-1β; PLA2, phospholipase A2; ROS, reactive oxygen species; TNF-α, tumour necrosis factor-α; PMA, phorbol 12-myristate 13-acetate; MPO, myeloperoxidase; EMSA, electrophoretic mobility-shift assay; CHX, cycloheximide; PDTC, pyrrolidine-dithiocarbamate; EASIA, enzyme amplified sensitivity immunoassay.

‡ To whom correspondence should be addressed.

important oxidants produced by activated neutrophils, and there is now a growing body of evidence suggesting that they play an important role in inflammation as well as in the pathophysiology of inflammatory bowel diseases [28].

Since it is now clearly established that micromolar concentrations of H_2O_2 can modulate cell function through the activation of cellular transcription factors (see [2] and [29] for reviews), we decided to investigate whether low concentrations of HOCl can function as a mediator in cell activation, with special emphasis on the NF-κB transcription factor, which is redox regulated. In this paper, it is shown, for the first time, that an important oxidant such as HOCl can, with a slow kinetic rate, also induce NF-κB heterodimer p50/p65 in a T-lymphocytic cell line through proteolysis of IκB-α and p105 inhibitors. In addition, it is shown that HOCl contributes to the release of tumour necrosis factor-α (TNF-α) in cell supernatants capable of propagating activation to non-induced cells.

MATERIALS AND METHODS

Cell culture and reagents

The ACH-2 T-lymphocytic cell line was obtained from the NIH AIDS Research and Reference Reagent Program (Bethesda, MD, U.S.A.), and cells were cultivated in RPMI 1640 + Glutamax I with 10% (v/v) fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Life Technologies, Gaithersburg, MD, U.S.A.). Cells were seeded in fresh medium 12–24 h before any experiment.

Chemicals

All chemicals were of reagent grade and were obtained from UCB (Brussels, Belgium) or from Sigma (St. Louis, MO, U.S.A.). NaClO was purchased from Aldrich (Brussels, Belgium) and was used to generate hypochlorous acid (HOCl) by adjusting the pH to 6.85 with diluted H_2SO_4 immediately before use. HOCl concentrations were determined using a molar absorption coefficient of 100 at 235 nm [30]. At pH 6.85, HOCl is partly ionized to hypochlorite anion, ClO^-, at a ratio of $HOCl/ClO^-$ of $\approx 1.8:1$.

Generation of oxidative stress

ACH-2 cells, at 6×10^5 per ml of culture medium, were cultivated in 25 cm² culture flasks before being treated with HOCl (0–500 μM). Before treatment with HOCl, cells were collected by centrifugation and resupended at the same concentration in the medium without fetal calf serum. At 30 min after HOCl addition, ACH-2 cells were refed with 10% (v/v) fetal calf serum, and $(4–5) \times 10^6$ cells were subsequently harvested at various times (between 30 and 180 min) for NF-κB electrophoretic mobility-shift analysis. After 48 h, ACH-2 cells were counted and cytotoxicity was estimated using Trypan Blue exclusion.

Nuclear protein extraction

The method used for the extraction of nuclear proteins is described elsewhere [31]. Briefly, at various times after the oxidative stress, $(3–5) \times 10^6$ ACH-2 cells were washed in 1 ml of cold PBS and centrifuged at 15000 ***g*** for 15 s, resuspended in 100–400 μl of cold hypotonic buffer [10 mM Hepes/KOH/2 mM $MgCl_2$/0.1 mM EDTA/10 mM KCl/1 mM dithiothreitol (DTT)/0.5 mM PMSF (pH 7.9)], left on ice for 10 min, then vortexed and centrifuged at 15000 ***g*** for 30 s. Aliquots of supernatant, containing the cytoplasmic proteins, were quickly frozen in liquid nitrogen and stored at −80 °C. The pellets of nuclei were gently resuspended in 15 μl of cold saline buffer [50 mM Hepes/KOH/50 mM KCl/300 mM NaCl/0.1 mM EDTA/10% (w/v) glycerol/1 mM DTT/0.5 mM PMSF (pH 7.9)] and left for 20 min on ice. After centrifugation (15000 ***g*** for 5 min at 4 °C), aliquots of supernatant, containing the nuclear proteins, were kept in liquid nitrogen and stored at −80 °C. Protein concentrations were measured with the bicinchoninic acid (BCA) kit supplied by Pierce (Rockford, IL, U.S.A.).

Electrophoretic mobility-shift assay (EMSA)

The EMSA method used is that described in [32], with slight modifications. Briefly, 5 μg of nuclear proteins were incubated for 30 min at room temperature with 0.2 ng of ^{32}P-labelled oligonucleotidic probe, 1 μg of BSA and 1.25 μg of poly(dI-dC)·poly(dI-dC) (Pharmacia Biotech Benelux, Rozendaal, The Netherlands) in 20 mM Hepes/KOH/75 mM NaCl/1 mM EDTA/5% (v/v) glycerol/0.5 mM $MgCl_2$/1 mM DTT (pH 7.9) buffer (final volume 10 μl). DNA–protein complexes were then resolved on a non-denaturing 6% polyacrylamide (4% for supershifts) gel run for 4 h at 180 V in TBE {0.5 × [2.5 mM Tris/2.5 mM H_3BO_3/2 mM EDTA (pH 8.5)]}. The gel was then dried and autoradiographed on a Fuji X-ray film (General Electrics, Antwerp, Belgium). For competition experiments, unlabelled probe (wild type or mutated) was added in excess (50×) in the binding buffer. The oligonucleotide probe (Eurogentec, Seraing, Belgium) was labelled by in-filling with the Klenow DNA polymerase (Boehringer, Mannheim, Germany) as described elsewhere [32]. Probe (100 ng) was labelled with 3 μCi of [α-^{32}P]dATP and [α-^{32}P]dCTP (3000 Ci/mmol; Du Pont De Nemours International, Brussels, Belgium) and unlabelled dTTP and dGTP (Boehringer, Mannheim, Germany), then purified on a Sephadex G-25 (Pharmacia Biotech Benelux) column and stored at −20 °C until use. Specific radioactivity was always $\geqslant 10^8$ c.p.m./μg. The sequences of the probes used in this work were:

wild-type NF-κB probe:

5′-GGTTACAAGGGACTTTCCGCTG

TGTTCCCTGAAAGGCGACGGTT-5′

mutated NF-κB probe:

5′-GGTTACAACTCACTTTCCGCTG

TGTTGAGTGAAAGGCGACGGTT-5′

AP-1 probe:

5′-CTAGAGGTGTCTGACTCATGCTTTA

TCCACAGACTGAGTACGAAATTCGA-5′

Exposure of ACH-2 cells to hyperthermia

Exponentially growing ACH-2 cells (0.6×10^6 per ml of complete culture medium) were placed in a water bath at 37 °C, with the temperature increased to 42 °C over a period of 30 min. ACH-2 cells were then maintained at 42 °C for 40 min before being slowly cooled down to 37 °C within 30 min. At each time point during the various phases, aliquots of $(4–5) \times 10^6$ cells were taken for NF-κB assay. ACH-2 cells in culture medium made hyperthermic following this procedure were then subjected to an oxidative stress mediated by HOCl (100–300 μM). Between 30 and 180 min after treatment with HOCl, nuclear extracts were prepared for NF-κB analysis.

Exposure of ACH-2 cells to TNF-α before HOCl

TNF-α (Boehringer, Germany) (0–1000 units/ml) was directly added to complete media, using 0.6×10^6 ACH-2 cells per ml of growth medium. At 90 min after treatment with TNF-α, ACH-2 cells were subjected to an oxidative stress mediated by HOCl (100–300 μM), and 3 h later nuclear extracts were prepared for NF-κB analysis.

Western blotting

The IκB-α subunit, p65 and p105–p50 were detected by Western blot analysis using specific antibodies. Cytoplasmic extracts were prepared at various times after HOCl-mediated treatment by hypotonic lysis, pelleting of the nuclei and collection of the supernatant fraction [32]. Cytoplasmic proteins were added to a loading buffer [10 mM Tris/HCl (pH 6.8)/1 % (w/v) SDS/25 % (v/v) glycerol/0.1 mM 2-mercaptoethanol/0.03 % (w/v) Bromophenol Blue], boiled and electrophoresed on a 10 %-polyacrylamide/SDS gel and electro-transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.). Filters were incubated in primary antibody for 60 min at 37 °C (1:2000 dilution) and in peroxidase-conjugated goat anti-rabbit IgG (1:250 dilution) for 60 min at 37 °C, and finally analysed using an enhanced chemiluminescence system (ECL; Amersham, Slough, U.K.) using Fuji X-ray films.

TNF-α and interleukin-1β (IL-1β) detection

The release of TNF-α or IL-1β in cell supernatants taken from ACH-2 cells, pretreated with either 100 μM H_2O_2 or 200 μM HOCl, was quantitatively assayed by immunoassays [TNF-α and IL-1β enzyme amplified sensitivity immunoassay (EASIA), Medgenix, Fleurus, Belgium]. Cytokine measurements were performed in triplicate, and values were directly compared with an internal standardization curve. The sensitivity of the EASIA tests was 3 and 2 pg/ml for TNF-α and IL-1β respectively.

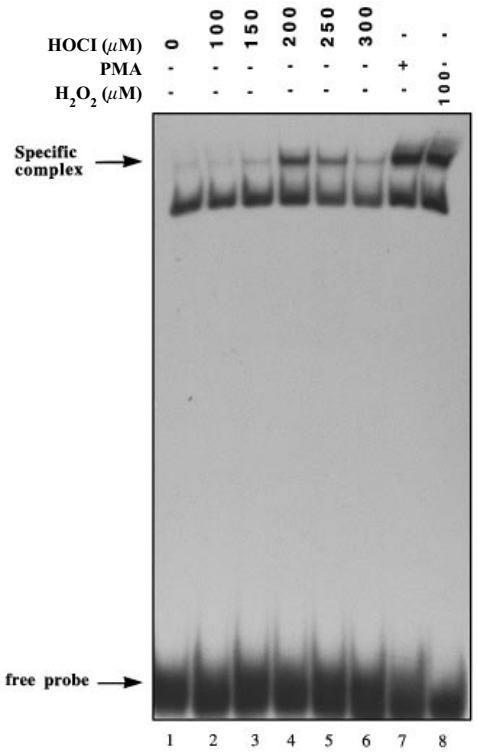

Figure 1 NF-κB induction in ACH-2 cells by treatment with HOCl

Effect of HOCl on NF-κB DNA-binding activity in ACH-2 cells. Induction of a nuclear κB enhancer DNA-binding protein by treatment of ACH-2 cells with increasing concentrations of HOCl (0–300 μM), PMA (1 μM) and H_2O_2 (100 μM). Nuclear extracts were prepared 120 min after the reaction and equal amounts of proteins were mixed with a ^{32}P-labelled probe encompassing the κB elements of the HIV-1 enhancer. Samples were loaded on 6 % native polyacrylamide gels and electrophoresed at 150 V. An autoradiogram of the gel is shown. Arrows indicate the positions of the specific complex and of the free probe.

RESULTS

HOCl induces the activation of NF-κB binding factor in a T-lymphocytic cell line

H_2O_2-mediated oxidative stress has been reported to strongly activate the transcription factor NF-κB in lymphocytes or in various transformed cell lines [1,33]. We performed EMSA in order to determine whether HOCl-mediated oxidative stress could lead to a similar induction of this transcription factor in a T-lymphocytic cell line. Within 120 min following the oxidative stress mediated by HOCl, a factor capable of binding to the κB sites of the HIV-1 enhancer could be detected in the nuclear extracts of ACH-2 cells (Figure 1). The level of induction was directly related to the concentration of HOCl (Figure 1). Analysis of the NF-κB induction by phosphorimaging revealed that the intensity of the complex varied with HOCl concentration in a dose-dependent manner; maximal induction was observed between 200 and 250 μM HOCl (Figure 1, lanes 4 and 5) where a maximal stimulation factor of 10.5 could be detected. At 300 μM HOCl, the band intensity decreased, probably due to a cytotoxic effect. The specificity for these κB sites was shown by the disappearance of this complex through competition with an excess of unlabelled probe (Figure 2, lane 5) but not by an excess of unlabelled AP-1 probe (Figure 2, lane 6). Another complex, showing greater electrophoretic mobility (Figures 1 and 2), seemed to be induced with the same kinetic characteristics, but was not competed out by the wild-type probe (Figure 2). Thus, the latter complex did not seem to involve a nuclear factor which binds specifically to the κB sites.

The intensity of the specific κB complex induced by HOCl is lower than that induced by phorbol 12-myristate 13-acetate (PMA) (Figure 1, lane 7) or by H_2O_2 in a similar concentration range (Figure 1, lane 8), demonstrating that while HOCl should be considered as an oxidative species capable of inducing NF-κB, the nuclear translocation of this complex is less efficient than with other ROS such as H_2O_2 [1,33] or singlet oxygen [34,35].

The identity of the HOCl-activated DNA–protein complex with an NF-κB probe was further investigated in supershift experiments (Figure 2). We examined whether the HOCl-activated NF-κB complex could react with antisera raised against the DNA-binding p50 and p65 subunits of NF-κB as well as with c-Rel. The anti-p50 and anti-p65 sera clearly gave rise to a characteristic supershift of the retarded complex, demonstrating unambiguously that the HOCl-activated factor is p50- and p65-containing NF-κB (Figure 2, lanes 2 and 3). No supershift of the specific complex could be observed either with c-Rel (Figure 2, lane 4), with p52 antibodies, or with an unrelated serum (results not shown).

Nuclear appearance of NF-κB has been shown to be quite rapid when induction is carried out with various membrane-targeting mediators, such as IL-1, phorbol ester or lipopolysaccharide [19,20]. In order to determine whether NF-κB induction occurs in a similar time scale, ACH-2 cells were treated

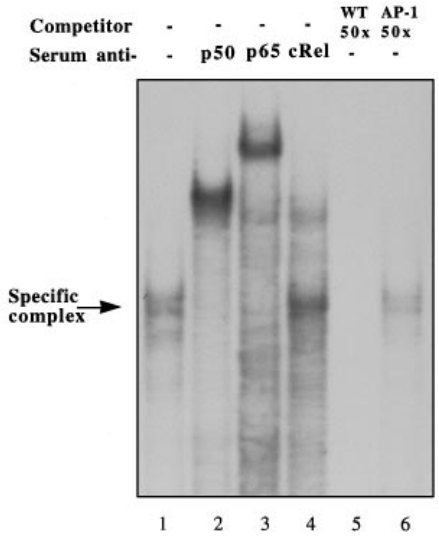

Figure 2 NF-κB induced by HOCl is the heterodimer p50/p65

Immunoreactivity of the HOCl-inducible protein-κB enhancer complex. Nuclear extracts from ACH-2 cells treated with HOCl (200 μM) were either mixed directly with the ^{32}P-labelled κB probe or incubated with antisera specific for p50, p65 or c-Rel before being mixed with the ^{32}P-labelled κB probe. Samples were then loaded on a 4% polyacrylamide native gel and electrophoresed as in Figure 1. Competition with an unlabelled oligonucleotide (50 M excess), encompassing NF-κB sites, reveals the position of the specific NF-κB complex. The specific NF-κB band could not be competed out with a 50 M excess of a probe encompassing the AP-1 element of the collagenase gene promoter. WT, wild-type probe.

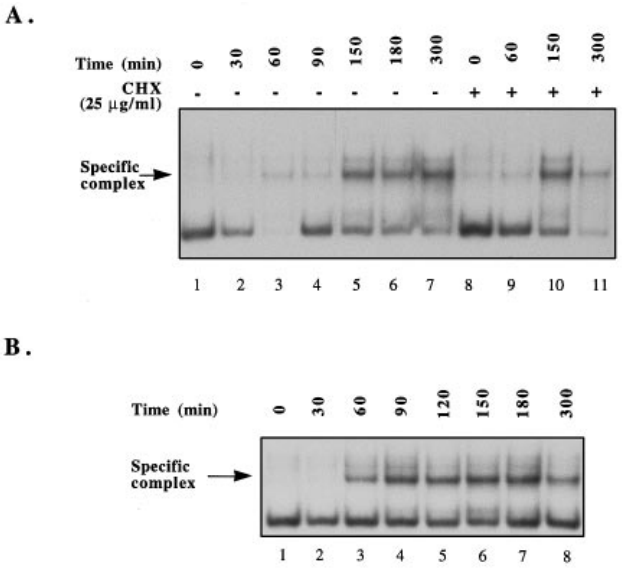

Figure 3 Time-course experiments of H_2O_2- or HOCl-mediated NF-κB induction

(**A**) NF-κB activation by HOCl is a post-transcriptional event. Evolution of the NF-κB complex induced in ACH-2 cells by treatment with HOCl (200 μM). Cells were taken at various times after treatment (0–300 min) and used to prepare nuclear extracts to be analysed by EMSA as described in Figure 1. A time-course experiment was carried out in the absence or in the presence of 25 μg/ml CHX. (**B**) Time-course experiment of H_2O_2-mediated NF-κB induction. ACH-2 cells were treated with 100 μM H_2O_2. Cells were taken at various times after the treatment (0–300 min) and used to prepare nuclear extracts to be analysed by EMSA as described in Figure 1.

with HOCl (200 μM) and nuclear extracts were prepared at various time points (between 0 and 300 min) before being analysed by EMSA with a κB probe. Figure 3(A) shows that very shortly after the stress (0–30 min), no NF-κB-specific complex could be detected. The minimal induction time required to detect a specific NF-κB complex turned out to be 60 min (Figure 3A, lane 3), this complex still being observable for longer periods up to 300 min (Figure 3A, lane 7). These data reveal that NF-κB induction mediated by HOCl is significantly slower than that observed with other inducers acting directly at the cell membrane, where maximal NF-κB induction occurred within 5–30 min [19,20]. Indeed, NF-κB induction by H_2O_2 was rather similar to that measured by HOCl: it was detectable after 60 min (Figure 3B, lane 3) and was sustained up to 300 min (Figure 3B, lane 8).

Another characteristic of NF-κB is its activation by a post-translational mechanism involving the release of the inhibitory subunit IκB from a cytoplasmic inactive form [36]. In order to investigate whether the activation of NF-κB by HOCl involves a post-translational mechanism, the oxidative stress was performed in the presence of cycloheximide (CHX), a protein synthesis inhibitor (Figure 3A). When ACH-2 cells alone were treated with 25 μg/ml CHX, only a very weak activation of NF-κB was seen. On the other hand, NF-κB activity induced by HOCl at 200 μM was not affected by the presence of CHX, indicating that up to 150 min after the stress, NF-κB was activated by a post-translational mechanism (Figure 3A, lanes 8–10). At a longer time (300 min), the intensity of the band was decreased, demonstrating that part of the induction at this longer time might be due to a transcriptional mechanism (Figure 3A, lane 11).

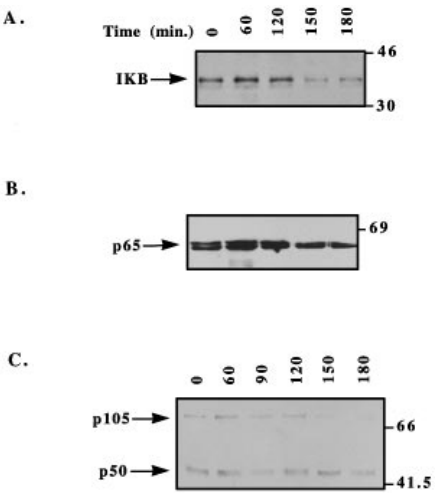

Figure 4 Proteolysis of inhibitory subunits induced by HOCl

Western blot analysis of the evolution of the members of the NF-κB protein family after stress mediated by HOCl (200 μM). (**A**) IκB-α, (**B**) p65 and (**C**) p50 and its precursor p105. Cytoplasmic extracts were prepared from untreated cells and at several time points after treatment with HOCl. The same amount of protein was loaded on an SDS/10%-polyacrylamide gel and electrophoresed before being transferred to a membrane and revealed by luminescence.

Western blot analysis of NF-κB family members

We also followed the appearance of NF-κB in the nucleus (Figure 3A) in connection with the disappearance of IκB-α (formely MAD3) in cytoplasmic extracts (Figure 4A). ACH-2 cells were treated with HOCl under the same conditions as

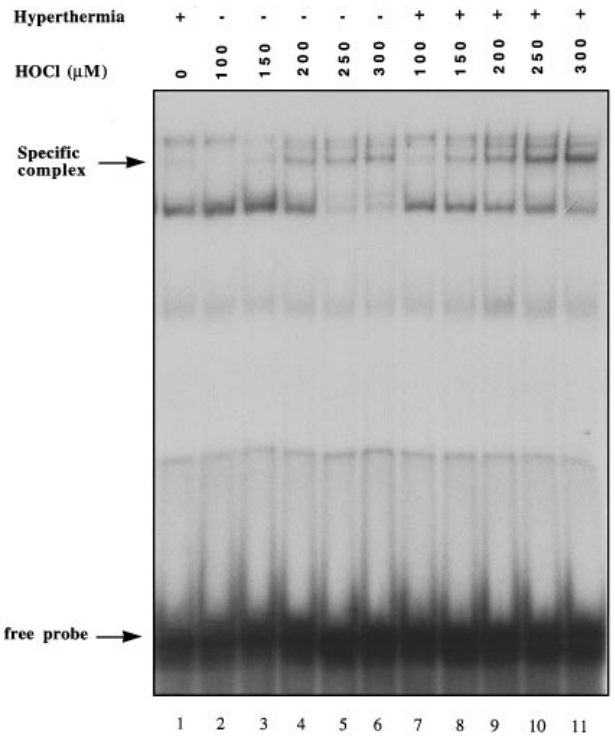

Figure 5 Hyperthermia and HOCl can synergistically induce NF-κB

Induction of a nuclear NF-κB enhancer DNA-binding protein by treatment of ACH-2 cells with increasing concentrations of HOCl (100–300 μM) after a round of hyperthermia at 42 °C, or maintained at 37 °C. Hyperthermia was carried out by raising the temperature from 37 °C to 42 °C over a period of 30 min and maintaining cells at 42 °C for 40 min before cooling the cells down. Samples were treated as in Figure 1 and analysed on a 4%-polyacrylamide native gel and electrophoresed.

mentioned above, then placed in culture medium. Aliquots were prepared various times after the stress to follow the disappearance of IκB-α in conjunction with the induction of NF-κB. In both untreated cells and at very short times after the stress, IκB-α-specific IgG detected a single 38 kDa band on Western blots. Between 120 and 150 min after the stress, the amount of IκB-α decreased significantly in cytoplasm, coinciding with the appearance of NF-κB activity in the nucleus. These results confirmed the temporal relationship between the two biochemical events. Similarly, analysis of the cytoplasmic content of p50 and its precursor p105 revealed that the amount of p105 slowly decreased and disappeared completely 180 min after the stress (Figure 4C). The kinetics of IκB-α proteolysis and p105 cleavage occurred concomitantly with the nuclear appearance of NF-κB, demonstrating that these two inhibitory molecules are involved in sequestering p50/p65 in the cytoplasm of ACH-2 cells. On the other hand, the amount of cytoplasmic p65 remained constant after HOCl-mediated stress (Figure 4B).

Hyperthermia sensitizes ACH-2 cells to HOCl treatment

Since the accumulation of inflammatory cells in a damaged tissue site is accompanied by the release of ROS, pro-inflammatory cytokines and a possible rise in temperature [37,38], we tested the role of hyperthermia as a cofactor capable of enhancing the efficacy of HOCl in activating NF-κB. ACH-2 cells were first subjected to a slow increase in temperature, from 37 to 42 °C in 30 min, the cells then being maintained at 42 °C for 40 min. The temperature was then restored to 37 °C within 30 min. Nuclear extracts were taken at various phases of temperature variation and were analysed by a gel-retardation assay using an NF-κB probe. No retarded bands corresponding to the NF-κB-specific complex could be detected on the gel (results not shown), demonstrating that this factor was not significantly activated during and after the hyperthermia. In a second step, ACH-2 cells were subjected to a hyperthermic stress similar to that described above before being stressed directly by various HOCl concentrations as soon as the temperature had returned to 37 °C. Nuclear extracts were then prepared 180 min after the end of the hyperthermia for analysis by a gel-retardation assay. As shown in Figure 5, the NF-κB-specific retarded band appeared clearly at low HOCl concentrations, i.e. between 100 and 150 μM. In cells not exposed to hyperthermia, NF-κB was not detectable at these HOCl concentrations. At higher HOCl concentrations (200–300 μM), the NF-κB band was clearly 3-fold less intense in non-hyperthermic cells compared with cells subjected to one round of hyperthermia.

Antioxidants block NF-κB induction by HOCl

Taken together, our data suggest that HOCl induced NF-κB activity in ACH-2 cells through a signal transduction pathway involving the intracellular release of ROS, because the kinetics of induction very closely resemble those observed when H_2O_2 is added to either lymphocyte or monocyte [11,39]. We thus investigated the nature of the intermediates suspected of mediating HOCl-dependent NF-κB induction in ACH-2 cells.

The free radical scavenger pyrrolidine-dithiocarbamate (PDTC) and nordihydroguaiaretic acid [40] were able to suppress NF-κB activation, in a dose-dependent manner after HOCl stimulation of ACH-2 cells (Table 1), the inhibition being almost complete at 100 μM PDTC. Since the generation of intracellular ROS could proceed through the involvement of phospholipase A2 (PLA2), we tested the functional importance of these enzymes on HOCl-mediated NF-κB activation by using an inhibitor. The involvement of PLA2 was evaluated by using *p*-bromophenacylbromide (BPB), an irreversible inhibitor of PLA2 [41]. To this end, ACH-2 cells were pretreated for 60 min with increasing concentrations of BPB and then stimulated with HOCl. Non-toxic concentrations of BPB strongly inhibited, in a dose-dependent manner, NF-κB activation by HOCl, demonstrating the importance of PLA2 in the pathway (Table 1).

TNF-α is released in the supernatants of cells treated with HOCl or H_2O_2

In order to characterize the release of cytokines in the cell supernatant after an oxidative stress mediated either by H_2O_2 or HOCl, IL-1β and TNF-α levels were measured in cell supernatants. The amount of IL-1β released by ACH-2 cells after these oxidative stresses was very low and was only very weakly stimulated by either H_2O_2 or HOCl compared with the background levels (results not shown). On the other hand, less than 5 pg/ml TNF-α was detectable in the supernatant of untreated ACH-2 cells during the first 8 h of culture. This amount was increased when cells were treated with both chemicals. With H_2O_2, the amount of TNF-α released was stimulated more than 20-fold until 8 h after the stress, before returning to the level observed in untreated ACH-2 cells after 24 h (Figure 6). The same behaviour was observed with HOCl, except that the amplitude was lower, with maximal stimulation being ~ 5-fold after 8 h. Addition of 25 μg/ml CHX before the stress mediated by H_2O_2 or HOCl abolished the release of TNF-α in the supernatant (results not shown). These results indicate that a

Table 1 Inhibition of HOCl-mediated NF-κB induction by antioxidants

ACH-2 cells treated with 200 μM HOCl for 180 min were either preincubated or not with various inhibitors: a PLA2 inhibitor, BPB, the free radicals scavenger PDTC and nordihydroguaiaretic acid (NDGA). Results show percentages of NF-κB inducibility in comparison with HOCl induction, which was considered to be 100%.

Inhibitor	Inhibitor (μM)	NF-κB inducibility (%)
–	–	100
PDTC	60	89
PDTC	100	14
NDGA	30	14
NDGA	50	5.3
BPB	2	24.4
BPB	5	0.1

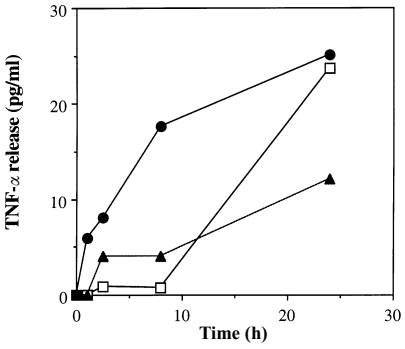

Figure 6 TNF-α is released after treating ACH-2 cells with either H_2O_2 or HOCl

Time-course of TNF-α release following ACH-2 cell treatment with 200 μM HOCl (▲) or 100 μM H_2O_2 (●); (□) represents the time-course of TNF-α release in untreated ACH-2 cells. Aliquots of the cell supernatants were directly taken for TNF-α quantification by EASIA. Data shown are the average of eight independent experiments.

small amount of TNF-α can be found in the supernatant of ACH-2 cells and that both H_2O_2 or HOCl can rapidly stimulate its release.

TNF-α plays a crucial role in NF-κB induction by both H_2O_2 and HOCl

In order to determine the functionality of the TNF-α released in cell supernatants in terms of NF-κB inducibility, we treated ACH-2 cells with equivalent TNF-α concentrations to those released in supernatants after treatment with H_2O_2 or HOCl, i.e. 100 and 33 pg/ml respectively (or 16.6 and 5 units/ml). Nuclear extracts were prepared 30, 60 and 120 min after induction and analysed by EMSA. As shown in Figure 7(A), a rather important specific NF-κB induction was detectable in the nucleus of ACH-2 cells treated under these conditions. On the other hand, supernatants of ACH-2 cells pretreated with either H_2O_2 (100 μM) or HOCl (200 μM) were collected and transferred onto untreated ACH-2 cells before being used to prepare nuclear extracts 30, 60 or 120 min after supernatant addition. As shown in Figure 7(B), a clear NF-κB band can be induced 60 min after addition of the supernatant taken from ACH-2 cells treated with H_2O_2 for 180 min. Similar observations can be made with supernatants

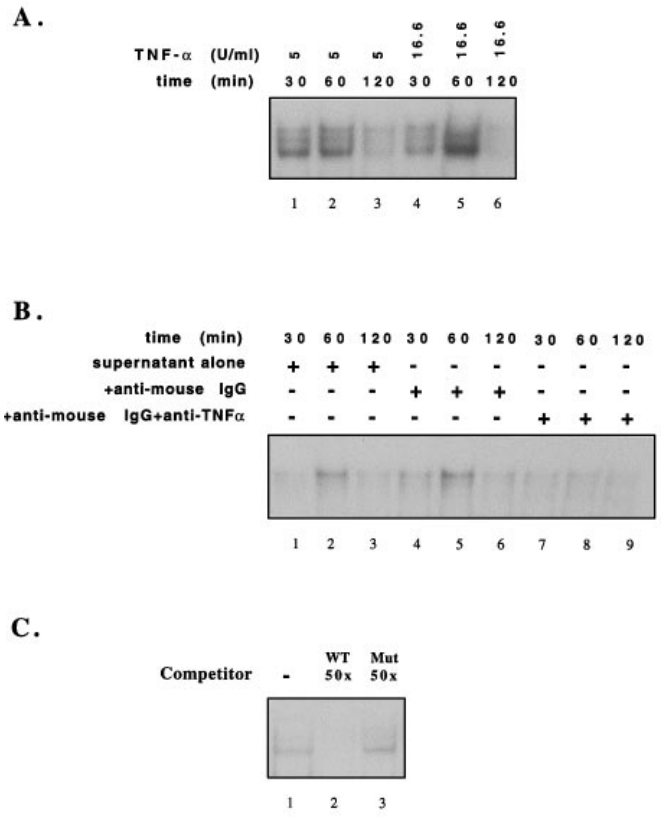

Figure 7 TNF-α released by treating cells either with H_2O_2 or HOCl is biologically active

(**A**) Low TNF-α concentrations can induce NF-κB. Medium of ACH-2 cells was supplemented with 5 and 16.6 units/ml TNF-α, and nuclear extracts were prepared 30, 60 and 120 min after the treatment. An equal amount of protein was mixed with a ^{32}P-labelled probe encompassing the κB elements of the HIV-1 enhancer. Samples were loaded on 6% native polyacrylamide gels and electrophoresed at 150 V. The autoradiogram of the gel is shown. (**B**) TNF-α detected in H_2O_2- or HOCl-treated cell supernatant has conserved its biological properties. Lanes 1, 2 and 3 show an NF-κB induction time-course experiment when supernatant was directly transferred onto untreated ACH-2 cells. In columns 7, 8, and 9 supernatant was depleted of TNF-α using an anti-TNF-α monoclonal antibody (Boehringer, Mannheim, Germany). (**C**) Competition with an unlabelled oligonucleotide (50 M excess) encompassing NF-κB sites reveals the position of the specific NF-κB complex. The specific NF-κB band could not be competed out with a 50 M excess of the mutated NF-κB probe. WT, wild-type probe; mut, mutated probe.

collected from ACH-2 cells treated for 180 min with HOCl, except that NF-κB induction was somewhat less intense. It should be noted that traces of H_2O_2 remaining in cell supernatants were eliminated by treating supernatants with 10 units/ml catalase. To verify whether the effect was due to TNF-α release subsequent to oxidative stress, supernatants were treated with Sepharose beads to which either anti-TNF-α monoclonal antibody or an irrelevant antibody was attached. The supernatants thus treated were added to ACH-2 cells and nuclear extracts were prepared after 30, 60 or 120 min. As shown in Figure 7(B), the treatment of cell supernatants taken from cells treated with H_2O_2, with the beads linked to an anti-TNF-α antibody, abolished NF-κB induction, while beads alone did not modify NF-κB induction by supernatant transfer. The specificity of the complex was shown by its disappearance through competition with an excess of unlabelled probe, but not with an excess of unlabelled mutated probe (Figure 7C). These results demonstrate

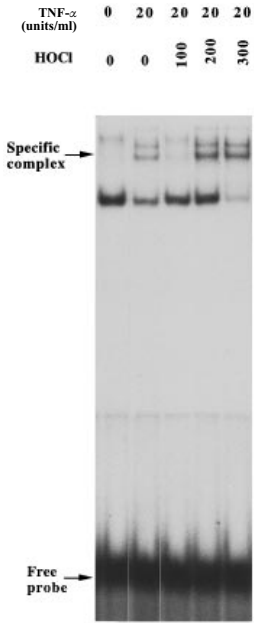

Figure 8 HOCl and TNF-α can cooperate to induce NF-κB

Induction of a nuclear NF-κB by treatment of ACH-2 cells with increasing concentrations of HOCl (100–300 μM) after a pretreatment with 20 units/ml TNF-α. Nuclear extracts were prepared 180 min after treatment with HOCl, and an equal amount of protein was mixed with a ^{32}P-labelled NF-κB probe. Samples were analysed as in Figure 1.

that TNF-α released after treatment with H_2O_2 or HOCl is functional and is an important secondary messenger which could be propagated to uninduced cells.

Pretreatment with TNF-α sensitizes ACH-2 cells to HOCl

To corroborate the demonstration that TNF-α played a crucial role in NF-κB induction by HOCl, ACH-2 cells were pretreated with TNF-α before being stressed with HOCl in order to reveal potential additive or synergistic effects between these two agents. ACH-2 cells were first treated with TNF-α (0, 20 or 200 units/ml) for 90 min. After induction, nuclear extracts were prepared and analysed by gel-retardation assay. As shown in Figure 8, a specific NF-κB complex could be clearly seen, even at TNF-α concentrations as low as 20 units/ml; this stimulation was slightly increased for higher TNF-α concentrations. When these cells were prestimulated with TNF-α at either 20 or 200 units/ml and stressed 90 min later with HOCl ranging from 100 to 300 μM, the induction of NF-κB was significantly higher than in non-pretreated cells (Figure 8). These results again demonstrate that cell pretreatment with TNF-α, which is known to generate intracellular ROS, renders ACH-2 cells more sensitive to subsequent stress mediated by HOCl, in terms of NF-κB nuclear induction.

DISCUSSION

In this work, we have shown that exposure of a T-lymphocyte cell line to an oxidizing treatment mediated by HOCl leads to activation of the NF-κB complex. The form of NF-κB induced by HOCl is the heterodimer p50/p65. This induction presents the same characteristics as NF-κB induction by PMA, lectins or cytokines [19,20], except that induction was slower in the case of HOCl. This may occur, either because HOCl alters one of the components (e.g. a cytoplasmic signalling protein) involved in activation, or because HOCl is not produced close enough to (i.e. does not directly damage) the cellular component from which the induction of NF-κB is initiated. However, activation by HOCl seems to be completely achieved after 4 h, possibly because several different activation pathways could well be required for full NF-κB activation. Thus, it is perhaps likely that an identical sensor molecule is activated by both H_2O_2 and HOCl, triggering IκB-α and p105 degradation and allowing p50/p65 to migrate inside the cell nucleus to activate genes harboring NF-κB responsive elements in their promoters [1,4]. The candidate sensory molecule would be oxidized by both HOCl and H_2O_2 and would transmit the message, leading to the degradation of the inhibitory subunits, retaining inactive NF-κB in the cytoplasm.

The cellular damage generated by HOCl is as diversified as that caused by exposure to H_2O_2, if it is assumed that H_2O_2 acts through generation of hydroxyl radicals in Fenton reactions. With a pK_a of 7.53, HOCl exists in essentially equal concentrations as the conjugate base hypochlorite (OCl^-) [30,42]. HOCl/OCl^- is a powerful biologically relevant oxidizing agent and can introduce damage to protein, lipid, carbohydrate and nucleic acid [43–45]. Furthermore, reaction of HOCl with amino and imino acids is known to generate long-lived *N*-chloroamines [27,46]. Although *N*-chloroamines exhibit a lower oxidizing potential than HOCl, their much longer lifetimes may enable such species to cause damage at distant sites [27,47]. In constrast, HOCl is thought to react close to its site of production [44,45]. Thus, addition of HOCl directly to the culture medium would damage T-lymphocyte membranes, modifying proteins and generating lipid peroxidation. These two kinds of oxidation products, i.e. protein modifications and lipid peroxidation products, have been recognized as being stimuli for NF-κB activation (see [4,48] for reviews). In particular, it has recently been shown that the treatment of Jurkat T-cells with millimolar concentrations of H_2O_2 generates the association between the ζ and ε chains of the T-cell receptor with ZAP-70 kinase [21,22]. This event occurs very rapidly after treatment, and is suspected to be crucial in the transduction pathways initiated by H_2O_2. Because HOCl is known to generate chlorinated adducts on proteins [44,48], it can be envisaged that these oxidation products could favour the formation of protein–protein interactions such as those between tyrosine kinases and membrane receptors. These events would take place at the cellular membrane and would be initiated by oxidative damage generated by HOCl. HOCl is also known to generate DNA-oxidation products. It has been suggested by several authors that DNA damage [35,49,50], especially thymidine dimers induced by UV light, could also be an event triggering a transduction pathway leading to IκB-α phosphorylation [49,50]. Thus, in summary, it can be proposed that HOCl is an oxidant that behaves somewhat like H_2O_2 and can activate NF-κB through various transduction pathways initiated at several cellular localizations.

In an attempt to further characterize the events involved in NF-κB activation by HOCl, we have used inhibitors known to act in a signal-transduction pathway yielding ROS [51]. Inhibition of PLA2 was shown to prevent NF-κB activation by HOCl. We further observed that HOCl-induced NF-κB activation was completely abolished by antioxidants such as PDTC or nordihydroguaiaretic acid, which concurs with the idea that intracellular ROS generation may be the ultimate step leading to

IκB-α or p105 phoshorylation. Further experiments are needed to fully characterize all the intermediates involved in this pathway, especially the redox-regulated kinase which is activated by intracellular ROS and suspected of being involved in IκB-α or p105 phosphorylation. Nonetheless, the fact that inhibitors other than antioxidants can prevent NF-κB induction by HOCl suggests that intracellular ROS are generated subsequent to the initial reaction mediated by HOCl. Several studies have shown that activation of NF-κB in response to other stimuli, such as cytokines, lipopolysaccharide, or anti-CD 28, can be abolished by antioxidants, suggesting that ROS may represent widely used secondary messenger molecules in lymphocytes or monocytes [1,51–54].

Within the spectrum of systemic reactions to inflammation, several physiological responses are regarded as being associated with acute inflammation. One of these is the alteration of the temperature set point in the hypothalamus and the generation of febrile response [37,55]. Three cytokines that are released from the site of injury (IL-1, TNF and IL-6) are considered to regulate the febrile response, possibly as a protective mechanism, and to mediate fever through the induction of prostaglandin E2 [37]. Since fever and oxidants released by inflammatory cells may exert synergistic actions in modifying cellular metabolism, and more specifically gene expression, we have investigated these effects on NF-κB induction. When T-cells undergo one round of hyperthermia no clear induction of NF-κB seems to occur. However, when the heat-stressed cells were subjected to a subsequent stress with HOCl a more important NF-κB induction could be detected. These results provide clear evidence of increased susceptibility to HOCl, suggesting that hyperthermia can constitute a phase which can significantly worsen the damage induced by a second oxidative stress. This shows that synergistic effects between fever and inflammatory processes might occur. Similarly, a synergism between TNF-α and HOCl has been also demonstrated in this paper. Because TNF-α is known to cause the intracellular release of ROS [56], it seems very likely that cellular antioxidant defences are transiently depressed by TNF-α treatment, rendering these cells more susceptible to a subsequent stress, mediated in this case by HOCl. Mitochondria play a crucial role in causing TNF-induced cytotoxicity in TNF-sensitive cells [56,57]. Presumably, upon TNF-α stimulation, mitochondrial radicals are formed at the ubiquinone site of the respiratory chain, where electrons are directly transferred from ubisemiquinone to molecular oxygen. This one-electron transfer results in the formation of $O_2^{-\bullet}$ as the primary reactive oxygen species of mitochondria, and is probably the cause of the observed synergistic effect between hyperthermia or TNF-α and HOCl.

Consistent with these findings, another important aspect of this work has been to demonstrate that oxidative stress induced by the addition of H_2O_2 or HOCl contributes significantly to the release of TNF-α in cell supernatants during the first 8 h following stress. TNF-α release in response to oxidative stress is a very important observation, because we have shown that this molecule is biologically active and capable of activating, even at low doses, non-induced cells in terms of NF-κB translocation. The release of this pro-inflammatory cytokine should contribute significantly to amplification and also propagation of the activation message to non-induced cells, triggering a generalized pro-oxidant state in distant cells.

In this paper, we have shown for the first time that HOCl, which is produced by activated neutrophils, can contribute significantly to the establishment of pro-oxidant conditions in a T-lymphocytic cell line, either directly, by triggering a transduction pathway which in turn leads to the nuclear translocation of NF-κB, or indirectly, by contributing to the processing and release of TNF-α in cell supernatants. Given that HOCl has a rather short diffusion radius in biological media because of its rather important reactivity [58], the fact that TNF-α can be released in response to its reaction with T-cells makes it possible to understand how local oxidation can have long-distance effects.

This study was supported by grants from the Belgian National Fund for Scientific Research (NFSR, Brussels, Belgium), the Télévie Research Program funded by the Belgian NFSR, the Sidaction Research Program (Paris, France) and the Concerted Action Program (Communauté Française de Belgique). J.P. is a Research Director at the Belgian NFSR. S.S. is supported by a concerted action program from the 'Communauté Française de Belgique'. We wish to thank Dr. V. Bours for his gift of the oligonucleotides and antibodies used in this work. We are grateful to the NIH AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious diseases, NIAID, Bethesda, U.S.A.) for providing the ACH-2 cell line.

REFERENCES.

1 Schreck, R., Rieber, P. and Baeuerle, P. A. (1991) EMBO J. **10**, 2247–2258
2 Remacle, J., Raes, M., Toussaint, O., Renard, P. and Rao, G. (1995) Mutation Res. **316**, 103–122
3 Schreck, R. and Baeuerle, P. A. (1991) Trends Cell Biol. **1**, 39–42
4 Schreck, R., Albermann, K. and Baeuerle, P. A. (1992) Free Radical Res. Commun. **17**, 221–237
5 Bose, Jr., H. (1992) Biochim. Biophys. Acta **1114**, 1–17
6 Brownell, E., Mittereder, N. and Rice, N. R. (1989) Oncogene **4**, 935–942
7 Bours, V., Burd, P. K., Brown, K., Villalobos, J., Park, S., Ryseck, R. P., Bravo, R., Kelly, K. and Siebelnist, U. (1992) Mol. Cell. Biol. **12**, 685–695
8 Bours, V., Villalobos, J., Burd, P. R., Kelly, K. and Siebelnist, U. (1990) Nature (London) **348**, 76–80
9 Ghosh, S., Gifford, A. M., Riviere, L. R., Tempst, P., Nolan, G. P. and Baltimore, D. (1990) Cell **62**, 1019–1029
10 Kieran, M., Blank, V., Logeat, F., Vandekerkove, J., Lottspeich, F., LeBail, O., Urban, M. B., Kourilsky, P., Baeuerle, P. A. and Israel, A. (1990) Cell **62**, 1007–1018
11 Baeuerle, P. A. and Baltimore, D. (1991) in The Hormonal Control Regulation of Gene Transcription, (Cohen, P. and Foulkes, J., eds.), pp. 423–446, Elsevier Science Publishers, Amsterdam, The Netherlands
12 Beg, A. A. and Baldwin, Jr., A. (1993) Genes Dev. **7**, 2064–2070
13 Mercurio, F., DiDonato, J., Rosette, C. and Karin, M. (1992) DNA Cell Biol. **11**, 523–537
14 Meyer, R., Hatada, E. N., Hohmann, H. P., Haiker, M., Bartsch, C., Rothlisberger, U., Lahm, H. W., Schlaeger, E. J., van Loon, A. P. and Scheidereit, C. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 966–970
15 Neri, A., Chang, C. C., Lombardi, M., Salina, M., Corradini, P., Maiolo, A. T., Chaganti, R. S. and Dalla-Favera, R. (1991) Cell **67**, 1075–1087
16 Mercurio, F., DiDonato, J., Rosette, C. and Karin, M. (1993) Genes Dev. **7**, 705–718
17 Rice, N., McKichan, M. L. and Israel, A. (1992) Cell **71**, 243–253
18 Legrand-Poels, S., Vaira, D., Pincemail, J., Van de Vorst, A. and Piette, J. (1990) AIDS Res. Hum. Retroviruses **6**, 1389–1397
19 Henkel, T., Machleidt, T., Alkalay, I., Krönke, M., Ben-Neriah, Y. and Baeuerle, P. A. (1993) Nature (London) **365**, 182–185
20 Brown, K., Park, S., Kanno, T., Franzoso, G. and Siebenlist, U. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 2532–2536
21 Schieven, G. L., Mittler, R. S., Nadler, S. G., Kirihara, J. M., Bolen, J. B., Kanner, S. B. and Ledbetter, J. A. (1994) J. Biol. Chem. **269**, 20718–20726
22 Chan, A. C., Iwashima, M., Turck, C. W. and Weiss, A. (1992) Cell **71**, 649–662
23 Schieven, G. L., Kirihara, J. M., Myers, D. E., Ledbetter, J. A. and Uckun, F. M. (1993) Blood **82**, 1212–1220
24 Segal, A. W. and Abo, A. (1993) Trends Biochem. Sci. **18**, 43–47
25 Weiss, S. J. (1989) New Engl. J. Med. **320**, 365–376
26 Mayeno, A. N., Curran, A. J., Roberts, R. L. and Foote, C. S. (1989) J. Biol. Chem. **264**, 5660–5668
27 Weiss, S. J., Lampert, M. B. and Test, S. T. (1983) Science **222**, 625–628
28 Grisham, M. B. and Granger, D. N. (1988) Dig. Dis. Sci. **33**, 6S–15S (suppl.)
29 Burdon, R. H. (1995) Free Radicals Biol. Med. **18**, 775–794
30 Morris, J. C. (1966) J. Phys. Chem. **70**, 3798–3805
31 Andrews, N. C. and Faller, D. V. (1991) Nucleic Acids Res. **19**, 2499
32 Ryseck, R.-P., Bull, P., Takamiya, M., Bours, V., Siebenlist, U., Dobrzansky, P. and Bravo, R. (1992) Mol. Cell. Biol. **12**, 674–684
33 Schreck, R., Meier, B., Männel, D. N., Dröge, W. and Baeuerle, P. A. (1992) J. Exp. Med. **175**, 1181–1194
34 Piret, B., Legrand-Poels, S., Sappey, C. and Piette, J. (1995) Eur J Biochem. **228**, 447–455
35 Legrand-Poels, S., Bours, V., Piret, B., Pflaum, M., Epe, B., Rentier, B. and Piette, J. (1995) J. Biol. Chem. **270**, 6925–6934

36 Baeuerle, P. A. and Baltimore, D. (1988) Science **242**, 540–546
37 Baumann, H. and Gauldie, J. (1994) Immunol. Today **15**, 74–80
38 Fey, G. H. and Fuller, G. M. (1987) Mol. Biol. Med. **4**, 323–329
39 Sappey, C., Legrand-Poels, S., Best-Belpomme, M., Favier, A., Rentier, A. and Piette, J. (1994) AIDS Res. Hum. Retroviruses **10**, 1451–1460
40 Rillema, J. A. (1984) Leukotrienes Med. **16**, 89–94
41 Lister, M. D., Glaser, K. B., Ulevitch, R. J. and Dennis, E. A. (1989) J. Biol. Chem. **264**, 8520–8528
42 Gordon, A. J. (1972) The Chemist's Companion: A Handbook of Practical Data, Techniques and References, John Wiley and Sons, New York
43 Test, S. T. and Weiss, S. J. (1986) Adv. Free Radical Biol. Med. **2**, 91–116
44 Albricht, J. M., Mc Carthy, C. A. and Hurst, J. K. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 210–214
45 Harrison, J. E., Watson, B. S. and Schultz, J. (1978) FEBS Lett. **92**, 327–332
46 Weiss, S. J., Klein, R., Slivka, A. and Wei, M. (1988) J. Clin. Invest. **70**, 598–607
47 Siebelnist, U., Franzoso, G. and Brown, K. (1994) Annu. Rev. Cell Biol. **10**, 405–455
48 Klebanoff, S. (1988) in Inflammation: Basic Principles and Clinical Correlates (Gallin, J. I. and Snyderman, R., eds.), Raven Press, New York
49 Stein, B., Rahmsdorf, H. J., Steffen, A., Litfin, M. and Herrlich, P. (1989) Mol. Cell. Biol. **9**, 5169–5181
50 Yarosh, D. B., Alas, L., Kibitel, J., O'Connor, A., Carrier, F. and Fornace, Jr., A. J. (1993) J. Invest. Dermatol. **100**, 790–799
51 Los, M., Schenk, H., Hexel, K., Baeuerle, P. A., Dröge, W. and Schulze-Ostoff, K. (1995) EMBO J. **14**, 3731–3740
52 Roederer, M., Staal, F. J. T., Raju, P. A., Ela, S. W., Herzenberg, L. A. and Herzenberg, L. A. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 4884–4888
53 Meyer, M., Schreck, R. and Baeuerle, P. A. (1993) EMBO J. **12**, 2005–2015
54 Schenk, H., Klein, M., Erdbrügger, W., Dröge, W. and Schulze-Osthoff, K. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 1672–1676
55 Dinarello, C. A., Cannon, J. G. and Wolf, S. M. (1988) Rev. Infect. Dis. **10**, 34–64
56 Schulze-Osthoff, K., Bakker, A. C., Vanhaesebroeck, B., Bayert, R., Jacobs, W. and Fiers, W. (1992) J. Biol. Chem. **267**, 5317–5323
57 Schulze-Osthoff, K., Bayert, R., Vandevoorde, V., Haegeman, G. and Fiers, W. (1993) EMBO J. **12**, 3095–3104
58 Folkes, L. K., Candeias, L. P. and Wardman, P. (1995) Arch. Biochem. Biophys. **323**, 120–126

Received 20 June 1996/12 September 1996; accepted 24 September 1996