Ozone induction of cytokine-induced neutrophil chemoattractant (CINC) and nuclear factor-κb in rat lung: inhibition by corticosteroids

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Abstract **We determined in rat lung whether ozone exposure was associated with the expression of the chemokine, cytokine-induced neutrophil chemoattractant (CINC), and of the transcription factor, NF-κB. CINC mRNA expression peaked at 2 h after cessation of ozone exposure, and returned to basal levels by 24 h. DNA-binding activity of NF-κB showed a marked increase after ozone, maximal at 2 h. Dexamethasone inhibited CINC mRNA and NF-κB expression, together with neutrophilic inflammation. Our data supports the concept that ozone leads to NF-κB activation which increases CINC mRNA expression. These series of events could lead to neutrophilic inflammation.**

Key words: Ozone; Chemokine; CINC mRNA; Lung inflammation; NF-κB

1. Introduction

Migration of leukocytes from blood into tissues is a complex phenomenon involving chemoattraction, adhesion of cells to endothelium and transendothelial migration. Over the past 10 years, there have been advances in terms of the discovery of several families of adhesion molecules and pro-inflammatory cytokines. A family of cytokines with a variety of proinflammatory properties referred to as chemokines has the ability of attracting and activating leukocytes in vitro [1]. This family of cytokines can be divided into two large groups depending on whether or not there is an intervening amino acid between the first two conserved cysteines yielding the C-X-C and C-C subgroups, respectively. The C-X-C chemokines of which IL-8 is the best known member are active principally towards neutrophils. More recently, another member of this family has been described, cytokine-induced neutrophil chemoattractant (CINC), first purified from an epithelioid clone of a normal rat kidney line [2]. CINC has been shown to be active as a neutrophil chemoattractant both in vitro and in vivo [3,4].

Exposure of animals to ozone induces infiltration of neutrophils into the airways with an increase in airway's responsiveness [5,6]. Ozone exposure also induces the expression of the chemokine MIP-2 in mouse and rat lung [7,8]. In human airway epithelial cells exposed to ozone, an increase in the production of interleukin-6 and interleukin-8 has been demonstrated [9]. Transcriptional activation of several cytokine genes for C-X-C chemokines including IL-8, MGSA/gro and MIP-2 appears to be dependent on the transcription regulating factor, nuclear factor-κB (NF-κB), in their promoter regions [10,11]. The aim of this study was to examine the effect of ozone exposure on the expression of another C-X-C chemokine, CINC, in Brown Norway rat lung. Recent cloning and sequencing of the CINC gene has shown a binding site for the transcription regulating factor, nuclear factor-κB in its promoter region [12]. To determine whether any increase in CINC transcription was associated with the activation of NF-κB-binding to the CINC promoter, we examined NF-κB-binding activity to nuclear extracts purified from rat lung after exposure to ozone. To examine further the association of NF-κB-binding and CINC expression, we studied the effect of corticosteroid treatment on these parameters. Because activated glucocorticoid receptors are known to prevent NF-κB-binding [13], we also studied whether corticosteroids could inhibit both NF-κB-binding and CINC expression, in addition to neutrophilic inflammation, following ozone exposure.

2. Materials and methods

2.1. Experimental protocol

Ozone was generated by passing laboratory air through a Sander Ozoniser (Model IV; Sander, Germany) as previously described [7]. Four groups (each group had 6–9 animals) of pathogen-free Brown-Norway rats (250–350 g; Harlan-Olac, Essex, UK) were used in this study. Rats were either exposed to ozone (OZ, 3 ppm for 6 h) or to filtered air (CTRL). In experiments involving the synthetic glucocorticoid dexamethasone (DEX), it was administered (3 mg/kg given i.p.) 1 h prior to filtered air (DEX) or ozone (DEX/OZ) exposures. After 2, 8 or 24 h following exposure, rats were sacrificed, and bronchoalveolar lavage was performed, followed by removal of lungs. Lavaged lungs were then used for mRNA extraction and electrophoretic mobility shift assays. The retrieval and differential cell counts were performed as previously described [5].

2.2. Reverse transcription-polymerase chain reaction (RT-PCR) and sequencing

For the preparation of the CINC cDNA, a Brown-Norway rat was administered lipopolysaccharide (*Escherichia coli*; Sigma; 100 μg intratracheally). Total cellular RNA from lung recovered from LPS-stimulated rat was isolated according to the method of Chomczynski and Sacchi [14] and reversely transcribed at 42°C for 60 min. PCR was performed as previously described [7]. The primers were designed from the published sequences for CINC [15] and GAPDH cDNAs [16]. The PCR products were electrophoresed in 2% agarose gels to visualize the CINC and GAPDH bands. The sizes of the PCR products generated were 205 base pairs (bp) for CINC and 309 bp for GAPDH. The PCR product was excised and purified using Geneclean II (Stratech, Luton, UK). The CINC PCR product was also analysed by sequencing. Cycle sequence was performed on 100 ng of PCR product and 1 pmol of each primer. Cycling conditions were 30 s at 95°C, 30 s at 55°C and 30 s at 72°C using exo pfu- (Stratagene, Cambridge, UK). The purified products show a 100% homology with the published sequence of rat CINC cDNA [15] and were then used as cDNA probes for Northern blot analysis.

2.3. Northern blot analysis

The 205 bp CINC PCR product and the 1272-bp *Pst*I fragment from rat GAPDH cDNA were labelled by random priming using [α-^{32}P] dCTP (3000 Ci/mmol; Amersham, UK). Total cellular or messenger RNAs from rat lung or bronchoalveolar lavage cells were subjected to

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electrophoresis on a 1% agarose/formaldehyde gel and blotted onto Hybond-N membranes (Amersham, UK). The prehybridization and hybridization were carried out at 42°C as previously described [7]. Each blot was washed to a stringency of 0.1 × SSC/0.1% SDS for 30 min at 60°C and exposed at −80°C for 1–7 days to Kodak X-OMAT S film. Autoradiographic bands were quantified by densitometric scanning (Quantity One software, PDI, New York, NY).

2.4. Electrophoretic mobility shift assays

Nuclear proteins were extracted from lung tissues as previously described [17]. Double-stranded oligonucleotides encoding the consensus target sequence of NF-κB (and flanking regions) present in the CINC promoter (5′-CTCC**GGGAATTTCC**CTGGC-3′) (R&D systems, Abingdon, UK) were end-labelled using [α-^{32}P]ATP and T4 polynucleotide kinase. 10 μg of nuclear protein from each sample was incubated with 50,000 cpm of labelled oligonucleotide in 25 μl incubation buffer (4% glycerol, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM Tris-HCl, pH 7.5, 0.8 mg/ml sonicated salmon sperm DNA) for 20 min at 25°C. Protein–DNA complexes were separated on a 6% polyacrylamide gel using 0.25 × Tris-Borate-EDTA running buffer. The retarded band was detected by autoradiography and quantified by laser densitometry. Specificity was determined by addition of excess unlabelled double-stranded oligonucleotides and by using a non-specific competitor consisting of the transcription factor activator protein-1 or AP-1.

2.5. Data analysis

Data are presented as mean ± S.E.M. Statistical analysis of results was performed by Mann–Whitney *U*-test for stepward comparison. *P* values less than 0.05 were considered to be significant.

3. Results

3.1. Ozone induction of CINC mRNA expression in rat lung

Ozone-exposed rats (3 ppm for 6 h) demonstrated a significant increase in neutrophil counts in bronchoalveolar lavage fluid at 2, 8 and 24 h post-ozone exposure (Fig. 1). This increase was maximal at 8 h and remained higher up to 24 h post-exposure. There was no significant difference in the total cell counts, macrophages, eosinophils, or lymphocytes recovered in BAL fluid in animals exposed to ozone or filtered air.

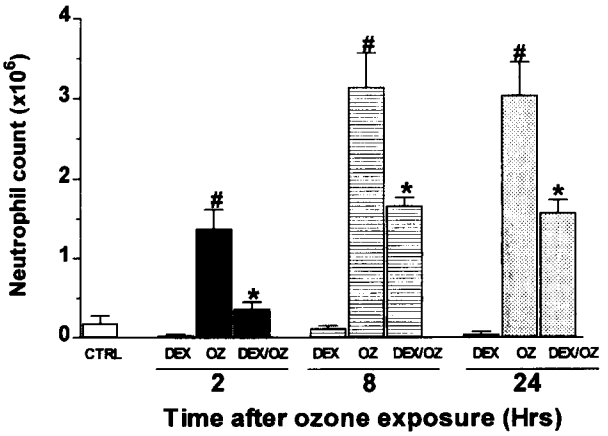

Fig. 1. Effect of ozone and/or dexamethasone on neutrophil accumulation in bronchoalveolar lavage fluid (BALF). Time course of neutrophil accumulation in BALF of rats exposed to ozone (3 ppm for 6 h) at 2, 8 and 24 h after exposure. Rats were exposed to filtered air (CTRL) or ozone (OZ). 1 h prior to ozone (DEX/OZ) or to filtered air (DEX), dexamethasone was administered i.p. (3 mg/kg). There was an increase in neutrophils recovered from 2 to 24 h which can be prevented by dexamethasone Data shown as mean ± S.E.M. of 6 to 9 animals in each group. $^{\#}P < 0.05$ compared to control (CTRL); $^{*}P < 0.05$ compared to ozone (OZ).

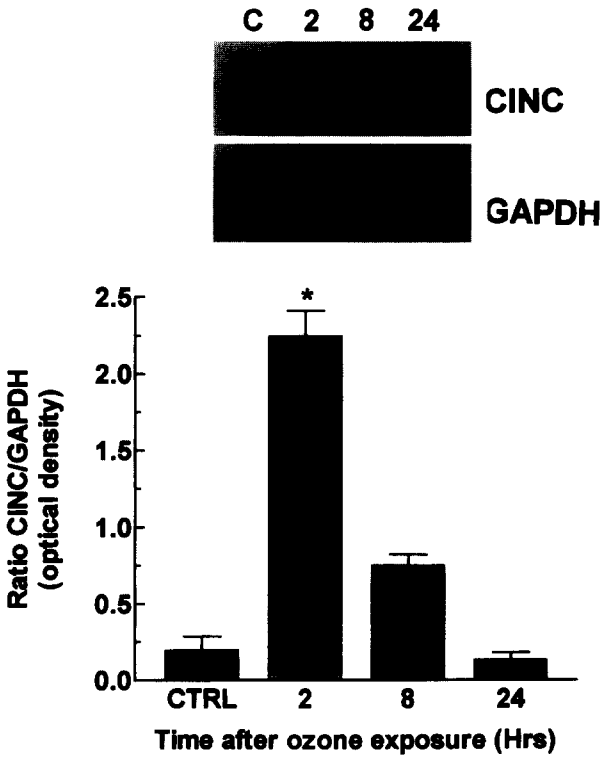

Fig. 2. Time course of ozone-induced CINC mRNA expression in rat lung. Northern blot showing CINC mRNA expression in mRNA extracted from whole lung homogenate obtained from rats at 2, 8 and 24 h after exposure to ozone (3 ppm for 6 h). As controls (C, CTRL) rats inhaled filtered air only. The upper panel shows a representative Northern blot for CINC mRNA expression from two rats in each experimental group, together with the GAPDH mRNA expression used as an internal standard. The CINC mRNA signals expressed as a ratio to GAPDH mRNA as measured by densitometric scanning are shown in the lower panel. Values are the mean ± S.E.M. of at least 6 animals. Maximal expression of CINC mRNA occurred 2 h after exposure, returning towards baseline values by 8 and 24 h. $^{*}P < 0.05$ compared to control (C, CTRL).

Ozone exposure resulted in a marked increase in the expression of CINC mRNA 2 h after exposure and rapidly declined thereafter (Fig. 2). CINC mRNA signal was barely detectable in RNA obtained from animals exposed to filtered air only. A similar profile of CINC mRNA expression was found in Northern analysis of RNA extracted from bronchoalveolar lavage cells.

3.2. Inhibition by dexamethasone of ozone-induced CINC mRNA expression in lung

Dexamethasone alone had no effect on the level of CINC mRNA expression over the time course investigated. However, it almost completely inhibited (87–92% reduction) ozone-induced CINC mRNA expression to control levels 2 and 8 h after ozone exposure (Fig. 3). The inhibitory effect of dexamethasone was also observed at the level of neutrophils recovered in BAL fluid. Dexamethasone significantly reduced ozone-induced neutrophilia in bronchoalveolar lavage fluid up to 24 h after exposure without any effect on total cell number, eosinophils, lymphocytes or macrophages (Fig. 1).

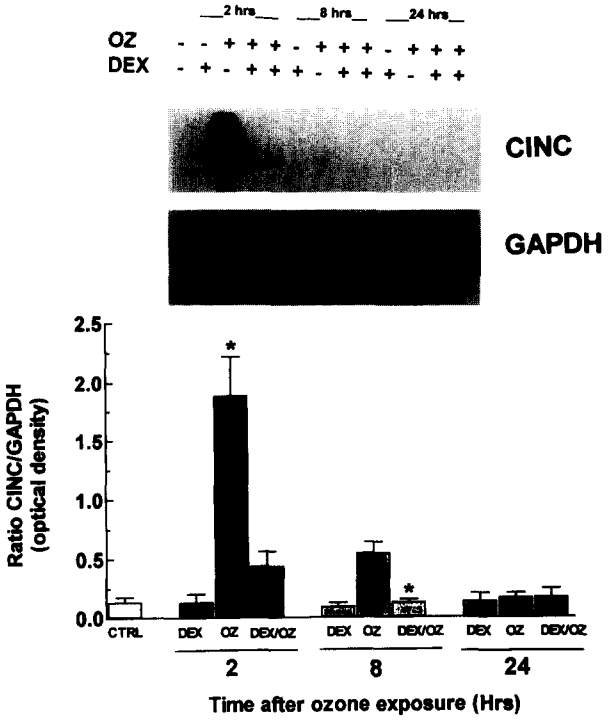

Fig. 3. Effect of dexamethasone on ozone-induced CINC mRNA expression. Northern blot of CINC mRNA expression in mRNA purified from rat lung after exposure to ozone (OZ) or to filtered air (CTRL). 1 h prior to ozone (DEX/OZ) or to filtered air (DEX), dexamethasone was administered i.p.w (3 mg/kg). The lungs were then harvested 2, 8 and 24 h after ozone exposure and assayed for CINC mRNA expression The *upper panel* shows the CINC mRNA from rats in each experimental group, together with the GAPDH mRNA expression. The *lower panel* shows the mean ± S.E.M. densitometric measurements of the autoradiograms obtained in at least 6 animals in each experimental group. The CINC mRNA signals were expressed as a ratio to GAPDH mRNA to account for differences in loading or transfer of the mRNA. Ozone induced a significant increase in CINC mRNA expression 2 h postexposure [$*P < 0.05$ compared to control (CTRL)], an effect suppressed by dexamethasone. 8 h after exposure there was a significant reduction in CINC mRNA expression by dexamethasone ($*P < 0.05$ compared to ozone at 8 h).

3.3. *Ozone-induced NF-κB-binding activity and its inhibition by dexamethasone*

Electrophoretic mobility shift assays on nuclear extracts showed a marked increase in NF-κB DNA-binding activity following ozone exposure (Fig. 4). This activity was maximal at 2 h and declined thereafter to control levels after 24 h postozone exposure (Fig. 4). A significant level of NF-κB-binding activity was still seen at 8 h after the cessation of ozone exposure. Dexamethasone alone did not have any effect on the baseline NF-κB DNA-binding activity but attenuated this activity in ozone-exposed rats towards control levels at 2 and 8 h after exposure to ozone.

4. Discussion

CINC was first described as an endotoxin-inducible chemotactic factor produced by rat renal epithelioid cells [2] and has been shown to have chemoattractant effects for neutrophils. We have shown that ozone, an environmental pollutant which can induce pulmonary inflammation characterised by a predominant neutrophilia, can markedly upregulate the expression of CINC in rat lung. This effect was transient, being maximal at 2 h, and levels of CINC mRNA returned to baseline values at 24 h after exposure. This upregulation of CINC mRNA expression was blocked by prior treatment with the corticosteroid, dexamethasone, which also inhibited ozone-induced neutrophilic inflammation in the lungs.

The mechanisms by which CINC mRNA is upregulated following ozone exposure are unclear. Secondary messages such as IL-1 and TNF-α have been shown to stimulate CINC production in vitro from epithelioid cells from rat renal glomeruli [18,19]. In the rat, ozone exposure has been shown to enhance the production of these cytokines, IL-1 and TNF-α from alveolar macrophages [20]. Because CINC mRNA was upregulated also in rat bronchoalveolar lavage cells following ozone exposure, it is possible that IL-1 and TNF-α are the stimuli for CINC mRNA expression by alveolar macrophages and other cells in the airways and lung parenchyma.

NF-κB is a member of a novel family of transcription regulatory factors showing a common structural motif for DNA-binding and dimerization. It regulates the transcription of many genes particularly those of cytokines, NF-κB-binding motifs

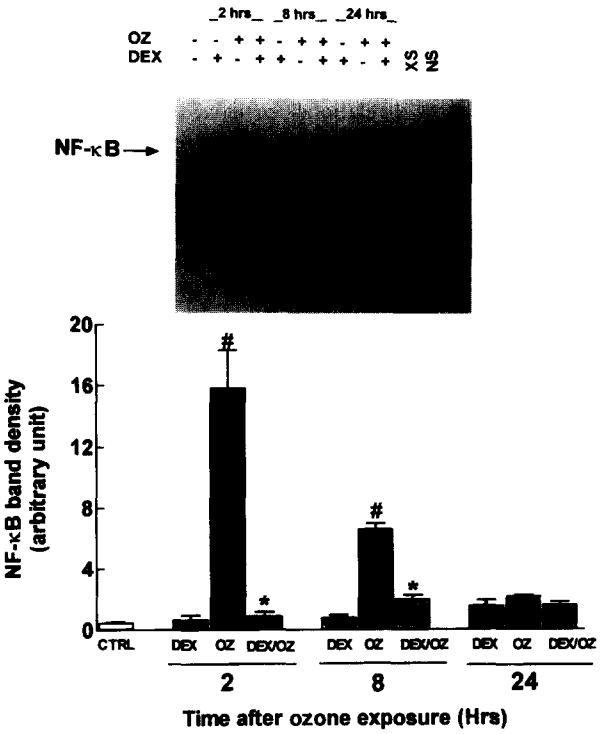

Fig. 4. Effect of ozone and dexamethasone on NF-κB-binding activity. The top panel shows an electrophoretic gel mobility shift assay showing NF-κB-binding activity (arrowed) in 10 μg rat lung cell nuclear proteins from individual animals exposed to filtered air (CTRL) or to ozone (3 ppm for 6 h) in the presence or absence of dexamethasone (Dex) pretreatment for various time points after exposure. The legend is similar to that described in Fig. 3. The bottom panel represents densitometric scanning of the band shift data. Ozone (2 and 8 h postexposure) produced a marked increase in NF-κB-binding activity [$\#P < 0.05$ compared to control (CTRL)]. This increase was entirely abrogated by pretreatment with dexamethasone. The specificity of binding was confirmed by the addition of 100-fold excess unlabelled NF-κB oligonucleotide (XS) and by using a non-specific (NS) competitor (activator protein 1 or AP-1). Values are the mean ± S.E.M. of 6 to 8 animals.

have been described in the proximal promoter regions of cytokine genes such as MIP-2, TNF-α, IL-8, GM-CSF, MGSA/GRO, MIP-1α and IL-6 genes, and appear to be important in regulating their transcription [21–26]. More recently, with the identification and cloning of the CINC gene, an NF-κB motif has also been described in the upstream region, and no other *cis*-acting regulatory elements were identified on up to 1 kb upstream region that has been cloned [10]. This suggest that NF-κB is likely to be involved in CINC gene transcription. In unstimulated cells, NF-κB is bound to an inhibitor, I-κB, which keeps the complex localised to the cytoplasm. However, after stimulation, NF-κB dissociates from I-κB and translocates to the nucleus, where it can bind to specific sequences in the promoter and induce transcription [25]. Our data shows that following ozone exposure NF-κB-binding activity was increased in rat lung nuclear extracts within 2 h after cessation of exposure.

Since both TNF-α and IL-1 can induce NF-κB [27–29], it is tempting to postulate that ozone may induce TNF-α and IL-1 which in turn increase NF-κB-binding to cause CINC mRNA upregulation. As ozone is a powerful oxidising agent, it is also possible that it can induce an increase in NF-κB-binding directly since hydrogen peroxide and other oxidants have been shown to activate rapidly NF-κB in certain cell lines in vitro [30,31]. Thus, the possibility of a direct effect of ozone on NF-κB cannot be excluded.

We used corticosteroids in our study in order to determine whether there was a relationship between the expression of CINC mRNA, NF-κB-binding and neutrophilic inflammation. Corticosteroids have been previously found to inhibit NF-κB-binding in vitro [13]. The CINC gene promoter contains no glucocorticoid response elements (GRE) [10] which suggests that the effect of corticosteroids on CINC mRNA is mediated via a direct inhibition of transcription regulatory factors such as NF-κB to the CINC gene promoter. Such direct interactions of the activated steroid receptor with NF-κB has been described in in vitro studies performed on human lung, peripheral blood mononuclear cells and in cultured cells [13,17,32].

In summary, we have shown that CINC mRNA expression is closely linked to NF-κB DNA-binding activity following ozone exposure in vivo, with their concomitant suppression by corticosteroids. This suggests that CINC may be involved in the neutrophilic chemoattraction response to ozone.

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References

[1] Baggiolini, M., Dewald, B. and Moser, B. (1994) Adv. Immunol. 55, 97–179.
[2] Watanabe, K., Kinoshita, S. and Nakagawa, H. (1989) Biochem. Biophys. Res. Commun. 161,1093–1099.
[3] Watanabe, K., Koizumi, F., Kurashige, Y., Tsurufuji, S., and Nakagawa, H. (1991) Exp. Mol. Pathol. 55,30–37.
[4] Iida, M, Watanabe, K., Tsurufuji, M., Takaishi, K., Iizuka, Y. and Tsurufuji, S. (1992) Infect. Immun. 60,1268–1272.
[5] Tsukagoshi, H., Haddad, E.-B., Sun, J., Barnes, P.J. and Chung, K.F. (1995) J. Appl. Physiol. 78, 1015–1022.
[6] Holtzman, M.J., Fabbri, L.M., O'Byrne, P.M., Gold, B.D., Aizawa, H., Walters, E.H., Alpert, S.E. and Nadel, J.A. (1983) Am. Rev. Resp. Dis. 127, 686–690.
[7] Haddad, E.-B., Salmon, M., Sun, J., Liu, S, Das, A., Adcock, I., Barnes, P.J. and Chung, K.F. (1995) FEBS Lett. 363, 285–288.
[8] Driscoll, K.E., Simpson, L., Carter, J., Hassenbein, D. and Leikauf, G.D. (1993) Toxicol. Appl. Pharmacol. 119, 306–309.
[9] Devlin, R.B., McKinnon, K.P., Noah, T., Becker, S. and Koren, H.S. (1994) Am. J. Physiol. 266, L612-L619.
[10] Mukaida, N. and Matsushima, K. (1992) Cytokine 4, 41–53.
[11] Anisowicz, A., Messineo, M., Lee, S.W. and Sager, R. (1991) J. Immunol. 147, 520–527.
[12] Konishi, K., Takata, Y., Yamamoto, M., Yomogida, K., Watanabe, K., Tsurufuji, S. and Fukioka, M. (1993) Gene 126, 285–286.
[13] Ray, A. and Prefontaine, K.E. (1994) Proc. Natl. Acad. Sci. USA 91, 752–756.
[14] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–160.
[15] Huang, S., Paulauskis, J.D. and Kobzik, L. (1992) Biochem. Biophys. Res. Commun. 184, 922–929.
[16] Fort, P., Marty, L., Piechaczyk, M., Sabraity, S.E., Dani, C., Jeanteur, P. and Blanchard, J.M. (1985) Nucleic Acids Res. 13, 1431–1442.
[17] Adcock, I.M., Brown, C.R., Gelder, C.M., Shirasaki, H., Peters, M.J. and Barnes, P.J. (1995) Am. J. Physiol. 268, C331–C338.
[18] Watanabe, K. and Nakagawa, H. (1987) Biochem. Biophys. Res. Commun. 149, 989–994.
[19] Watanabe, K. and Nakagawa, H. (1990) Nephron 54, 169–175.
[20] Pendino, K.J., Shuler, R.L., Laskin, J.D. and Laskin, D.L. (1994) Am. J. Resp. Cell Mol. Biol. 11, 279–286.
[21] Grove, M. and Plumb, M. (1993) Mol. Cel. Biol. 13, 5276–5289.
[22] Danoff, T.M., Lalley, P.A., Chang, Y.S., Heeger, P.S., and Neilson, E.G. (1994) J. Immunol. 152, 1182–1189.
[23] Hiscott, J., Marois, J., Garoufalis, J., D'Addario, M., Roulston, A., Kwan, I., Lacoste, J., Nguyen, H., Bensi, G. (1993) Mol. Cel. Biol. 13, 6231–6240.
[24] Grilli, M., Chiu, J.J. and Lenardo, M.J. (1993) Int. Rev. Cytol. 143, 1–62.
[25] Bauerle, P.A. (1991) Biochim. Biophys. Acta 1072, 63–80.
[26] Widmer, K.R. Manogue, Cerami, A. and Sherry, B. (1993) J. Immunol. 150, 4996–5012.
[27] Hohmann, H.P., Brockhaus, M., Baeuerle, P.A., Remy, R., Kolbeck, R. and van Loon, A.P. (1990) J. Biol. Chem. 265, 22409–22417.
[28] Messer, G., Weiss, E.H. and Baeuerle, P.A. (1990) Cytokine 2, 389–397.
[29] Osborn, L., Kunkel, S. and Nabel, G.J. (1989) Proc. Natl. Acad. Sci. USA 86, 2336–2340.
[30] Schreck, R., Rieber, P. and Baeuerle, P.A. (1991) EMBO J. 10, 2247–2258.
[31] Toledano, M.B. and Leonard, W.J. (1991) Proc. Natl. Acad. Sci. USA 88, 4328–4332.
[32] Adcock, I.M., Shirasaki, H., Gelder, C.M., Peters, M.J., Brown, C.R. and Barnes, P.J. (1994) Life Sci. 55, 1147–1153.