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Inhibition by antioxidants of nitric oxide synthase expression in murine macrophages: role of nuclear factor κB and interferon regulatory factor 1

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1 In view of the potential deleterious effects of high amounts of nitric oxide (NO) produced by the inducible isoform of NO synthase (iNOS) in inflammation, the prevention of the expression of this enzyme represents an important therapeutic goal. In cytokine-stimulated cells, activation of nuclear factor κB (NF-κB) is crucial for the increase in iNOS gene expression. Since NF-κB activation appears to involve a redox-sensitive step, we have investigated whether three structurally unrelated antioxidants, 5,7-dihydroxyflavone (chrysin), 3,4-dichloroisocoumarin (DCI) and N-acetyl 5-hydroxytryptamine (N-acetylserotonin, NAS), affect iNOS expression in cultured RAW 264.7 monocyte/macrophages stimulated with bacterial lipopolysaccharide (LPS, 140 ng ml^{-1}) and interferon-γ (IFNγ, 5 u ml^{-1}).

2 During a 6 h incubation period neither LPS nor IFNγ alone exerted a significant effect but when combined, caused a prominent increase in nitrite formation, iNOS mRNA and protein abundance. Co-incubation with chrysin (50 μM), DCI (50 μM) or NAS (1 mM) markedly attenuated this increase in iNOS gene expression.

3 DCI, but not chrysin or NAS, prevented the activation of NF-κB in cells exposed to LPS plus IFNγ for 30 min. In contrast, all three antioxidants significantly blunted the DNA-binding activity of interferon regulatory factor 1 (IRF-1), which mediates the synergistic effect of IFNγ on iNOS gene expression in cells treated for 2 h with LPS plus IFNγ.

4 DCI thus appears to inhibit iNOS gene expression at the transcriptional level by preventing the activation of both NF-κB and IRF-1. The inhibitory effect of DCI on NF-κB activation, however, does not seem to be related to its antioxidative properties, since DCI, unlike chrysin or NAS, is a potent serine protease inihbitor which stabilizes the inactive NF-κB complex by protecting the inhibitory IκB-α subunit from proteolytic degradation.

5 The virtually identical inhibitory effect of chrysin, DCI and NAS on the activation of IRF-1 points to a redox-sensitive step in the activation of this transcription factor, which in contrast to NF-κB requires *de novo* protein synthesis.

6 Since iNOS gene expression in human cells and tissues usually requires the combination of several cytokines, antioxidants such as chrysin and NAS which do not interfere with the activation of NF-κB may be of therapeutic value for selectively inhibiting the enhanced expression of this enzyme in inflammation.

Keywords: Antioxidants; inducible NO synthase; gene expression; IRF-1; NF-κB; RAW 264.7 monocyte/macrophages

Introduction

Nitric oxide (NO) plays an important role in vascular homeostasis both in health and disease. In addition to the calcium/calmodulin-dependent constitutive isoform of NO synthase, e.g. in endothelial and neuronal cells, a calcium-dependent NO synthase can be induced in macrophages, vascular smooth muscle cells (VSMC) and other cells following exposure to certain cytokines, microbes or microbial products. In general, expression of this inducible NO synthase (iNOS) may be viewed as the result of a generalized or localized inflammatory response resulting from infection or tissue injury. The high production of NO generated by this enzyme can exert both protective and deleterious effects (Morris & Billiar, 1994). It follows, therefore, that the selective inhibition of expression of this enzyme represents an important therapeutic goal.

Changes in NO formation in iNOS-expressing cells are usually correlated with similar changes in iNOS mRNA abundance, indicating that a major part of iNOS regulation occurs at the level of transcription. The promoter region of the iNOS gene contains several bindings sites for transcription factors such as nuclear factor κB (NF-κB) and activator protein-1 as well as for various members of the C/EBP, ATF/CREB and Stat family of transcription factors, (Lowenstein *et al.*, 1993; Xie *et al.*, 1993; Chartrain *et al.*, 1994). Of these transcription factors only activation of NF-κB has been shown to mediate the enhanced expression of the iNOS gene in macrophages exposed to bacterial lipopolysaccharides (LPS) (Mülsch *et al.*, 1993; Sherman *et al.*, 1993; Xie *et al.*, 1994) as well as in interleukin-1β-stimulated VSMC (Schini-Kerth *et al.*, 1995) and mesangial cells (Eberhardt *et al.*, 1994). Moreover, the synergistic effect of interferon-γ (IFNγ) on iNOS expression in LPS-stimulated macrophages (Lorsbach *et al.*, 1993) appears to be mediated by the activation of interferon regulatory factor 1 (IRF-1) (Kamijo *et al.*, 1994; Martin *et al.*, 1994).

Recently, the hypothesis has been put forward that an increase in the cellular production of reactive oxygen species (oxidative stress) mediates the cytokine-induced activation of NF-κB in various cell types (Grimm & Baeuerle, 1993). This hypothesis is based on observations that potential antioxidants such as pyrrolidine dithiocarbamate or N-acetylcysteine are capable of inhibiting the cytokine-induced activation of NF-κB which can be mimicked by exogenous hydrogen peroxide

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and pyrogallol, a generator of superoxide anions (Adcock *et al.*, 1994). Therefore, we have investigated whether three structurally different but equally effective antioxidants are capable of inhibiting the NF-κB-mediated expression of the iNOS gene in RAW 264.7 macrophages stimulated with LPS and IFNγ.

Methods

Cell culture and experimental protocol

RAW 264.7 mouse macrophages (ATCC No. TIB 71; 2.5×10^6 cells per well) were cultured in 6-well plates in 2 ml Dulbecco's Modified Eagle's Medium (Sigma) containing 4 mM L-glutamine, 17.7 mM D-glucose, 50 u ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, and 10% foetal calf serum (Pan Systems, Aidenbach, Germany) for 18 h. Thereafter the medium was replaced by 2 ml Minimum Essential Medium (without phenol-red; Biochrom, Berlin, Germany) containing 4 mM L-glutamine, 1.7 mM D-glucose, 44 mM sodium bicarbonate, 1 mM sodium pyruvate, 50 u ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, and 5% foetal calf serum to which 140 ng ml^{-1} LPS and/or 5 u ml^{-1} IFNγ were added. The incubation was continued for 6 h, and in some experiments for 2–24 h, followed by harvesting of the cells and analysis of the nitrite concentration in the conditioned medium (100 μl plus 400 μl Griess reagent) as described by Saville (1958). In some experiments, incubations were terminated after 30–120 min to prepare nuclear extracts (Schreiber *et al.*, 1989).

Cell viability was assessed by the uptake of the fluorescent dyes, calcein AM (Molecular Probes, Eugene, Oreg., U.S.A.) and alamarBlue (alamar, Sacramento, CA, U.S.A.), and usually exceeded 95%, irrespective of the antioxidant treatment.

RT-PCR analysis

Total RNA was isolated as described by Chromczynski & Sacchi (1987).

Reverse transcription (RT) The following RT components were added to the reaction vials: 2 μg of total RNA, 4 μl 5 × reaction buffer (Gibco, Eggenstein, Germany), 1 μl deoxynucleotide mixture (dATP, dCTP, dGTP, dTTP; 2.5 mM), 1 μl olig-dT (200 ng μl^{-1}), 1 μl DL-dithiothreitol (0.1 mM), 0.5 μl RNase inhibitor (Pharmacia, Freiburg, Germany) and 1 μl superscript reverse transcriptase (Gibco) in a total volume of 20 μl. The vials were incubated for 60 min at 37°C, thereafter the RT reaction was terminated by heating at 95°C for 7 min.

Polymerase chain reaction (PCR) The sequence of the two iNOS-specific primers (MWG-Biotech, München, Germany) was 5′-TCA TTG TAC TCT GAG GGC TGA CAC A-3′ (sense) and 5′-GCC TTC AAC ACC AAG GTT GTC TGC A-3′ (antisense), and the predominant cDNA amplification product was predicted to be 900 bp in length. The sequence of the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)-specific primers (MWG-Biotech) was 5′-TAT GAC AAC TCC CTC AAG AT-3′ (sense) and 5′-AGA TCC ACA ACG GAT ACA TT-3′ (antisense), and the predominant cDNA amplification product was predicted to be 320 bp in length. RT–PCR of GAPDH served as a positive control. The PCR was carried out with 6 μl of the RT reaction mixture, 5 μl 10 × reaction buffer (Pharmacia), 1 μl deoxynucleotide mixture, 20 pmol each of the four primers and 0.8 units Taq DNA polymerase (Pharmacia) in a total volume of 50 μl. The samples were overlayed with mineral oil (60 μl) and placed in a Strategene RoboCycler 40 (Stratagene, La Jolla, CA, U.S.A.) which was programmed as follows: (1 ×) incubation for 5 min at 95°C (denaturation), 1 min at 52°C (annealing) and 90 s at 72°C (extension); (25 ×) incubation for 1 min at 95°C, 1 min at 52°C and 90 s at 72°C; (1 ×) incubation for 1 min at 95°C, 1 min at 52°C and 7 min at 72°C (final extension). The PCR products (20 μl) were size-fractionated by agarose (1.5%) gel electrophoresis, stained with ethidium bromide and visualized by use of an ultraviolet transilluminator (Bachofer, Reutlingen, Germany).

For Southern blot analysis, gels were denatured, neutralized, blotted onto Hybond-N nylon membranes (Amersham) and heated at 80°C for 2 h. Prehybridization (5 h at 42°C) and hybridization (overnight at 42°C) were performed in 50% formamide, 5 × SSC (0.75 M NaCl, 75 mM sodium citrate), 10 × Denhardt's solution (0.2% BSA, 0.2% Ficoll, 0.2% polyvinylpyrrolidone), 0.5% sodium dodecylsulphate and 250 μg ml^{-1} non-homologous salmon sperm DNA. The blots were hybridized with the purified RT–PCR amplification products of iNOS and GAPDH which had been labelled with ^{32}P by random priming (Feinberg & Vogelstein, 1983) followed by autoradiography. The intensity of the iNOS- and GAPDH-specific bands was quantified by densitometry (Pharmacia densitometer equipped with a Kappa CCD video camera and the ImageMaster software).

Electrophoretic mobility shift analysis (EMSA)

Aliquots of nuclear protein (3 μg) were incubated with the IRF-1(5′-GAA AAT GAA ATT-3′; Santa Cruz Biotechnology, Heidelberg, Germany)- or NF-κB(5′-AGT TGA GGG GAC TTT CCC AGG C-3′; Promega from Serva, Heidelberg, Germany)-specific double-stranded oligonucleotides which had been labelled with ^{32}P by random priming (Feinberg & Vogelstein, 1983). Binding experiments were performed with 1–4 μl nuclear extract, 3 μl 5 × binding buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 25% glycerol (w/v), 5 mM EDTA), 1 μg poly dI-dC (Pharmacia) as non-specific competitor DNA, and the oligonucleotide (5000 c.p.m.) in a total volume of 15 μl for 30 min at ambient temperature. Non-denaturing polyacrylamide gel (4%) electrophoresis was performed with 1 × TBE buffer, pH 8.0 followed by autoradiography. To monitor the specificity of the binding reaction, the assay was performed in parallel with the same samples in the presence of a 100–1000 fold excess of the non-labelled oligonucleotide. Densitometry was used to quantify the intensity of the labelled DNA-protein complexes. In some experiments determining the effects of the antioxidants on the activation of NF-κB, a supershift analysis was performed by preincubation of nuclear extracts from LPS plus IFNγ stimulated RAW 264.7 cells with 1 μg of a specific polyclonal anti-p65 (Rel A) antibody (Santa Cruz Biotechnology) for 12 h at 4°C.

Western blot analysis

Immunoblot analysis of iNOS protein was performed essentially as described previously (Hecker *et al.*, 1994). Protein extracts (10,000 g supernatant) were separated by electrophoresis (10–50 μg protein per lane) on 8% polyacrylamide gels in the presence of sodium dodecylsulphate and then transferred onto nitrocellulose membranes. The loading and transfer of equal amounts of protein in each lane was verified by staining of the protein bands with Ponceau S (0.2% in 3% trichloroacetic acid, v/v). After extensive washing with distilled water to remove the protein stain, the immobilized iNOS protein was visualized by subsequent incubation with a polyclonal anti-iNOS antibody (kindly provided by Dr M. Marletta, University of Michigan, Ann Arbor, Mich., U.S.A.) and a secondary polyclonal peroxidase-conjugated anti-rabbit antibody (Amersham), followed by staining with the enhanced chemiluminescence (ECL) technique developed by Amersham. The antibody was specific for iNOS and did not cross-react with the constitutive NOS isoforms from rat brain or porcine aortic endothelial cells. The intensity of the iNOS-specific bands was quantified by densitometry.

Determination of superoxide anion (O_2^-) production

RAW 264 macrophages (2.5×10^6 cells per ml) were transferred to a plastic cuvette and incubated in 0.9 ml of HEPES (10 mM)-buffered salt solution (1.8 mM $CaCl_2$, 2.7 mM KCl, 0.5 mM $MgCl_2$, 137 mM NaCl, 0.36 mM NaH_2PO_4, 5 mM D-glucose), pH 7.4 at 37°C in a luminometer (Lumac Biocounter M2010 from Abimed, Langenfeld, Germany). Lucigenin dissolved in HEPES-buffered salt solution (0.1 ml corresponding to a final concentration of 0.25 mM) was added and the incubation continued for 2 min. Thereafter O_2^- production was stimulated by the addition of phorbol 12,13-myristate acetate (PMA, 1 μM) and monitored by lucigenin-enhanced chemiluminescence for 10 min. The assay was calibrated by monitoring the chemiluminescence signal of known amounts of O_2^- generated by xanthine oxidase (0.3–10 mu) and xanthine (50 μM) which had been previously determined spectrophotometrically by monitoring the reduction of ferricytochrome c (Ohara *et al.*, 1993). The chemiluminescence assay was specific for O_2^-; no light emission was recorded in the presence of authentic NO or H_2O_2.

Materials

Lipopolysaccharide (from *Escherichia coli*, 0111:B4) N-acetyl 5-hydroxytryptamine (N-acetylserotonin, NAS), 5,7-dihydroxyflavone (chrysin) and 3,4-dichloroisocoumarin (DCI) were obtained from Sigma (Deisenhofen, Germany), mouse interferon-γ (IFNγ) from Boehringer Mannheim (Germany) and the isotopes from Amersham (Braunschweig, Germany).

Statistical analysis

Unless indicated otherwise, all data in the figures and text are expressed as means ± s.e.mean of n observations. Statistical evaluation, unless indicated otherwise, was performed by one-way analysis of variance followed by a Bonferroni t test for multiple comparisons with a P-value <0.05 considered statistically significant.

Results

O_2^- formation in PMA-stimulated cells

In the presence of the three antioxidants, the PMA-induced oxidative burst, i.e. the generation of O_2^- by the macrophages was either strongly reduced (NAS) or even abolished (chrysin, DCI; Figure 1). Chrysin appeared to be the most potent antioxidant, completely inhibiting the PMA-induced O_2^- production at concentrations ≤ 1 μM (not shown). This effect of the antioxidants did not seem to involve an inhibition of protein kinase C (PKC) activation by the phorbol ester, since chrysin, DCI or NAS did not significantly affect the activity of a semi-purified PKC preparation from rat brain in concentrations up to 100 μM (not shown). Moreover, chrysin (50 μM) and NAS (1 mM) also strongly reduced the generation of O_2^- by xanthine oxidase (20 mu) in the presence of 0.5 mM xanthine (58% and 90% inhibition respectively), presumably due to scavenging of O_2^-, while DCI had no such effect. No acute increase in O_2^- formation was detected in RAW 264.7 cells exposed to LPS (140 ng ml^{-1}) plus IFNγ (5 u ml^{-1}) for up to 30 min (not shown).

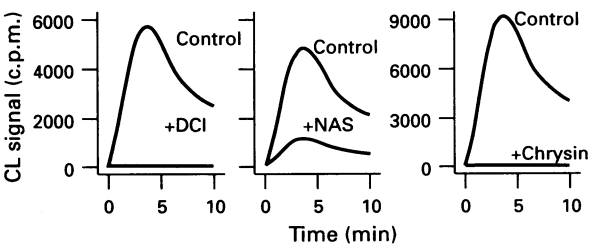

Figure 1 Effects of chrysin (50 μM), 3,4-dichloroisocoumarin (DCI, 50 μM) and N-acetylserotonin (NAS, 1 mM) on the time-dependent generation of O_2^- in RAW 264.7 cells stimulated with PMA (1 μM), as detected by lucigenin-enhanced chemiluminescence (CL). The figure shows typical original traces of at least three further experiments performed in the same manner. The CL signal corresponds to the following amounts of O_2^- produced over 10 min: Control, 24.1 nmol, +DCI, 0.1 nmol; Control 20.5 nmol, +NAS, 4.9 nmol; Control, 39.0 nmol, +Chrysin, 0.1 nmol.

iNOS expression and activity

Exposure of the macrophages to LPS plus IFNγ led to a time-dependent increase in the concentration of nitrite in the conditioned medium which reached a maximum at 24 h (not shown). After 6 h a prominent increase in nitrite release was detected in cells treated with LPS plus IFNγ, while LPS or IFNγ alone had only a minimal effect (Figure 2).

Co-incubation of LPS plus IFNγ stimulated cells with chrysin (50 μM), DCI (50 μM) or NAS (1 mM) resulted in a marked reduction in nitrite concentration in the medium (Figure 3). This effect was concentration-dependent, as determined with DCI (15 μM, IC_{50}) and NAS (0.26 mM, IC_{50}), and correlated with the parallel reduction in iNOS protein abundance in these cells (Figure 4a and b). According to RT–PCR analysis, chrysin (50 μM), DCI (50 μM) and NAS (1 mM) also markedly attenuated the increase in iNOS-specific mRNA in cells stimulated with LPS plus IFNγ (Figure 5). Similar results were also obtained by Northern blot analysis with an iNOS-specific cDNA probe, kindly provided by Dr C Nathan (Cornell University, New York, N.Y., U.S.A.; not shown).

Neither DCI (up to 100 μM) nor NAS (up to 5 mM) significantly affected iNOS activity in the cytosolic fraction of RAW 264.7 cells exposed to LPS plus IFNγ for 24 h, as determined by the conversion of [^{3}H]-arginine to [^{3}H]-citrulline (not shown).

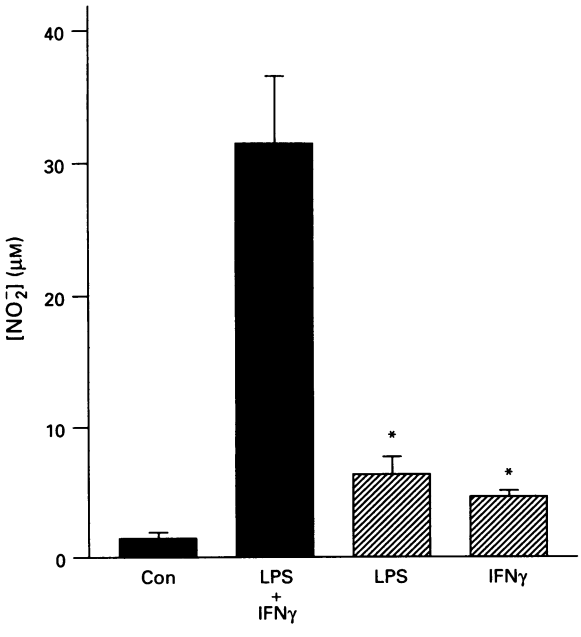

Figure 2 Synergistic effect of LPS (140 ng ml^{-1}) and IFNγ (5 u ml^{-1}) on nitrite (NO_2^-) formation by RAW 264.7 cells over a period of 6 h, as judged by the increase in the concentration of nitrite in the medium ($n=3$, $^*P<0.05$ vs. LPS + IFNγ). Con, unstimulated control cells.

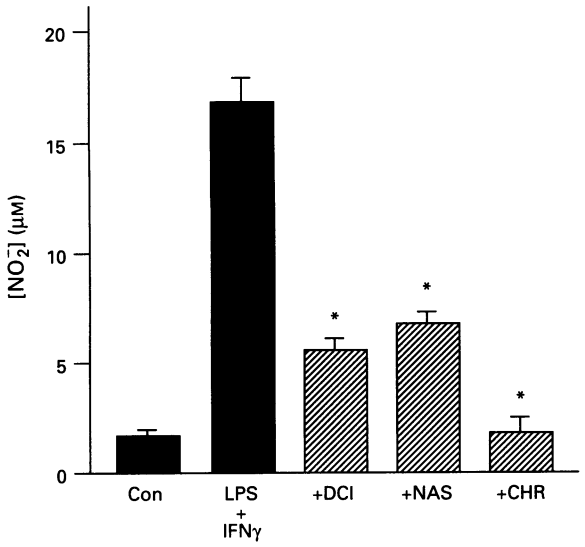

Figure 3 Effects of chrysin (CHR, 50 μM), DCI (50 μM) and NAS (1 mM) on nitrite (NO_2^-) formation by RAW 264.7 cells exposed to LPS plus IFNγ for 6 h ($n=16$; $^*P<0.05$ vs. LPS + IFNγ). Con, unstimulated control cells.

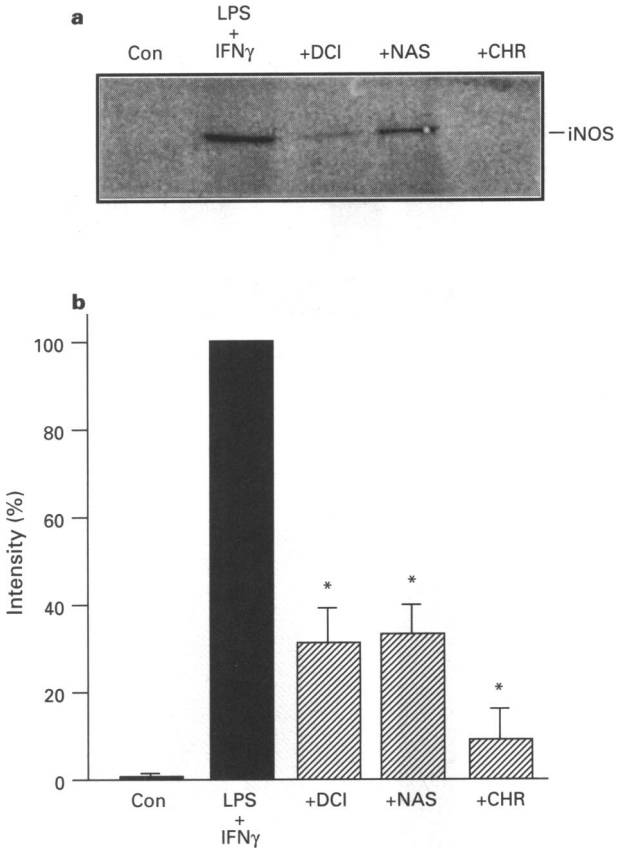

Figure 4 Western blot analysis of the effects of chrysin (CHR, 50 μM), DCI (50 μM) and NAS (1 mM) on the LPS plus IFNγ stimulated increase in iNOS protein abundance. (a) Typical Western blot. (b) Statistical summary of five separate experiments. Changes in iNOS protein abundance according to densitometric analysis are expressed as percentage of the level of the stimulated control cells ($^*P<0.05$ vs. LPS + IFNγ by one sample two-sided t test).

Activation of NF-κB

Two faint NF-κB-specific DNA-protein complexes were identified in nuclear extracts of unstimulated macrophages, the intensity of which was markedly increased in extracts from cells exposed to LPS plus IFNγ (Figure 6a and b). Treatment of these extracts with a specific anti-p65 antibody resulted in a supershift of the slower migrating DNA-protein complex, suggesting that this complex comprises the p65/p50 heterodimer of NF-κB (not shown). Consequently, the faster migrating DNA-protein complex is likely to represent the p50/p50 homodimer which has been previously detected in nuclear extracts of LPS-stimulated RAW 264.7 cells (Xie *et al.*, 1994; Goldring *et al.*, 1995).

Neither the p65-containing DNA-protein complex nor the p50/p50-like complex were detectable in nuclear extracts from LPS plus IFNγ stimulated cells which had been treated with DCI (50 μM), suggesting that the coumarin derivative abolished both the basal and stimulated binding of NF-κB to the oligonucleotide (Figure 6a and b). NAS (1 mM) had no appreciable efect (Figure 6a and b), while in some experiments (Figure 6a) the intensity of the two NF-κB-specific DNA-protein complexes was increased rather than decreased in nuclear extracts of RAW 264.7 cells exposed to LPS plus IFNγ in the presence of chrysin (50 μM).

Activation of IRF-1

Although several non-specific DNA-protein complexes were present in nuclear extracts of unstimulated RAW 264.7 cells (see also Martin *et al.*, 1994), no IRF-1-specific complex was detectable. However, when the cells were exposed to LPS plus IFNγ for 2 h, a prominent IRF-1-specific DNA-protein complex was revealed (Figure 7a). The intensity of this IRF-1-specific complex was markedly reduced when the cells were stimulated in the presence of DCI (50 μM), NAS (1 mM) or chrysin (50 μM) (Figure 7a and b).

Discussion

This study demonstrates that three equally effective, but structurally dissimilar antioxidants inhibit iNOS gene expression in RAW 264.7 cells at the transcriptional level either by preventing the activation of NF-κB and/or that of IRF-1.

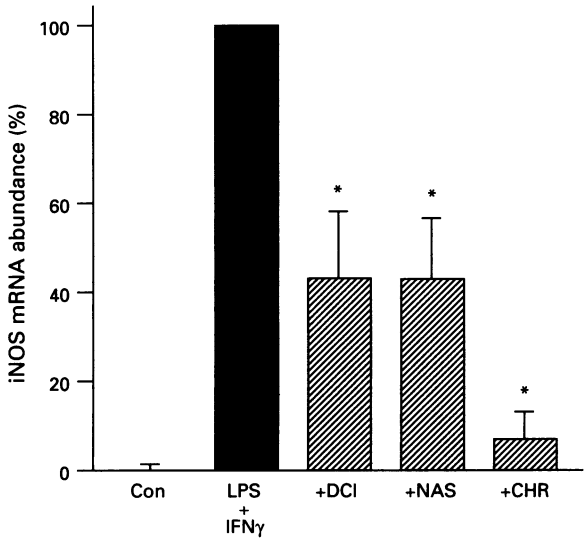

Figure 5 RT–PCR analysis of the effects of chrysin (CHR, 50 μM), DCI (50 μM) and NAS (1 mM) on the increase in iNOS mRNA abundance in RAW 264.7 cells stimulated with LPS plus IFNγ for 6 h. Changes in iNOS mRNA abundance according to densitometric analysis of the Southern blots were normalized on the basis of the mRNA abundance of GAPDH and are expressed as percentage of the level of the stimulated control cells ($n=6$, $^*P<0.05$ vs. LPS + IFNγ). Con, unstimulated control cells.

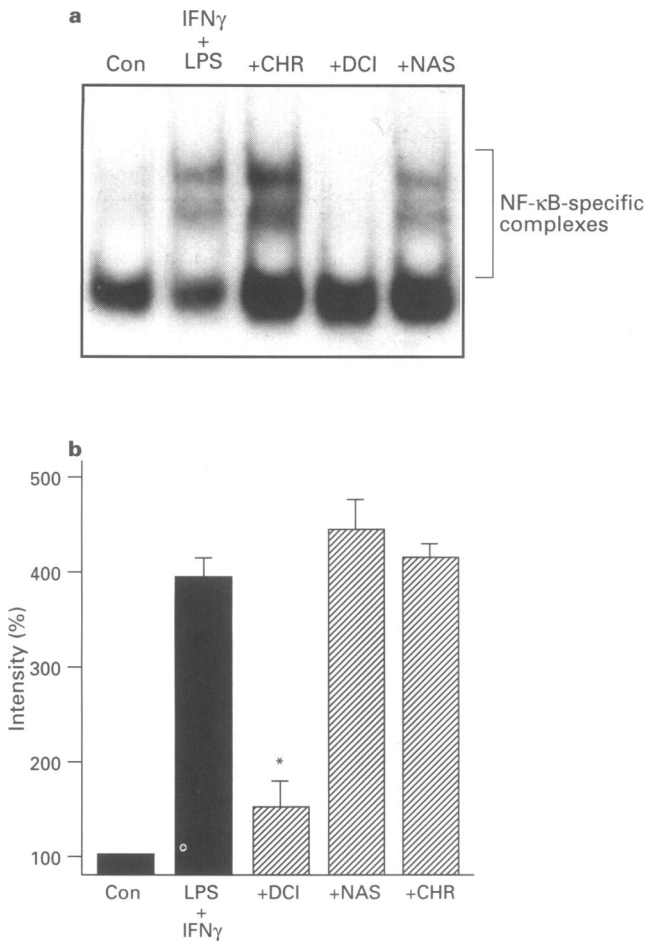

Figure 6 Effects of chrysin (CHR, 50 μM), DCI (50 μM) and NAS (1 mM) on the NF-κB-specific DNA-protein complex formation in nuclear extracts of RAW 264.7 cells stimulated with LPS plus IFNγ for 30 min. (a) Typical EMSA. (b) Statistical summary of five separate experiments following densitometric quantification of the two NF-κB-specific bands (*$P<0.05$ vs. LPS + IFNγ). Con, unstimulated control cells.

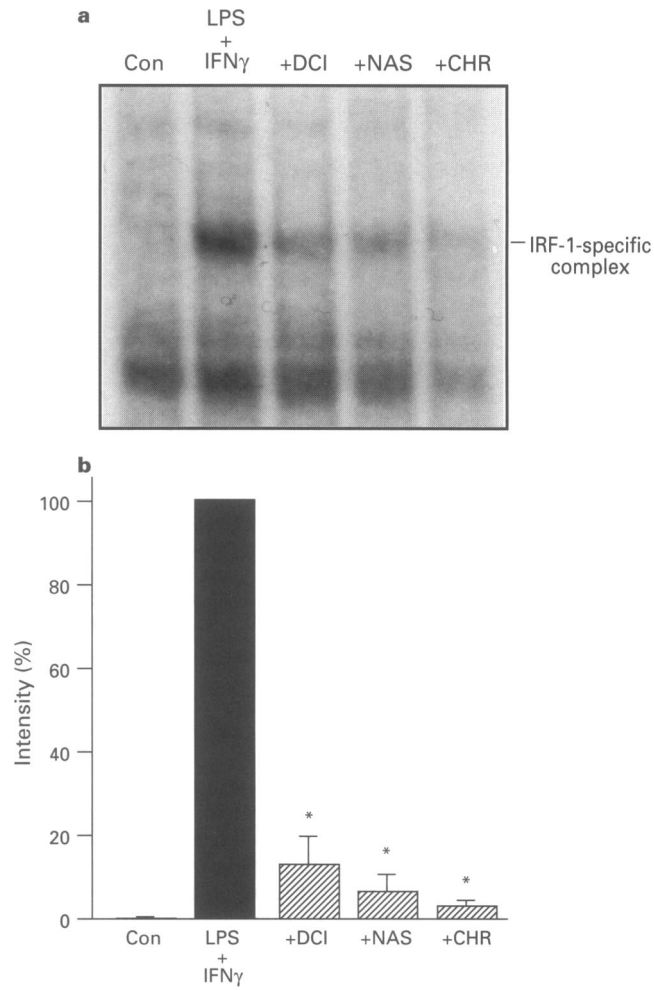

Figure 7 Effects of chrysin (CHR, 50 μM), DCI (50 μM) and NAS (1 mM) on the IRF-1-specific DNA-protein complex formation in nuclear extracts of RAW 264.7 cells stimulated with LPS plus IFNγ for 2 h. (a) Typical EMSA. (b) Statistical summary of three separate experiments. Changes in IRF-1-specific DNA-protein complex abundance according to densitometric analysis are expressed as percentage of the level of the stimulated control cells (*$P<0.05$ vs. LPS + IFNγ by one sample two-sided t test). Con, unstimulated control cells.

Antioxidants such as the coumarin derivative cloricromene (Zingarelli *et al.*, 1993), curcumin (Brouet & Oshima, 1995) or flavones (Król *et al.*, 1995) have recently been reported to inhibit iNOS expression in LPS-stimulated macrophages, but their mechanism of action remained obscure. A likely target for these antioxidants seems to be the signal transduction cascade leading to the activation of NF-κB, which in many cell types, including LPS-stimulated RAW 264.7 cells, is regarded as a crucial step in the initiation of iNOS gene expression (Mülsch *et al.*, 1993; Sherman *et al.*, 1993; Xie *et al.*, 1994; Eberhardt *et al.*, 1994; Adcock *et al.*, 1994; Schini-Kerth *et al.*, 1995). Indeed, NF-κB activation by many if not all stimuli has been proposed to be mediated by an increased formation of reactive oxygen intermediates (Grimm & Baeuerle, 1993). This hypothesis is suppported by findings that (i) antioxidant candidates such as N-acetylcysteine, vitamin E derivatives, dithiocarbamates and other metal chelators inhibit the activation of NF-κB in various cells stimulated in a variety of ways and (ii) that hydrogen peroxide (Grimm & Baeuerle, 1993; Siebenlist *et al.*, 1995) and O_2^- (Adcock *et al.*, 1994) are capable of activating NF-κB in some cell lines.

In the present study, no increase in O_2^- formation was detected when RAW 264.7 cells were stimulated with LPS plus IFNγ, while PMA elicited a pronounced oxidative burst. The phorbol ester, on the other hand, failed to cause an activation of NF-κB or an increase in iNOS gene expression (M. Hecker *et al.*, unpublished observation). Chrysin, DCI and NAS, but not the well-known NF-κB inhibitor, pyrrolidine ditiocarbamate (M. Hecker *et al.*, unpublished observation), effectively suppressed the PMA-induced increase in O_2^- formation, and this effect could not be attributed to a blockade of PKC activity by the antioxidants.

In spite of these comparable antioxidative effects, only DCI inhibited the LPS-mediated activation of NF-κB, thus questioning the concept of a redox-sensitive activation of NF-κB. Indeed, unlike chrysin or NAS, which failed to exert a significant inhibitory effect on chymotrypsin or elastase activity at the concentrations employed in this study (M. Hecker *et al.*, unpublished observation), DCI is a potent serine protease inhibitor (Harper *et al.*, 1985) which has been shown to protect the inhibitory IκB-α subunit from proteolytic inactivation (Henkel *et al.*, 1993). This stabilization of IκB-α not only prevents the active NF-κB complex (p50/RelA heterodimer) from entering the nucleus, but may also lead to a removal of NF-κB from its binding sites at the iNOS gene (Siebenlist *et al.*, 1995). It is not clear, however, whether DCI stabilizes IκB-α by blocking its proteolysis or by preventing its phosphorylation which appears to be crucial for the targeting of IκB-α to the proteasome

(Finco *et al.*, 1994). Irrespective of the precise mechanism of action by which DCI prevents the activation of NF-κB in LPS-stimulated RAW 264.7 cells, this effect is unlikely to be related to its antioxidative properties.

The lack of effect of chrysin and NAS on the activation of NF-κB and the strong synergism between LPS and IFNγ in the induction of iNOS gene expression prompted us to search for a potential antioxidant target in IFNγ signalling. Moreover, NF-κB cannot be the sole determinant of gene expression, but must function in concert with one or several other transcriptions factors, some of which may also be regulated in activity (Siebenlist *et al.*, 1995 and references cited therein). One of these additional transcription factors is IRF-1 which has been shown to be responsible for the synergistic effect of IFNγ (Lorsbach *et al.*, 1993) on iNOS gene expression stimulated by LPS in mouse macrophages and RAW 264.7 cells (Kamijo *et al.*, 1994; Martin *et al.*, 1994).

Treatment of the RAW 264.7 cells with LPS plus IFNγ resulted in a marked activation of IRF-1 that was strongly inhibited in the presence of the three antioxidants. Although the precise mechanism of action underlying this effect of the antioxidants remains to be determined, this finding points to a redox-sensitive step in the activation of IRF-1 which unlike NF-κB is not constitutively present in the cytosol in an inactive form, but synthesized *de novo* following exposure to IFNγ (Harada *et al.*, 1990). Considering the strong synergism between LPS and IFNγ under our experimental conditions, it is conceivable that blocking the activation of either NF-κB or IRF-1 or both will have the same impact on iNOS gene expression, hence explaining the similar inhibitory effect of the three antioxidants.

Since iNOS gene expression in human cells and tissues usually requires the combination of several cytokines (Morris & Billiar, 1994), antioxidants such as chrysin and NAS which do not interfere with the activation of NF-κB may be of greater therapeutic value than, for example, DCI, for selectively inhibiting the enhanced expression of this enzyme in acute and chronic inflammation. Moreover, the insight into the precise mechanism of action of these compounds may also help to design strategies for selectively interfering with the expression of pro-atherosclerotic chemokines and adhesion molecules in endothelial cells, the induction of which is also controlled by NK-κB in concert with other transcription factors such as ATF-2 and IRF-2 (Collins *et al.*, 1995).

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