Peroxide-mediated chromatin remodelling of a nuclear factor κ B site in the mouse inducible nitric oxide synthase promoter

Junping WEI, Hongtao GUO, Chengjiang GAO and Paul C. KUO¹

Department of Surgery, Duke University Medical Center, Durham, NC 27710, U.S.A.

Hepatocyte expression of iNOS (inducible nitric oxide synthase) and synthesis of nitric oxide convey protective antioxidant functions in models of sepsis, shock and reperfusion. However, the underlying redox-sensitive mechanisms that regulate hepatocyte expression of iNOS and its antioxidant functions are largely unknown. Activity of the transcription factor NF- κ B (nuclear factor κ B) is known to be redox-modulated. In this regard, the mouse hepatocyte iNOS promoter has NF- κ B-binding sites at nt – 1044 to – 1034 and at nt – 114 to – 104, which are considered to be critical for iNOS expression in response to pro-inflammatory cytokine stimulation. The relative contribution of these two NF- κ B-binding sites in the mouse iNOS promoter to hepatocyte iNOS promoter activity in the context of oxidative stress has not been characterized previously. In addition, although the *cis*- and *trans*-

regulatory factors controlling mouse hepatocyte iNOS expression have been well-characterized, the local changes in chromatin structure that accompany activation of iNOS gene transcription have not been considered. In the present study, we demonstrate that (1) in the absence of exogenous oxidative stress, the NF- κ B site at nt – 114 is inactive and (2) peroxide-mediated oxidative stress induces hyperacetylation and enhanced accessibility of the restriction enzyme to this NF- κ B region. Our results suggest that chromatin structural changes activate this NF- κ B site and increase interleukin-1 β -stimulated iNOS expression in the presence of oxidative stress.

Key words: chromatin, iNOS (inducible nitric oxide synthase), NF- κ B (nuclear factor κ B), nitric oxide, peroxide.

INTRODUCTION

States of shock or sepsis commonly initiate a complex cellular cascade of interlocking redox modulatory systems, which detoxify electrophiles and regulate key cellular functions such as nucleotide synthesis, gene transcription and translation, post-translational protein modification, enzyme activation, cell-cycle regulation and signal transduction [1–6]. In this regard, hepatocyte expression of iNOS (inducible nitric oxide synthase) and synthesis of nitric oxide (NO) convey protective antioxidant functions in models of sepsis, shock and reperfusion injury [7,8]. This effect is independent of both the oxidant species and the specific pro-inflammatory cytokine that characterizes these pathophysiological states. However, the underlying redox-sensitive mechanisms that regulate hepatocyte expression of iNOS and its antioxidant functions are largely unknown.

Activity of the transcription factor NF- κ B (nuclear factor κ B) is known to be redox-modulated [9–11]. In this regard, the mouse hepatocyte iNOS promoter has NF- κ B-binding sites at nt – 1044 to – 1034 and nt – 114 to – 104, which are considered to be critical for iNOS expression in response to pro-inflammatory cytokine stimulation. The relative contribution of these NF- κ B-binding sites to hepatocyte iNOS promoter activity in the context of oxidative stress has not been characterized previously. In addition, although the *cis*- and *trans*-regulatory factors controlling mouse hepatocyte iNOS expression have been well-characterized, the local changes in chromatin structure that accompany activation of iNOS gene transcription have not been considered. In the present study, we demonstrate that (1) in the absence of exogenous oxidative stress, the NF- κ B site at nt – 114 is inactive and (2)

peroxide-mediated oxidative stress induces hyperacetylation and chromatin structural changes, which activate this NF- κ B site and increase IL-1 β (interleukin-1 β)-stimulated iNOS expression.

MATERIALS AND METHODS

Materials

The mouse iNOS promoter (1745 bp; GenBank® accession no. L09126) was a gift from Professor Carl Nathan (Cornell University, Ithaca, NY, U.S.A.). The I κ B- α dominant-negative (DN) vector system was purchased from BD Biosciences (San Jose, CA, U.S.A.).

Cell culture

CCL 9.1 mouse hepatocytes and ANA-1 macrophages were maintained in Dulbecco's modified Eagle's medium with 10 % (v/v) heat-inactivated foetal calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin.

Induction of NO synthesis

IL-1 β (1000 units/ml) was used in the absence of foetal calf serum to induce NO synthesis. In selected instances, H₂O₂ (100 μ M) or the competitive substrate inhibitor of iNOS, L-NMMA (*N*^G-monomethyl-L-arginine; 100 μ M), was added. After incubation for 6 h at 37 °C in 5 % CO₂, the supernatants and cells were harvested for assays.

Abbreviations used: CAT, chloramphenicol acetyltransferase; ChIP, chromatin immunoprecipitation; CREB, cAMP-response-element-binding protein; CBP, CREB-binding protein; DN, dominant negative; DTT, dithiothreitol; HAT, histone acetyltransferase; HDAC, histone deacetylase; iNOS, inducible nitric oxide synthase; IL-1 β , interleukin-1 β ; L-NMMA, *N*^G-monomethyl-L-arginine; MNase, micrococcal nuclease; NF- κ B, nuclear factor κ B; NP40, Nonidet P40; RE, restriction enzyme; TNF- α , tumour necrosis factor- α ; TSA, trichostatin A.

¹ To whom correspondence should be addressed (e-mail kuo00004@mc.duke.edu).

RNA preparation and Northern-blot analysis

Total RNA was isolated using TRIzol® reagent (Gibco BRL, Rockville, MD, U.S.A.). The RNA samples (10 µg/lane) were fractionated by electrophoresis on a 1% agarose formaldehyde gel and transferred on to a Hybond-C nylon membrane (Amersham Biosciences, Piscataway, NJ, U.S.A.). A ³²P-dATP-labelled probe was constructed based on the mouse iNOS cDNA sequence (GenBank® accession no. XM147149). Hybridization was performed at 42 °C for 18 h in ULTRAhyb hybridization buffer (Ambion, Austin, TX, U.S.A.). After hybridization, filters were washed twice and subjected to autoradiography. cDNA probes were prepared by random primer labelling, followed by purification using a Sephadex G-50 mini-column (BioMax, Odenton, MD, U.S.A.).

Transient-transfection analysis

Hepatocytes were transfected using the DEAE-dextran technique [12]. After the cells were washed twice with media, 10 µg of plasmid DNA containing the mouse iNOS promoter construct was added per 10⁷ cells to 1 ml of media without serum and containing DEAE-dextran (250 µg/ml) and 50 mM Tris (pH 7.4) and prewarmed to 37 °C. The suspension was incubated at 37 °C for 45–60 min, followed by a 1 min shock with 10% (v/v) DMSO at room temperature (24 °C). The cells were washed, distributed to 100 mm plates, each with approx. 5 × 10⁶ cells in 10 ml of complete medium, and incubated at 37 °C in 5% CO₂. After at least 24 h, the medium was changed, and IL-1β (1000 units/ml), H₂O₂ or IL-1β + H₂O₂ was added. In selected instances, catalase (100 µM haem) was used to inactivate H₂O₂. After approx. 6 h, the cells were washed with ice-cold PBS, resuspended in 0.25 mM Tris (pH 7.8) and subjected to three cycles of freezing and thawing. Lysates were centrifuged (11700 g for 10 min at 4 °C); the supernatant was heated at 65 °C for 10 min to inactivate CAT (chloramphenicol acetyltransferase) inhibitors and then centrifuged as above. The supernatant was assayed for CAT activity using a CAT ELISA technique (Boehringer Mannheim, Indianapolis, IN, U.S.A.). Transfection efficiency was normalized by co-transfection of a β-galactosidase reporter gene with a constitutively active early SV40 promoter. All values are expressed in terms of pg of CAT/ml.

Plasmid constructs

PCR-based site-directed mutagenesis was performed on the two NF-κB sites (NF-κB-2 at nt -114 and NF-κB-1 at nt -1044) in the context of the full-length iNOS promoter-reporter plasmid to generate a series of mutant iNOS promoter plasmids (see Figure 1). The resulting mutants were DNA-sequenced to confirm the extent of mutagenesis. The IκB-α DN expression vector system, containing a functionally silent mutant IκB-α and a constitutively active IκB-α expression vector, was purchased from BD Biosciences.

Preparation of nuclear protein fractions

Briefly, IL-1β-stimulated cells were washed and scraped into PBS and centrifuged at 4500 rev./min (rotor diameter, 16.5 cm) for 5 min in a microfuge. The pelleted cells were suspended in buffer A [10 mM Tris, pH 7.5/1.5 mM MgCl₂/10 mM KCl/0.5% NP40 (Nonidet P40)] at approx. 10 times the packed cell volume and lysed by gentle pipetting. Nuclei were recovered by microcentrifugation at 7000 rev./min (rotor diameter, 16.5 cm) for 5 min. The

Figure 1 Schematic representation of mutations of NF-κB-1 and -2 sites in the mouse iNOS promoter

Boldface and upper-case letters represent the nucleotides of interest. Wt, wild-type; M, mutated.

supernatant was collected and stored at -80 °C and represents the cytoplasmic and membrane protein fractions. Nuclear proteins were extracted at 4 °C by gentle resuspension of the nuclei (at approx. two times the packed nuclear volume) of a buffer containing 20 mM Tris (pH 7.5), 10% (v/v) glycerol, 1.5 mM MgCl₂, 420 mM NaCl and 0.2 mM EDTA, followed by 30 min of platform rotation. The nuclear protein suspension was cleared by microcentrifugation at 13000 rev./min (rotor diameter, 16.5 cm) for 15 min. The supernatants were collected and frozen at -80 °C or directly used in gel-shift assays. All buffers contained the following additions: 1–2 µg/ml each of aprotinin, chymostatin, leupeptin, pepstatin, 0.2 mM PMSF, 0.5 mM DTT (dithiothreitol) and 0.1 mM sodium vanadate. All steps were performed on ice or at 4 °C. Protein concentrations were measured by the Bio-Rad protein assay, using BSA as a standard.

Electrophoretic mobility-shift assays

DNA probes were prepared by end-labelling with [γ -³²P]dATP (DuPont/NEN, Boston, MA, U.S.A.) and T₄ polynucleotide kinase (Boehringer Mannheim) and purified in TEN (10 mM Tris/HCl, pH 8.0/10 mM EDTA/150 mM NaCl solution) by using G-50 resin columns (Whatman, Clifton, NJ, U.S.A.). Typically, 5 µl (10–20 µg) of nuclear proteins was incubated with approx. 100000 c.p.m. of ³²P-labelled oligonucleotides (approx. 0.5 ng) for 2 h at room temperature. The nuclear proteins and various oligonucleotide probes were incubated in a buffer containing 10 mM Tris (pH 7.5), 10% glycerol and 0.2% NP40. Additionally, 2–4 µg of poly(dI/dC) (Boehringer Mannheim) was included as a non-specific competitor DNA. Protein–DNA complexes were resolved on 4% non-denaturing polyacrylamide gels in 0.4 × TBE (Tris/borate/EDTA) running buffer (450 mM Tris/borate/1 µM EDTA, pH 8.0). After electrophoresis, gels were dried and subjected to autoradiography. Antibody supershift experiments included the addition of 2 µl of various antibodies, all of which were purchased from Santa Cruz Biotechnology. Purified recombinant p50 and p65 NF-κB proteins were purchased from Promega (Madison, WI, U.S.A.).

MNase (micrococcal nuclease) analysis

Cell nuclei were resuspended in MNase digestion buffer (10 mM Tris, pH 7.4/15 mM NaCl/60 mM KCl/0.15 mM spermine/0.5 mM spermidine) containing CaCl_2 (1 mM). MNase (stock solution, 25 units/ μl) was added, and the reaction mixture was incubated at room temperature. The reaction was terminated by the addition of 80 μl of MNase digestion buffer and 20 μl of stop buffer (100 mM EDTA and 10 mM EGTA). For restriction enzyme (RE) digestion, cell nuclei were washed with an RE buffer (10 mM Tris, pH 7.4/50 mM NaCl/10 mM MgCl_2 /0.2 mM EDTA/0.2 mM EGTA/1 mM 2-mercaptoethanol/0.15 mM spermine/0.5 mM spermidine). Nuclei were resuspended in the specific buffer for RE digestion (50 μl). Digestion reactions were initiated with 2 μl of RE and the reaction mixture was incubated at 37 °C for 10 min. After digestion with nucleases, the total volume of the reaction mixture was brought to 200 μl by adding double-distilled water. Then, the reaction was stopped and genomic DNA was isolated. For Southern-blot analysis, the purified genomic DNA (15–25 μg) was incubated overnight at 37 °C with an excess of RE. The DNA was then cleaned by phenol/chloroform extraction and analysed on a 1% agarose gel, followed by transfer and hybridization with a ^{32}P -dCTP-radio-labelled probe.

RNA preparation and Northern-blot analysis

Total RNA was isolated from the cells using TRIzol® reagent (Gibco BRL). The RNA samples (10 μg /lane) were fractionated by electrophoresis on a 1% agarose formaldehyde gel and transferred on to a Hybond-C nylon membrane. A ^{32}P -dATP-labelled 600 bp probe was constructed based on the mouse iNOS promoter sequence. Hybridization was performed at 42 °C for 18 h in ULTRAhyb hybridization buffer (Ambion, Austin, TX, U.S.A.). After hybridization, filters were washed twice and subjected to autoradiography.

RE accessibility assay

Cells were washed with ice-cold PBS and resuspended in 1.5 vol. of lysis buffer (10 mM Tris/HCl/10 mM NaCl/3 mM MgCl_2 /0.2% NP40/10 mM DTT, pH 7.5) and incubated for 10 min at 4 °C. Nuclei were collected by centrifugation at 600 g for 2 min, resuspended in wash buffer (10 mM Tris/HCl/10 mM NaCl/3 mM MgCl_2 /10 mM DTT, pH 7.5), and collected again by centrifugation. Nuclei were resuspended in $1 \times$ New England Biolabs RE buffer 2 at 2×10^7 nuclei/0.2 ml. After adding RE, aliquots were incubated for 45 min at 37 °C. Reactions were terminated by adding 10 mM Tris/HCl, 25 mM EDTA and 1% SDS (pH 7.5). Proteinase K (0.4 mg/ml) was added, and samples were incubated overnight at 37 °C. DNA was purified by multiple phenol/chloroform extractions and one extraction with chloroform, followed by ethanol precipitation. Equal amounts of DNA (15 μg) were digested with secondary REs. Samples were resolved on a 1.1% agarose gel, and Southern-blot analysis was performed.

ChIP (chromatin immunoprecipitation) assay

Chromatin from hepatocytes was fixed and immunoprecipitated using the ChIP assay kit (Upstate Biotechnology, Charlottesville, VA, U.S.A.) according to the manufacturer's instructions. The purified chromatin was immunoprecipitated using anti-p65 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-(acetyl

histone H3) (Upstate Biotechnology) or rabbit non-immune serum. The input fraction corresponded to 0.1 and 0.05% of the chromatin solution before immunoprecipitation. After DNA purification, the presence of the selected DNA sequence was assessed by PCR. The PCR programme was 94 °C for 4 min, followed by 94 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s for a total of 28 cycles and then 72 °C for 7 min. PCR products were resolved on 10% (w/v) acrylamide gels. The average size of the sonicated DNA fragments subjected to immunoprecipitation was 500 bp as determined by ethidium bromide gel electrophoresis. ChIP assays addressing NF- κ B-1 (nt – 1044) utilized the PCR primers TGTACCTTAGACAAGGCAAAACA and TGAGTTCTAGGACAACTAGGGCT, resulting in a 260 bp fragment, whereas NF- κ B-2 (nt – 114) utilized AAAGTGCAAATGAGAGAACAGACAG and TGTTTTGCATAAAGTCACTGTTCC, resulting in a 312 bp fragment.

DNase hypersensitivity assay

Cells were trypsinized and pooled in separate centrifuge tubes and then washed with 30 ml of medium in the presence of serum. The cells were pelleted at 1500 rev./min for 5 min at 4 °C and then gently resuspended in 10 ml of ice-cold solution A (150 mM sucrose/80 mM KCl/35 mM Hepes, pH 7.4/5 mM K_2HPO_4 /5 mM MgCl_2 /0.5 mM CaCl_2). After re-centrifugation, the pellets were resuspended in 4.0 ml of ice-cold solution A. The cells were permeabilized at room temperature with 4.0 ml of 0.1% lysophosphatidylcholine (Calbiochem) in solution A, triturated, incubated on ice for approx. 2 min and diluted to 40 ml with ice-cold solution A. Permeabilized cells were centrifuged at 1800 rev./min (rotor diameter, 16.5 cm) for 5 min at 4 °C. The pellets were resuspended in 3 ml of ice-cold solution A and placed on ice before immediate DNase I digestion.

DNase I digestion and genomic DNA isolation

Duplicate sets of tubes containing increasing amounts (range 0–40 units/ml) of DNase I (Worthington Biochemical, Lakewood, NJ, U.S.A.) were prepared on ice. A permeabilized cell suspension (300 μl) from control or treated samples was added, gently mixed and incubated for 4 min in a 37 °C water bath. The cells were lysed by the addition of 300 μl of freshly prepared DNA lysis buffer (4% SDS/0.2 M EDTA/800 μg /ml proteinase K). DNase I-digested genomic DNA was purified by sequential organic extraction, followed by RNase digestion and ethanol precipitation. Genomic DNA was resuspended to a final concentration of approx. 1 $\mu\text{g}/\mu\text{l}$ in $1 \times$ Tris/EDTA. To ensure that DNase I digestion had occurred, all samples were size-fractionated and stained with ethidium bromide. Loss of high-molecular-mass DNA and the appearance of a continuous gradient of smaller-molecular-mass DNA were evidence of DNase I cleavage. DNase I-cleaved DNA (15 μg) from control and cytokine-induced cells were digested with an appropriate RE and size-fractionated on either 0.8% high gelling temperature agarose (FMC, Rockland, ME, U.S.A.) or 2.5% NuSieve 3:1 gels (FMC) overnight at 40 V. The gels were washed, and DNA was electrotransferred on to Hybond N+ nylon membranes (Amersham Biosciences). Hybridization and washing were performed as described previously, followed by Southern-blot analysis. The HS site analysis was reproduced in three independent experiments.

Statistical analysis

Results are expressed as the means $\pm$ S.E.M. for 3 or 4 experiments. Analysis was performed using the Student's *t* test; *P* < 0.05 was considered significant.

Figure 2 Transient-transfection assays of wild-type (Wt) and mutated (M) mouse iNOS promoter-reporter constructs in mouse hepatocytes

Hepatocytes were transfected using the DEAE-dextran technique. IL-1 β (1000 units/ml), H₂O₂ (100 μ M) or IL-1 β + H₂O₂ was added. In selected instances, catalase (100 μ M haem) was used to inactivate H₂O₂. The supernatant was assayed for CAT activity using a CAT ELISA technique (Boehringer Mannheim, Indianapolis, IN, U.S.A.). Transfection efficiency was normalized by co-transfection of a β -galactosidase reporter gene with a constitutively active early SV40 promoter. All values are expressed as pg of CAT/ml. Results are expressed as the means $\pm$ S.E.M. for 3 or 4 experiments. Analysis was performed using the Student's *t* test; *P* < 0.05 was considered significant. (A) *P < 0.01: Wt, IL-1 β versus control or H₂O₂; Wt1 + M2, IL-1 β versus control or H₂O₂. **P < 0.01: Wt, IL-1 β + H₂O₂ versus control, IL-1 β , H₂O₂ or IL-1 β + H₂O₂ + catalase. #*P* < 0.01: Wt, IL-1 β + H₂O₂ + catalase versus control or H₂O₂; Wt1 + M2, IL-1 β + H₂O₂ + catalase versus control or H₂O₂. (B) *P < 0.01: Wt, IL-1 β

RESULTS

Differential contribution of NF- κ B sites to redox-induced iNOS promoter activity

We have demonstrated previously that oxidative stress in the form of peroxide or superoxide significantly enhances IL-1 β -dependent hepatocyte iNOS promoter activation and gene transcription [13–15]. DNA binding and transactivation activities of NF- κ B are essential components of this observation. To determine the relative roles of NF- κ B-1 (nt – 1044) and NF- κ B-2 (nt – 114) binding sites in redox-dependent hepatocyte iNOS expression, transient-transfection analysis was performed in cells using the wild-type and mutated variants (Figure 1) of the mouse iNOS promoter. Normalized CAT activity was determined in control cells or IL-1 β -, H₂O₂- or IL-1 β + H₂O₂-treated cells (Figure 2). Mutations in NF- κ B-1 (M1 + Wt2, where M stands for mutated and Wt for wild-type), NF- κ B-2 (Wt1 + M2) and both positions (M1 + M2) were initially examined (Figure 2A). As shown previously, treatment with IL-1 β increased CAT activity by 10-fold in the wild-type iNOS promoter; treatment with IL-1 β + H₂O₂ resulted in an additional 3-fold increase in CAT activity over that caused by IL-1 β stimulation, which was ablated by the addition of catalase. Mutation of the NF- κ B-2 site in the Wt1 + M2 construct produced a similar increase in activity with IL-1 β treatment, but no additional activation was found in the IL-1 β + H₂O₂ cells. Mutation of the NF- κ B-1 site in the M1 + M2 or M1 + M2 constructs eliminated all responses to either IL-1 β or IL-1 β + H₂O₂ stimulation.

Additional transient-transfection studies were then performed using constructs in which the entire 11 nt sequence of NF- κ B-1 was substituted in the NF- κ B-2 position, Wt1 + M1, M1 + Wt1, Wt1 + M1 and M1 + M1 (Figure 2B). In these experiments, the Wt1 + Wt1 construct was associated with significantly enhanced activity in the presence of IL-1 β , approx. 3-fold higher than that noted with the wild-type iNOS promoter. In addition, there was no augmentation in the activity of the Wt1 + Wt1 construct in the presence of IL-1 β + H₂O₂. The further addition of catalase did not alter Wt1 + Wt1 CAT activity. In the Wt1 + M1 construct, IL-1 β -mediated activity was not significantly different from that of the wild-type construct. No additional change was seen in the presence of IL-1 β + H₂O₂ or IL-1 β + H₂O₂ + catalase treatment. Finally, no activity was present under any condition with the M1 + Wt1 construct. Further transient-transfection assays were performed using constructs in which the entire 11 nt sequence of NF- κ B-2 was substituted in the NF- κ B-1 position, Wt2 + M2, M2 + Wt2, Wt2 + M2 and M2 + M2. In these studies, no CAT activity was found in any of the constructs under any of the treatment conditions (results not shown). These results suggest that NF- κ B-1 is required for iNOS promoter augmentation by IL-1 β and NF- κ B-2 is required for redox-induced augmentation of iNOS promoter activity in the presence of both IL-1 β and H₂O₂. In addition, NF- κ B-2 cannot function in the presence of a mutated NF- κ B-1 site.

Nuclear protein-binding characteristics of NF- κ B-1 and -2 DNA sequences

To determine the *in vivo* nuclear protein-binding characteristics of NF- κ B-1 and -2 sites, ChIP assays were performed using a

versus control or H₂O₂; Wt1 + M1, IL-1 β versus control or H₂O₂; Wt1 + M1, IL-1 β versus control or H₂O₂. #*P* < 0.01: Wt, IL-1 β + H₂O₂ versus control, IL-1 β , H₂O₂ or IL-1 β + H₂O₂ + catalase; Wt1 + Wt1, IL-1 β + H₂O₂ versus control or H₂O₂; Wt1 + M1, IL-1 β + H₂O₂ versus control or H₂O₂. **P < 0.01: Wt, IL-1 β + H₂O₂ + catalase versus control or H₂O₂; Wt1 + Wt1, IL-1 β + H₂O₂ + catalase versus control or H₂O₂; Wt1 + M1, IL-1 β + H₂O₂ + catalase versus control or H₂O₂.

Figure 3 ChIP assays

Chromatin from hepatocytes was fixed and immunoprecipitated using the ChIP assay kit (Upstate Biotechnology) according to the manufacturer's instructions. The purified chromatin was immunoprecipitated using 10 μ g of anti-p65 (Santa Cruz Biotechnology), anti-(acetyl histone H3) or 5 μ l of rabbit non-immune serum. The input fraction corresponded to 0.1% of the chromatin solution before immunoprecipitation. ChIP assays addressing NF- κ B-1 utilized the PCR primers TGTACCTTAGACAAGGCAAAACA and TGAGTTCTAGGACA-ACTAGGGCT, whereas NF- κ B-2 utilized AACTGCAAATGAGAGAACAGACAG and ATGCAT-TATTACGTCACTCTGTGG. The average size of the sonicated DNA fragments subjected to immunoprecipitation was 500 bp as determined by ethidium bromide gel electrophoresis. Blots are representative of three experiments. NS, non-specific. ChIP assays with (a) anti-p65 antibody and (b) anti-(acetyl histone H3) antibody.

p65 antibody (Figure 3a). No p65 binding was found in control or H₂O₂-treated cells. In the presence of IL-1 β , we noted binding to the NF- κ B-1 segment only. In the presence of IL-1 β + H₂O₂, p65 protein binding was present at both NF- κ B-1 and -2 sites. In contrast with the gel-shift results, these ChIP assays indicate that NF- κ B binds *in vivo* to the NF- κ B-1 site on IL-1 β stimulation. After the addition of oxidative stress, NF- κ B binds *in vivo* to both NF- κ B-1 and -2. These results suggest that NF- κ B-2 binding occurs only in the setting of both IL-1 β and peroxide stimulation. The ChIP assays for NF- κ B-2 binding were then repeated using anti-(acetyl histone H3) antibody to immunoprecipitate the DNA after shearing (Figure 3b). The DNA was then amplified using promoters specific for the NF- κ B-2 region. Again, no signal was noted in the presence of either IL-1 β or H₂O₂ alone; however, in the presence of IL-1 β + H₂O₂, acetylation was observed, suggesting that histone acetylation in the vicinity of NF- κ B-2 occurs on IL-1 β + H₂O₂ stimulation.

Identification of DNase-hypersensitive sites relative to NF- κ B-1 and -2 sequences

To determine whether nucleosome repression may cause steric interference at the NF- κ B-2 site in the absence of oxidative stress, DNase I hypersensitivity experiments were performed on permeabilized cells (Figure 4a). Digestion of genomic DNA with *Bgl*II resulted in a 4266 bp segment containing the iNOS promoter having the two NF- κ B sites and spanning the region from nt - 3927 to nt + 339; NF- κ B-1 resides at nt - 1044 and NF- κ B-2 at nt - 114. A probe P1, spanning nt + 105 to + 309, was designed to complement DNA at the 3'-edge of the *Bgl*II fragment. After stimulation with IL-1 β , two DNA fragments, 4.3 and 1.4 kb, were found, suggesting the presence of one DNase-hypersensitive site corresponding to the region of NF- κ B-1 (Figure 4b). In the presence of IL-1 β + H₂O₂, a different pattern emerged, with a 4.4 kb full-length fragment, a transitory 1.4 kb fragment and a smaller 500 bp segment, suggesting that the NF- κ B-2 region became DNase-sensitive in this setting (Figure 4c). A second probe P2 (nt - 894 to - 479) was then selected to complement a segment of genomic DNA between NF- κ B-1 and -2. Similar to that noted with probe P1 after IL-1 β stimulation, a hypersensitive site corresponding to the region of NF- κ B-1 was detected by Southern-blot experiments with two DNA fragments, 4.4 and 1.4 kb, after stimulation with IL-1 β (results not shown). In contrast, after IL-1 β + H₂O₂ stimulation, a different pattern emerged, with only the full-length 4.4 kb fragment being attenuated with increasing concentrations of DNase (Figure 4d). No other bands were found, suggesting that the segment of DNA complementary to P2 was digested by DNase in the presence of IL-1 β + H₂O₂. These results suggest that the NF- κ B-1 region is DNase-sensitive in the presence of IL-1 β , whereas both NF- κ B-1 and -2 regions exhibit sensitivity on IL-1 β + H₂O₂ stimulation.

RE accessibility and MNase analysis of the iNOS promoter region

DNase I hypersensitivity is not precise, and it is difficult to determine the exact region in which DNase I digestion occurs. Therefore we measured RE accessibility to map more precisely the chromosomal site. First, to demonstrate that the iNOS promoter region is assembled into nucleosomes, the isolated nuclei from unstimulated control cells were digested with MNase. MNase digests genomic DNA at the internucleosomal region, causing a DNA ladder to be revealed on Southern-blot analysis when using probe P1, as described above. After digestion with MNase (10 units for 5 min), a DNA ladder appeared, whereas MNase digested naked DNA much more efficiently and the DNA ladder was not observed (Figure 5a). These results suggest that the iNOS promoter region is incorporated into nucleosomes.

Then, RE accessibility was utilized to map more precisely the chromosomal region surrounding NF- κ B-2. Cell nuclei were treated with a limiting concentration of the REs *Hinf*I and *Pst*I (Figure 5b). These two REs cut in the vicinity of NF- κ B-2 (nt - 114); *Hinf*I cuts at nt - 100 and *Pst*I cuts at nt - 61. Genomic DNA was digested to completion *in vitro* with a different RE *Bgl*II that recognizes sites on either side of the *Hinf*I and *Pst*I restriction sites. To normalize the reaction, the results are also presented as the ratio of nuclear cleavage to *in vitro* cleavage. An increase in the ratio after cell activation would suggest that a nucleosome becomes more accessible to RE cleavage and thus remodelled. In comparison with IL-1 β -treated cells, IL-1 β + H₂O₂ activation leads to a strong and reproducible increase in nuclear cleavage by both *Hinf*I and *Pst*I (Figure 5c). In comparison with unstimulated controls and IL-1 β -treated cells, nuclear cleavage at the *Hinf*I and *Pst*I sites increased by more than 13- and 6-fold respectively in the presence of IL-1 β + H₂O₂. In cells

Figure 4 DNase hypersensitivity assay

Stimulation of mouse hepatocytes with IL-1 β + H₂O₂ results in the appearance of a DNase-hypersensitive band in the NF- κ B-2 region of the iNOS promoter. Nuclei from cells were digested with the designated concentration of DNase I for 4 min. Southern-blot analysis was conducted using the probes P1 (nt + 105 to + 309) and P2 (nt - 894 to - 479) derived from the designated region of the mouse iNOS promoter. Results are representative of three independent experiments. (a) Schematic representation *Bgl*II fragment of iNOS promoter with the location of promoters P1 and P2. (b) DNase hypersensitivity blot of IL-1 β -stimulated hepatocytes using the probe P1. (c) DNase hypersensitivity blot of IL-1 β + H₂O₂-stimulated hepatocytes using the probe P1. (d) DNase hypersensitivity blot of IL-1 β + H₂O₂-stimulated hepatocytes using the probe P2.

treated with either IL-1 β + H₂O₂ and catalase or with H₂O₂ alone, the degree of nuclear cleavage by *Hinf*I and *Pst*I reverts to levels not different from that of IL-1 β -stimulated cells or unstimulated control cells. Peroxide-mediated oxidative stress significantly enhances RE access at NF- κ B-2 in the presence of IL-1 β stimulation. In addition, remodelling of the NF- κ B-2 region is independent of binding at the NF- κ B-1 region as RE access does not increase in the presence of IL-1 β alone. Northern-blot analysis confirmed that increased RE accessibility correlated with enhanced iNOS mRNA expression in the presence of both IL-1 β and oxidative stress (Figure 5d). Our results indicate that chromatin remodelling in this NF- κ B-2 region in the setting of IL-1 β + H₂O₂ treatment is associated with increased iNOS mRNA expression.

In the setting of IL-1 β stimulation, we have demonstrated previously [15] that mouse hepatocytes increase iNOS promoter activity and expression of iNOS mRNA and protein with the addition of peroxide-mediated oxidative stress, whereas ANA-1 cells do not. We then examined RE accessibility to chromatin in the region of NF- κ B-2 in the ANA-1 mouse macrophage cell line (Figure 6). In contrast with the findings in hepatocytes, the RE accessibility of chromatin in the vicinity of NF- κ B-2 from ANA-1 cells was not altered in the presence of IL-1 β + H₂O₂. This suggests that the absence of enhanced peroxide-mediated iNOS promoter activation in ANA-1 cells may be the result of the absence of a remodelling response. Conversely, these

results provide a functional correlation for chromatin remodelling in peroxide-mediated enhancement of hepatocyte iNOS mRNA expression.

RE accessibility and NF- κ B activity

We then sought to determine whether remodelling occurs in the NF- κ B-2 region independently of NF- κ B activation or NO synthesis. Cells were first transfected with a constitutively active I κ -B α expression vector to inhibit NF- κ B activation or a control I κ -B α DN mutant vector that is functionally silent. Subsequently, cells were stimulated with IL-1 β or IL-1 β + H₂O₂. In the presence of the control I κ -B α DN mutant vector, the RE accessibility assays performed indicate that the access of *Hinf*I and *Pst*I to the NF- κ B-2 region is significantly enhanced in IL-1 β + H₂O₂ in comparison with that of IL-1 β -stimulated cells (Figure 7). However, inhibition of NF- κ B activation with a constitutively active I κ -B α expression vector altered the extent of RE access to levels equivalent to that of unstimulated controls. However, inhibition of NF- κ B activation will also inhibit NO synthesis. To determine whether chromatin remodelling in the NF- κ B-2 region in the presence of IL-1 β + H₂O₂ is dependent on the presence of NO, cells were stimulated with IL-1 β or IL-1 β + H₂O₂ in the presence or absence of the competitive substrate inhibitor of iNOS, L-NMMA (100 μ M). RE accessibility assays were repeated. In this instance,

Figure 5 MNase and RE accessibility assays

(a) The NF- κ B-2 region of the iNOS promoter is incorporated into nucleosomes. Nuclei from cells treated with IL-1 β + H₂O₂ and naked genomic DNA were digested with 50 units of MNase for 10 min. Genomic DNA was isolated and separated on an agarose gel. Southern-blot analysis was conducted using the probe P1 (nt +105 to +309). A nucleosome array was observed, and the first three bands are indicated. The blot is representative of three experiments. (b) Schematic representation of cut sites of the REs (*HinfI*, *PstI* and *BglII*) utilized for RE accessibility assays. (c) RE accessibility increases at the NF- κ B-2 region of the iNOS promoter after IL-1 β + H₂O₂ stimulation. Nuclei were digested with specified REs (20 units) for 10 min at 37 °C. Genomic DNA was isolated and then completely digested with *BglII*. Southern-blot analysis was performed using the probe P1 (nt +105 to +309). The upper band represents the fragment derived from *in vitro* digestion with *BglII*, whereas the lower band represents the fragment derived from nuclear digestion with *HinfI* or *PstI*. Intensity of the band representing the nuclear-cleavage or *in vitro* cleavage product was determined by scanning. The ratio of nuclear cleavage to *in vitro* cleavage is presented. Results are representative of three independent experiments. (d) Northern-blot analysis demonstrates that enhanced RE access is associated with increased iNOS mRNA expression. Total RNA was isolated from cells using TRIzol® reagent (Gibco BRL). The RNA samples (10 μ g/lane) were fractionated by electrophoresis on a 1% agarose formaldehyde gel and transferred on to a Hybond-C nylon membrane. A ³²P-dATP-labelled 600 bp probe was constructed based on the mouse iNOS cDNA sequence (GenBank® accession no. XM147149). Results are representative of three independent experiments.

the enhanced RE access noted in the presence of IL-1 β + H₂O₂ was not altered by the inhibition of NO synthesis. These results indicate that chromatin remodelling in the NF- κ B-2 region in the presence of both IL-1 β and H₂O₂ is dependent on NF- κ B, but not NO.

DISCUSSION

In the present study, we examined the relative contribution of two NF- κ B-binding sites to redox-induced enhancement of IL-1 β -induced iNOS promoter activation in mouse hepatocytes. We have demonstrated previously that peroxide or superoxide will significantly increase IL-1 β -mediated iNOS promoter activity

[14,16]. The NF- κ B-1 site at nt -1044 was absolutely necessary for IL-1 β -induced iNOS promoter activation in the presence or absence of peroxide. Binding of NF- κ B (p50-p65) to the NF- κ B-2 site at nt -114 and augmentation of iNOS promoter activity occurred only in the presence of both IL-1 β and peroxide treatment. In this setting, there was both increased acetylation and RE accessibility in the region of the NF- κ B-2 site, suggesting alteration of local nucleosomal conformation. This observation depended on the presence of NF- κ B, but was NO-independent. Redox-dependent change in the local chromatin structure and its role in iNOS promoter activation have not been examined previously. Our results indicate that (1) in the absence of exogenous oxidative stress, the NF- κ B-2 site at nt -114 is inactive and

Figure 6 RE accessibility at NF- κ B-2 of iNOS not altered by IL-1 β + H₂O₂ stimulation

ANA-1 mouse macrophages were stimulated, as described above. Nuclei were digested with specified REs (20 units) for 10 min at 37 °C. Genomic DNA was isolated and then completely digested with *Bgl*II overnight. Southern-blot analysis was performed using the probe P1 (nt + 105 to + 309). The upper band represents the fragment derived from *in vitro* digestion with *Bgl*II, whereas the lower band represents the fragment derived from nuclear digestion with *Hinf*I or *Pst*I. The intensity of the band representing the nuclear-cleavage or *in vitro* cleavage product was determined by scanning. The ratio of nuclear cleavage to *in vitro* cleavage is presented. Results are representative of three independent experiments.

Figure 7 RE accessibility dependent on NF- κ B, but independent of NO, at NF- κ B-2 after IL-1 β + H₂O₂ stimulation

Mouse hepatocytes were stimulated, as described above. In selected instances, cells transfected with an expression vector for IkB- α , a control vector labelled IkB- α DN or co-incubated with the substrate inhibitor of iNOS, L-NMMA (100 μ M). Nuclei were digested with specified REs (20 units) for 10 min at 37 °C. Genomic DNA was isolated and then completely digested with *Bgl*II overnight. Southern-blot analysis was performed using the probe P1 (nt + 105 to + 309). The upper band represents the fragment derived from *in vitro* digestion with *Bgl*II, whereas the lower band represents the fragment derived from nuclear digestion with *Hinf*I or *Pst*I. The intensity of the band representing the nuclear-cleavage or *in vitro* cleavage product was determined by scanning. The ratio of nuclear cleavage to *in vitro* cleavage is presented. Results are representative of three independent experiments.

(2) peroxide-mediated oxidative stress induces hyperacetylation and chromatin structural changes, which activate this NF- κ B site and subsequently increase IL-1 β -stimulated iNOS expression.

Nucleosome remodelling and transcription factor access to the target promoter sites of genes is increasingly viewed as an important regulatory mechanism for the transcriptional activation of genes. The extent of histone acetylation is directly related to the levels of gene transcription [17,18]. The degree of histone acetylation is mediated by compounds with HAT (histone acetyltransferase) activity and a family of HDAC (histone deacetylase) enzymes that inhibit acetylation [17,18]. The central chromatin core is comprised of four core histone proteins (H2A, H2B, H3 and H4) and DNA is tightly coiled around it [17,18]. Acetylation of the histone core of the chromatin promotes unwinding of DNA, allowing the access of transcription factors and co-activators to target gene promoter sites, thus initiating or enhancing transcription [17,18]. In contrast, the deacetylation of the histone core enhances DNA winding, inhibits access of transcription factors to DNA targets and, as a result, down-regulates gene transcription [17,18]. In the presence of IL-1 β + H₂O₂, we found increased acetylation of the NF- κ B-2 promoter region in association with enhanced NF- κ B binding and iNOS promoter activity.

The role of chromatin remodelling and histone acetylation in iNOS promoter activation induced by oxidative stress and IL-1 β has not been examined previously. However, Gilmour et al. [19] have found that reactive oxygen species and TNF- α (tumour necrosis factor- α) increase the activation of activator protein 1 and NF- κ B, and regulate chromatin remodelling, leading to IL-8 expression in lung cells. Peroxide increased HAT activity and the level of acetyl histone H4 associated with the promoter region of IL-8. Histone acetylation regulates IL-8 and IL-6 gene expression, whereas IL-8 gene silencing is associated with increased HDAC activity [17,19–21]. The oxidant and TNF- α -stimulated expression of IL-6/IL-8 is regulated by NF- κ B-dependent HAT activity [19–22]. Ito et al. [23,24] have shown a role for histone acetylation and deacetylation in IL-1 β -induced TNF- α release in alveolar macrophages derived from cigarette smokers. These authors suggest that oxidants may play an important role in the modulation of HDAC and inflammatory cytokine gene transcription.

Co-regulatory (co-activator and co-repressor) proteins have been shown to be required for the regulation of gene expression by many transcription factors. These proteins are thought to function by facilitating or bridging the sequence-specific activators to the basal transcriptional machinery as well as by altering chromatin structure. Co-activator proteins include CBP [CREB (cAMP-response-element-binding protein)-binding protein] and its structural homologue p300, SRC1 (steroid receptor co-activator 1) and p/CAF (p300/CBP-associated factor) [25–28]. Consistent with their role in altering chromatin structure, many co-activator proteins contain HAT domains that are capable of acetylating lysine residues in the N-terminal tails of the core histones.

In our setting, both IL-1 β -induced activation of iNOS and the associated enhancement due to the concomitant presence of oxidative stress are NF- κ B-dependent [27]. Recruitment of co-activators such as CBP and p300 by NF- κ B is important in linking promoter activation to the transcriptional machinery [27]. In addition, the co-activator proteins associated with NF- κ B transcription factor complexes have been demonstrated to take part in chromatin remodelling. Recent work has shown that NF- κ B-dependent gene expression requires the function of transcriptional co-activator proteins. The CBP and p300 co-activators interact with the p65 subunit of NF- κ B to enhance its ability

to activate transcription [29–31]. Inducible phosphorylation of p65 by the protein kinase A catalytic subunit stimulates NF- κ B-dependent gene expression by enhancing the interaction of p65 with CBP [31,32]. Two other co-activators are also known to be involved in the regulation of the transactivation function of NF- κ B. The HAT function of the p/CAF co-activator was shown to be required for the activation of NF- κ B-dependent gene expression and the SRC-1 co-activator protein was shown to interact with the p50 subunit of NF- κ B to potentiate NF- κ B-mediated transactivation [33,34]. Thus interaction with transcriptional co-activators is important in mediating the transactivation potential of NF- κ B. Moreover, the differential association of NF- κ B with co-activator proteins and, possibly, with co-repressor proteins in unstimulated cells as well as in cells in which NF- κ B has been activated probably determines the level of activation or repression of NF- κ B-regulated genes. Certainly, in the setting of IL-1 β + H₂O₂ stimulation, our results demonstrate that enhanced acetylation of histone H3 at the NF- κ B-2 site is associated with enhanced RE access and augmentation of iNOS promoter activation. The specific co-activator that may be involved in our observations is unknown, but is the subject of ongoing investigation. Nevertheless, in the region of NF- κ B-2, it is possible that the nuclear milieu established by IL-1 β and oxidative stress enhances histone acetylation and remodelling through the actions of NF- κ B and an as yet unidentified co-activator with associated HAT activity or inhibition of a co-repressor with HDAC activity.

With respect to the potential role for HDAC, Ashburner et al. [21] demonstrated that trichostatin A (TSA) inhibition of HDAC activity increased the expression of an NF- κ B-dependent reporter gene and HDAC1 and HDAC2 regulate NF- κ B-dependent transcription; HDAC1 can interact directly with the p65 subunit of NF- κ B to exert its co-repressor function. In addition, Ashburner et al. [21] show that TSA increased basal and induced expression of the NF- κ B-dependent IL-8 gene, and this was associated with hyperacetylation of the IL-8 promoter due to the inhibition of HDAC activity. These authors suggest that NF- κ B transactivation function is regulated by interaction with HDAC co-repressor proteins to repress basal expression of NF- κ B-regulated genes. They go on to suggest that the specific activating signal for NF- κ B may determine the nature of the interaction of NF- κ B with co-activator proteins such as CBP and/or co-repressor proteins such as HDAC1 and HDAC2. A potent activation signal, such as oxidative stress, may result in a higher affinity of CBP for p65 and potentially weaker interactions with HDAC proteins, resulting in higher levels of expression. Conversely, weaker signalling with decreased phosphorylation of p65 would result in less binding of CBP (or other co-activators) to p65 and a greater amount of HDAC associated with p65, giving a lower level of expression. Alternatively, HDACs may also dissociate from p65, allowing more CBP (or other transcriptional co-activators) to bind. These authors did not address the associated issue of chromatin remodelling after the induced activation of the IL-8 gene.

Although histone acetylation and remodelling have not been addressed previously with iNOS and oxidative stress, Yu et al. [35] have examined the role of acetylation in cytokine-mediated iNOS promoter activation in mouse mesangial cells and RAW 264.7 macrophages. In contrast with our findings, they found that TSA inhibited promoter activation of both iNOS and an NF- κ B element promoter. NF- κ B-binding activity was unaltered. Furthermore, they found that HDAC2 co-immunoprecipitated with NF- κ B p65. Hyperacetylation diminished cytokine-mediated iNOS activation. Ashburner et al. [21] did not examine remodelling and RE access in their system. In a different cell type, our results support the more commonly held paradigm of histone-acetylation-mediated

enhancement of transcription. Nevertheless, the bifunctional role of acetylation and deacetylation has been described in many cell systems.

In summary, we examined the relative contribution of two NF- κ B-binding sites to redox-induced enhancement of IL-1 β -induced iNOS promoter activation in mouse hepatocytes. There was both increased acetylation and RE accessibility in the region of the NF- κ B-2 site, suggesting alteration of local nucleosomal conformation. A redox-dependent change in the local chromatin structure and its role in iNOS promoter activation have not been examined previously. Our results suggest the role of yet another level of regulation in iNOS gene transcription.

REFERENCES

- Powis, G., Breihl, M. and Oblong, J. (1995) Redox signalling and the control of cell growth and death. *Pharmacol. Ther.* **68**, 149–173
- Halliwell, B. (1995) Antioxidant characterization. *Biochem. Pharmacol.* **49**, 1341–1348
- Harris, E. D. (1992) Regulation of antioxidant enzymes. *FASEB J.* **6**, 2675–2683
- Schulze-Osthoff, K., Los, M. and Baeuerle, P. A. (1995) Redox signalling by transcription factors NF- κ B and AP-1 in lymphocytes. *Biochem. Pharmacol.* **50**, 735–741
- Sen, C. K. and Packer, L. (1996) Antioxidant and redox regulation of gene transcription. *FASEB J.* **10**, 709–720
- Jaiswal, A. K. (1994) Antioxidant response element. *Biochem. Pharmacol.* **48**, 439–444
- Mayer, B., Schrammel, A., Klatt, P., Koesling, D. and Schmidt, K. (1995) Peroxynitrite induced accumulation of cyclic GMP in endothelial cells and stimulation of purified soluble guanylyl cyclase. *J. Biol. Chem.* **270**, 17355–17360
- Sergent, O., Griffon, B., Morel, I., Chevanne, M., Dubos, M. P., Cillard, P. and Cillard, J. (1997) Effect of nitric oxide on iron-mediated oxidative stress in primary rat hepatocyte culture. *Hepatology* **25**, 122–127
- Baldwin, A. S. (1994) Control of gene expression by NF- κ B and I κ B. In *Transcription: Mechanisms and Regulation* (Conaway, R. C. and Conaway, J. W., eds.), pp. 443–547, Raven Press, New York
- Schreck, R., Rieber, P. and Baeuerle, P. A. (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF- κ B transcription factor and HIV-1. *EMBO J.* **9**, 2247–2259
- Baldwin, A. S. (1996) The NF- κ B and I κ B proteins: new discoveries and insights. *Annu. Rev. Immunol.* **14**, 649–683
- Meszaros, K. S., Aberle, S., White, M. and Parent, J. B. (1995) Immunoreactivity and bioactivity of lipopolysaccharide binding protein in normal and heat inactivated sera. *Infect. Immunol.* **63**, 363–365
- Kuo, P. C. and Abe, K. Y. (1995) Interleukin-1 induced production of nitric oxide inhibits benzenetriol mediated oxidative injury in rat hepatocytes. *Gastroenterology* **109**, 206–216
- Kuo, P. C., Abe, K. Y. and Schroeder, R. A. (1997) Oxidative stress increases hepatocyte iNOS gene transcription and promoter activity. *Biochem. Biophys. Res. Commun.* **234**, 289–292
- Guo, H., Cai, C. Q. and Kuo, P. C. (2002) Hepatocyte nuclear factor-4 α mediates redox sensitivity of inducible nitric oxide synthase gene transcription. *J. Biol. Chem.* **277**, 5054–5060
- Kuo, P. C., Abe, K. and Schroeder, R. A. (2000) Superoxide enhances interleukin 1 β -mediated transcription of the hepatocyte-inducible nitric oxide synthase gene. *Gastroenterology* **118**, 608–618
- Rahman, I. (2002) Oxidative stress, transcription factors and chromatin remodelling in lung inflammation. *Biochem. Pharmacol.* **64**, 935–942
- Struhl, K. (1998) Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev.* **12**, 599–606
- Gilmour, P. S., Rahman, I., Donaldson, K. and MacNee, W. (2003) Histone acetylation regulates epithelial IL-8 release mediated by oxidative stress from environmental particles. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **284**, L533–L540
- Vanden Berghe, W., De Bosscher, K., Boone, E., Plaisance, S. and Haegeman, G. (1999) The nuclear factor- κ B engages CBP/p300 and histone acetyltransferase activity for transcriptional activation of the interleukin-6 gene promoter. *J. Biol. Chem.* **274**, 32091–32098
- Ashburner, B. P., Westerheide, S. D. and Baldwin, Jr, A. S. (2001) The p65 (RelA) subunit of NF- κ B interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. *Mol. Cell. Biol.* **21**, 7065–7077

- 22 Rahman, I., Gilmour, P. S., Jimenez, L. A. and MacNee, W. (2002) Oxidative stress and TNF- α induce histone acetylation and NF- κ B/AP-1 activation in alveolar epithelial cells: potential mechanism in gene transcription in lung inflammation. *Mol. Cell. Biochem.* **234**, 239–248
- 23 Ito, K., Jazrawi, E., Cosio, B., Barnes, P. J. and Adcock, I. M. (2001) p65-activated histone acetyltransferase activity is repressed by glucocorticoids: mifepristone fails to recruit HDAC2 to the p65–HAT complex. *J. Biol. Chem.* **276**, 30208–30215
- 24 Ito, K., Lim, S., Caramori, G., Chung, K. F., Barnes, P. J. and Adcock, I. M. (2001) Cigarette smoking reduces histone deacetylase 2 expression, enhances cytokine expression, and inhibits glucocorticoid actions in alveolar macrophages. *FASEB J.* **15**, 1110–1112
- 25 Bannister, A. J. and Kouzarides, T. (1996) The CBP co-activator is a histone acetyltransferase. *Nature (London)* **384**, 641–643
- 26 Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H. and Nakatani, Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell (Cambridge, Mass.)* **87**, 953–959
- 27 Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J. et al. (1997) Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature (London)* **389**, 194–198
- 28 Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H. and Nakatani, Y. (1996) A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature (London)* **382**, 319–324
- 29 Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y. and Collins, T. (1997) CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2927–2932
- 30 Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, K., Beach, D. H. and Nabel, G. J. (1997) Regulation of NF- κ B by cyclin-dependent kinases associated with the p300 coactivator. *Science* **275**, 523–527
- 31 Zhong, H., Voll, R. E. and Ghosh, S. (1998) Phosphorylation of NF- κ B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol. Cell* **1**, 661–671
- 32 Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P. and Ghosh, S. (1997) The transcriptional activity of NF- κ B is regulated by the I κ B-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell (Cambridge, Mass.)* **89**, 413–424
- 33 Sheppard, K. A., Rose, D. W., Haque, Z. K., Kurokawa, R., McInerney, E., Westin, S., Thanos, D., Rosenfeld, M. G., Glass, C. K. and Collins, T. (1999) Transcriptional activation by NF- κ B requires multiple coactivators. *Mol. Cell. Biol.* **19**, 6367–6378
- 34 Lee, S. K., Kim, H. J., Na, S. Y., Kim, T. S., Choi, H. S., Im, S. Y. and Lee, J. W. (1998) Steroid receptor coactivator-1 coactivates activating protein-1-mediated transactivations through interaction with the c-Jun and c-Fos subunits. *J. Biol. Chem.* **273**, 16651–16654
- 35 Yu, Z., Zhang, W. and Kone, B. C. (2002) Histone deacetylases augment cytokine induction of the iNOS gene. *J. Am. Soc. Nephrol.* **13**, 2009–2017

Received 13 August 2003/22 October 2003; accepted 5 November 2003

Published as BJ Immediate Publication 5 November 2003, DOI 10.1042/BJ20031235