Enhanced DNA Binding and Activation of Transcription Factors NF-κB and AP-1 by Acetaldehyde in HEPG2 Cells*

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Juan Román, América Giménez, José María Lluís, Marta Gassó, Mireia Rubio, Joan Caballeria, Albert Parés, Joan Rodés, and José C. Fernández-Checa‡
From the Liver Unit, Institut Malalties Digestives y Instituto de Investigaciones Biomédicas August Pi Sunyer, Consejo Superior de Investigaciones Científicas, Barcelona 08036, Spain

Because transcription factors NF-κB and activator protein-1 (AP-1) are known to regulate gene expression, we have analyzed the role of acetaldehyde in the activation of NF-κB and AP-1 in HepG2 cells. Binding activity and transactivation of NF-κB and AP-1 were determined by gel retardation assays and transfection of a luciferase reporter construct controlled by NF-κB and AP-1 binding sites, respectively. Acetaldehyde enhanced the DNA binding of NF-κB and AP-1 by 1 and 4 h, respectively, increasing the NF-κB- and AP-1-dependent luciferase expression. Supershift assays revealed the presence of NF-κB heterodimers p65/p50 and p50/p52, whereas nuclear c-Jun levels correlated with the DNA binding of AP-1. The enhanced binding of NF-κB to DNA by acetaldehyde in intact cells was accompanied by the proteolytic degradation of IκB-α. However, the addition of acetaldehyde to cytosolic extracts from untreated Hep G2 cells did not affect the DNA binding of AP-1 but activated the NF-κB heterodimer p65/p50 in the absence of IκB-α degradation. Preincubation of HepG2 cells with protein kinase C inhibitors abolished the enhanced DNA binding of NF-κB and AP-1 caused by acetaldehyde. Hence, these findings uncover a previously unrecognized role for acetaldehyde in the activation of NF-κB and AP-1, which may be of relevance in the alcohol-induced liver disease.

The mechanisms underlying alcohol-induced liver disease (ALD) remains incompletely understood. Nevertheless, it is widely recognized that the oxidative metabolism of ethanol elicits a complex interplay of factors, including mitochondrial dysfunction, autoimmune-mediated cell injury, oxidative stress, and overproduction of inflammatory cytokines, which contribute to the development of the disease (1–4).

Acetaldehyde, a product of the oxidative metabolism of ethanol, is a very reactive intermediate that has been suggested to have a pathogenic role in ALD (1, 2). Indeed, there is a correlation between acetaldehyde generation within the liver and cell injury. The impairment of the low Km mitochondrial acetaldehyde dehydrogenase in perivenous hepatocytes indicates the existence of an acetaldehyde gradient along the liver acinus being greater in the perivenous zone of the liver, the area where most of the ethanol-induced liver injury is seen in both alcoholic patients and experimental animal models (2, 5–7). Furthermore, previous in vivo and in vitro studies have revealed the ability of acetaldehyde to form covalent adducts with various proteins that are thought to lead to altered liver function and structure (1, 2). In addition to these effects, it has been shown that acetaldehyde increases the transcription of collagen in several cell types (8–11) mediated by enhanced DNA binding of transcription factors NF-1 and SP-1 to specific sites located in the promoter of the α2(I) collagen gene (12, 13).

In eukaryotes, regulation of gene expression can be dramatically enhanced by the binding of sequence-specific DNA-binding proteins to promoter cis-acting elements. NF-κB and AP-1 are transcription factors whose role in gene regulation is widely recognized (14, 15). The DNA binding subunits of NF-κB belong to a multigene family that comprise several members including p50, p65 (Rel A), c-Rel, p-52, and RelB. These proteins share a domain required for DNA binding, forming homo- and heterodimers, IκB binding, and nuclear localization (15–17). On the other hand, AP-1 is a transcriptional activator composed of members of the Jun and Fos families. These proteins associate to form homo- and heterodimer complexes through a leucine zipper domain that bind to a common site (18–21). The final step in the gene regulation of both transcription factors is the translocation of the active complex to the nuclei followed by the binding to specific DNA sequences. However, unlike the AP-1 complex, NF-κB activation combines several mechanisms used by other transcription factors such as phosphorylation at specific residues of the inhibitory subunit IκB followed by its degradation and translocation of the active NF-κB into the nuclei. Both transcription factors become activated by a wide variety of stimuli, including growth factors, cytokines, UV irradiation, and oxidative stress (18, 21, 22). NF-κB and AP-1 control the inducible expression of a variety of genes that are involved in immune response as well as inflammatory and cellular defense mechanisms, including cytokines, cytokine receptors, cell adhesion molecules, and hematopoietic growth factors (15, 20–22).

Previous studies in experimental animal models of ALD have shown that overexpression of pro-inflammatory cytokines, e.g. tumor necrosis factor-α, is a critical event in the progression of ALD as it has been shown that neutralization of tumor necrosis factor-α attenuates hepatic necrosis and inflammation caused by chronic ethanol feeding (23–26). On the other hand, as transcription factors NF-κB and AP-1 are recognized to up-

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† To whom correspondence should be addressed: Liver Unit Hospital Clinic i Provincial, Villarroel 170, Barcelona 08036, Spain. Tel.: 34-3-2275709; Fax: 34-3-4515272; E-mail: checa@medicina.ub.es.
‡ The abbreviations used are: ALD, alcoholic liver disease; NF-κB, transcription factor κB; AP-1, activator protein-1; PBS, phosphate-buffered saline; H7, 1-(5-isquinolinesulfonyl)-2-methyl-piperazine; PMSF, phenylmethylsulfonyl fluoride; PKC, protein kinase C; TLCK, 1-chloro-3-tosylamido-7-amino-l-2-heptanone; IL, interleukin; EMSA, electrophoretic mobility shift assay.

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regulate the expression of pro-inflammatory cytokines, hence, it can be inferred that both NF-κB and AP-1 would be of relevance in the development of ALD. However, because the role of acetaldehyde on NF-κB and AP-1 activation has not been definitively established, the purpose of the present study was to investigate the DNA binding and transactivation of NF-κB and AP-1 and the mechanisms involved in HepG2 cells cultured in the presence of acetaldehyde.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Incubations**—The human hepatoblastoma cell line, HepG2, was obtained from the European Collection of Animal Cell Cultures (Salisbury, Wilts, UK) and grown at 37°C in 5% CO2 in Dulbecco’s modified Eagle’s medium containing high glucose levels. Culture medium was supplemented with 10% heat-inactivated fetal bovine serum, 2 mm l-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). A 174 mm stock solution of acetaldehyde was prepared daily in PBS. The cell cultures were then incubated with acetaldehyde at a wide range of concentration (50–200 μM) in serum-free medium in 25-cm² flasks for up to 24 h, and the flasks were kept tightly closed with parafilm to prevent evaporation of acetaldehyde. In some cases, cells were preincubated with protein kinase C inhibitors, H7 or calphostin C, and the effect of acetaldehyde on activation of AP-1 and NF-κB was examined as described below.

**Nuclear Extract Preparation**—Nuclear extracts of HepG2 cells (3 × 10⁶) were prepared by lysing cells with Nonidet P-40 followed by differential centrifugation as described before (27, 28). Cells were washed twice with ice cold PBS and collected with a rubber policeman after 1, 4, and 24 h of incubation with acetaldehyde. Cells were resuspended in buffer 10 mM Hepes, pH 7.8, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml TLCK and kept on ice for 15 min. Then, cells were lysed with Nonidet P-40 (10%), and the nuclear pellet was recovered after centrifugation at 13,000 g at 4°C for 30 s. The nuclear pellet was resuspended in ice-cold buffer containing 20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml TLCK and stored at −80°C.

**Electrophoretic Mobility Shift Assays**—Activation of transcription factors NF-κB and AP-1 was examined using consensus oligonucleotides of NF-κB (5'-AGTTGAGGGGACCTTCCCCAGG-3') and AP-1 (5'-GCGTGTTGATGACCCGGAA-3'), respectively. Probes were labeled by T4 polynucleotide kinase as described (27, 28). Binding reactions included 10 μg of nuclear extracts or cytosolic fractions in incubation buffer (10 mM Tris-HCl, pH 7.5, 40 mM NaCl, 1 mM EDTA and 4% glycerol), 1 μM of poly(dI-dC), and labeled oligonucleotide (30,000 cpm). The mixture was electrophoresed, and the gel was dried and autoradiographed at −70°C overnight.

Nuclear extracts were determined by competition experiments using a molar excess of unlabeled NF-κB or AP-1 to the nuclear extracts before the addition of the labeled probes. In some cases, supershift assays were done using human antibodies for p65, p50, p52, c-Rel, and RelB. Antibodies were used kindly provided by Dr. Nancy Rice (National Institutes of Health, Bethesda) (29). Furthermore, nuclear extracts were incubated with antibody anti-c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA) to immunoprecipitate the AP-1 complex containing c-Jun before the addition of labeled AP-1 oligonucleotide.

**Transfection of HepG2 Cells with NF-κB and AP-1 Luciferase Constructs**—To measure the transactivating activity of NF-κB and AP-1, HepG2 cells were transfected with a luciferase reporter construct controlled by NF-κB and AP-1 binding sites using LipofectAMINE® reagent (Life Technologies, Inc.). In each experiment, six 35-mm wells containing 6 × 10⁵ cells were transfected with 1.5 μg of a DNA construct composed of a luciferase reporter gene under the control of four tandem κB (pNF-κB-Luc) or AP-1 (pAP-1-Luc) binding sites upstream of the herpes simplex virus thymidine kinase (CLONTECH) and 1.5 μg of the pSV-β-galactosidase vector (Promega) as control to monitor transfection efficiency. Alternatively, the 5′-flanking region of the IL-8 gene spanning from base pairs −133 to −44, encompassing the AP-1, NF-κB, and κB binding sites, was subcloned into the luciferase expression vector as described (30). DNA constructs were diluted in OPTIMEM (Life Technologies, Inc.), and the cells were incubated for 5 h at 37°C. 24 h after transfection, cells were incubated with acetaldehyde, okadaic acid, or phorbol 12-myristate 13-acetate for 2–4 h. Following incubation cells were harvested in 350 μl of 1X reporter lysis buffer (Promega), and supernatants (20 μl) were used to determine luciferase activity using an EG&G/Berthold Lumat LB 9507 lumimeter.

**Determination of Nuclear c-Jun Levels**—To determine the presence of c-Jun in nuclei, 15 μg of nuclear proteins obtained as described were run on 12% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) at a dilution of 1:1000 in T-PBS for 1 h at room temperature and incubated with the antibody anti-c-Jun at a dilution of 1:1000 in T-PBS for 1 h at room temperature. Membranes were washed four times with T-PBS and incubated with a secondary antibody (donkey anti-rabbit) for 1 h at room temperature. The blots were stained with enhanced chemiluminescence ECL kit (Amersham Pharmacia Biotech), in which membranes were exposed to Hyperfilms ECL.

**Cell-free Activation of NF-κB and AP-1**—To determine the effect of acetaldehyde on NF-κB and AP-1 DNA binding in a cell-free system, a cytosolic fraction was prepared from untreated HepG2 cells. Cells (5 × 10⁶/ml) were resuspended in an hypotonic buffer (10 mM Tris, pH 7.4, containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml TLCK) followed by 10 up and down passes in a glass Teflon homogenizer. Homogenization was verified by examination on a hemocytometer with a phase contrast microscope to confirm the absence of intact cells. The resulting homogenate was then centrifuged at 100,000 × g for 1 h at 4°C. The resulting cytosolic extracts were then incubated with acetaldehyde, formamide, or deoxycholate for 30 min, and then activation of NF-κB and AP-1 was analyzed by EMSA as described above.

**Statistical Analyses**—Statistical analyses of mean values for multiple comparisons were made by one-way analysis of variance (ANOVA) followed by Fisher’s test.

**RESULTS AND DISCUSSION**

**Acetaldehyde Enhances DNA Binding of NF-κB and AP-1**—Transfection factors NF-κB and AP-1 are known to regulate expression of pro-inflammatory cytokines through the binding of these proteins to specific regulatory sites located in the promoter/enhancer of the target gene (14–16, 31). However, because the role of acetaldehyde in the activation of these transcription factors has not been fully characterized, we examined the effect of acetaldehyde on activation of NF-κB and AP-1 in HepG2 cells. Nuclear extracts from HepG2 cells were isolated at various times after acetaldehyde addition, and NF-κB and AP-1 activation were determined as binding of members of these transcription factors to consensus κB and AP-1 oligonucleotides. Acetaldehyde activated NF-κB as demonstrated by the retarded complexes detected by EMSA using labeled κB oligonucleotide, an effect that was significant for complexes I and II (Fig. 1). Similarly, an enhanced DNA binding of AP-1 in nuclear extracts prepared from HepG2 incubated in the presence of acetaldehyde was detected (Fig. 2). However, the enhanced DNA binding of NF-κB and AP-1 caused by acetaldehyde occurred with distinct kinetics, being detected within 1 and 4 h, respectively, of acetaldehyde treatment of HepG2 cells (Figs. 1 and 2). To determine the specificity of the enhanced DNA binding for either transcription factor induced by acetaldehyde, an experiment corresponding to an unlabeled oligonucleotide was used (Figs. 1 and 2). The retarded complexes of NF-κB and AP-1 were displaced by unlabeled κB and AP-1 oligonucleotides, respectively. A similar enhanced DNA binding of NF-κB and AP-1 was observed at lower acetaldehyde concentrations, 50–150 μM, as shown recently (32). Thus, these findings reveal the ability of acetaldehyde to increase the binding to specific DNA sequences of NF-κB and AP-1. Similar to
these findings with HepG2 cells, primary cultured rat hepatocytes displayed enhanced DNA binding of NF-κB and AP-1 following exposure to acetaldehyde (not shown).

To determine the presence of individual components of NF-κB, different human antibodies against individual members of the NF-κB family were used. Nuclear extracts were incubated with labeled κB consensus oligonucleotide and with polyclonal antibodies against p50, p52, p65, c-Rel, and RelB. p50, p52, and p65 antibodies resulted in supershifted bands, indicating that acetaldehyde activated the heterodimers p50/p65 and p50/p52, denoted by the arrows in Fig. 3. Interestingly, a lower molecular weight complex not shifted by any of the antibodies used was also detected, the identity of which remains to be established. However, this undetermined complex was specific for κB binding as it was displaced by an excess of unlabeled κB oligonucleotide (Fig. 1). The product of the proto-oncogene c-jun is a component of transcription factor AP-1 (18–20). Hence, we assessed its presence in the activated AP-1 complex by determining the levels of c-Jun in nuclear extracts prepared from acetaldehyde-treated HepG2 cells. As shown, acetaldehyde increased c-Jun levels, an effect that was dependent on the dose of acetaldehyde (Fig. 4). The presence of c-Jun in nuclear extracts correlated with the greater DNA binding of AP-1 caused by increasing concentrations of acetaldehyde (Fig. 4), suggesting the presence of c-Jun in the activated AP-1 complex. Indeed, the immunoprecipitation from nuclear extracts using antibody anti-c-Jun abolished the binding of AP-1 to DNA (not shown).

Acetaldehyde Stimulates κB- and AP-1-controlled Gene Expression—Although the above findings indicate that acetaldehyde enhanced the binding activity of NF-κB and AP-1 using EMSA, this approach fails to underscore the potential of acetaldehyde to regulate gene expression. Thus, to determine unambiguously whether the enhanced DNA binding of NF-κB and AP-1 caused by acetaldehyde stimulates κB- and AP-1-controlled gene expression, we examined the transactivation potential of these transcription factors in HepG2 cells using a luciferase reporter gene construct containing κB or AP-1 binding sites upstream of the herpes simplex virus thymidine kinase promoter. As seen, acetaldehyde induced the κB- and AP-1-dependent luciferase expression similar to okadaic acid and phorbol 12-myristate 13-acetate, known activators of κB and AP-1, respectively (Fig. 5). Similar findings were observed using a luciferase expression vector containing a 5′-flanking fragment of the IL-8 gene encompassing AP-1, NF-IL6, and κB binding sites (30) (not shown). Therefore, these findings show that acetaldehyde enhances DNA binding of NF-κB and AP-1, which activate the transcription of synthetic and natural promoters, indicating that these transcription factors may be components of a complex of additional transcriptional regulators, which trigger the inducible expression of target genes.

As wild type HepG2 cells do not express the enzymes required for the activation of AP-1, we used human acute myelogenous leukemia cells (HL-60) for additional investigations. As shown, acetaldehyde induced the expression of κB- and AP-1-controlled genes such as cyclooxygenase-2, inducible nitric oxide synthase, and IL-8, similar to the findings in HepG2 cells (Fig. 6). Therefore, these findings indicated that acetaldehyde stimulates κB- and AP-1-controlled gene expression in HepG2 cells.
Kupffer cells versus HepG2 cells, or acetaldehyde concentration may have determined the differential outcome obtained. Although these studies suggested that acetaldehyde may form an adduct with IκB-α making the protein less susceptible to degradation, the inhibitory effect of acetaldehyde on the lipopolysaccharide-induced NF-κB activation appeared to be significant only at low acetaldehyde concentration (25–50 μM) (40). Nevertheless, although these findings of Jokelaiben et al. (40), are intriguing and imply that acetaldehyde may function as a NF-κB repressor hence playing a potential anti-inflammatory role in ALD, the in vivo significance of their observation is uncertain as up-regulation of tumor necrosis factor-α, a gene regulated by NF-κB, is a hallmark feature of ALD and is recognized as a key factor for the development of the disease (26, 41–43).

**Activation of NF-κB by Acetaldehyde in Cytosolic Extracts**—To address whether degradation of IκB-α is required for NF-κB activation and if acetaldehyde may exert a direct role in regulating the DNA binding of NF-κB, cytosolic extracts from untreated HepG2 cells were prepared and then incubated with acetaldehyde monitoring the DNA binding of NF-κB and IκB-α levels. As shown, incubation of cytosolic extracts with formamide increased the binding of NF-κB to DNA in agreement with previous reports (44). Similar results were obtained using deoxycholate (not shown). Interestingly, acetaldehyde mimicked the effect elicited by formamide as revealed by the similar pattern of DNA binding of NF-κB in cytosolic extracts (Fig. 7). However, this process required a high acetaldehyde concentration, as acetaldehyde below 1 mM failed to enhance DNA binding of NF-κB (not shown). The activation of NF-κB in cytosolic extracts was a specific event as in intact cells, because an excess of unlabeled κB oligonucleotide displaced the DNA binding of NF-κB (not shown). Furthermore, the identification of individual components of NF-κB was performed in supershift assays using specific antibodies. Incubation of cytosolic extracts after acetaldehyde exposure with antibodies against p65 and p50 revealed a pattern of retarded complexes that differed from that obtained in nuclear extracts, as the heterodimer p65/p50 was the only complex detected (Figs. 3 and 7A). In addition, because acetaldehyde signaled the degradation of IκB-α in intact cells resulting in NF-κB activation, we determined the levels of IκB-α in cytosolic extracts incubated with acetaldehyde. The levels of IκB-α remained unchanged, despite the fact that acetaldehyde activated NF-κB (Fig. 7B). Having shown an increased DNA binding of NF-κB in cytosolic extracts caused by acetaldehyde addition, we next examined the effect of acetaldehyde in the DNA binding of AP-1 in this paradigm. The addition of acetaldehyde at a wide range of concentrations failed to enhance the binding of AP-1 to DNA (Fig. 7C).

Such an outcome contrasts with the lack of effect of acetaldehyde to enhance the DNA binding of AP-1 at the same concentration range. Although the significance of the preceding findings is unclear because of the exceedingly high, supraphysiological, acetaldehyde concentration required for this process, it could be speculated that such a putative, additional pathway for NF-κB activation by acetaldehyde may play a role in advanced stages of the disease accompanied by impaired mitochondrial acetaldehyde dehydrogenase activity, facilitating the accumulation of acetaldehyde, similar to the marked increase of acetaldehyde concentration in the livers from chronic ethanol-fed rats following inhibition of acetaldehyde dehydrogenase (45).

**Role of PKC in Acetaldehyde-induced NF-κB and AP-1 Activation**—Phosphorylation is involved in the regulation of the activity of many transcription factors. It may regulate subcellular localization, dimerization, binding to DNA, or the trans-
activation potential. Phosphorylation is an important regulator of NF-κB activation through the ubiquitin-dependent degradation of the inhibitor IκB-α after phosphorylation at specific serine residues (31, 37–39). On the other hand, previous studies have shown that acetaldehyde induces the levels of c-Jun through a PKC-dependent mechanism and enhances murine α2(I) collagen promoter mediated by PKC activation (46, 47). Hence, we tested the role of PKC inhibitors on the effect of acetaldehyde in the DNA binding of NF-κB and AP-1 in HepG2 cells. HepG2 cells were preincubated with the PKC inhibitor H7, and the activation of NF-κB and AP-1 caused by acetaldehyde was assessed by EMSA. As shown, H7 abolished the DNA binding of NF-κB and AP-1 enhanced by acetaldehyde in HepG2 cells analyzing the differential effect of ethanol versus acetaldehyde in the activation of NF-κB and AP-1 (32). Taken together these data indicate that acetaldehyde itself plays a role in inducing gene expression through enhanced DNA binding of NF-κB and AP-1, implying that acetaldehyde, in addition to its recognized role in cell dysfunction, may also contribute to the increased expression of inflammatory cytokines, a critical event in the manifestations of the ethanol-induced cytotoxicity (26, 32, 42).

Recent observations have shown that disulfiram and ben-
FIG. 7. Effect of acetaldehyde on DNA binding of NF-κB in cytosol extracts. Cytosolic extracts from untreated HepG2 were prepared and then incubated with acetaldehyde or formamide for 30 min. A, activation of NF-κB was analyzed by EMSA using a specific κB-labeled oligonucleotide. The effects of formamide and acetaldehyde were compared with the activation of NF-κB using nuclear extracts prepared from HepG2 cultured in the presence of acetaldehyde as described in Fig. 1 and are denoted as Nuclear extracts. The identification of heterodimer p65/p50 using specific p50 and p65 antibodies is shown by the arrows. B, parallel aliquots of cytosolic extracts were used for Western blot analyses of IκB-α. C, cytosolic extracts from untreated HepG2 were prepared and then incubated with acetaldehyde or formamide for 30 min as in A. The effect of acetaldehyde on activation of AP-1 in cytosolic extracts of untreated HepG2 is compared with the AP-1 activation detected in nuclear extracts of HepG2 cells cultured in the presence of acetaldehyde as indicated in Fig. 2. Results are the mean ± S.D. of four individual experiments.

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