Cellular distribution of nuclear factor κ B binding activity in rat liver

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The cellular localization of nuclear factor kappa B (NF- κ B) binding activity in rat liver has been investigated using electrophoretic mobility shift assay on extracts of highly purified hepatocytes and Kupffer cells obtained from liver perfused *in vivo* with collagenase. Constitutive NF- κ B binding activity was demonstrated in nuclear extracts of control Kupffer cells, and this was not apparently influenced by injection of lipopolysaccharide (LPS) into rats 24 h before perfusion. In contrast, little nuclear NF- κ B binding activity was present in hepatocytes from control animals, although there was detectable inactive, inhibitor-bound, NF- κ B in the cytoplasm. However, nuclear NF- κ B binding activity was increased in hepatocytes from LPS-treated animals and after *in vitro* culture of control rat hepatocytes. Thus NF- κ B binding activity has been demonstrated in highly purified hepatocytes and appears to be inducible both *in vivo* and *in vitro*. These findings support a role for NF- κ B in hepatocyte gene regulation which may be important in the modulation of the hepatic acute phase response.

INTRODUCTION

The hepatic acute phase response, which is part of the systemic response to inflammation, involves a complex, co-ordinated alteration in the production of certain proteins, termed acute phase proteins, by the liver. Evidence suggests that synthesis of these proteins is controlled at both transcriptional and post-transcriptional levels [1] by cytokines. Interleukin-6 (IL-6) appears to be the most important of these [2], but both IL-1 and tumour necrosis factor- α (TNF- α) have also been implicated [3].

Nuclear factor kappa B (NF- κ B) was first identified in the nuclei of mature B lymphocytes as a transcription factor which binds an 11 bp DNA sequence in the κ light chain enhancer [4]. NF- κ B binding sites have subsequently been demonstrated in the regulatory regions of a number of other human genes, including IL-2, the IL-2 receptor α -chain, β_2 -microglobulin [5] and IL-6 [6], as well as the HIV (human immunodeficiency virus) enhancer region [7], suggesting that NF- κ B has a widespread gene regulatory function. In addition, several reports have indicated the presence of putative NF- κ B binding sites in the 5'-flanking regions of genes encoding various hepatic acute phase proteins, including human serum amyloid A [8], rat angiotensinogen [9] and mouse complement factor B [10]. These NF- κ B binding sites are in regions distinct from those previously identified as IL-6 responsive elements [11,12]. Thus the possibility exists that NF- κ B may have gene regulatory activity in hepatocytes, in particular in the transcriptional control of acute phase proteins.

Although NF- κ B-like binding activity has been demonstrated in nuclear protein extracts of both human hepatoma cell lines and whole rat liver [9,10], there have been no previous studies of NF- κ B distribution within the different cell types in the liver, such as parenchymal cells (hepatocytes) and non-parenchymal cells (Kupffer and endothelial cells). Kupffer cells are of macrophage lineage [13] and therefore could account entirely for the NF- κ B binding activity previously reported in extracts of whole liver. Here we report the presence of NF- κ B binding

activity in extracts of highly purified rat hepatocytes as well as in Kupffer cells, and demonstrate its inducibility both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Preparation of protein extracts from livers of saline- and lipopolysaccharide (LPS)-treated rats

Adult male Wistar rats, weighting 200–240 g, were injected intraperitoneally with a single sub-lethal dose (3 mg/kg body weight) of LPS (Sigma *Escherichia coli* 0127:B8) as a 1 mg/ml solution in sterile 0.9 NaCl; control rats were injected with the same volume of 0.9% NaCl. After 24 h the rats were killed and the left lobe of the liver removed. The liver tissue was sliced finely with a scalpel blade, rinsed with ice-cold buffer A [10 mM-Hepes (pH 7.9 at 4 °C) 1.5 mM-MgCl₂, 10 mM-KCl, 0.5 mM-dithiothreitol (DTT)] [14], suspended in 1 ml of cold buffer A and homogenized in a loose-fitting Dounce homogenizer. The suspension was transferred to a microcentrifuge tube and centrifuged at 10000 g for 30 s at 4 °C. After removal of supernatant the pellet was spun at 10000 g for 45 min at 4 °C; the volume of the resulting supernatant was adjusted to 1.5 ml in buffer D [20 mM-Hepes (pH 7.9), 20% (v/v) glycerol, 0.1 M-KCl, 0.2 mM-EDTA, 0.5 mM-phenylmethanesulphonyl fluoride, 0.5 mM-DTT] [14] and the protein concentration was determined by the Bradford method [15] (Bio-Rad).

Preparation of isolated rat Kupffer cells and hepatocytes

Adult male Wistar rats weighing 200–250 g were anaesthetized by intraperitoneal injection of pentobarbitone (60 mg/kg body weight). Following laparotomy the livers of such rats were perfused via the portal vein with a collagenase solution [16]. Briefly, the liver was perfused with 80 ml of Earl's Basal salt solution (without Ca²⁺) containing collagenase (Sigma type IV) at 0.5 mg/ml. After 30 min of perfusion the dispersed liver suspension was filtered through a 50 μ m-pore-size nylon gauze to

Abbreviations used: IL-6, interleukin-6; TNF- α , tumour necrosis factor- α ; NF- κ B, nuclear factor kappa B; LPS, lipopolysaccharide; EMSA, electrophoretic mobility shift assay; PMA, phorbol 12-myristate 13-acetate; I κ B, inhibitor of NF- κ B.

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remove tissue debris and aggregated cells. The filtrate was centrifuged at 50 *g* for 2 min at 4 °C, yielding a hepatocyte-enriched fraction in the pellet and non-parenchymal cells in the supernatant.

Each fraction was subsequently purified by differential centrifugation over Percoll (Pharmacia), as previously described [17]. The hepatocyte fraction was further purified to homogeneity by two cycles of negative selection with immunomagnetic beads (Dynabeads; Dynal Ltd.) coated with mouse monoclonal IgG2a antibody to rat macrophages (Serotec MCA 275), to remove all contaminating Kupffer cells. Control experiments confirmed binding of the antibody-coated beads to Kupffer cells but not to hepatocytes. The purity and viability of each hepatocyte preparation was checked by examining a suspension of cells stained with 0.2% Trypan Blue under light microscopy.

Short-term culture of hepatocytes

Pure hepatocytes obtained as above were suspended in serum-free RPMI 1640 tissue culture medium (Flow Laboratories) with or without 10% endotoxin-free fetal calf serum (Sigma) at a density of 5×10^5 cells/ml, with and without IL-6 (recombinant human type; 4 ng/ml). Culture flasks were incubated in a 37 °C shaking water bath with continuous oxygenation. Such cultures were maintained for a maximum of 6 h.

Cytokine stimulation of AML-193 cells

In an effort to determine if IL-6 regulates NF- κ B expression in a cell line known to proliferate in response to that cytokine, experiments were carried out on AML-193 cells, derived from a human acute myelogenous leukaemia line [18]. AML-193 cells were suspended in RPMI 1640 containing 10% fetal calf serum at a density of 5×10^6 cells/ml. Aliquots of 2×10^7 cells were incubated for 120 min in the presence of TNF- α (10 units/ml) (known to inhibit proliferation of these cells), IL-6 (20 ng/ml) or no added cytokine. After this incubation period cells were harvested for preparation of nuclear extracts as described below.

Preparation of nuclear and cytoplasmic extracts

Nuclear and cytoplasmic soluble protein extracts were prepared from both Kupffer cells and hepatocytes using a rapid version of the method of Dignam *et al.* [14] as previously described [5]. Cytoplasmic extracts were obtained by diluting the supernatant obtained after the first centrifugation with 3 vol. of buffer D [14]. Nuclear extracts of the AML-193 cells were prepared by the same method.

Detection of NF- κ B binding activity by electrophoretic mobility shift assay (EMSA)

NF- κ B binding activity was detected as previously described [7] using 20 μ g of cell protein extract (nuclear or cytoplasmic) in the presence of 1 μ g of dI,dC and a synthetic 32 P-radiolabelled double-stranded DNA κ B probe. Nonidet P-40 (1%) and sodium deoxycholate (0.2%) were added to cytoplasmic extracts to dissociate NF- κ B from its inhibitor [19] in some experiments.

RESULTS

NF- κ B in whole liver from control and LPS-treated rats

NF- κ B binding activity was demonstrated by EMSA in whole liver extracts from two control rats (Fig. 1, lanes 1–3) and two

LPS-treated rats (Fig. 1, lanes 4–6). In each case there were two separate κ B bands which were competed for by an excess of the unlabelled κ B probe, but not by an unrelated oligonucleotide (octamer site from the IL-2 enhancer). The two bands were considerably weaker and of lower molecular masses than corresponding bands obtained using the same quantity of nuclear

Fig. 1. NF- κ B binding activity in extracts of whole rat liver

Protein extracts were prepared from whole livers removed from control, saline-treated rats (lanes 1–3) and LPS-treated rats (lanes 4–6). NF- κ B was detected by EMSA using 20 μ g of protein extract in each lane and a synthetic 32 P-radiolabelled double-stranded DNA probe. Nuclear extracts of PMA-stimulated Jurkat T cells (lanes 7–9) were assayed for comparison. Competition studies were performed using either unlabelled κ B probe (lanes 2, 5 and 8) or an unrelated double-stranded DNA fragment from the IL-2 octamer site (lanes 3, 6 and 9).

Fig. 2. NF- κ B binding activity in extracts of purified Kupffer cells prepared from control rats

Nuclear (lanes 1–3) and cytoplasmic extracts without (lanes 4–6) and with (lanes 7–9) 0.2% sodium deoxycholate (DOC) treatment were assayed by EMSA. Competition studies were as described in Fig. 1.

Fig. 3. NF- κ B binding activity in extracts of purified hepatocytes prepared from control and LPS-treated rats

(a) Nuclear (lanes 1–3) and cytoplasmic extracts without (lanes 4–6) and with (lanes 7–9) DOC treatment of control rat hepatocytes were assayed by EMSA. (b) Nuclear extracts of hepatocytes from control rats (lanes 1–3) and LPS-treated rats (lanes 4–6) were compared by EMSA. Competition studies were as described in Fig. 1.

extract of phorbol 12-myristate 13-acetate (PMA)-stimulated Jurkat T cells (Fig. 1, lanes 7–9). The extracts from the two LPS-treated rats contained slightly more NF- κ B binding activity than those from control rats, the same quantity of protein extract yielding more intense bands on the same gel.

NF- κ B in nuclear and cytoplasmic extracts of purified rat Kupffer cells

NF- κ B binding activity in nuclear and cytoplasmic extracts of control rat Kupffer cells purified as described above, with and without treatment with deoxycholate to dissociate NF- κ B from its inhibitor I κ B, is shown in Fig. 2. There was NF- κ B binding activity present in both the nuclear (Fig. 2, lanes 1–3) and cytoplasmic compartments; the cytoplasmic extract yielded

Fig. 4. NF- κ B binding activity in nuclear extracts of control rat hepatocytes and AML-193 cells, incubated with and without exogenous cytokines

(a) Nuclear extracts prepared from control rat hepatocytes before (lanes 1–3) and after 3 h incubation in medium alone (lanes 4–6) or with added IL-6 (4 ng/ml) (lanes 7–9) were assayed by EMSA. (b) Nuclear extracts prepared from AML-193 cells after 2 h incubation in medium alone (lane 1), with added TNF- α (10 units/ml) (lane 2) or IL-6 (20 ng/ml) (lane 3) were assayed by EMSA.

more intense bands after deoxycholate treatment (lanes 7–9), suggesting that a significant proportion of NF- κ B was complexed with I κ B.

NF- κ B in nuclear and cytoplasmic extracts of purified hepatocytes from control and LPS-treated rats

Microscopy of hepatocyte suspensions, purified as described, confirmed the absence of any non-parenchymal cells. There was weak NF- κ B binding activity in nuclear extracts of hepatocytes prepared from saline-treated rats (Fig. 3a, lanes 1–3). However, inhibitor-bound NF- κ B was present in cytoplasmic extracts (Fig. 3a, lanes 7–9) as demonstrated by dissociation using de-

tergent agents. Nuclear extracts of hepatocytes prepared from LPS-treated rats (Fig. 3*b*, lanes 4–6) contained more NF- κ B binding activity than those from saline-treated animals (Fig. 3*b*, lanes 1–3). However, most of the NF- κ B binding activity from these cells was also in the bound, cytoplasmic form (results not shown).

Short-term culture of control rat hepatocytes with and without IL-6

Fig. 4(*a*) compares NF- κ B binding activity in control rat hepatocyte nuclear extract prior to culture (lanes 1–3) with that after 3 h in culture in serum-free medium in the absence (lanes 4–6) and in the presence (lanes 7–9) of IL-6 (4 ng/ml). There was a marked increase in NF- κ B binding activity in both groups of hepatocytes after 3 h in culture, but no additional increase was produced by adding IL-6. Identical results were obtained when hepatocytes were cultured in medium containing 10% fetal calf serum (not shown).

Effects of TNF- α and IL-6 on AML-193 cells

NF- κ B binding activity was detected in nuclear extracts of AML-193 cells incubated in the absence of exogenous cytokine (Fig. 4*b*, lane 1). Addition of TNF- α (known to cause growth arrest of such cells [20]) caused a marked increase in nuclear NF- κ B binding activity (Fig. 4*b*, lane 2). In contrast, the addition of IL-6 had no effect on nuclear NF- κ B binding activity over this time course (Fig. 4*b*, lane 3), although it did cause these cells to proliferate (results not shown).

DISCUSSION

Although NF- κ B is believed to be an important, inducible transcriptional activator of a wide variety of genes, its constitutive expression in the active, nuclear form has been demonstrated, to date, in only a limited set of cell types. These include mature B cells [4], mature monocytes and macrophages [21] and some T cell lines [22]. In other cell types it is present in an inactive, cytoplasmic form, bound to an inhibitor (I κ B) [19], but can be activated by a variety of stimuli, including cytokines, phorbol esters and viral transactivators [22]. This activation involves the rapid dissociation of cytoplasmic NF- κ B from I κ B, followed by translocation of the free NF- κ B to the cell nucleus. In addition, recent evidence suggests that transcription of NF- κ B is regulated by factors known to enhance NF- κ B binding activity [23].

The reported presence of NF- κ B binding sequences in the regulatory elements of hepatic acute phase response genes [8–10] implied a possible role for inducible NF- κ B in the transcriptional control of their synthesis. However, if NF- κ B is important in such regulation during the acute phase response, it would need to be present in its inactive, cytoplasmic form in resting hepatocytes and be rapidly activated in response to inflammation. We, therefore, wished to investigate the distribution of NF- κ B in liver tissue, which is composed of both parenchymal (hepatocytes) and non-parenchymal cells; the latter include endothelial cells and Kupffer cells which are of macrophage lineage. The techniques of rat liver collagenase perfusion, combined with centrifugation over Percoll and negative selection with immunomagnetic beads, enabled us to prepare highly purified Kupffer cells and hepatocytes in order to investigate the cellular distribution of NF- κ B within the liver.

Initial studies with crude whole liver extracts demonstrated the constitutive presence of NF- κ B in livers from healthy control rats. Such NF- κ B binding activity was increased in the livers of rats which had been injected with LPS 24 h before killing. LPS is known to be a potent stimulus *in vivo* for the acute phase

response and production of acute phase proteins by the liver. Previous experiments in our laboratory had demonstrated that acute phase protein (α_2 -macroglobulin and α_1 -acid glycoprotein) mRNA levels within the liver peak at 24 h after a single intraperitoneal injection of LPS (R. J. Sharma & G. E. Griffin, unpublished work). We therefore prepared hepatocytes from rats at this time after LPS injection in an attempt to optimize the chance of detecting a change in κ B binding activity. We have subsequently confirmed that there were significant rises in both plasma levels and hepatic mRNA of both of these acute phase proteins in the same LPS-treated rats at 24 h [24].

To determine which cells within the liver were responsible for this apparent induction of NF- κ B activity by LPS, we proceeded to purify subsets of liver cells and examine NF- κ B binding activity in nuclear and cytoplasmic extracts of such cells. In keeping with their macrophage lineage, Kupffer cells from control rats contained constitutively active nuclear NF- κ B. However, there was no apparent increase in the amount of NF- κ B in the LPS-treated rats (results not shown). In addition, inactive cytoplasmic NF- κ B was present in Kupffer cells from both the control and LPS-treated rats, as shown by deoxycholate treatment of the extracts.

In contrast, hepatocytes from control rats contained little nuclear NF- κ B binding activity, but the amount was increased in hepatocyte nuclear extracts prepared from LPS-treated rats. This suggests that NF- κ B activation is inducible *in vivo* in rat hepatocytes. To investigate inducibility of nuclear NF- κ B activity in hepatocytes *in vitro*, we examined the effect of short-term (6 h) culture of these cells in serum-free medium. There was a marked increase in nuclear NF- κ B binding activity after 3 h in culture, suggesting that the hepatocytes were activated by the culture procedure alone. Any additional stimulation by IL-6 may, therefore, have been masked. However, the absence of any apparent additional nuclear NF- κ B binding activity after culturing hepatocytes in the presence of IL-6 was not surprising. Although recombinant human IL-6 is known to be a potent stimulus of acute phase protein synthesis in cultured rat hepatocytes [25], known IL-6 responsive elements in acute phase response genes bear little resemblance to the putative NF- κ B binding sites [9]. In addition, IL-6 treatment did not influence nuclear NF- κ B binding activity in AML-193 cells, which are known to be responsive and to proliferate in response to this cytokine. In contrast, treatment of these cells with TNF- α , which suppresses their proliferation, produced a marked increase in nuclear NF- κ B binding activity. This finding indicates that NF- κ B binding activity is inducible in these cells under conditions of growth arrest.

We have demonstrated in this study that NF- κ B is present in both Kupffer cells and hepatocytes isolated from rat liver. In hepatocytes, NF- κ B was detected predominantly in its inactive, inhibitor-bound cytoplasmic form. However, active nuclear NF- κ B is inducible in hepatocytes both *in vivo* by administration of LPS endotoxin and *in vitro* during short-term cell culture after preparation from collagenase-perfused livers. It is highly likely that such preparation procedures may activate Kupffer cells and stimulate cytokine release which may in turn activate hepatocytes maximally, thus masking the effect of exogenous IL-6 added *in vitro*.

These data support the hypothesis that NF- κ B may have an important role in the hepatic acute phase response, perhaps in response to cytokines such as TNF- α and IL-1 which are known to activate NF- κ B in other cell types and to be released locally by Kupffer cells. The recent reports of the successful cloning of cDNAs encoding the DNA binding subunits of NF- κ B [26,27] will permit further studies of the distribution and regulation of this factor in liver cells.

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