Nuclear factor κB is required for the transcriptional control of type II NO synthase in regenerating liver

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A concerted activation of transcription factors involved in the transactivation of type II NO synthase (iNOS) gene occurred after partial hepatectomy (PH), resulting in the transient expression of iNOS. The corresponding mRNA and protein levels of iNOS reached a maximum at 4 h and 8 h post-PH respectively. This induction was preceded by an early and transient activation of nuclear factor κB (NF-κB). Analysis of the κB inhibitory (I) proteins showed an important role for IκBα in the process of NF-κB activation, whereas the contribution of IκBβ was less evident. Interferon regulatory factor 1, which has been described as an important activator of iNOS expression, was up-regulated after PH but failed to bind to the corresponding DNA binding sequences of the iNOS promoter. The transcriptional control of iNOS after PH, was compared with the events associated with the hepatic expression of this enzyme in animals challenged with lipopolysaccharide, showing a differential pattern of transcription-factor activation and IκB degradation between both models. Transfection of hepatoma cell lines with iNOS promoter constructs, followed by stimulation with post-PH sera, revealed the requirement of NF-κB activation for iNOS expression. These data suggest that there is an important role for the restricted NF-κB activation in the temporal pattern of iNOS expression in regenerating liver.

INTRODUCTION

In the aftermath of liver injury by surgical resection of different portions of tissue, the remnant liver initiates a series of reactions in an attempt to re-establish the hepatic-dependent homoeostasis in the organism and to promote cell growth to achieve restored liver function [1–4]. A great effort has been devoted to the identification of the primary stimuli that switch-on the regenerative process, and the view emerging from these studies is that there exists a complex network of interactions, which ultimately modulate a fine balance between factors compromised with cell proliferation, growth-arrest and differentiation [5–7]. Among the multiple changes observed in the immediate hours following partial hepatectomy (PH), an induction of type II NO synthase (iNOS) and the release of NO *in vivo* have been reported [8,9]. The expression of iNOS has been better characterized in murine cells than in rat or humans, and for this reason we have used a murine PH model to address the study of the molecular basis of iNOS expression in the remnant liver [8,10,11]. Also, because iNOS is expressed in liver in response to other systemic stresses such as endotoxin treatment, we examined both conditions to determine whether some responses are specific to each type of liver insult [12].

A 1.8 kb fragment of the promoter region of the murine iNOS gene has been cloned [10,11]. Analysis of the transcriptional activity of this promoter using deletional mutants revealed the essential role of the κB motifs in the control of iNOS expression [9,10,13,14]. Indeed, a rapid nuclear factor κB (NF-κB) activation has been reported in the liver remnant after PH [3,6]. For this reason, the analysis of NF-κB activation is important to establish the mechanisms that control iNOS expression in the course of liver regeneration after PH. NF-κB consists of a heterodimeric complex composed of two subunits of the NF-κB/c-Rel family, usually p50 and p65, which are retained in the cytoplasm as an inactive complex through the interaction with IκB inhibitory (I) proteins [15,16]. A specific regulation by extracellular factors of the IκBα and IκBβ isoforms has been reported, which in turn defines the temporal activation span of the NF-κB complex [17]. Our results show that iNOS is effectively induced in the remnant liver of partially hepatectomized mice, but this response is quantitatively lower than that elicited in animals treated with lipopolysaccharide (LPS). Regarding the transcriptional factors engaged in each model of iNOS induction, specificity exists in the hepatic response, depending on the mechanism of liver injury.

MATERIALS AND METHODS

Chemicals

Chemicals and biochemicals were from Sigma (St. Louis, MO, U.S.A.). Materials and chemicals for electrophoresis were from Bio-Rad (Richmond, CA, U.S.A.). LPS was from *Salmonella typhimurium*.

PH

Male Balb/c mice (25–30 g), maintained free of pathogens and fed *ad libitum*, were used. Liver resection was performed under light ether anaesthesia, and represented two-thirds of the mass of tissue. To maintain the same stress between the distinct animal

Abbreviations used: LPS, lipopolysaccharide; iNOS, type II NO synthase; NF-κB, nuclear factor κB; IκB, inhibitory κB; IRF-1, interferon regulatory factor 1; SIE, *sis* inducible element; EMSA, electrophoretic mobility-shift assay; PH, partial hepatectomy; CAT, chloramphenicol acetyltransferase.

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groups, surgery was carried out in 7 min for both sham and hepatectomized animals [8]. Sera from mice was obtained by cardiac puncture.

Animal treatment

Endotoxin administration was performed by intraperitoneal injection of 0.5 ml of a solution containing LPS from *S. typhimurium* (1 mg/kg body weight) in saline. Animals were killed at different times and liver extracts were prepared immediately.

Cell cultures

The murine hepatic cell line AT3F was derived from the tumorous liver of transgenic mice expressing the SV40 early genes under the direction of the liver-specific antithrombin III promoter [18]. The cells were maintained in Ham-F-12/Dulbecco's modified Eagle's medium supplemented with antibiotics and 10 % (v/v) fetal-calf serum. When cells were stimulated with mice serum, the dishes were washed twice with PBS and incubated in the absence of serum 14 h before mice serum addition.

Plasmids and transfection assays

A 1749 bp *Hin*cII fragment, corresponding to the 5′-flanking region of murine iNOS [10] fused to a promoterless chloramphenicol acetyltransferase (CAT) reporter gene (pNOS-CAT), was generously given by Dr. Q.-w. Xie and C. Nathan (Cornell University, Ithaca, NY, U.S.A.). A plasmid construct [pNOS($\kappa B^{-,-}$)-CAT vector] in which the two κB sites were mutated (nucleotides −971 to −961 and −85 to −75 respectively) was generated by PCR using oligonucleotide primers in which the GGG motif of the κB site was substituted by a CCG (donated by Dr. T. J. Evans; Royal Postgraduate Medical School, London [14]). AT3F hepatocytes were transfected using DOTAP (Boehringer) as described by the manufacturer. Upon transfection, the cells were maintained for 14 h with culture medium, followed by stimulation with serum from control, sham or hepatectomized animals (10 %). After 24 h of incubation, CAT activity was measured [18].

Preparation of total RNA and Northern blot analysis

Total cellular RNA was prepared from small, liquid-N_2-frozen portions of liver (50–100 mg of tissue) following the guanidinium isothiocyanate method [19]. Aliquots of RNA (20–35 μg) were size-separated by electrophoresis on a 0.9 % (w/v) agarose gel, and transferred to Nytran membranes (NY 13-N; Schleicher & Schüell) [20]. An 817 bp fragment (nucleotides 1–817) from the cDNA of macrophage iNOS was random-primed labelled with the Rediprime kit from Amersham (Amersham, Bucks, U.K.) and was used to detect the level of transcription [8]. A *Bam*HI/*Hin*dIII fragment of 1.4 kb of the cDNA of I$\kappa B\alpha$ was used as a probe to detect the mRNA levels and was generously given by Dr. J. Moscat (Universidad, Autónoma, Madrid, Spain) [21]. A 420 bp fragment of I$\kappa B\beta$ was synthesized by reverse-transcriptase PCR, using samples of RNA obtained from testis, due to the relative abundance in this tissue [17], and cloned into a pGEM-T vector (Promega). The oligonucleotide primer sequences were 5′-GGACACAGCCCTGCACTTGG-3′ (oligonucleotides 247–266) and 5′-GTAGCCTCCAGTCTTCATCA-3′ (oligonucleotides 668–649) from the published murine cDNA sequence (DDBJ/EMBL/GenBank Nucleotide Sequence Databases accession number U19799). After hybridization with the probes, the membranes were exposed to a micrograph film (Hyperfilm, Amersham). Quantification of the film was performed by laser densitometry (Molecular Dynamics) using the hybridization with a ribosomal 18 S probe as internal control.

Preparation of cytosolic and nuclear extracts

A modified procedure based on the method of Costa et al. was used [22]. The tissue (100 mg) was homogenized at 4 °C in a Dounce homogenizer in 8 vol. (w/v) of buffer A [10 mM Hepes (pH 7.9)/1 mM EDTA/1 mM EGTA/10 mM KCl/1 mM dithioerythritol/0.5 mM PMSF/aprotinin (2 μg/ml)/leupeptin (10 μg/ml)/7-amino-1-chloro-3-L-tosylamidoheptan-2-one (2 μg/ml)/5 mM NaF/1 mM $NaVO_4$/10 mM Na_2MO_4/0.15 mM spermine/0.5 mM spermidine/0.5 M sucrose/trypsin inhibitor (50 μg/ml)]. After 10 min of incubation in an ice-bath Nonidet P40 was added to reach 0.5 % (v/v) final concentration. The tubes were vortexed for 15 s and nuclei were sedimented by centrifugation at 800 ***g*** for 15 min. Aliquots of the supernatant were stored at −80 °C (cytosolic extract), and the nuclei pellet was washed twice with buffer A lacking detergent, and finally resuspended in 250 μl of buffer A supplemented with 20 % (v/v) glycerol and 0.4 mM KCl. The incubation was followed for 30 min at 4 °C with gentle vortexing. Nuclear proteins were extracted by centrifugation at 13000 ***g*** for 15 min and aliquots of the supernatant were stored at −80 °C. Proteins were measured using the Bio-Rad protein reagent.

Electrophoretic mobility-shift assays (EMSAs)

Synthetic oligonucleotides were prepared using a Pharmacia oligonucleotide synthesizer: NF-κB_p, corresponding to the proximal κB motif of the iNOS promoter [10,11] 5′-CCAACT-GGGGACTCTCCCTTTGGGAACA-3′; interferon regulatory factor 1 (IRF-1), corresponding to the IRF-1 motif of the murine iNOS promoter 5′-TCGACACTGTCAATATTTCACTTTCA-TAA-3′; *sis* inducible element (SIE), corresponding to the high-affinity SIE m67 site [23] 5′-GACAGTTCCCGTCAATC-3′; AP-1 (consensus), corresponding to the AP-1 motif of the albumin promoter [24] 5′-TTCCAAAGAGTCATCAG-3′. The oligonucleotides were annealed and end-labelled with the Klenow enzyme fragment in the presence of 50 μCi of [α-^{32}P]dCTP, and the other unlabelled dNTP in a final volume of 50 μl [21]. The DNA probes (5×10^4 d.p.m.) were incubated for 30 min at 4 °C with 5 μg of protein extract and 1 μg of poly(dI-dC)/ml, 5 % glycerol/1 mM EDTA/100 mM KCl/5 mM $MgCl_2$/1 mM dithiothreitol/10 mM Tris/HCl, pH 7.8, in a final volume of 20 μl. The incubation mixtures were processed by SDS/PAGE as described previously [21]. Analysis of competition with unlabelled oligonucleotides was performed using a 20-fold excess of double-stranded DNA in the binding reaction, and adding the nuclear extracts as the last step in the binding assay. Supershift assays were carried out after addition of the antibody (0.5 μg) to the binding reaction and incubation for 1 h at 4 °C [22]. Anti-p50 (human), anti-p65 (human) and anti-c-Rel (human) were generously given by Dr. N. R. Rice (National Cancer Institute, Frederick, MD, U.S.A.). Anti-IRF-1 (mice) was from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.

Western blot analysis

Cytosolic and nuclear extracts were obtained as described previously for the EMSAs. Proteins (30 μg and 10 μg per lane for cytosolic and nuclear extracts respectively) were size-separated in SDS/10 % PAGE. The gels were processed as recommended by the supplier of the antibodies (anti-iNOS from Transduction

Laboratories; anti-IκBα, anti-IκBβ and anti-IRF-1 from Santa Cruz Laboratories). After blotting onto a polyvinylidene fluorescein membrane (Millipore) proteins were revealed following the enhanced chemiluminescence technique (Amersham).

Statistical analysis

Results are means ± S.E.M. of the indicated number of experiments. Statistical significance was estimated using Student's *t* test for unpaired observations. A P value of less than 0.05 was considered to be significant.

RESULTS

iNOS expression in the remnant liver is preceded by NF-κB activation

To identify the transcription factors relevant for the expression of iNOS in animals that underwent PH, a timed programme of surgery was carried out and the levels of iNOS mRNA were followed by Northern blot analysis. As Figure 1(left) shows that the maximal iNOS mRNA levels (4.5 kb band) were observed 4 h after hepatectomy, followed by a drop in the steady-state levels after 8 h, and are virtually undetectable at 18 or 24 h post-PH. The protein levels were measured by Western blot using an antibody specific for iNOS. As Figure 1(right) shows, the maximal protein content was observed 8 h after PH and progressively decreased (29% of the maximal content after 24 h). These results extend to the mouse, the previous observation of iNOS induction after PH in the rat [8,9]. To address whether this iNOS expression correlates with a NF-κB activation, the binding of nuclear proteins to the κB sequences of the iNOS promoter was assayed. As Figure 2 shows, an intense peak of binding of nuclear proteins to the proximal κB sequence was observed 1 h after PH, followed by a marked decrease in the intensity of the bands. On analysis, the bands included p50–p50 homodimers that were always present, and p50–p65 heterodimers that were transiently induced after PH. This NF-κB activation was specific, since in sham-operated animals the band pattern was virtually identical with that of the controls. Because iNOS is highly expressed in the liver of animals intraperitoneally injected with LPS, experiments were undertaken to compare the activation process in the course of endotoxin treatment with that of PH. LPS treatment of mice elicited a higher increase in p50–p65 than after PH (Figure 2). Several dosages of *in vivo* administration of LPS were tested, obtaining the maximal iNOS expression and animal viability when used at 1 mg/kg body weight [25]. The maximal ratios corresponding to the [p50–p65]/[p50–p50] bands were 0.34 and 2.20 for PH-and LPS-treated animals respectively.

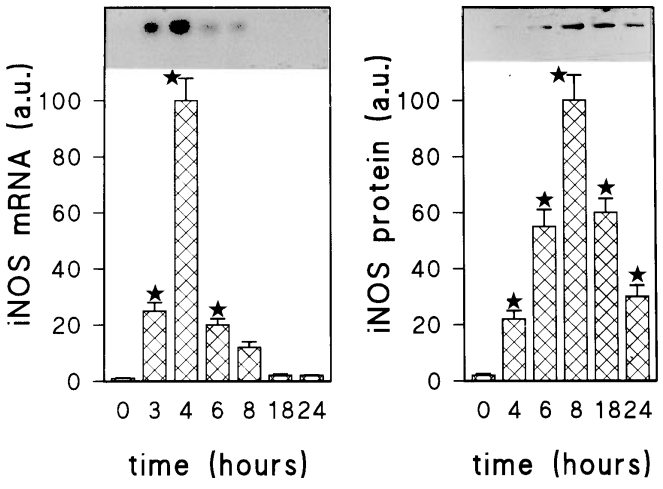

Figure 1 iNOS mRNA and protein levels after 2/3 PH

Mice were partially hepatectomized and samples of the remnant liver were analysed for the iNOS mRNA and protein levels. Results are means ± S.E.M. of four samples after normalization for the ribosomal 18 S content (left panel) or the mean ± S.E.M. of three experiments for the immunodetected iNOS by Western blot analysis (right panel). $^*P < 0.001$ compared with samples at 0 h.

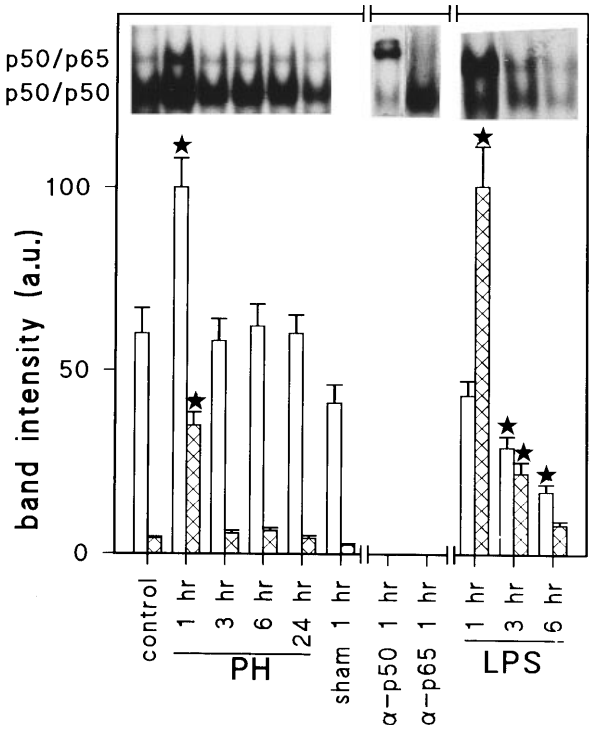

Figure 2 NF-κB is transiently activated after PH

Nuclear extracts from mice that underwent PH, sham, or intraperitoneal treatment with LPS were assayed using as probe the κB sequence of the murine iNOS promoter. Only two bands were detected and corresponded to p50–p50 (open bars) and p50–p65 (cross-hatched bars) complexes. Results show the densitometric analysis of the bands (± S.E.M.) corresponding to three experiments. $^*P < 0.01$ compared with samples from the control condition.

Characterization of the IκB proteins implicated in NF-κB activation in regenerating liver

The activation of NF-κB is largely dependent on the dissociation between the proteins of the c-Rel family and the inhibitory proteins termed IκB. The characterization of the changes in IκB proteins is of interest because the extent of the biological response in which NF-κB activation is involved depends on the time-course of IκB resynthesis [15–17]. For this reason, IκBα and IκBβ were analysed both at the mRNA and the protein levels. As Figure 3 shows, the levels of IκBα mRNA in the remnant liver after PH progressively increased to double at 6 h. However, in animals treated with LPS this up-regulation of the mRNA levels was more rapid (maximum at 1 h) and higher (4.7-fold increase) than in the hepatectomized animals. Regarding the protein levels (Figure 3, right) a decrease of IκBα was observed in hepatectomized animals during the initial 6 h, followed by a recovery when

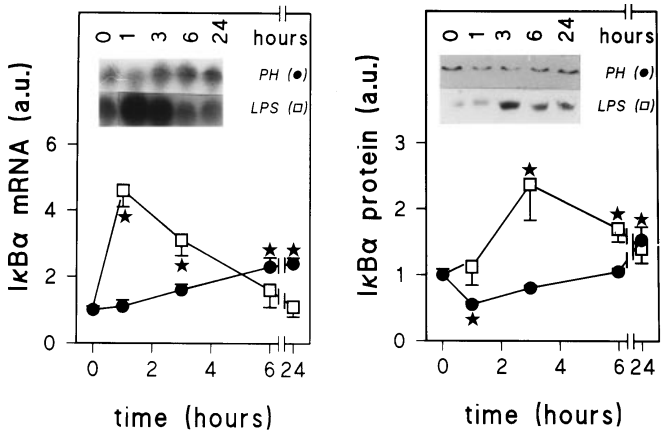

Figure 3 IκBα protein levels decrease after PH

The mRNA levels and the amount of protein corresponding to IκBα in animals after PH or intraperitoneal injection with LPS were determined in liver at the indicated times. Normalization for RNA lane charge in the Northern blot was carried out after hybridization of the membrane with a ribosomal 18 S probe. No significant changes were observed in sham animals. Results are means ± S.E.M. of three experiments. $^*P < 0.05$ compared with samples at 0 h.

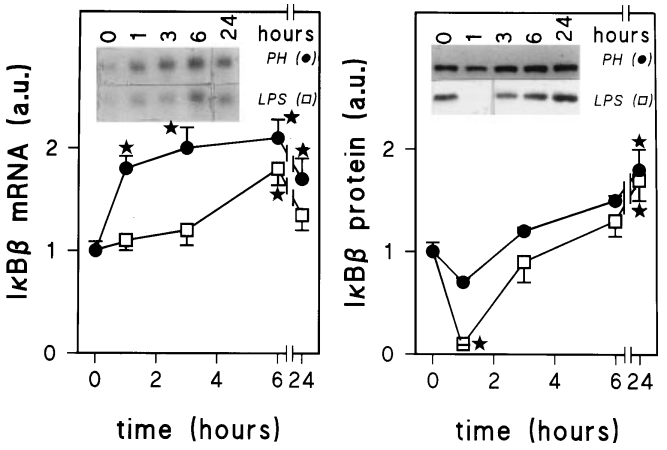

Figure 4 IκBβ protein is rapidly down-regulated in the liver of LPS-treated animals

The mRNA levels and protein content of IκBβ were determined at the indicated times. The Northern blot was normalized after hybridization with a ribosomal 18 S probe. Results are means ± S.E.M. of three experiments. $^*P < 0.05$ compared with samples at 0 h.

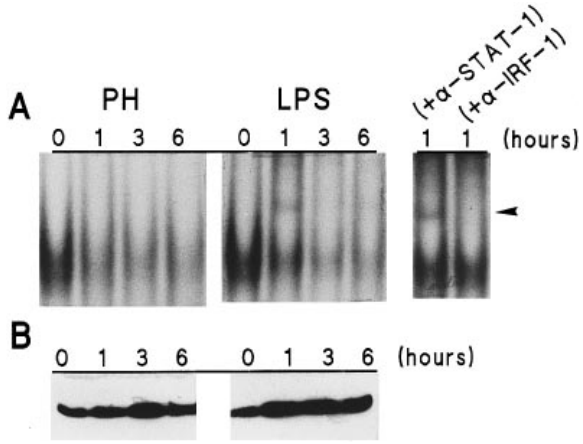

Figure 5 IRF-1 analysis in the remnant liver after PH

Nuclear extracts from animals hepatectomized or intraperitoneally injected with LPS were incubated with an oligonucleotide sequence containing the IRF-1 motif of the iNOS promoter (right arrowhead). To assess the specificity of the binding, incubation of the extracts with anti-IRF-1 antibody completely displaced the binding to the sequence of the arrow-indicated band (**A**). The amount of IRF-1 in cytosolic liver extracts was determined by Western blot using the same antibody as in the supershift assay (**B**).

assayed in the remnant liver 24 h after PH. When this protein was analysed in animals treated with LPS, a 2.2-fold accumulation was observed at 3 h, followed by a recovery of the basal levels at 24 h.

When IκBβ was studied (Figure 4), the amount of mRNA nearly doubled 1 h after PH, and remained higher than the control for up to 24 h after PH. However, in animals intraperitoneally injected with LPS, the mRNA levels remained essentially stable during 3 h, with a 175% increase at 6 h. The IκBβ protein levels of PH animals exhibited a moderate decrease (25%) at 1 h, followed by a progressive increase (175%) at 24 h. However, in animals treated with LPS, an important decrease in IκBβ (more than 90% of the control) was observed at 1 h, followed by a recovery at 3 h, and reaching the values of hepatectomized animals at 6 h. These results suggest the existence in the liver of a differential regulation of IκBα and IκBβ degradation and resynthesis, and therefore of the control of NF-κB activation, in response to distinct stimuli.

Characterization of nuclear factors potentially involved in iNOS expression

In addition to the analysis of NF-κB activation after PH, the binding of proteins to motifs present in the murine iNOS promoter was studied by EMSAs. The sequences used were the SIE element and the IRF-1 and AP-1 binding sites. An important synergism between the IRF-1 and the distal NF-κB motifs of the murine iNOS promoter has been described previously [10,11,13]. As Figure 5(A) shows, a specific band displaced by anti-IRF-1 antibody was detected in nuclear extracts of animals treated for 1 h with LPS. However, absence of specific binding was observed in nuclear extracts from remnant liver after PH. The nature of the specific band detected after LPS challenge agrees with the binding profile described in other cases, such as the LPS-dependent activation of the murine interleukin-10 promoter [26]. Interestingly, murine liver contains an important amount of IRF-1 that is slightly up-regulated in hepatectomized animals (at 3 and 6 h), and more intensely in LPS-treated animals (Figure 5B). The absence of IRF-1 binding to its sequence after PH might be due to the inability of this transcription factor to overpass the levels of IRF-2, which acts as a repressor and which is largely expressed in liver [27].

AP-1 activation was observed in samples 1 h after PH, and exhibited a maximum at 6 h (Figure 6). In animals treated with LPS the maximum was obtained at 1 h, but significant levels were still evident after 6 h. When the binding of nuclear proteins to the SIE motif was measured, an increase was observed in samples obtained 3 h after PH, whereas in animals treated with LPS, this binding was observed at 1 h and extended for longer periods (Figure 6). Since the synthesis of IRF-1 is regulated,

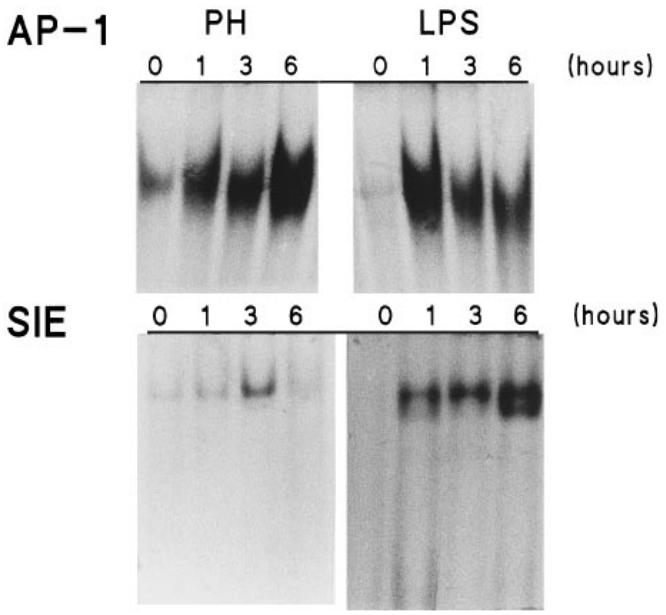

Figure 6 Binding of AP-1 and SIE to consensus motifs by EMSA

Hepatic nuclear extracts from animals that underwent PH or intraperitoneal injection with LPS were prepared at the indicated times and incubated with oligonucleotide sequences containing the AP-1 and SIE binding motifs. Results show a representative experiment out of four.

among other mechanisms, through the activation of SIE motifs in the promoter region of the gene [28], the up-regulation of IRF-1 in hepatectomized or LPS-treated animals (Figure 5B) is in agreement with the enhanced levels of binding to SIE motifs reported in Figure 6.

κB activation is required for the expression of iNOS by serum from PH animals

The results described above show qualitative and quantitative differences in the temporal pattern of activation of transcriptional factors that bind to the iNOS promoter either in the liver of two-thirds hepatectomized animals or after intraperitoneal LPS administration. To determine whether the expression of iNOS after PH might be reproduced using an *ex vivo* system, AT3F cells were transfected with a plasmid harbouring a 1.8 kb fragment of the iNOS promoter linked to a CAT reporter gene, and cells were stimulated with serum after PH or sham animals. As Figure 7 shows, the serum obtained from animals 3 h after PH induced a 4-fold increase in the CAT activity, and this effect was lost when serum after 8 h of PH was used. Sera from sham animals failed to significantly induce CAT activity. To investigate the role of the κB motifs in the promoter activity, AT3F cells were transfected with a promoter construct in which both κB sites were mutated. As Figure 7(right) shows, under these conditions the reporter activity was completely abrogated, indicating an essential role of the κB sites for the expression of iNOS in the course of PH. The effect of the PH serum was compared with serum from animals treated with LPS at 3 and 6 h, resulting in an important induction of the CAT activity when the κB sites were functional (Figure 7, insets). Transfection with a construct containing the thymidine kinase promoter spanning from nt −105 bp to +1 bp failed to express CAT activity in response to serum from PH or LPS-treated animals (results not shown).

DISCUSSION

Liver regeneration after PH is characterized by the existence of a precise temporal pattern of events that regulate entrance into the division cell cycle, at the time when the organ basically maintains its physiological functions [3]. In this regard, most of the changes in the expression of immediate–early genes that

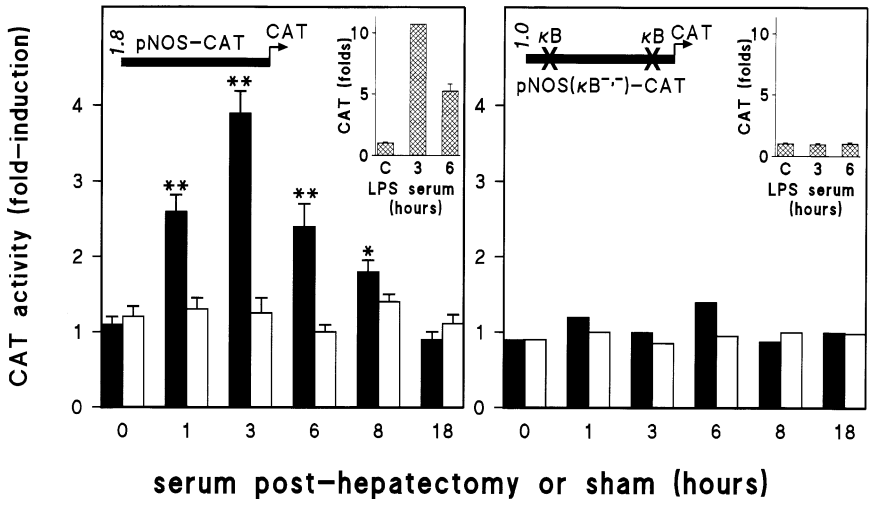

Figure 7 Post-hepatectomy serum stimulates iNOS promoter activity

The AT3F murine hepatoma cell line was transfected with a plasmid (pNOS-CAT) harbouring a 1.8 kb fragment of the iNOS promoter (left panel) or a plasmid [pNOS(κB$^{-,-}$)-CAT] with the two κB sites mutated (right panel). The cells were incubated with 10% (v/v) serum from hepatectomized (solid bars) or sham (open bars) animals, and CAT activity was measured after 24 h of incubation. Insets shows the CAT activity (fold) of AT3F cells stimulated with serum of LPS-treated rats obtained at the indicated times; C refers to control serum (hatched bars). Results are means ± S.E.M. of three experiments (left panel), or a representative experiment (right panel) after normalization for the efficiency of the transfection [18,37]. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with samples at 0 h.

follow PH [3,29] trigger the expression of proteins that directly participate in the control of liver regeneration, either by promoting cell growth or arrest [1,6], or by favouring the restoration of blood flow and diffusion of extracellular factors that mediate the synchronized regenerative process [8,9,30,31].

The induction of iNOS in liver in the course of several hepatic dysfunctions, such as hyperdynamic circulation and chemical aggression or during septic shock, appears to be a widely established response of the damaged organ [12,32]. iNOS expression is mainly controlled at the transcriptional level [10,13], and because of the synchrony of the regenerative process after PH, we have investigated the nature of the transcription factors responsible for the transactivation of this gene. Activation of NF-κB has been reported as an essential requirement for iNOS transcription [13,14]. In the remnant liver after PH, NF-κB activation occurs at the start of the regenerative process (as soon as 30 min post-PH), and rapidly falls in the hours following. According to these data, it might be concluded that this early and quantitatively important activation of NF-κB is sufficient to turn on iNOS transcription, which exhibits a peak of mRNA levels 4 h after PH, and the corresponding peak of iNOS immunoreactivity at 8 h. However, in terms of the duration of NF-κB activation, the requirements for iNOS expression could not be as restricted, as reflected by the EMSA profile. Using higher amounts of protein in EMSAs, FitzGerald et al. [33] reported the presence of p50–p65 complexes (the transcriptionally active molecules) 2 h after PH. Under our experimental conditions, overexposure of the film confirmed the presence of the p50–p65 complex in 3-h post-PH samples, but it was completely absent in 6-h post-PH samples (results not shown). The requirement of NF-κB activation for iNOS induction after PH was in agreement with the absence of effect of post-PH serum upon an iNOS promoter with mutated κB sites after transient transfection in a PH-responsive hepatoma cell line. Moreover, using the κB sites of the iNOS promoter, only two bands were detected in EMSAs after PH, whereas a third band, termed supercomplex by other groups [33,34], was reported when the sequence used for the binding corresponded to the κB motif present in the promoter of class I major histocompatibility complex H2 gene.

Our results also give information on the differential regulation of IκB proteins between PH and LPS treatment. The biological activity of the NF-κB/c-Rel proteins depends upon the interaction with the inhibitory IκB proteins, and we observed a prevalence of IκBα degradation after PH. IκBβ levels largely decreased after endotoxin treatment although, in samples obtained at 3 h, a complete recovery of the protein level was observed. This result is of interest because, using established cell lines, other groups reported a long-sustained fall in IκBβ upon LPS stimulation [17]. At the mRNA level, the important up-regulation of IκBα in animals treated with LPS could be related with the corresponding protein levels, and the same applies to IκBβ after PH. These results might provide a plausible explanation for the short-term activation of NF-κB after PH.

Activation of AP-1 following PH could be due to both an induction of c-*fos* [35] and an activation of Jun kinase [36]. Interestingly, the pattern of AP-1 activation after PH and LPS treatment was different. Whereas a progressive activation of AP-1 was observed after PH, the opposite occurred in animals treated with endotoxin. However, using deletional analysis of the iNOS promoter and following the activity of a reporter gene, agreement exists in proposing a minimal contribution of the AP-1 sites to the transactivation of the gene [10,14,21].

Finally, IRF-1, which appears to be very important for the expression of iNOS in macrophages and other cells [11,27,37], is constitutively expressed in liver, and despite an increase in the protein content after PH, we were unable to detect binding of this factor to the corresponding motif using EMSAs of nuclear extracts of the remnant liver. This result can be explained in view of the important content of IRF-2 in liver, which inhibits the activity of IRF-1 [27,37]. Moreover, in addition to the relative amounts of IRF-1 and IRF-2, IRF-1 activity appears to be qualitatively regulated by a yet-unidentified post-translational mechanism that increases its intrinsic DNA binding affinity, as has been described after some viral infections [27].

The function of NO in the regenerative process after PH remains to be established. Inhibition of NO synthesis with more-or-less specific pharmacological iNOS inhibitors resulted in an enhanced superoxide production by liver [38], and in an altered ploidy pattern in the remnant tissue [8]. Moreover, NO has proved to be an important regulator of IκB function [39], and in this way, it can prevent NF-κB activation once its synthesis is turned on. In this aspect, NF-κB activation is largely delayed (at least 3 h after PH; experiments in progress) in animals previously treated with dexamethasone, a condition in which IκB activity is up-regulated [40] and iNOS expression repressed [41]. In addition to these 'intracellular' effects, NO may favour hepatic circulation after hepatectomy, and contribute to the angiogenic activity in the remnant liver. PH experiments in animals with disrupted iNOS gene [42] could provide further clues to better understand the role of iNOS expression in regenerating liver. Using this genetic approach, in mice with targeted disruption of the interleukin-6 gene, it has been possible to show a critical role for this cytokine in liver regeneration [43].

In summary, our results support the conclusion that transient activation of NF-κB after-PH is essential for the expression of iNOS. Specific regulation of IκBα and IκBβ activity after PH may contribute to this transient expression of iNOS, a situation different from that for animals treated with LPS.

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