Differential regulation of nitric oxide synthase mRNA expression by lipopolysaccharide and pro-inflammatory cytokines in fetal hepatocytes treated with cycloheximide

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The effect of cycloheximide (CHX) on the mRNA expression of the cytokine-inducible, calcium-independent nitric oxide synthase (iNOS) was investigated in fetal hepatocytes stimulated with lipopolysaccharide (LPS) or pro-inflammatory cytokines. In the presence of CHX the LPS-dependent iNOS mRNA levels were reduced, whereas the response to pro-inflammatory cytokines was enhanced. Because iNOS transcription is highly dependent on the activation of nuclear factor κB (NF-κB), this factor was evaluated by electrophoretic mobility shift assays, and a close correlation between NF-κB activity and iNOS mRNA levels was observed. CHX itself potentiated the degradation of the IκBα and IκBβ inhibitory subunits (IκB is inhibitory κB) of the NF-κB complex, and therefore the loss of LPS-dependent iNOS mRNA expression cannot be attributed to a blockage in the activation of NF-κB. These results suggest the existence of a CHX-sensitive pathway in the expression of iNOS mediated by LPS, a mechanism that is not involved in the response to pro-inflammatory cytokines.

INTRODUCTION

Nitric oxide is a messenger molecule playing key roles in multiple physiopathological processes [1]. Synthesis of nitric oxide from L-arginine is catalysed by nitric oxide synthase (NOS). Three different NOS isoenzymes have been identified in mammalian tissues: two constitutively expressed Ca^{2+}/calmodulin-dependent NOS isoforms are mainly present in brain (nNOS) [1–3] and endothelia (eNOS) [4], and a third form, cytokine-inducible, Ca^{2+}-independent NOS (iNOS) is expressed in different activated cells such as macrophages and hepatocytes [5,6].

The promoter region of iNOS has been characterized in different species including man, mice and rat [7–9]. Sequence analysis of this promoter in rodents revealed the presence of at least 24 consensus motifs for binding of transcription factors, including ten copies of interferon-γ response elements, three of the γ-activated site, two of the interferon-stimulated response element, two of the nuclear factor κB (NF-κB), two of the activator protein-1 (AP-1) and two of the tumour necrosis factor-α (TNF-α) responsive element, among others [7,10]. Functional analysis using deletional mutants of the iNOS promoter revealed the presence of two important regulatory regions, each one containing a κB motif [10].

NF-κB is a heterodimer of proteins of the c-Rel family of transcription factors [11]. NF-κB proteins are constitutively present in the cell, but they are retained in the cytoplasm, associated with inhibitory proteins known as inhibitory κB (IκB) [11,12]. Activated NF-κB complexes are translocated to the nucleus in response to mitogens, cytokines and lipopolysaccharide (LPS) [13]. Activation of NF-κB requires phosphorylation and degradation of the IκB proteins (including the α, β, γ and the oncogen Bcl-3 proteins [14]). IκBβ has been cloned recently [15] and, together with IκBα, are the main regulators of NF-κB activity.

Recent reports have demonstrated that treatment of rat hepatocytes with cycloheximide (CHX) activated NF-κB and elicited an intense expression of the iNOS gene [16]. To gain insight into the mechanisms controlling iNOS transcription, fetal hepatocytes were stimulated with LPS and pro-inflammatory cytokines, and in the absence of protein synthesis. Our results show a selective action of CHX in the control of this transcriptional mechanism since it attenuated the response to LPS at the time that it enhanced the activation following either TNF-α or interleukin-1β (IL-1β) stimulation.

MATERIALS AND METHODS

Animals

Pregnant albino Wistar rats (300–350 g) fed on a standard laboratory diet were killed for the experiments between 9:00 and 10:00 h. Gestational age was confirmed by standard criteria [17]. Fetuses were delivered by caesarean section on the morning of day 21 of gestation and were immediately used.

Isolation of hepatocytes

Hepatocytes from fetal rat liver were prepared by a non-perfusion collagenase dispersion method that involved incubation of chopped fetal liver for 30 min at 37 °C with Ca^{2+}-free Krebs-bicarbonate buffer containing 0.5 mM EGTA under continuous gassing with carbogen (O_2/CO_2, 19:1) [18]. The cell pellet was resuspended and incubated for 60 min in the presence of 2.5 mM Ca^{2+} and 0.5 mg/ml collagenase. At the end of the incubation

Abbreviations used: NOS, nitric oxide synthase; iNOS, inducible (calcium-independent) nitric oxide synthase; CHX, cycloheximide; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; TNF-α, tumour necrosis factor-α; IL-1β, interleukin-1β; EMSA, electrophoretic mobility shift assay; AP-1, activator protein-1; IκB, inhibitory κB.

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period the cells were centrifuged and filtered through nylon membranes of 500, 100 and 50 μm mesh. Cell viability was always higher than 90 %. Cells were plated in 6 cm tissue culture dishes in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10 % (v/v) fetal calf serum. After 6 h of incubation to facilitate cell adhesion to the dish, the medium was aspirated, and the plates were washed with PBS and filled with 2 ml of Dulbecco's modified Eagle's medium. Cells were stimulated with LPS or cytokines, and additions were made so that the changes in the total incubation volume were < 2 %. When CHX was used, cells were pretreated for 1 h with this drug before stimulation.

Northern blot analysis

Total RNA was extracted by the guanidinium isothiocyanate method [19] and aliquots of RNA (30 μg) were separated by electrophoresis in a 0.9 % (w/v) agarose gel containing 2 % (v/v) formaldehyde [20]. After transference of the RNA to an NY 13-N membrane (Schleicher & Schuell), the levels of iNOS, IκBα and IκBβ were determined using an *Eco*RI–*Hin*dIII fragment from iNOS (murine) [5], a *Bam*HI–*Hin*dIII fragment from IκBα (human) [6] and a 422 bp IκBβ fragment respectively, obtained by reverse-transcriptase PCR using 1 μg of mouse testis RNA as template and oligonucleotides based on the published sequence [15]. Probes were labelled with [α-^{32}P]dCTP using the Random Primed labelling kit (Amersham). After hybridization, the membranes were exposed to an X-ray film. Quantification of the films was performed by laser densitometry using the hybridization with a probe specific for the 18 S rRNA as internal standard.

Preparation of cytosolic and nuclear extracts

A modified procedure based on the method of Schreiber et al. was used [21]. Cells (4×10^6) were washed with PBS and collected by centrifugation. Cell pellets were homogenized in 400 μl of buffer A [10 mM Hepes (pH 7.9)/1 mM EDTA/1 mM EGTA/10 mM KCl/1 mM dithiothreitol/0.5 mM PMSF/tosyl-lysyl-chloromethane (2 μg/ml)/5 mM NaF/1 mM $NaVO_4$/10 mM Na_2MO_4]. After 15 min at 4 °C, Nonidet P40 was added to reach 0.5 % (v/v) concentration. The tubes were gently vortexed for 15 s and nuclei were collected by centrifugation at 8000 ***g*** for 15 min. The supernatants were stored at −80 °C (cytosolic extracts) and the pellets were resuspended in 70 μl of buffer A, supplemented with 20 % (v/v) glycerol/0.4 M KCl, and gently mixed for 30 min at 4 °C. Nuclear proteins were obtained by centrifugation at 13000 ***g*** for 15 min; aliquots of the supernatant were stored at −80 °C. Protein content was assayed using the Bio-Rad protein reagent.

Electrophoretic mobility shift assays (EMSAs)

The oligonucleotide sequences corresponding to the proximal κB motif of the iNOS promoter (5′-CCAACTGGGGACTCTCC-CTTTGGGAACA-3′) [7] and to the AP-1 motif of the albumin promoter (5′-TTCCAAAGAGTCATCAG-3′) [13] were annealed with their complementary sequence by incubation for 5 min at 85 °C in 10 mM Tris/HCl (pH 8.0)/50 mM NaCl/10 mM $MgCl_2$/1 mM dithiothreitol. Aliquots of 50 ng of these oligonucleotides were end-labelled with Klenow enzyme. Nuclear extracts of 5 μg were incubated with 5×10^4 d.p.m. of the DNA probe, and the DNA–protein complexes were separated on native 6 % (w/v) polyacrylamide gels in 0.5 % (v/v) Tris/borate/EDTA buffer [6].

Western blot analysis

After determining the protein content of cytosolic extracts, samples were boiled in 250 mM Tris/HCl (pH 6.8)/2 % (w/v) SDS/10 % glycerol/2 % (v/v) β-mercaptoethanol. Proteins (30 μg) were size-separated by 10 % SDS/PAGE. Gels were blotted onto a polyvinylidene fluorescein membrane (Amersham) and processed as recommended by the supplier of the antibodies for IκBα and IκBβ (Santa Cruz Biotechnology). Proteins recognized by the antibodies were revealed by following the enhanced chemiluminescence technique (Amersham). At the end of the experiment the membranes were stained with Ponceau S reagent to confirm the protein charge after blotting.

Statistical analysis

Results are expressed as 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 < 0.05 was considered significant.

RESULTS

Differential effect of CHX on iNOS mRNA levels in hepatocytes stimulated with LPS and cytokines

Incubation of fetal hepatocytes for 4 h with LPS induced a large iNOS expression, whereas TNF-α and IL-1β elicited a moderate increase (11 % and 8 % of the effect of LPS respectively; Figure 1). Treatment of the cells with CHX was a sufficient condition to induce iNOS transcription (14-fold increase with respect to

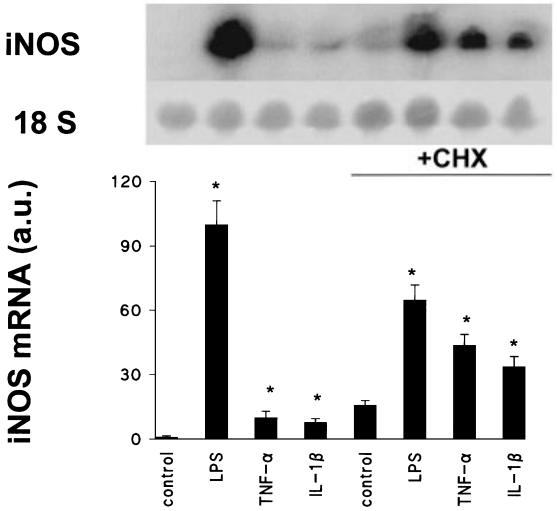

Figure 1 CHX increases the levels of iNOS mRNA in cells treated with TNF-α and IL-1β

Cultured fetal hepatocytes [(3–4) $\times 10^6$ cells] were stimulated for 4 h with 0.5 μg/ml LPS, 10 ng/ml TNF-α or 10 ng/ml IL-1β and the iNOS mRNA levels were measured by Northern blot. When the experiment was performed in the presence of 1 μg/ml CHX, the drug was added 1 h before stimulation. Quantitative analysis was carried out after normalization of the lane charge with a ribosomal 18S probe. Results are means ± S.E.M. of three experiments in which a pool of livers from fetuses obtained from three or four pregnant rats were used to obtain isolated hepatocytes. *Denotes $P < 0.005$ for stimulated cells compared with the respective control in the absence or presence of CHX.

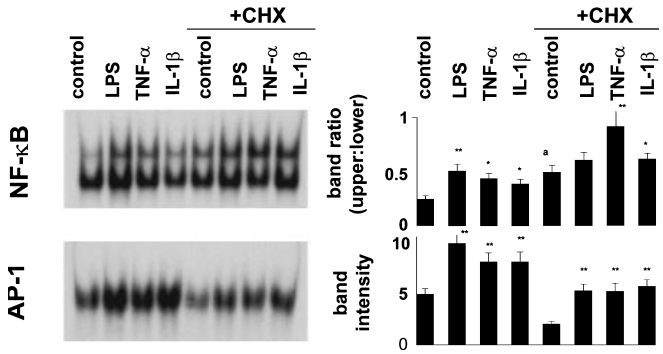

Figure 2 EMSA of nuclear extracts from fetal hepatocytes

Nuclear extracts from cultured fetal hepatocytes were incubated with sequences corresponding to the κB motif of the iNOS promoter or the AP-1 motif of the albumin promoter, and the protein interaction with these sequences was followed by EMSA. Hepatocytes were stimulated for 1 h as described in the legend to Figure 1. Results show a representative experiment out of four. The densitometric analysis of the bands corresponding to the protein–DNA complexes (means $\pm$ S.E.M.) is shown in the right panel. * and ** denote $P < 0.05$ and $P < 0.01$ respectively, for stimulated cells compared with the control in the absence or presence of CHX; [a] denotes $P < 0.05$ with respect to the control in the absence of CHX.

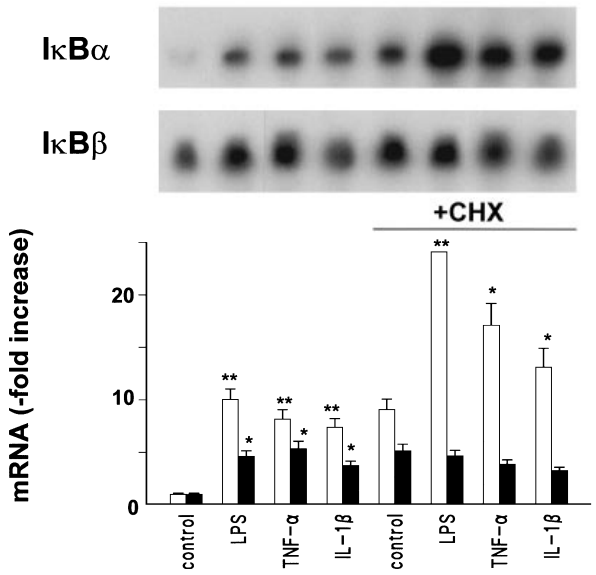

Figure 3 IκBα and IκBβ mRNA levels in hepatocytes incubated with LPS and pro-inflammatory cytokines

Total RNA from cultured fetal hepatocytes incubated for 1 h as described in the legend to Figure 1 were analysed by Northern blot using specific IκBα (open bars) or IκBβ (solid bars) probes. The RNA lane charge was normalized with a ribosomal 18S probe. Results show the relative mRNA content (mean $\pm$ S.E.M.) of three experiments considering unity to be the amount of RNA corresponding to the control condition. * and ** denote $P < 0.01$ and $P < 0.001$ compared with the respective control in the absence or presence of CHX.

control cells). Moreover, the effect of TNF-α and IL-1β on iNOS mRNA levels was potentiated in the presence of CHX. However, the response to LPS decreased under this condition (35% decrease with respect to the level in the absence of CHX; Figure 1). At the enzyme activity level, a close correlation was observed between iNOS mRNA and the synthesis of nitrite by the cells, a process completely blocked in the presence of CHX (results not shown).

Effect of CHX on NF-κB and AP-1 activities

To gain insight into the mechanisms involved in this differential expression of iNOS mRNA on treatment with CHX, the activity of relevant transcription factors that participate in the transactivation of this gene was analysed [6,7,10]. NF-κB was followed by EMSA using the proximal κB motif of the murine iNOS promoter. AP-1 activity was followed using the AP-1-binding motif of the albumin promoter. As Figure 2 shows, a basal level of NF-κB, characterized by a predominant lower-band complex, was detected by EMSA in the nuclear extracts of untreated cells. Stimulation with LPS, TNF-α or IL-1β further increased the levels of the upper-band complex of NF-κB, exhibiting a peak at 1 h. CHX by itself elicited an increase in the activation of NF-κB in control cells and specifically potentiated the activation of NF-κB caused by TNF-α and IL-1β (2- and 1.5-fold increase in the upper/lower-band ratio respectively). Interestingly, CHX did not affect the amount of NF-κB detected in cells treated with LPS. Analysis of the proteins present in the NF-κB complexes by supershift assays and by Western blot, using specific antibodies, revealed that the complexes detected by EMSA correspond to p50/p65 heterodimers and p50/p50 homodimers for the upper and lower bands respectively. Figure 2 also shows the binding of nuclear extracts to a consensus AP-1 sequence. Stimulation of fetal hepatocytes with LPS or cytokines increased AP-1 binding after 4 h, and treatment with CHX attenuated this response. The quantitative analysis of the band intensity ratios of NF-κB (upper/lower bands) and the amount of AP-1 binding is given in the right-hand panel of Figure 2.

Expression of IκBα and IκBβ in fetal hepatocytes stimulated with LPS and cytokines

NF-κB activation requires phosphorylation, targeting and degradation of the IκB components of the heteromeric complexes. Therefore the measurement of the IκBα and IκBβ mRNA and protein levels provides useful criteria for the assessment of their rate of resynthesis and the turn-off of the NF-κB activation process. As Figure 3 shows, basal levels of IκBα mRNA were very low, but a rapid up-regulation was observed after incubation of fetal hepatocytes for 1 h with LPS, TNF-α and IL-1β. This increase in the mRNA levels was potentiated when cells were pretreated with CHX, even under control conditions (9-fold increase). An up-regulation in the IκBβ mRNA level was observed in response to LPS and cytokines, although the changes were lower than those of IκBα because, under similar experimental conditions, the basal levels of IκBβ mRNA were 3- to 4-fold higher (Figure 3; [15]). In the presence of CHX the IκBβ mRNA levels exhibited a 5-fold increase in non-stimulated cells, a process that was not affected by stimulation of the cells with LPS or pro-inflammatory cytokines.

CHX decreases IκBα and IκBβ protein levels in fetal hepatocytes

The amount of IκBα and IκBβ in the cytosol of fetal hepatocytes was determined at 1 h and 4 h on challenge of fetal hepatocytes with LPS, TNF-α and IL-1β, in the absence or presence of CHX. As Figure 4 shows, LPS induced the complete degradation of IκBα at 1 h, followed by a complete recovery at 4 h. TNF-α and IL-1β were not as effective as LPS in promoting IκBα degradation. However, treatment with CHX was sufficient to trigger IκBα degradation, in agreement with the NF-κB activation observed in Figure 2. When the IκBβ levels were measured, an

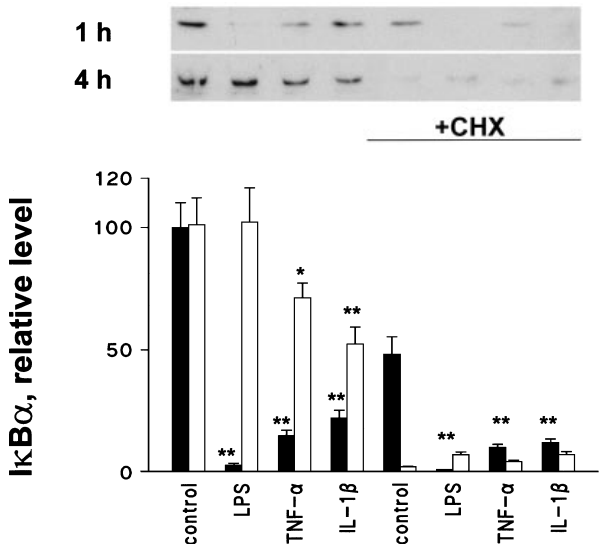

Figure 4 CHX decreases IκBα protein levels in fetal hepatocytes

Cytosolic extracts from hepatocytes incubated for 1 h (solid bars) and 4 h (open bars) with the indicated stimuli were analysed by Western blot using a specific anti-IκBα antibody. A 37 kDa protein was detected and the band intensities were expressed as the percentage of the value corresponding to the control condition at each time. Results are means ± S.E.M. of three experiments. * and ** denote $P < 0.01$ and $P < 0.001$ compared with the respective control in the absence of CHX.

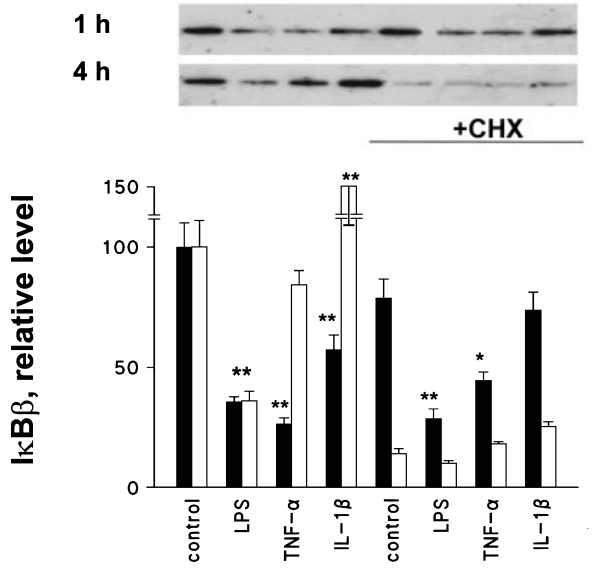

Figure 5 CHX decreases IκBβ protein levels in fetal hepatocytes

The same extracts analysed in Figure 4 were used to determine the IκBβ content in the cytosol of cells treated with the indicated stimuli. A 43 kDa protein was detected and the intensity of the bands was quantified. Results are means ± S.E.M. of three experiments. * and ** denote $P < 0.01$ and $P < 0.001$ compared with the respective control in the absence or presence of CHX.

important decrease was observed in cells incubated with LPS both at 1 h and 4 h, whereas this effect was transient (1 h) in cells stimulated with TNF-α and IL-1β. Interestingly, an up-regulation (152 %) was observed at 4 h in cells treated with IL-1β (Figure 5). When cells were treated with CHX, a time-dependent decrease in the amount of IκBβ protein was observed. Moreover, in the presence of CHX the degradation of IκBβ induced by LPS and TNF-α was still observed in samples obtained 1 h after stimulation. The effect of CHX was more pronounced after 4 h of incubation of the cells (Figure 5).

Table 1 LPS abrogates the over-expression of iNOS mRNA elicited by cytokines in cells treated with CHX

Hepatocytes were stimulated for 4 h with 1 μg/ml LPS, 10 ng/ml TNF-α and 20 ng/ml IL-1β, and the iNOS mRNA was determined by Northern blot. When cells were treated with CHX, this was added 1 h before cell stimulation. Results are means ± S.E.M. of three experiments after normalization of the blots with an 18S ribosomal probe. *$P < 0.001$ compared with the 'none' condition; **$P < 0.001$ compared with the condition in the absence of CHX.

	iNOS mRNA levels (a.u.)	
CHX ...	−	+
None	3 ± 1	16 ± 2**
LPS	100 ± 9*	61 ± 5*,**
TNF-α	10 ± 2*	45 ± 4*,**
IL-1β	8 ± 1*	34 ± 3*,**
TNF-α + IL-1β	91 ± 8*	84 ± 7*
LPS + TNF-α	112 ± 10*	53 ± 5*,**
LPS + TNF-α + IL-1β	115 ± 9*	58 ± 5*,**

LPS abrogates the effect of TNF-α and IL-1β on iNOS mRNA expression in the presence of CHX

To investigate the mechanism by which CHX differentially affects the levels of iNOS mRNA in response to LPS or to inflammatory cytokines, hepatocytes were treated simultaneously with various stimuli. As Table 1 shows, incubation with TNF-α and IL-1β synergistically acted on iNOS mRNA expression, an effect that was minimally enhanced by LPS. In cells treated with CHX, TNF-α plus IL-1β induced iNOS mRNA, as in the absence of the protein inhibitor. However, when LPS was present in this incubation, the levels of iNOS mRNA decreased, indicating a dominant effect of LPS on the mechanism of signalling elicited by the inflammatory cytokines.

DISCUSSION

Incubation of fetal hepatocytes with LPS induced a high iNOS expression, similar to that described previously in adult and neonatal hepatocytes [6,16]; however, TNF-α and IL-1β elicited a moderate expression. In these cells, CHX was used as a tool to investigate the effect of protein synthesis abrogation on the transcriptional control of iNOS. Our data show that CHX by itself induced iNOS transcription in fetal hepatocytes and reduced the transcription elicited by LPS, but interestingly, it enhanced the effects of TNF-α and IL-1β in a synergistic way.

CHX and other protein-synthesis inhibitors have been widely used as an experimental approach to analyse the contribution of protein synthesis to the intracellular signalling of different cell-triggering pathways. Moreover, the use of CHX is of special interest in those cases in which the catalytic activity of the gene product modulates its own transcription, as occurs in the case of iNOS: NO stabilizes IκBα, blocking its degradation and therefore preventing a further activation of NF-κB, which is essential for iNOS transcription [6,10,22]. In addition to this, CHX by itself affects the basal rate of transcription of various genes, and in this regard, it has been postulated that CHX can induce the over-expression of specific genes through mechanisms involving,

among other possibilities, the loss of labile transcriptional repressors, as well as the fall of mRNA-degrading enzymes [23]. This is the case with iNOS mRNA, since it contains multiple copies of the AUUUA consensus sequence, characteristic of unstable messages [24]. In addition to this, CHX, like other protein-synthesis inhibitors, may activate the transcription through their ability to interact with molecules involved in intracellular signalling, acting as a second-messenger agonist [25]. In agreement with this mechanism, the up-regulation of iNOS mRNA levels elicited by CHX can be explained by the observed activation of NF-κB. Superinduction of NF-κB by CHX has been reported in various cell types [16,26], although the mechanisms directing this process remain uncharacterized. The degradation of IκBα and IκBβ elicited by CHX, as well as the inhibition of their resynthesis, can contribute to generate a sustained activation of NF-κB that could be enhanced by other stimuli (TNF-α and IL-1β), which might explain the up-regulation of iNOS mRNA elicited by pro-inflammatory cytokines. However, this situation does not apply to LPS, which exhibits a NF-κB activity similar to that of cells in the absence of CHX. The attenuation in the rate of iNOS transcription in the presence of LPS and CHX suggests the existence of other mechanisms, distinct from NF-κB activation, which may contribute to iNOS transcription [10]. Indeed, treatment of fetal hepatocytes with LPS and TNF-α in the presence of CHX was unable to restore the response observed in the absence of CHX, suggesting that the attenuated expression of iNOS mRNA elicited by LPS was independent of the blockage of an autocrine, TNF-α-dependent pathway. Furthermore, the resynthesis of IκBβ observed in stimulated fetal hepatocytes is of interest since, after an important degradation, complete in the case of LPS, a rapid transcription was observed in these cells 1 h after stimulation; however, in lymphoid cells, a lengthy delay (at least 24 h) in IκBβ resynthesis was observed, allowing a sustained activation of NF-κB [15].

In addition to NF-κB, the activity of other transcription factors that might participate in the control of iNOS expression was investigated. Only the results of AP-1 deserve an additional comment, since they increased on treatment with LPS and cytokines, but decreased in the presence of CHX. These results revealed a minimal contribution of this transcription factor to the expression of iNOS in hepatocytes, and were in agreement with previous reports using deletional analysis of the iNOS promoter [6].

The potentiation of the effect of TNF-α by CHX has been reported in other cases, such as the induction of apoptotic cell death in T-cells and macrophages [27]. With respect to IL-1β, an enhanced NF-κB activity in the presence of CHX can also be proposed as the mechanism of increased iNOS transcription. In addition to this, we have observed a systematic up-regulation of IκBβ in these cells which may contribute to a rapid reset of NF-κB activity in response to this stimulus, and therefore might explain the lower effect of IL-1β when compared with TNF-α.

In conclusion, the results reported in this work show an important degradation of IκBα and IκBβ in the presence of CHX, which results in NF-κB activation and iNOS transcription. Moreover, the use of CHX allows us to propose the existence of a dominant pathway, sensitive to CHX, which is not engaged when cells are stimulated with pro-inflammatory cytokines.

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