Epidermal growth factor inhibits cytokine-dependent nitric oxide synthase expression in hepatocytes

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Received 27 April 1995; revised version received 28 May 1995

Abstract **Liver cells express a wide range of extracellular receptors involved in the control of cell growth and arrest that can be studied ex vivo. Incubation of primary cultures of hepatocytes with IL-1β, TNF-α or lipopolysaccharide promotes the expression of the inducible form of nitric oxide synthase and NO release, a process that is inhibited to a different extent by incubation of the cells with EGF. In addition to this growth factor, IL-6 and TGF-β also inhibited NO synthesis. Therefore, EGF by itself or in combination with other cytokines may be involved in the down-regulation of the NO synthesis that occurs in the early steps of liver regeneration.**

Key words: Nitric oxide; Hepatocyte: Epidermal growth factor; Lipopolysaccharide

1. Introduction

Cell growth and differentiation require the coordinate presence of extracellular factors and their corresponding functional plasma membrane receptors. In the case of liver, at least in the adult animal, most of the cell population is growth arrested in the G_1/G_0 phase of the division cell cycle, although hepatocytes express receptors for different cytokines and growth factors and display one of the highest transcriptional rates in the organism [1,2]. In this regard, liver cells possess a high number of functional EGF receptors that in the presence of EGF mediate an important proliferative response either in vivo or when hepatocytes are kept in primary culture, especially if additional signaling (i.e. c-*myc* induction) occurs [2–4]. Therefore, a role for EGF has been proposed in liver development and differentiation although the ability of the fetus to produce EGF has been questioned by several authors, proposing a maternal origin for the EGF present in fetal and neonatal liver [5,6].

Recently, it has been shown that in the early steps of liver regeneration NO is released in the tissue and although the physiological significance of this process is a matter of debate, a role for NO in the decrease of liver damage and in regeneration has been proposed [7–9]. NO is released after induction of iNOS by endotoxins, such as LPS, or by cytokines produced in the course of the liver injury, especially TNF-α. iNOS expression is controlled by a complex regulatory mechanism involving several different *trans* activating factors [10] and, presumably, the temporal pattern of release of inhibitory cytokines may block NO synthesis after liver regeneration.

In this work we have investigated whether EGF may modulate the NO release in primary cultures of hepatocytes stimulated with an array of cytokines. Our results show an inhibitory role for EGF in the induction of iNOS by IL-1β and TNF-α and suggest that this growth factor by itself or in combination with other cytokines may participate in the down-regulation of the iNOS induced by some of the cytokines involved in the regenerating response of liver after pathological or surgical injury [11]. A similar role for EGF has been proposed in keratinocytes, where this growth factor antagonizes NO synthesis and the release of reactive oxygen intermediates [12], and in hippocampal neurons, where it prevents NO toxicity [13].

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Abbreviations: LPS, lipopolysaccharide; iNOS, nitric oxide synthase; EGF, epidermal growth factor.

2. Materials and methods

2.1. Materials

[α-^{32}P]dCTP (6000 Ci/mmol) was from Amersham. Tissue culture dishes were from Costar or Falcon and culture media from Biowhitaker. Substrates, cytokines, enzymes and coenzymes were obtained from Boehringer, Sigma or Merck. Hormones were tested for endotoxin content that was below 1 ng/mg using the *Limulus polyphemus* test (Sigma).

2.2. Isolation of adult and fetal hepatocytes

Adult hepatocytes were prepared from 3-month-old male rats using a recirculating perfusion medium containing 1 mg/ml of collagenase H, and following a previous protocol [8]. Fetal hepatocytes were prepared from chopped livers of 21-day-old fetuses using a non-perfusion collagenase dispersion method that involved incubation 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) [14]. To deplete the preparation of Kupffer cells, the cells were kept for 1 h in Costar plastic dishes (4×10^5 cells/cm^2) and the non-adherent cells were transferred to collagen-coated Falcon dishes, 2 h in the presence of 10% FCS and 4 additional hours in phenol red- and serum-free DMEM.

2.3. Measurement of nitrites and nitrates

NO release was determined spectrophotometrically by the accumulation of nitrite and nitrate in the medium (phenol red-free) as follows: 250 μl of culture medium were transferred to 1.5 ml Eppendorf tubes and the nitrate was reduced to nitrite with 0.5 units of nitrate reductase (Boehringer) in the presence of 50 μM NADPH, 5 μM FAD [8]. The excess of NADPH was oxidized in the presence of 0.2 mM pyruvate and 1 μg of lactate dehydrogenase. Nitrite was determined with Griess reagent [15] by adding 1 mM sulfanilic acid and 100 mM HCl (final concentration). After incubation for 5 min the tubes were centrifuged and 200 μl of supernatant were transferred to a 96-well plate. After a first reading of the absorbance at 548 nm, 1 mM of naphthylenediamine was added and the absorbance at 548 nm was compared with a standard of $NaNO_2$.

2.4. RNA extraction and analysis

Total RNA ($3–4 \times 10^6$ cells) was extracted following the guanidinium thiocyanate method [16]. After electrophoresis in a 0.9% agarose gel containing 2% formaldehyde the RNA was transferred to Nytran membranes and the level of iNOS mRNA was determined using an *Eco*RI/*Hin*dIII fragment from the iNOS cDNA [8], labeled with [α-^{32}P]dCTP using the Random Primed Labeling Kit (Boehringer). Quantification of the films was performed by laser densitometry (molecular dynamics), using the hybridization with a β-actin probe (0.6 kb *Eco*RI/*Hin*dIII fragment isolated from a VC 18 vector) as an internal standard.

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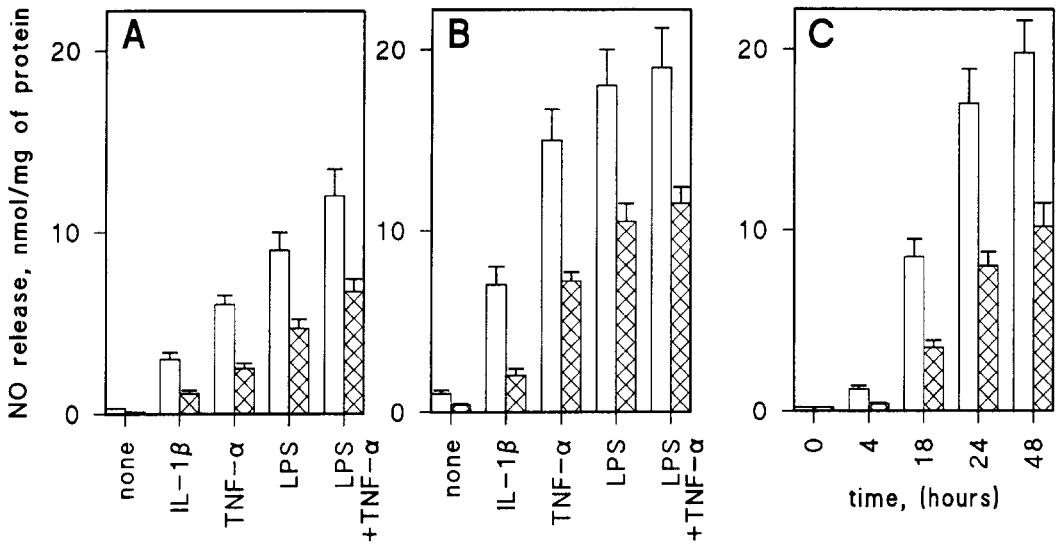

Fig. 1. EGF inhibits cytokine-dependent NO release. Primary cultures of fetal (A) or adult (B and C) hepatocytes were incubated with 10 ng/ml of IL-1β or TNF-α or 0.5 μg/ml of LPS in the absence (open bars) or presence (cross-hatched bars) of 10 nM EGF and the NO release was measured after 24 h. The time course for NO release in cells treated with TNF-α is shown in C. Results show the mean ± S.E.M. of 3 cell cultures.

2.5. iNOS enzyme activity assay

iNOS activity was measured after partial purification by 2′,5′-ADP-Sepharose chromatography and was followed by the production of [U-^{14}C]citrulline from [U-^{14}C]arginine [17]. The reaction mixture contained 50 μM [U-^{14}C]arginine (0.3 μCi), 20 mM HEPES (pH 7.5), 0.5 mM NADPH, 0.1 mM DTE, 10 μM 6*R*-5,6,7,8-tetrahydro-L-biopterin (BH_4, Dr. B. Schirck's Laboratories, Jona, CH) (200 μl of incubation volume). After 10 min of incubation, 1 ml of ice-cold 10 mM EGTA, 0.1 mM citrulline, 10 mM Pipes; pH 5.5 was added and 1 ml of this mixture was applied to a 1 ml Dowex AG50W-X8 column (Na^+ form). [U-^{14}C]Citrulline was eluted in 3 ml of water and the radioactivity was counted. The enzyme activity was expressed as the difference of product formation in the absence or presence of 0.25 mM N^G-methyl-L-arginine in the reaction mixture [8].

3. Results

3.1. EGF antagonizes NO release in hepatocytes treated with IL-1β, TNF-α or LPS

Because EGF receptors vary along development [5,18] we used fetal and adult hepatocytes to determine the effect of EGF on cytokine-dependent NO synthesis. Exposure of cultured 21-day-old fetal (Fig. 1A) or adult (Fig. 1B) hepatocytes to IL-1β, TNF-α or LPS promoted the release of NO-derived products (nitrites and to a lesser extent nitrates) after 24 h of culture. This effect was roughly twice in adult than in fetal hepatocytes under identical experimental conditions. When fetal or adult hepatocytes were incubated with EGF and then stimulated with these iNOS inducers, a blockage in the synthesis of NO was observed and the relative inhibitory effect exerted by EGF was independent of the developmental stage of the hepatocytes. Therefore, no differences between fetal and adult hepatocytes can be observed in the response to EGF in this experimental system; and because of the higher response of adult hepatocytes, these cells were used in the following experiments. Fig. 1C shows the time course for NO release in adult hepatocytes incubated with TNF-α as a representative cytokine, and the maximal rate of release was obtained between 18 and 24 h of incubation. The inhibitory effect exerted by EGF on this model was constant along this time, and similar results were obtained for IL-1β and LPS (not shown).

To study the inhibitory effect of EGF on the NO release elicited by stimulatory cytokines a titration was carried out using a constant saturant EGF concentration and increasing amounts of IL-1β or TNF-α. As Fig. 2 shows, EGF inhibited 85% and 60% of the NO synthesis elicited by concentrations below 2 ng/ml of IL-1β and TNF-α, respectively. However, concentrations of EGF up to 100 nM were unable to inhibit more than 60% of the NO release elicited by a saturant concentration of TNF-α, suggesting that several intracellular signaling pathways operate to promote iNOS expression in response to cytokines and only some of them are affected through EGF signaling.

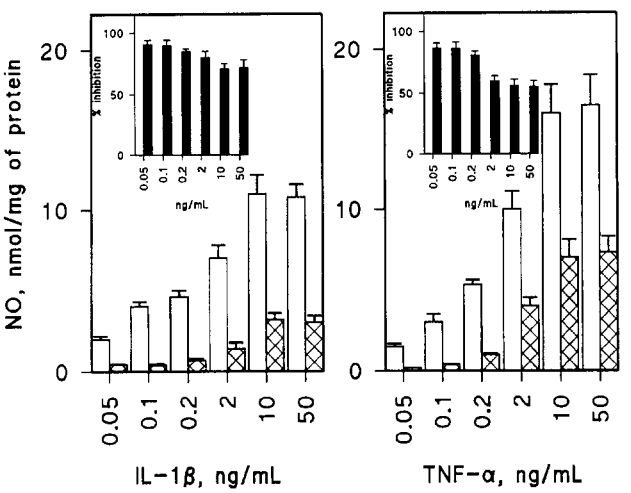

Fig. 2. Inhibition by EGF of NO release in cells treated with IL-1β or TNF-α. Adult hepatocytes were incubated in the absence (open bars) or presence (cross-hatched bars) of 10 nM EGF and the indicated cytokine concentration and the NO release were followed for a period of 24 h. Insets show the corresponding inhibition exerted by EGF for each panel. Results correspond to the mean ± S.E.M. of 3 experiments.

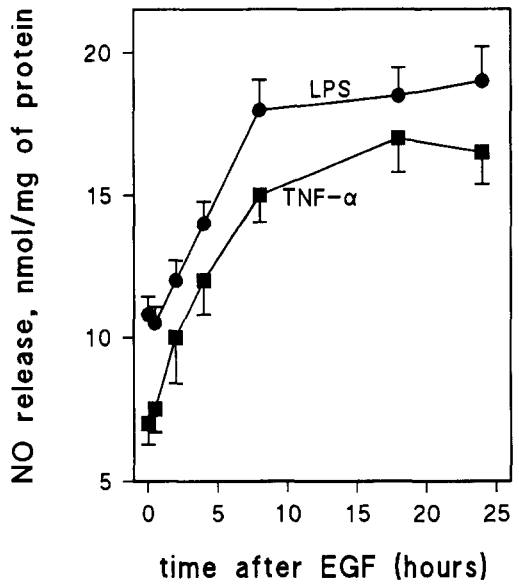

Fig. 3. Desensitization of EGF receptors in the inhibition of NO release. Hepatocytes were incubated with 10 nM EGF for the indicated periods followed by stimulation with TNF-α or LPS. The NO release during the following 24 h was measured. Results show the mean ± S.E.M. of 3 experiments.

Since EGF binding to the cell involves a rapid internalization of the membrane EGF receptors and a concomitant desensitization of the cell to this growth factor, hepatocytes were incubated for different times with EGF followed by triggering with TNF-α or LPS and the NO release was measured 24 h later. As Fig. 3 shows a time-dependent desensitization to the inhibitory effect of EGF was observed for the induction elicited by TNF-α or LPS revealing the necessity of a functional EGF receptor to mediate the inhibitory response. In addition to EGF, other cytokines relevant to hepatocyte function were tested. TGF-β resulted very effective in preventing iNOS induction by IL-1β, TNF-α and LPS, whereas moderate inhibitory effects were observed when cells were stimulated with IL-6 (Table 1). Therefore, the cooperative response to combinations of EGF with TGF-β or IL-6 might provide mechanisms to improve iNOS inhibition by these cytokines.

3.2. EGF antagonizes iNOS expression in response to cytokines

To ensure that the inhibitory effect exerted by EGF on the cytokine induction of iNOS was due to a decreased expression of this enzyme, mRNA levels and enzyme activity were measured in hepatocytes. As Fig. 4 shows, decreased mRNA levels of iNOS were observed in the response to cytokines in cells treated with EGF. Accordingly, a lower iNOS activity was present in cells incubated with EGF and then stimulated with IL-1β, TNF-α or LPS, in agreement with data on NO release to the medium.

Table 1
Modulation of NO release by TGF-β and IL-6

Addition	IL-1β (10 ng/ml)	TNF-α (10 ng/ml)	LPS (0.5 μg/ml)
None	10.5 ± 0.8	16.3 ± 1.0	18.7 ± 1.4
TGF-β, 10 ng/ml	2.6 ± 0.3	5.8 ± 0.7	11.6 ± 0.9
+ EGF, 10 ng/ml	0.2	1.1 ± 0.1	2.1 ± 0.2
IL-6, 10 ng/ml	6.2 ± 0.5	8.8 ± 0.7	14.2 ± 1.1
+ EGF, 10 ng/ml	1.5 ± 0.1	1.7 ± 0.2	4.8 ± 0.3

Cultured hepatocytes were incubated for 24 h with the indicated cytokines, and the release of NO was measured. Results show the means ± S.E.M. of 3 cell preparations.

4. Discussion

The ability of hepatocytes to express iNOS in response to cytokines is now well accepted, despite the controversy that existed in previous reports [7,8,19]. The synthesis of NO in regenerating liver after partial hepatectomy or in the liver of animals suffering septic shock is probably the result of the concerted action of various cytokines, among them TNF-α, that are released during the early steps following liver injury [1,20,21]. However, this release of NO is important in the first 24 h of the regenerative response, and both NO synthesis and iNOS expression rapidly decrease after this period to be completely absent in normal liver [7,8]. Therefore, NO release in liver is restricted to events associated with commitment for cell growth, and preliminary experiments suggest that in its absence the distribution of cells in the cell cycle is affected [8].

Our results clearly show that EGF efficiently counteracts the iNOS expression elicited by IL-1β, and to a lower extent than that triggered by TNF-α and LPS. Indeed, these results also provide information regarding the complexity of the molecular mechanisms that operate to promote iNOS expression, suggesting that cytokines such as TNF-α and bacterial products such as LPS recruit several different signaling pathways in their mechanism of action, only part of them blocked by EGF signaling. Alternatively, the partial inhibition of EGF might be due to the time-limited EGF signaling because of the rapid internalization and degradation of the ligand-receptor complex of this growth factor. In addition, the coordinated action of EGF with

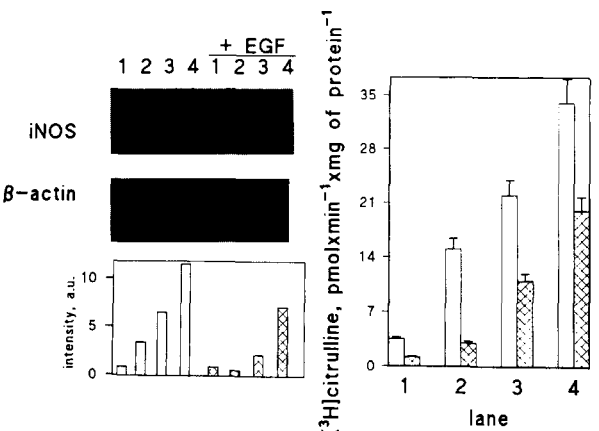

Fig. 4. EGF inhibits iNOS expression by cytokines. Primary cultures of hepatocytes were incubated for 6 h in the absence or presence of 10 nM EGF and 10 ng/ml of IL-1β (lane 2), TNF-α (lane 3) or 500 ng/ml of LPS (lane 4), and the mRNA was analyzed by Northern blot to determine iNOS expression (in arbitrary units, bottom panel). Alternatively, iNOS activity was determined in cell cultures after 24 h of incubation with these cytokines in the absence (open bars) or presence (cross-hatched bars) of EGF (right panel). Results show a representative experiment (Northern blot) or the mean ± S.E.M. of 3 experiments (enzyme activity).

other cytokines that inhibit iNOS expression provides alternative mechanisms to reinforce iNOS expression blockage since distinct intracellular signaling pathways are involved. This is the case for TGF-β that has ben reported as an inhibitor of iNOS expression in various cell types such as macrophages [22,23].

Finally, the regulation of iNOS expression could be controlled by the balance between cytokines that induce and repress its synthesis. According to this view, the fact that liver expresses an important number of receptors for EGF could be pharmacologically exploited to specifically repress or attenuate liver NO production under pathological conditions (i.e. septic shock).

Acknowledgements: The authors thank O.G. Bodelón and E. Lundin for technical help, and Dr Q.-w. Xie and C.F. Nathan for the generous gift of the iNOS probe. M.C. was a recipient of a fellowship from the Spanish Ministry of Science and Education. This work was supported by Grant PB92-070 from CICYT, Spain.

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