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Involvement of nitric oxide synthesis in hepatic perturbations induced in rats by a necrogenic dose of thioacetamide

Carmen Díez-Fernández, Nuria Sanz, Lisardo Boscá, Sonsoles Hortelano & [1]María Cascales

Instituto de Bioquímica (CSIC-UCM), Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain

1 The biological actions of nitric oxide (NO), a highly diffusible and short-lived radical, range from signal transduction to cytotoxicity. The present study investigated whether NO is released in the course of liver necrosis and regeneration induced by a single necrogenic dose of thioacetamide (6.6 mmol kg^{-1} body wt) to rats. Samples of liver were obtained at 0, 3, 12, 24, 48, 72 and 96 h after thioacetamide administration.

2 Inducible nitric oxide synthase (iNOS) activity was determined in purified liver homogenates and a sharp 6 fold increase ($P<0.001$) in iNOS activity was recorded at 48 h of intoxication, followed by a slight but progressive increase at 72 and 96 h. Changes in the expression of iNOS, as detected by its mRNA levels, were parallel to the NOS enzyme activity. Hepatocyte NO synthesis showed a progressive increase at 24, 48 and 72 h, to 8 ($P<0.001$), 13 ($P<0.001$) and 13 ($P<0.001$) times the initial values, respectively.

3 In isolated Kupffer cells, where initial NO release was ten fold higher than in hepatocytes, a progressive increase was detected from 48 h which reached two fold of initial at 72 h of intoxication (192%, $P<0.001$). Hepatic cyclic GMP concentration did not change significantly. However, mitochondrial aconitase activity decreased markedly at 12 and 24 h of intoxication showing a sharp increase towards normal values at 48 h which was maintained at 72 and 96 h.

4 As protein kinase C (PKC) is one of the likely candidates to mediate iNOS expression, translocation (activation) of PKC was assayed in hepatocytes, and a significant two fold increase ($P<0.001$) between 48 and 96 h after thioacetamide intoxication was observed. When peritoneal macrophages from control rats were incubated with serum from thioacetamide-treated rats, a sharp increase in NO release was detected with serum obtained at 48 h, reaching at 96 h a value four fold ($P<0.001$) that of the control.

5 These results suggest that iNOS activity and NO release play a role in the pathophysiological mechanisms that trigger post-necrotic hepatocellular regeneration following thioacetamide administration.

Keywords: Nitric oxide; nitric oxide synthase; thioacetamide; necrosis; regeneration

Introduction

There is an increasing interest in the field of pharmacology in the ability of mammalian liver to grow after the loss of its own cells due to the action of hepatotoxic substances. The proliferative response immediately following experimentally induced cytotoxicity provides a useful model to study the phenomenon of cell proliferation in liver and to consider the factors which trigger and stop cell division in response to chemical attack (Mehendale, 1991; Díez-Fernández *et al*., 1993; Mehendale *et al*., 1994; Cascales *et al*., 1994; Steer, 1995; Mangipudy *et al*., 1995). In the regulation of the post-necrotic regenerating process, a multistep chain of events is involved, in which reactive oxygen species, growth factors and cytokines play an important role in the transition from the quiescent state of adult hepatocytes to the proliferative state of regenerating cells (Braun *et al*., 1988; Mehendale, 1991; Mehendale *et al*., 1994).

The acute liver injury induced by necrogenic doses of thioacetamide is characterized by a severe perivenous necrosis immediately followed by hepatocellular regeneration (Díez-Fernández *et al*., 1993; Mangipudy *et al*., 1995). The biotransformation of this hepatotoxic agent by the microsomal FAD-mono-oxygenase system (Dyroff & Neal, 1983; Chieli & Mavaldi, 1984) is followed by oxidative stress, lipid peroxidation and increases in cytosolic calcium (Díez-Fernández *et al*., 1996). Necrosis develops as a consequence of the biotransformation of the drug. In this process, reactive oxygen species are generated (Arthur *et al*., 1985), together with the release of growth factors and cytokines, either by the hepatocytes or by Kupffer, endothelial and fat storing cells. Growth factors and cytokines may potentially induce nitric oxide synthase (NOS) expression in different cell types, e.g. macrophages. The accumulation of activated macrophages in the liver amplify the inflammatory response contributing to hepatic necrosis induced by hepatotoxic drugs (Shiratori *et al*., 1988; Laskin, 1991, 1992; Laskin *et al*., 1995; Ródenas *et al*., 1995).

Recent evidence suggests that nitric oxide (NO) is an important regulator of some liver functions (Milbourne & Bygrave, 1995). The liver as a venous organ is able to store a significant volume of blood that can be released into the circulatory system in stress conditions. This vascular control has been shown to be strongly influenced by NO (Moy *et al*., 1992). Moreover, the liver has a high potential to produce NO which appears to be involved, not only in hepatocellular functions, but also in liver immunological damage and inflammation.

During liver regeneration, a role for NO, released by the action of NOS, has been proposed by several authors (Obolenskaya *et al*., 1994a,b; Decker & Obolenskaya, 1995; Hortelano *et al*., 1995) and NO is implicated in the shift between the quiescent and the proliferative status. Protein kinase C (PKC) is activated in the course of liver regeneration and proliferating cells exhibit a higher particulate-to-cytosolic PKC activity ratio than quiescent cells (Houweling *et al*., 1989; Alessenko *et al*., 1992). Moreover, PKC has been shown to be involved in the induction of NOS activity in rat hepatocytes treated with phorbol esters (Hortelano *et al*., 1992).

[1]Author for correspondence at: Instituto de Bioquimíca (CSIC-UCM), Facultad de Farmacia, Universidad Complutense, Plaza Ramón y Cajal sn, 28040 Madrid, Spain.

The purpose of the present study was to investigate the potential role of NO in the pathophysiological response of the liver to hepatotoxins. A study was therefore performed to examine the involvement of NO release in the processes of liver injury and of post-necrotic regeneration induced by the administration to rats of a single necrogenic dose of thioacetamide (TAM).

Methods

Animal treatment and processing of the samples

Adult male Wistar rats (180 to 220 g), supplied with food (SanderSA, Barcelona) and water *ad libitum* and exposed to a 12 h light-dark cycle (starting at 08 h 00 min), were given intraperitoneally a single dose of thioacetamide (6.6 mmol kg^{-1} body wt) freshly dissolved in 0.9% NaCl. This dose was chosen on the basis of previous studies showing significant increases in serum enzymes: aspartate and alanine aminotransferases and severe centrilobular necrosis evidenced by histopathological study (Díez-Fernández *et al*., 1993; 1996). Control rats received 0.5 ml of 0.9% NaCl (Díez-Fernández *et al*., 1993). Rats were killed by cervical dislocation at 0, 12, 24, 48, 72 and 96 h following treatment and samples were obtained. Blood was collected from the heart and kept at 4°C for 24 h, centrifuged at 3,000 r.p.m. for 5 min and serum was obtained as the supernatant, as described previously (Díez-Fernández *et al*., 1993). Liver samples were quickly freeze-clamped *in situ*, by use of stainless tongs cooled in liquid nitrogen, and kept at −80°C until processed. Each experiment was performed following the international criteria for the use and care of experimental animals research outlined in the 'Guide for the Care and Use of Laboratory Animals' published by the National Institute of Health (N.I.H., 1985).

Isolation and culture of hepatocytes and Kupffer cells

Hepatocytes and Kupffer cells were isolated from thioacetamide-treated animals by the collagenase perfusion technique as previously described (Seglen, 1993; Díez-Fernández *et al*., 1996). An hepatocyte-enriched population was obtained by differential centrifugation (40 g for 2 min, three times), whereas centrifugation of the supernatants at 200 *g* yielded an enriched population of non-parenchymal cells (Kupffer cells, lipocytes and endothelial cells). The yield of hepatocytes from thioacetamide-treated animals at maximum necrosis was approximately 50% when compared to control. The viability of hepatocytes was detected by trypan blue exclusion (<90%), and the purity determined under phase contrast light microscopy was 95%. Kupffer cells were purified from the enriched population of non parenchymal cells by density gradient fractionation and selective substrate adherence following the method described by Friedman (1993). Briefly, density gradient fractionation by larex (arabino galactan) was obtained by centrifuging the gradients at 20,000 r.p.m. for 25 min at 25°C. Lipocytes were eliminated from the top (6% larex) and Kupffer and endothelial cells were obtained from the 8–12% and 12–15% larex gradient interphases. The selective adherence method was immediately applied following Friedman (1993) because of the more adherent nature of Kupffer cells when compared to endothelial cells. The purity of the Kupffer cell fraction was recognizable under phase contrast light microscopy by the 'fried egg' appearance of these cells and proved to be 93%. Cells were seeded at similar densities and cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of foetal calf serum (FCS) (Hortelano *et al*., 1995).

Preparation and culture of macrophages

Macrophages were obtained from the peritoneal cavity of control rats and seeded on plastic dishes at $1 \times 10^6/cm^2$. Experiments were carried out in phenol-red free DMEM supplemented with 0.5 mM arginine and 10% FCS (Hortelano *et al*., 1995).

Enzyme assays

Mitochondrial aconitase activity was measured spectrophotometrically at 340 nm, in the presence of 20 mM citrate, 0.5 mM NADP, 0.5 mM $MnCl_2$, 50 mM Tris HCl (pH 7.4) and 1 unit of isocitrate dehydrogenase (Rose & O'Connel, 1967). Protein kinase C activity was assayed in cytosolic and particulate fractions from hepatocytes after purification through a DE52 column with histone H1 as substrate (Kikkawa *et al*., 1982). One unit of PKC activity was defined as the incorporation of 1 pmol of phosphate into histone H1 min^{-1}. Nitric oxide synthase activity was measured after partial purification by 2′,5′-ADP-sepharose chromatography following the production of [U-^{14}C]-citrulline from [U-^{14}C]-arginine. One unit of NOS activity was defined as the amount of protein that released 1 nmol of [U-^{14}C]-citrulline min^{-1} (Bredt & Snyder, 1990; Yui *et al*., 1991; Schmidt *et al*., 1992).

mRNA analysis of iNOS

Total RNA was extracted from pieces of liver by the guanidinium isothiocyanate method (Chirgwin *et al*., 1979) and analysed by Northern blotting. iNOS mRNA levels were detected with an 817 bp fragment from the cDNA of macrophage NOS (Xie *et al*., 1992). The membranes were exposed to Hyperfilm-M and the bands were quantified by laser densitometry (Molecular Dynamics) by use of the hybridization with a 18S ribosomal probe as an internal control.

Other assays

Guanosine 3′:5′-cyclic monophosphate (cyclic GMP) was assayed in liver samples by a specific binding kit, following the recommendations of the supplier (Amersham). The concentration of NO in cultured cells was detected by the accumulation of nitrite and nitrate (total NO), as follows: 200 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 in the presence of 50 μM NADPH, 5 μM FAD. In order to eliminate any interference, excess NADPH was oxidized in the presence of 0.2 mM pyruvate and 1 μg of lactate dehydrogenase. Nitrites were quantified with the Griess reagent (Green *et al*., 1982) measuring the absorbance at 548 nm. In order to detect the presence of cytokines, turnover necrosis factor α (TNFα) levels were determined in serum from thioacetamide-treated rats. TNFα specific bioassay was carried out following the instructions in the Amersham kit. Protein was measured according to the method of Bradford (1975) with bovine serum albumin as standard.

Chemicals

[U-^{14}C]-arginine was from New England Nuclear (Boston, MA). Adenosine 2′,5′-bisphosphate-sepharose was from Pharmacia (Uppsala, Sweden). Dowex AG50W-X8 (Na^+-form) was from Bio-Rad (Richmond, CA). Nitrate reductase from *Aspergillus* and other enzymes were from Boehringer (Mannheim, FRG). Tetrahydrobiopterine (BH_4) was from Dr B. Schircks Laboratories (Jona, Switzerland). [α-^{32}P]-dCTP and [γ-^{32}P]-ATP were from Amersham. Arginine derivatives and other chemicals and biochemicals were from Sigma (St. Louis, MO) or Merck (Darmstadt, FRG). Tumour necrosis factor α [(n)TNFα]ELISA system was from Amersham.

Statistical analysis

Values were obtained as the mean ± s.d. of four different experimental observations (four animals). Student's *t* test was performed for statistical evaluations, and the statistical significance considered as $P < 0.001$.

Results

NOS activity in liver of thioacetamide treated rats

To determine whether the administration of thioacetamide could affect NOS activity, samples of liver were homogenized and an aliquot was partially purified through a 2′,5′ ADP-sepharose column, and after elution assayed for the presence of NOS activity in the absence of Ca^{2+} and calmodulin. Figure 1 shows that the increase in hepatic NOS activity, measured by the formation of [U-^{14}C]-citrulline from [U-^{14}C]-arginine, began at 24 h, reaching at 48 h of intoxication, 600% ($P<0.001$) of the control value. A progressive increase at 72 and 96 h, to 690% ($P<0.001$) and 760% ($P<0.001$), respectively, was detected (Figure 1). The time course of these values indicates that NOS activity does not change during liver necrosis but its activity increases during post-necrotic regeneration. As NOS activity was evaluated in samples of whole liver, hepatocytes, non-parenchymal cells as well as bone marrow derived macrophages that infiltrate the liver as injury develops, were present.

Analysis of the inducible m-NOS RNA after thioacetamide treatment

To confirm that the isoenzyme of NOS was the inducible form, we determined the levels of mRNA by use of a cDNA probe specific for the iNOS of macrophages (Xie *et al*., 1992). iNOS mRNA levels were determined at several time points after thioacetamide administration. Northern blot analysis of hepatic RNA from thioacetamide-treated and control animals revealed the presence of a unique species of 4.4 kb in the thioacetamide-treated animals at 48–72 h that was absent in the other samples (Figure 2). These results are parallel to and consistent with the values of NOS activity described above.

Time course of NO release in hepatocytes and Kupffer cells isolated from thioacetamide-treated animals

Hepatocytes and Kupffer cells were separately obtained from the liver of thioacetamide-treated rats in order to evaluate the relative contribution of both types of cells to NO release. Figure 3 shows the pattern of NO release both in hepatocytes and Kupffer cells obtained from animals following thioacetamide treatment. In hepatocytes the initial NO release was very low and a sharp and significant 8 fold increase ($P<0.001$) was

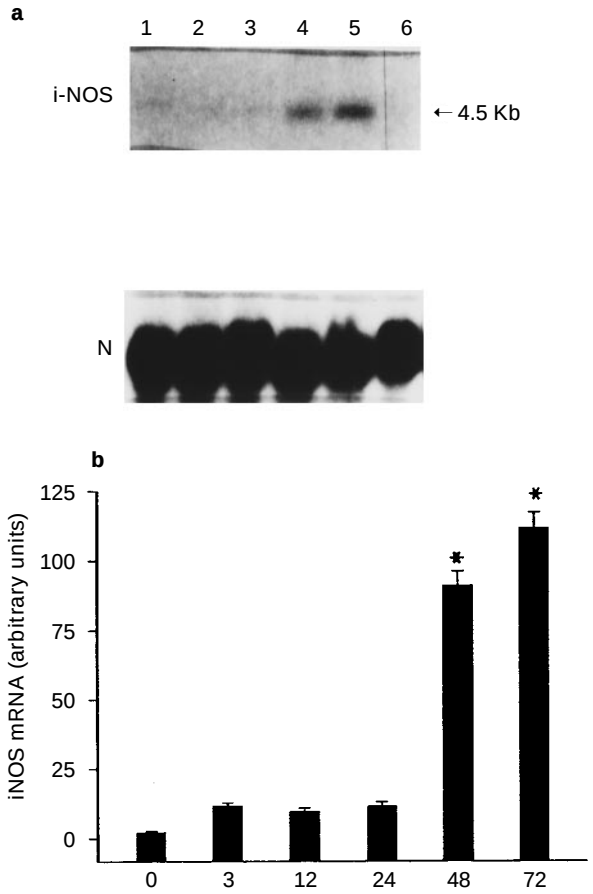


Figure 2 Northern blot analysis of inducible nitric oxide (NOS) synthase mRNA in liver from animals after thioacetamide treatment. (a) Total RNA (25 μg) from control (lane 6) and 3, 12, 24, 48 and 72 h after thioacetamide injection (lanes 1 to 5, respectively) were size fractionated in 0.9% agarose gel and blotted onto a Nytran membrane. The membrane was hybridized with an inducible NOS probe and with an 18S ribosomal probe (N) to normalize the charge of the lane. (b) The densitometric analysis of the bands is shown. *$P<0.001$.

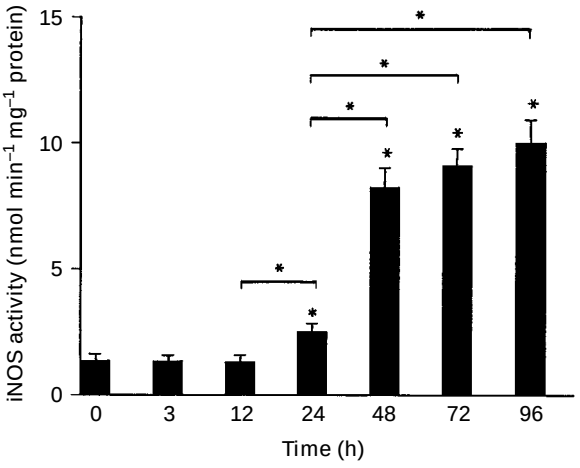


Figure 1 Determination of nitric oxide synthase (NOS) during necrosis and regeneration of liver. Samples of liver, obtained from rats at 0, 3, 12, 24, 48, 72 and 96 h after thioacetamide treatment (6.6 mmol kg^{-1}), were homogenized and NOS activity was measured after partial purification by 2′-5′-ADP-sepharose chromatography, by the release of [U-^{14}C]-citrulline from [U-^{14}C]-arginine. All reactions were carried out at 30°C. Results are given as nmol min^{-1} mg^{-1} protein and are the means±s.d. of four separate observations. *$P<0.001$.

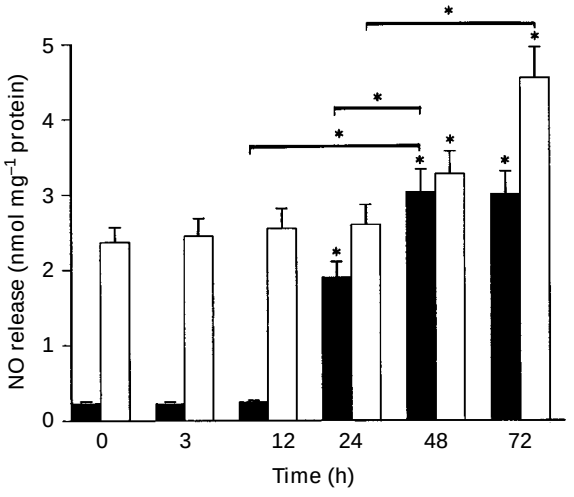


Figure 3 Time course of NO release by hepatocytes (solid columns) and Kupffer cells (open columns) from thioacetamide-treated rats. Hepatocytes and Kupffer cells were isolated from liver of rats at 0, 3, 12, 24, 48 and 72 h of thioacetamide administration (6.6 mmol kg^{-1} body weight) and maintained in culture for 24 h. The concentration of nitrite released into the medium was measured following the method of Griess (Green *et al*., 1982). Results, expressed as nmol of NO mg^{-1} protein, are the means±s.d. of four independent experiments (four rats). *$P<0.001$.

observed at 24 h of intoxication. At 48 h and 72 h the production of NO progressively increased to 13 ($P<0.001$) and 13 fold ($P<0.001$), respectively. In Kupffer cells NO release values were initially ten fold higher than in hepatocytes, and at 48 and 72 h an increase to 143% and 191% ($P<0.001$) of control was observed. The smaller increase of NO release in Kupffer cells, only two fold when compared to hepatocytes, can be explained by the effect of collagenase perfusion which activates Kupffer cells leading to a very high initial NO release.

NO release in hepatocytes and in ex vivo *intraperitoneal macrophages from control rats after incubation with serum from thioacetamide-treated rats*

The presence in the serum of cytokines and growth factors have been detected immediately following necrosis (Fausto *et al*., 1995; Webber *et al*., 1994). Some of these factors are characterized by a potential ability to induce iNOS in certain cells, such as macrophages (Moncada *et al*., 1991). Accordingly, the ability of serum collected at 0, 12, 24, 48, 72 and 96 h after thioacetamide injection to induce the expression of NOS and NO release was assayed *ex vivo* in primary cultures, either of peritoneal macrophages or hepatocytes from control rats. As Figure 4 shows, when macrophages were incubated in the presence of serum obtained from rats after thioacetamide-treatment, significant time-dependent changes in NO release, versus macrophages incubated with serum of untreated rats, were observed. Minimum values for the production of nitrites in the medium were detected with samples of serum obtained at 24 h post-treatment (12% of control, $P<0.001$) and significant increases were detected at 48, 72 and 96 h, 120%, 310% ($P<0.001$) and 390% ($P<0.001$) of control, respectively. When hepatocytes from control rats were incubated in the presence of serum from rats following thioacetamide administration, significant changes in NO release were also observed. These changes were parallel to those obtained in cultures of peritoneal macrophages, but the values were one order of magnitude lower. A progressive increase in NO release was detected with serum obtained after 48 h of intoxication reaching, at 72 and 96 h, values significantly higher when compared to the values obtained with serum from untreated rats (Figure 4).

The NO-dependent cyclic GMP synthesis and aconitase activity

Considering that NO production activates the soluble form of guanylate cyclase (Schneider *et al*., 1994), and inhibits mitochondrial aconitase (Hausladen & Fridovich 1994), experiments were performed in order to measure both the hepatic concentration of cyclic GMP and the mitochondrial activity of aconitase (Figure 5). Cyclic GMP concentrations were very close to control, with no significant variations throughout the intoxication process. These results are in agreement with those obtained by other authors (Billiar *et al*., 1992) who have described an efflux of cyclic GMP from the cell to the extracellular medium. This special permeability of the membrane to cyclic GMP would explain the low levels of cyclic GMP found in our system. Mitochondrial aconitase, a dehydratase whose [4Fe-4S] prosthetic group is inactivated by superoxide and peroxynitrite (Hausladen & Fridovich, 1994; Castro *et al*., 1994) was also determined. The results in Figure 5 show that aconitase activity decreased significantly at 12 and 24 h to 63% and 51% ($P<0.001$) respectively and increased thereafter towards control values.

TNFα levels in serum from thioacetamide-treated rats

TNFα concentration was determined in serum of thioacetamide-treated rats at 0, 3, 12, 24, 48, 72 and 96 h after intoxication. The basal levels of TNFα in serum of control rats were undetectable. However, 3 h after thioacetamide administration, TNFα appeared (3.1 ± 0.3 pg ml^{-1}), at 12 h was increased (57.0 ± 5.0 pg ml^{-1}) 18 fold compared to the level at 3 h, and at 24 h decreased to very low levels. A second peak was detected at 48 h (40.0 ± 5.0 pg ml^{-1}) and at 72 h a sharp decrease was again registered. At 96 h the level of TNFα was undetectable. These biphasic changes indicate that TNFα expression is initiated early after intoxication and the highest concentration precedes the time of maximum necrosis. The second peak at 48 h parallels the peak of regeneration (DNA replication). These two waves of TNFα in serum, the first preceding necrosis and the second coinciding with DNA replication, are not reflected in NO stimulation. Further experiments should be done while inhibiting NO release to clarify these results.

Protein kinase C activity in hepatocytes isolated from rats after thioacetamide administration

Since NOS is induced in cultured hepatocytes by phorbol esters, the specific activator of PKC, the time course of PKC activity was assayed in samples of hepatocytes after thioace-

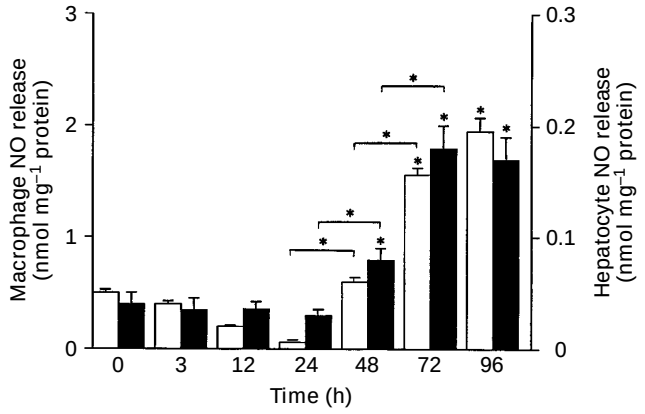


Figure 4 NO release in hepatocytes (solid columns) and in peritoneal macrophages (open columns) incubated in the presence of serum from thioacetamide-treated rats. Serum obtained from thioacetamide-treated rats (6.6 mmol kg^{-1} body weight), at 0, 3, 12. 24, 48, 72 and 96 h of intoxication, were added (10% v:v) to primary cultures of hepatocytes or peritoneal macrophages from control animals and incubated for 24 h. The release of NO was measured by the Griess method (Green *et al*., 1982). Results, expressed as nmol of NO mg^{-1} protein, show the means$\pm$s.d. of four separate experimental observations. *$P<0.001$.

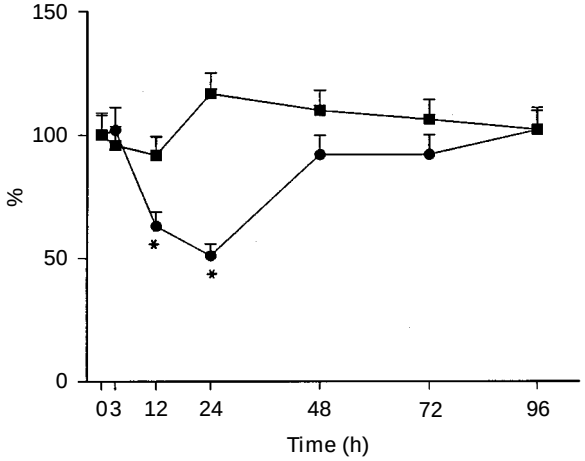
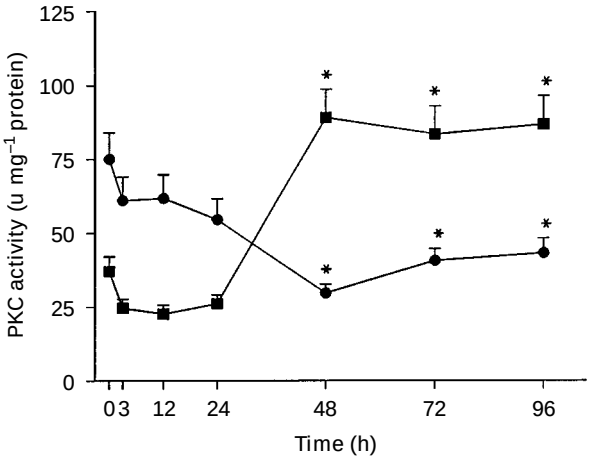


Figure 5 Cyclic GMP concentration and aconitase activity in livers of thioacetamide-treated rats. The concentration of cyclic GMP was measured in samples of rat liver obtained at 0, 3, 12, 24, 48, 72 and 96 h after injection of thioacetamide (6.6 mmol kg^{-1} body weight). Results for cyclic GMP (■) and for mitochondrial aconitase (●) are expressed as percentages of the control value (0.48 ± 0.06 pmol g^{-1} liver for cyclic GMP and 21.52 ± 3 nmol of substrate transformed min^{-1} mg^{-1} protein for aconitase activity). Values are the means$\pm$s.d. of four experimental observations. *$P<0.001$.



pFigure 6 Protein kinase C (PKC) activity in hepatocytes isolated from thioacetamide-treated rats. PKC activity was measured in hepatocytes isolated from thioacetamide-treated rats (6.6 mmol kg^{-1} body weight) at 0, 3, 12, 24, 48, 72 and 96 h of intoxication, after homogenization and centrifugation to separate the soluble (●) and particulate (■) fractions. One unit of PKC was defined as that incorporating 1 pmol phosphate into histone H1 min^{-1}. Results are means ± s.d. of four independent cell preparations. $*P<0.001$.

tamide administration. The activation of PKC results in changes in its subcellular localization between soluble and membrane bound forms. Figure 6 shows that particulated PKC increased significantly at 48, 72 and 96 h with values 240% ($P<0.001$), 225% ($P<0.001$) and 235% ($P<0.001$) versus control, respectively. Soluble activity decreased to a minimum, at 48 h, of 39% ($P<0.001$) of control value.

Discussion

Chemical models of liver injury are well established areas of research of considerable interest both at pathophysiological and pharmacological levels (Steer, 1995; Mehendale *et al*., 1994; Díez-Fernández *et al*., 1993) to study cellular mechanisms responsible for initiation of hepatotoxicity and for recovery from toxic injury. In this respect, thioacetamide was used experimentally to induce liver necrosis, regeneration and functional recovery. In our earlier experiments, when thioacetamide was administered to rats, acute injury developed in a process culminating in necrotic cell death that peaked at 24 h of intoxication, followed immediately by a series of responses that triggered cell DNA replication and cell division, at 48 h, to restore initial liver cellularity and functionality at 96 h (Díez-Fernández *et al*., 1993; 1996). The degree of liver necrosis was evaluated by serum alanine aminotransferase activity and liver regeneration by the flow cytometric analysis of the hepatocyte population involved in DNA synthesis and by calculating the mitotic index on liver slices under light microscopy (Díez-Fernández *et al*., 1993). Treatment of rats with sublethal doses of hepatotoxins is associated with an extensive mononuclear cell infiltration in the liver (Laskin, 1990, 1992). Macrophages migrate to liver attracted by chemotactic factors released by the injured hepatocytes and activated by inflammatory stimuli, and products released both by parenchymal and non-parenchymal cells. It has been proposed that the release of cytotoxic and proinflammatory mediators from macrophages amplifies the inflammatory response and contributes to hepatic necrosis induced by hepatotoxins. Kupffer cells, the resident macrophages in the liver, when activated, promote hepatic damage through the release of toxic secretory products and reactive oxygen species (Shiratori *et al*., 1988; Laskin, 1990; 1992; Castro *et al*., 1994; Laskin *et al*., 1995; Ródenas *et al*., 1995).

Arginine-dependent NO formation, catalysed by NOS, plays a key role in many biological systems. Reactive oxygen species and some cytokines, produced in the inflammatory processes, are good promoters of the expression of the inducible form of NOS (Ding *et al*., 1988; Spitzer, 1994; Ródenas *et al*., 1995). Opinions differ among research groups concerning the ability of hepatocytes to express inducible NOS. Thus, some studies, by use of the endotoxin model of NOS induction, showed that this ability occurs in the hepatic non-parenchymal cells (Kupffer and endothelial cells), whereas other studies demonstrated that the induction of NOS occurs also in hepatocytes (Billiar *et al*., 1990a,b; Knowles *et al*., 1990; Hortelano *et al*., 1995). However, from our results in post-necrotic liver and from the results obtained by other authors in post-hepatectomized liver, it can be proposed that the presence of cytokines, such as TNFα in serum, can induce iNOS mRNA expression and NOS activity in hepatocytes, with no requirement of macrophages for the process (Geller *et al*., 1993; Spitzer, 1994; Wang & Liu, 1995). Bearing in mind these data, it is possible to suggest that, in our experimental model of thioacetamide-induced liver damage, the response of Kupffer and parenchymal cells is cooperative. In this cooperation the Kupffer cells, through the release of TNFα, may stimulate NOS expression in hepatocytes (Decker & Obolenskaya, 1995), as well as positively modulate liver regeneration (Akerman *et al*., 1992). On the other hand, cytotoxic effects of TNFα are partially mediated by means of an autocrine enhanced generation of reactive oxygen species by activated Kupffer cells (Hortelano, 1995).

Our results showed that hepatic NOS activity increases sharply parallel to post-necrotic liver regeneration and that NO is released either by hepatocytes or Kupffer cells isolated from regenerating liver and cultured independently. Both hepatic NOS activity and iNOS mRNA levels were detected in the post necrotic regenerating liver, indicating that this is a local effect. However, the degree of contribution of Kupffer cells and hepatocytes to the total NOS activity and NO synthesis was different, since in normal conditions Kupffer cells, although less abundant than hepatocytes, produced about one order of magnitude more NO than the hepatocytes. This initial difference could be due to the effect of collagenase perfusion activating Kupffer cells, since when these cells were cultured independently for 24 h, the difference disappeared. In either case, NO may easily diffuse through the cells and the type of cell where this molecule is generated does not affect its ability to induce changes in the neighbouring cells.

On consideration that mechanisms may operate in extrahepatic tissues, in the present study we investigated NOS induction by peritoneal macrophages isolated from control rats incubated in the presence of serum obtained at different time points from thioacetamide-treated animals. These macrophages, when cultured *ex vivo*, developed NOS expression that released enormous amounts of NO, in the range of that observed from macrophages obtained from lipopolysaccharide (LPS)-treated animals (Nathan & Hibbs, 1991). By measuring nitrite release in the culture medium, we tested the time-course of the ability of the rat serum, collected following thioacetamide-treatment, to induce iNOS in cultures of macrophages. The amount of NO produced was dependent on the time of sampling the serum. Spontaneous production of NO by cultured peritoneal macrophages progressively decreased when incubated in the presence of serum obtained from animals at 3 and 12 h of intoxication reaching, at 24 h, a value of 12% vs control. Similar results, although one order of magnitude lower, were obtained with isolated hepatocytes instead of peritoneal macrophages, which indicates that hepatocytes are able to induce NOS in response to cytokines. These results suggest that NO release could also be involved in hepatocyte cell injury since it can react with the superoxide anion and generate peroxynitrite, a highly reactive species that can be a potent endogenous inhibitor of NO (Gryglewski *et al*., 1986). Peroxinitrite is also involved in inactivating aconitase and in mobilizing mitochondrial calcium with a parallel and sustained deenergization of mitochondria which causes cell death (Castro *et al*., 1994; Richter *et al*., 1994). The serum obtained at 48,

72 and 96 h after intoxication was especially potent in inducing NOS and NO release in macrophage or hepatocyte cultures, suggesting that the cytokines or growth factors involved in the expression of iNOS were present in the serum of thioacetamide-treated rats after 48 h, coinciding with hepatocellular regeneration.

With respect to the events involved in the proliferation of liver cells and the restoration of hepatic function, it has been shown that, following the loss of liver cells, several changes occur in the subcellular distribution of protein kinase C. It has been shown that exposure of cultures of isolated hepatocytes to phorbol esters, the pharmacological activators of protein kinase C, promoted the expression of iNOS and the release of NO in the culture medium (Hortelano *et al*., 1992). PKC is involved in the induction of NOS and can down-regulate the activity of NOS by direct phosphorylation of the enzyme at different serine sites (Bredt *et al*., 1992). However, NO, when overproduced, can inhibit both PKC and NOS, acting as a negative feedback modulator of its synthesis. PKC, in addition to its activation by Ca^{2+}, diacylglycerol and arachidonic acid (Nishizuka, 1992), may also be activated by modifications induced by oxidants (Gopalakrisna *et al*., 1993). The changes in PKC distribution between soluble and particulate fractions were measured in liver of thioacetamide-treated rats, and the redistribution of PKC showed an increase in the activity of the particulate fraction at 48–96 h as a result of the translocation from the cytosol to the membranes.

Some studies suggest that reactive nitrogen intermediates induce tissue injury, while others support the concept that NO release plays a hepatoprotective role. Furthermore, it has been suggested that, since NO release can exert either protective or regulatory functions in the cells at low concentrations (Hortelano *et al*., 1992; Gopalakrisna *et al*., 1993) or toxic effects at higher concentrations, its synthesis by NOS is tightly regulated. Our results led us to assign a specific role to NO release in hepatocytes in the post-necrotic regeneration induced by xenobiotics. From our point of view this is a general phenomenon triggered by liver injury and inflammation. The mechanism involved is still unknown, but a role for increasing blood flow through the organ, as well for inducing the expression of specific metabolic responses required for the recovery of hepatic function must also be considered (Kubes, 1992; Hasselgren, 1993). Further experiments should be performed during inhibition of NOS activity and NO release, in order to study the xenobiotic-induced necrogenic and regenerating processes in the absence of NO release. The results obtained in the present study indicate that iNOS is expressed and PKC is activated in the post-necrotic regenerating liver. The kinetics of NO synthesis in this experimental model reveal a complex regulatory mechanism in the course of the regenerative process.

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