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Liver Ischemic Preconditioning Is Mediated by the Inhibitory Action of Nitric Oxide on Endothelin

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The concerted involvement of both NO and endothelin in the protective effect of preconditioning against hepatic ischemia- reperfusion induced injury has been evaluated in this study. Thus hepatic ischemia-reperfusion or preconditioning plus ischemia-reperfusion was induced in rats and the effect of nitric oxide administration or inhibition with addition of the endothelin antagonist Bosentan was evaluated. Results show that the increases in plasma GPT release after ischemia-reperfusion were prevented after preconditioning. Inhibition of nitric oxide abolished the effect of preconditioning, addition of the endothelin antagonist abolished the injurious effect of NO inhibition. Also, increased synthesis of endothelin has been detected after ischemia-reperfusion, and addition of NO or preconditioning prevented this increase, suggesting that increases of NO inhibit endothelin synthesis. Altogether this indicates that hepatic preconditioning is mediated by the inhibitory action of nitric oxide on endothelin levels. © 1996 Academic Press, Inc.

Ischemic preconditioning is an inducible and potent endogenous mechanism by which repeated episodes of brief ischemia and reperfusion (I/R) confer a state of protection against subsequent sustained ischemia-reperfusion injury (1). Although the mechanism of preconditioning is not yet known, some hypotheses have recently been tested (2). It has been suggested that organ protection depends on the release of endothelial substances such as nitric oxide (NO) (3). This mediator has been reported to be protective in several models of ischemia (4, 5). Along this line, inhibition of NO was found to abolish the protective effect of preconditioning in dog heart (4) and a recent study from our group has demonstrated the implication of nitric oxide in rat intestinal preconditioning and the protective effect of this mediator in front the injury associated to ischemia-reperfusion (5). However, there are also conflicting reports in the literature such as that claiming no effect on preconditioning after NO inhibition, in rat heart (6).

On the other hand, the existence of a relationship between NO generation and endothelin (ET) biosynthesis has been described. The enhanced release of NO in ischemia-reperfusion could induce the synthesis of ET (7), and inhibition of endothelin release by NO has been described in endothelial cells from isolated perfused organs (8,9).

It is know that ET-1 may play an important role in modulating the disturbances in hepatic microcirculation in the injury associated to ischemia-reperfusion (10-11). Also, the in vivo beneficial effects of an ET antagonist such as Bosentan in animal models of I/R have further suggested a role for ET in this pathology (12).

This work was performed to examine the concerted involvement of both NO and endothelin in the protective effect of preconditioning against hepatic ischemia- reperfusion induced injury.

MATERIALS AND METHODS

The study was performed with male Wistar rats (8 for each group) weighing between 250 and 300 g. All animals (including controls) were anaesthetized with urethane (10 mg/Kg, i.p.) and placed in a supine position on a heating pad for maintenance of body temperature between 36°C and 37°C. In order to induce hepatic ischemia, laparotomy

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was performed and the blood supply to the right lobe of the liver was interrumped by placement of a bulldog clamp at the level of hepatic artery and portal vein. Reflow was initiated by removal of the clamp (13). Blood samples were obtained 90 min after reperfusion and processed to determine plasma levels of glutamic-pyruvic transaminase (GPT), and tissue levels of endothelin and NO synthase. All studies performed are in concordance with the European Union regulations for experimental animals.

The protective effect of ischemic preconditioning as well as the role of NO and endothelin was tested with the following groups (figure 1):

Group 1. **Control.** Animals were subjected to anaesthesia and laparotomy.

Group 2. **Ischemia Reperfusion (I/R).** Animals were subjected to a 90 min of right lobe hepatic ischemia, followed by 90 min reperfusion.

Group 3. **Preconditioning (Prec.).** Previous to the ischemic period (as in group 2), animals were subjected to 10 min of ischemia and 10 min reperfusion.

Group 4. **Ischemia Reperfusion + NO donor (I/R+NO).** Animals subjected to a 90 min ischemia, were treated with the NO-donor spermine NONOate (10 mg/Kg in PBS pH 7.4, i.v.) 5 min before ischemia.

Group 5. **Preconditioning + NAME (Prec.+ NAME).** Animals subjected to 90 min ischemia with previous preconditioning (as in group 3) were treated with L-NAME (10 mg/Kg, i.v.) 5 min before preconditioning.

Group 6. **Preconditioning + NAME + Bosentan.** Same as group 5 but with previous administration of Bosentan (Ro 46-2005) (10 mg/Kg, i.v.).

Blood and liver samples were obtained at 90 min of reperfusion.

The evaluation of hepatic injury was performed by enzymatic determinations of GPT in blood plasma using a commercial kit from Boheringer Manheim (Munich, Germany).

For the analysis of endothelin, frozen tissue specimens were homogenized in 5 ml of 6% acetic acid and immediately boiled for 10 min to inactivate proteases (14, 15). The homogenate was centrifuged at 25.000 g for 30 min, and extraction of samples was performed on Sep Pack C18 Cartridges pretreated with 5 ml of methanol, and 5 ml of acidified water at pH 3.0. After washing with 10 ml of 0.1% trifluoroacetic acid (TFA), endothelin was eluted with 3 ml of methanol/water/TFA (90/10/0.1). The cartridge eluates were vacuum evaporated and the resulting dried residues were resuspended in EIA buffer for subsequent EIA measurements. Endothelin production was determined using a commercial Kit of endothelin enzyme Immunoassay (Cayman Chemical, Ann Arbor, MI, USA).

NOS activity was evaluated after partial purification by 2′,5′ADP-Sepharose chromatography. Briefly, citrulline release was measured at pH 7.4 in a buffer that contained 20 mM Hepes, 10 μM [U-^{14}C] arginine (0.3 μCi) and 0.5mM NADPH (16). The activity was assayed either in the presence of 100 μM Ca^{2+} or 1 mM EGTA. The rate of production in the presence of 100 μM Ca^{2+} corresponded to total NOS, and the rate of production in absence of Ca^{2+} and in presence of 1 mM EGTA corresponded to inducible NOS. The measured activity, nonspecifically inhibited by 1 mM N^{w}-nitro-L-arginine (L-NNA), was substracted in both reactions (with Ca^{2+} or with EGTA). Constitutive NOS was calculated from the difference between total and inducible NOS.

Liver samples were taken for histopathology. These were fixed in 10% neutral buffered formalin, embebbed in paraplast and 5um sections, and stained with hematoxylin and eosin according to standard procedures. Sections were evaluated by light microscopic examination.

Data are expressed as means ± S.E.M. Comparisons between groups were made by the Student's t test, an associated probability of $p<0.05$ was considered to be significant.

RESULTS AND DISCUSSION

As shown in Figure 1 significant increases in GPT levels were observed in the group subjected to ischemia and reperfusion (I/R). These increases were significantly lower when ischemia was preceded by preconditioning (Prec.), this indicating the effectiveness of preconditioning in preventing hepatic I/R.

Inhibition of NO synthesis (Prec.+NAME) (figure 1) was found to abolish the beneficial effects of preconditioning. In addition, administration of nitric oxide donors previous to the I/R (I/R+NO) (figure 1) results in the same effects on GPT release as observed in the preconditioned group. Altogether these results clearly point to a role for NO as a mediator in the process of hepatic preconditioning.

The assay of NOS activity (figure 2) reflects the presence of NOS activity independent of Ca^{2+} (corresponding to the inducible enzyme). This activity was significantly increased both in the I/R group and in the preconditioned group, while the constitutive enzyme remains unmodified with respect to control group. Taking into account that preconditioning does not

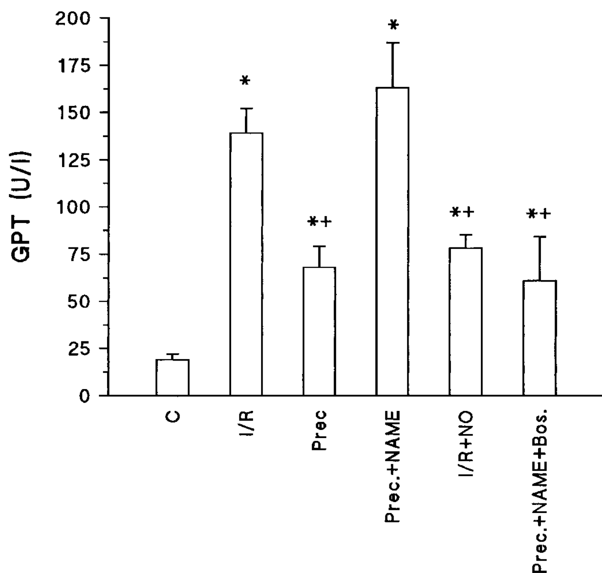


FIG. 1. GPT levels (U/l) in the following experimental groups: Group I: Control, Group 2. Ischemia Reperfusion (I/R), Group 3. Preconditioning (Prec.). Group 4. Ischemia Reperfusion + NO donor (I/R+NO). Group 5. Preconditioning + NAME (Prec.+ NAME). Group 6. Preconditioning + NAME + Bosentan. *=$p<0.05$ vs Control; +=$p<0.05$ vs Ischemia reperfusion.

promote any difference in the induction of NOS with respect to I/R group, it appears unlikely that preconditioning could be dependent on NOS induction. Previously, we have detected a transient increase in NO generation, only immediately after intestinal preconditioning (5). Consequently, it could be suspected that increases in intracellular Ca^{2+} concentration as a consequence of brief I/R (17) would induce NO generation by the action of constitutive NO synthase (18). Thus, we postulate herein that this brief pulse of NO could be determining for the phenomenon of preconditioning, through a mechanism yet to be determined.

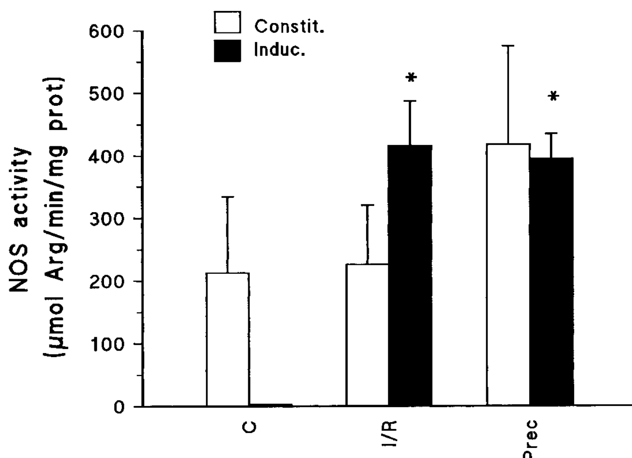


FIG. 2. NOS activity in the following groups: Group I: Control, Group 2. Ischemia Reperfusion (I/R), Group 3. Preconditioning (Prec.). *=$p<0.05$ vs Control

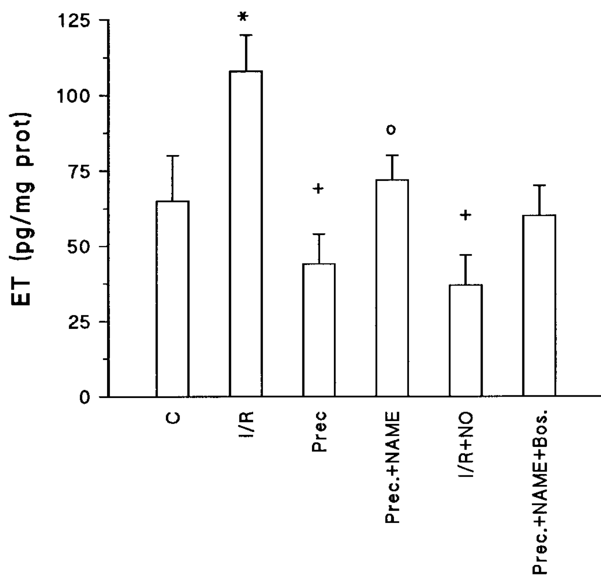


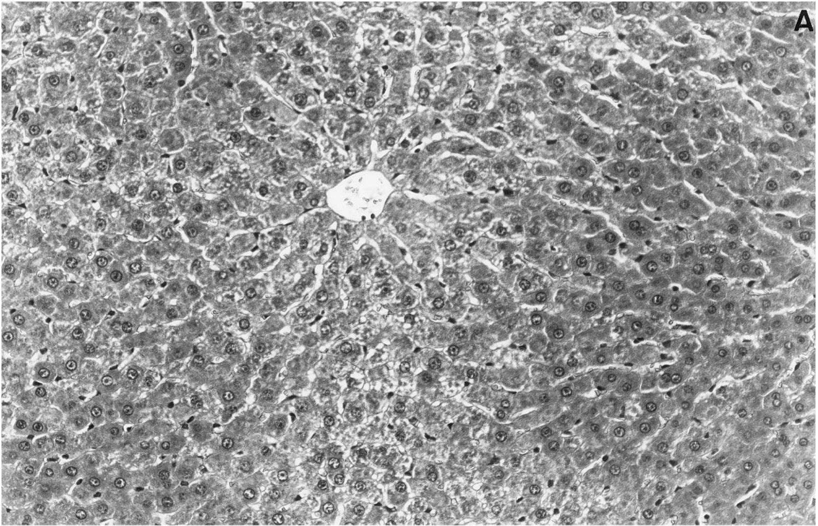
FIG. 3. Endothelin levels (pg/mg protein) in the following experimental groups: Group I: Control, Group 2. Ischemia Reperfusion (I/R), Group 3. Preconditioning (Prec.). Group 4. Ischemia Reperfusion + NO donor (I/R+NO). Group 5. Preconditioning + NAME (Prec.+ NAME). Group 6. Preconditioning + NAME + Bosentan. *=$p<0.05$ vs Control; +=$p<0.05$ vs Ischemia reperfusion; o=$p<0.05$ vs Preconditioning

On the other hand, a relationship between NO generation and endothelin biosynthesis has been established. In this sense, the NO produced in heart ischemia-reperfusion was able to induce the synthesis of ET (7). In addition, an inhibition of endothelin release by NO has been described in endothelial cells and in isolated perfused organs (8,9). Thus, it could be argued that NO could modulate organ injury sustained in ischemia-reperfusion through its action on endothelin. Our results do indeed suggest that the mechanism of action of NO in hepatic preconditioning is dependent of endothelin synthesis, as shown below.

As indicated above, inhibition of NO synthesis (Prec+NAME) (figure 1) was found to abolish the beneficial effects of preconditioning as shown by an increase in plasma GPT levels of the same order as that observed after I/R. However, addition of the endothelin receptor antagonist Bosentan to this group (Prec.+NAME+Bosentan) returns GPT levels to those observed in preconditioned group. Thus, by itself inhibition of NO fails to induce the injury when endothelin effects are inhibited by Bosentan.

Figure 3 depicts the endothelin levels measured in all experimental groups. I/R induces a significant increase in the release of endothelin, which is prevented by preconditioning, and addition of NO donors. On the other hand inhibition of NO release by NAME opposes the effect of preconditioning on ET release. Consequently, it appears that NO exerts its beneficial effect through its inhibitory action on endothelin synthesis. Accordingly, inhibition of NO in preconditioned rats results in an increase in the release of endothelin (figure 3). Nevertheless, this increase does not achieve the levels of I/R group, suggesting that there could be other mediators involved in this process.

FIG. 4. Histological lesions **A:** Control, normal hepatic parenchyma. **B** Ischemia Reperfusion (I/R), area of hepatic necrosis. **C** Preconditioning (Prec.), no apparent hepatic lesions. **D** Ischemia Reperfusion + NO donor (I/R+NO), no apparent hepatic lesions. **E** Preconditioning + NAME (Prec.+ NAME), area of hepatic necrosis. **F** Preconditioning + NAME + Bosentan, picnotic nuclei of scattered hepatocytes. Hematoxylin and eosin (× 215).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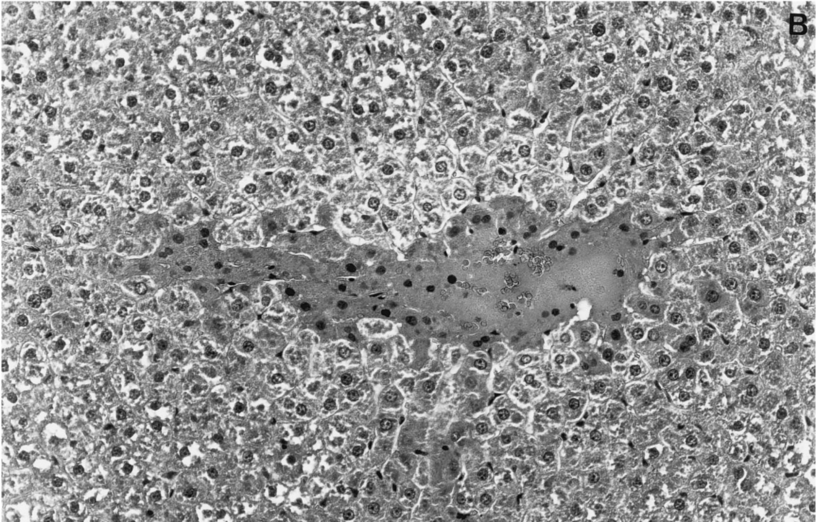
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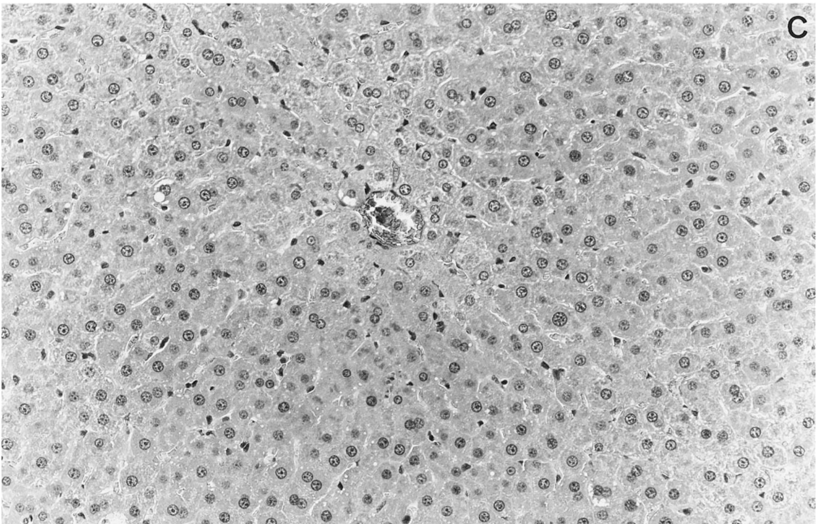
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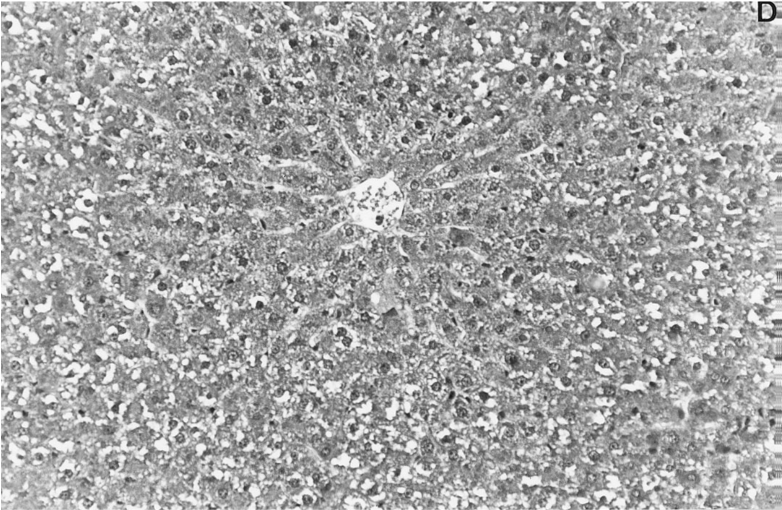
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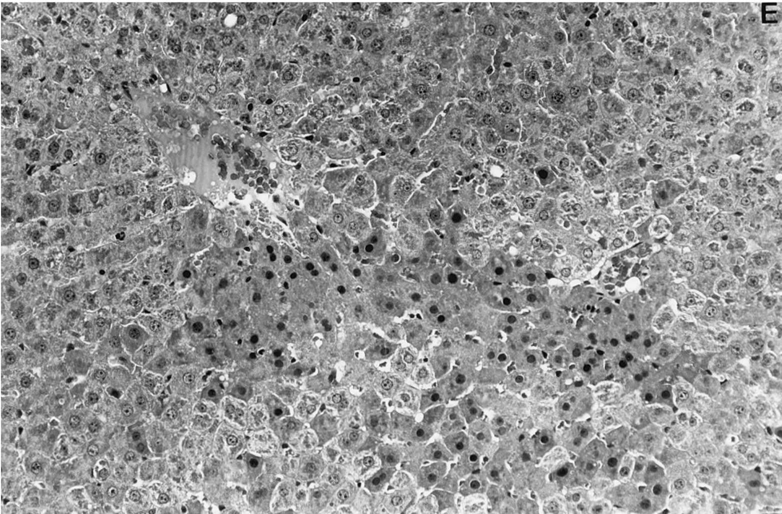
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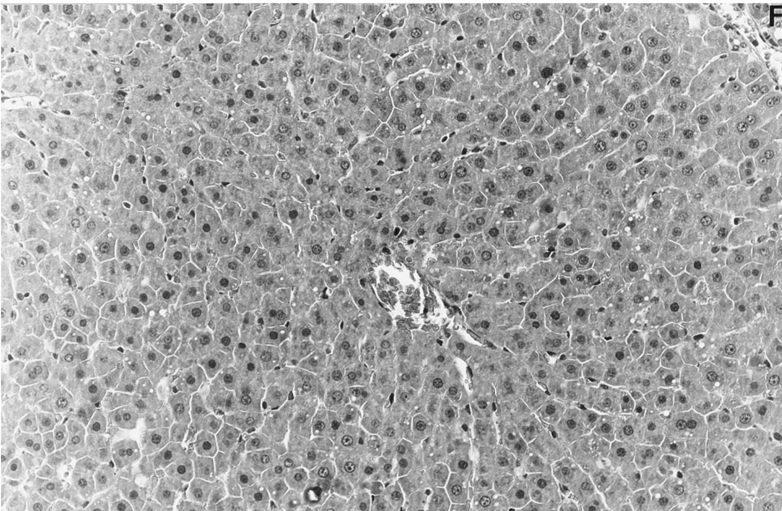
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In line with the biochemical findings, the microscopic study of the liver shows that preconditioning prior to I/R (figure 4C), preconditioning previous to I/R + NAME+ Bosentan (figure 4F), or NO addition (figure 4D) prevented tissue damage induced by ischemia reperfusion (figure 4B), showing minimal lesions which consisted of incipient necrosis of isolated hepatocytes (cytoplasmatic eosinophilia and nuclear picnosis) and scattered sinusoidal polymorphonuclear infiltration. By the contrary, in the liver of animals from both ischemia-reperfusion (figure 4B) or preconditioning previous to I/R +NAME (figure 4E) multiple extensive necrosis of hepatocytes randomly distributed through the parenchyma were observed. Occasionally polymorphonuclear infiltration associated with these lesions was present.

In conclusion, this work suggests that the mechanism of hepatic preconditioning is mediated by the inhibitory action of nitric oxide on endothelin levels.

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