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Intestinal Preconditioning Is Mediated by a Transient Increase in Nitric Oxide

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The effect of ischemic preconditioning on the intestine, as well the implication of nitric oxide and prostacyclin in this process has been evaluated. Thus, intestinal ischemia-reperfusion was induced in rats, and the protection conferred by previous preconditioning was evaluated. In addition, the effect of nitric oxide inhibition and the administration of nitric oxide were determined. Results show that the increases observed in LDH release after ischemia-reperfusion were prevented after preconditioning. Inhibition of nitric oxide synthesis abolished the protective effect of preconditioning, and nitric oxide administration replicated this effect. Also, an increased synthesis of nitric oxide has been detected after preconditioning. Increases in 6 keto $PGF_{1\alpha}$ were independent of nitric oxide. Altogether indicates that preconditioning is triggered by an initial increase in nitric oxide synthesis.

Repetitive short periods of ischemia, separated by intermittent reperfusion, render the heart (1) and the liver (2) more tolerant to subsequent longer ischemic episodes, and attenuate the injury observed after ischemia-reperfusion. Such a protective effect which has been called ischemic preconditioning has been commonly studied in the heart (3, 4). The preconditioning response may depend on the release of endogenous protective substances including adenosine, prostacyclin, nitric oxide (NO) and bradykinin (5).

It is known that L-arginine dependent production of NO may influence gut integrity in response to different challenges. With respect to intestinal ischemia-reperfusion injury, most of the alterations elicited by this process can be induced by inhibition of NO synthase in normal rats (6, 7). Also, administration of NO donors provides significant protection in front of dysfunctions associated with ischemia-reperfusion (8). In addition, a role for prostaglandins in maintaining mucosal integrity in the gastrointestinal tract is well documented. As with NO, inhibition of prostaglandin synthesis renders the mucosa more susceptible to injury (9). There are evidences to suggest that there may be cooperation in maintaining mucosal integrity in some circumstances (10).

In order to determine a possible role for NO and prostacyclin as mediators in intestinal ischemic preconditioning, we measured NO and prostacyclin production. In addition, the effect of NO donors as well as NO inhibitors in the development of tissue injury has been evaluated.

METHODS

The study was performed with male Sprague Dawley rats (Ifa Credo) weighting between 250 and 300 g. Animals were fasted for 24 hours before surgery. After laparotomy, proximal and distal portions of the small bowel of each rat were cannulated and secured with 5-0 silk ligatures, and continuously perfused with saline solution (100 ml/h) at 37°C.

The experimental design is shown in figure 1. Rats were assigned in three experimental groups ($n = 3 \times 10$). Group I.- Control, animals subjected to ether anaesthesia and laparotomy during 135 min. Group II.- Ischemic group, animals subjected to 90 min of intestinal ischemia, followed by 30 min of reperfusion. Group III.- Preconditioned group, same as group II but with previous ischemic preconditioning with 10 min ischemia and 5 min reperfusion. Control group was maintained, without ischemia, for 135 min.

Samples of perfusate (2 ml) were obtained at time: 0 min (sample 1; previous to manipulation); 15 min (sample 2; end of preconditioning); 105 min (sample 3; end of ischemia); 110 min (sample 4; after 5 min of reperfusion) and 135 min

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(sample 5; after 30 min of reperfusion). All were immediately aliquoted, frozen and maintained at −40°C until their analytical determination.

At the end of the protocol, tissue samples were obtained and fixed in 10% neutral buffered formalin, Paraplast-embedded, cut into 5 μm sections and stained with haematoxylin-eosin according to standard procedures. Sections were evaluated by light microscopy.

In order to evaluate the role of NO in the preconditioning process, a second series of experiments was performed. Animals were assigned to the ischemic group (n = 10) or to the preconditioned group (n = 10). L-NAME was i.v. administered (10 mg/kg) in the preconditioning group, and, by contrast in the ischemic group, the NO donor spermine NONOate was administered i.v. (10 mg/kg; resuspended in PBS pH = 7.4) previous to the start of the protocol. Samples of perfusate were obtained as depicted in the first series of animals (figure 1).

In the third series of animals tissue levels of NO were determined. For this purpose ischemic and preconditioned groups were performed, but animals were killed to obtain tissue samples (n = 8) at the following times: 0 min, 15 min (at the end of preconditioning), 105 min (end of ischemia) and 135 min (after 30 min of ischemia). Tissue samples were immediately frozen and maintained at −40°C.

LDH activity in the perfusate was determined using a commercial kit from Boheringer Manheimm (Munich, Germany).

For the analysis of prostanoids, 1 ml of perfusate was processed through preactivated C18 solid phase cartridges from Waters Assoc. (Milford, MA, U.S.A.). Cartridges were washed with H_2O at pH = 4.0 and retained prostanoids were eluted with 4 ml of methyl formate (11). Methyl formate extracts containing prostanoids were evaporated to dryness in a centrifugal rotary evaporator, and the residues reconstituted with 500 μl of 100 mM Tris-HCI buffer (pH = 7.4) for subsequent RIA measurements by using specific antisera.

NO production in pancreas tissue was determined by tissue accumulation of nitrite and nitrate, using a modification of the method previously described (12). Briefly, tissue specimens were homogenized in 2 ml of 100 mM TRIS-HCI, pH 4.0, at 4°C. Homogenates (1ml) were centrifuged at 100.000 × g during 60 min., 200 μl of supernatants were transferred to Eppendorf tubes. Nitrate was reduced to nitrite with 0.5 units of nitrate reductase in the presence of 50 μM of NADPH and 5 μM of FAD. The excess of NADPH was oxidized in the presence of 0.2 mM pyruvate and 1 μg of lactate dehydrogenase. Nitrite was determined with Greiss reagent by adding 1 mM sulfanilic acid, and 100 mM HCI. After 5 min. of incubation, tubes were centrifuged and 150 μl of supernatant were transferred to a 96-well microtiter plate. After a first reading of the absorbance at 595 nm, 50 μl of naphthylenediamine were added. After 15 min. incubation the absorbance was compared with a standard of $NaNO_2$.

Total protein concentration in homogenates was determined using a commercial kit from BioRad (Munich, Germany).

Data are expressed as means ± s.e.m. Mean of differents groups were compared using a one-way analysis of variance. Student's *t* test was performed for evaluation of significant differences between groups. Significant differences were assumed when $p<0.05$.

RESULTS AND DISCUSSION

Ischemic preconditioning could be defined as a protective mechanism towards ischemia-reperfusion injury and consists of previous short periods of ischemia followed by reperfusion. Figure 2 (top) depicts the LDH activity measured in the perfusate. LDH release was increased in the ischemic group at the end of the ischemia. Also LDH values were further increased after 5 and

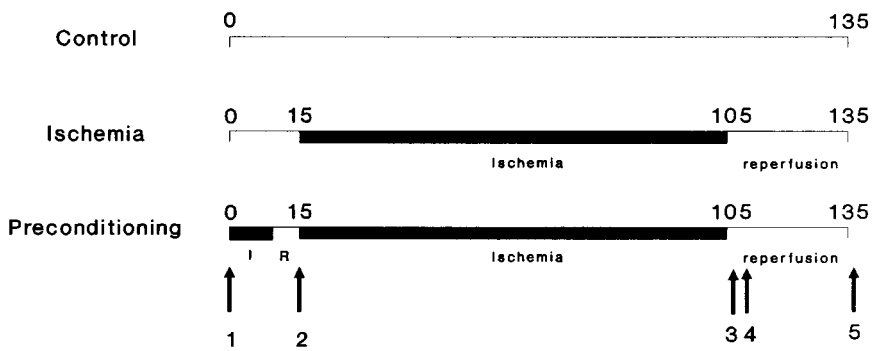


FIG. 1. Experimental protocol. Control rats were perfused for 135 min. Ischemic group underwent a single 90-minute occlusion of the superior mesenteric artery and the portal vein followed by a 30-minute reperfusion. Preconditioned rats underwent one 10-minute mesenteric artery and portal vein occlusion followed by a 5-minute reperfusion before the sustained 90-minute ischemia and 30-minute reperfusion. Samples numbered 1 to 5 were obtained at time 0 (1), at the end of preconditioning (2), at the end of ischemia (3), at the start of reperfusion (4), and at the end of reperfusion (5).

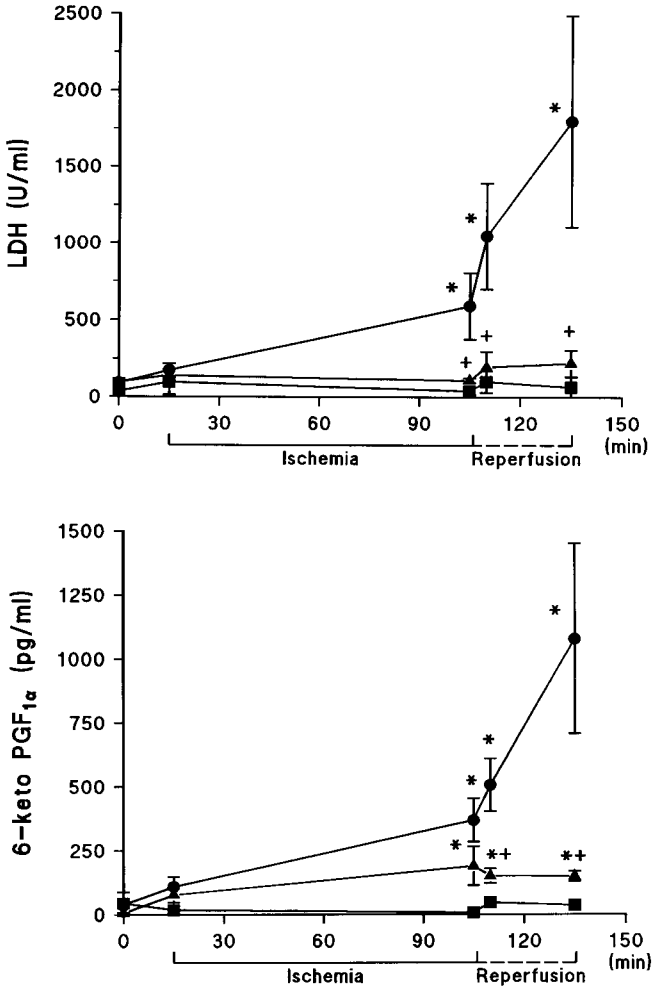


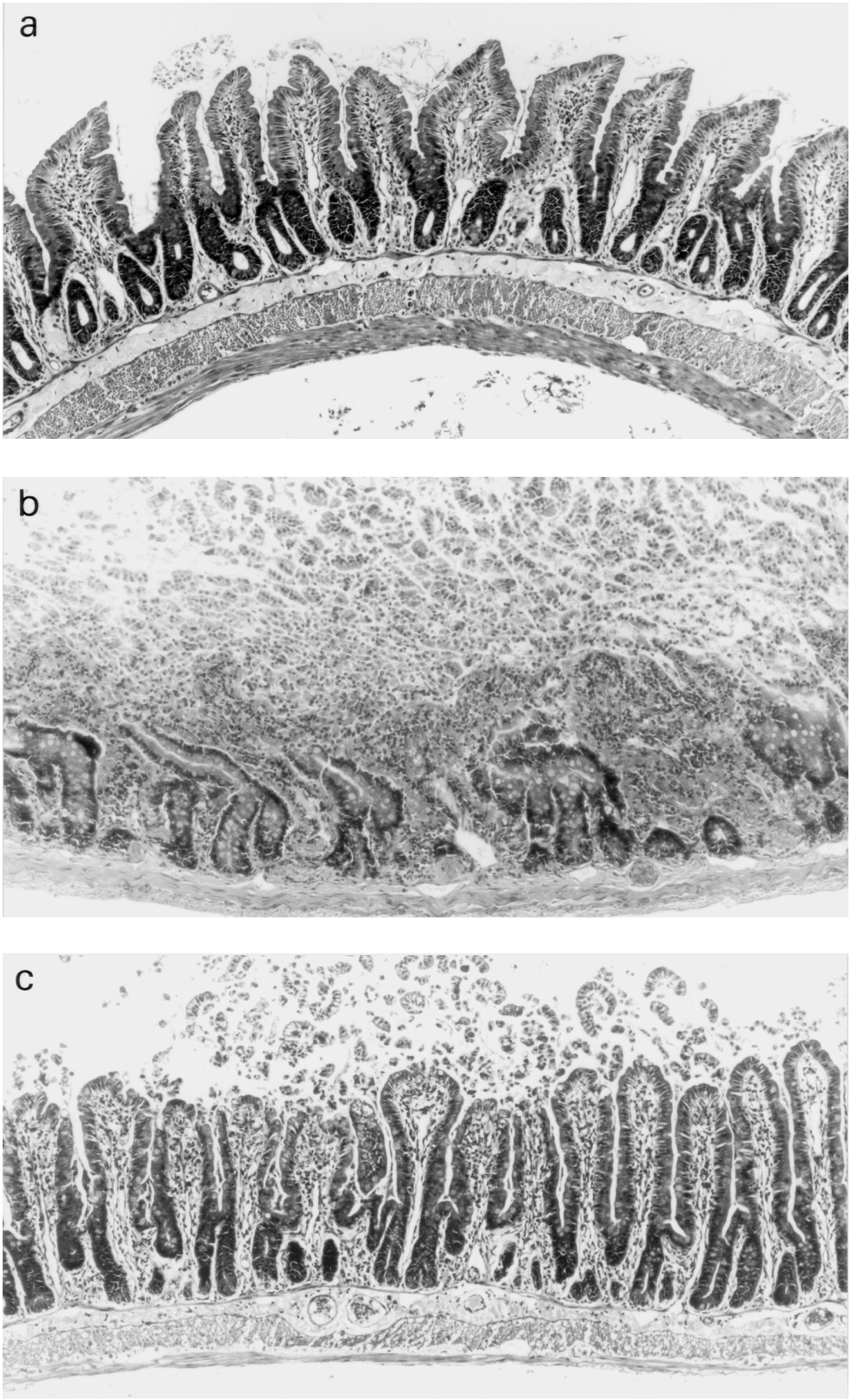
FIG. 2. Profiles of LDH (top) and 6-keto $PGF_{1\alpha}$ (bottom) release in the three experimental groups (n = 10). (■) Control; (●) ischemia; (▲) Preconditioning. (*) $p<0.05$ vs. control; (+) $p<0.05$ vs. ischemia.

30 min post-reperfusion, reflecting the progression of tissue damage. In contrast, when preconditioning was carried out before the ischemia, no increases in LDH release were detected at any time. This fact indicates the effectiveness of preconditioning in preventing intestinal ischemia-reperfusion damage.

Similar results were obtained for 6-keto $PGF_{1\alpha}$ (the stable metabolite of prostacyclin) in the perfusate (figure 2 bottom). At the end of the ischemic period, 6-keto $PGF_{1\alpha}$ levels were significantly higher in the ischemic group when compared to the control group. During reperfusion, the release of this metabolite showed increased levels. Preconditioning reduces the release of 6-keto $PGF_{1\alpha}$ but, in contrast with the LDH results, in this case, levels are significantly higher than those found in the control group.

In concordance with the LDH results, histological features (figure 3) show that preconditioning partially prevented tissue damage induced by intestinal ischemia-reperfusion.

When the NO synthase was inhibited with L-NAME, the effect of preconditioning with respect to LDH release was abolished (figure 4, top). In this case, LDH showed increased levels after ischemia and after reperfusion despite the preconditioning, suggesting a key role for NO in the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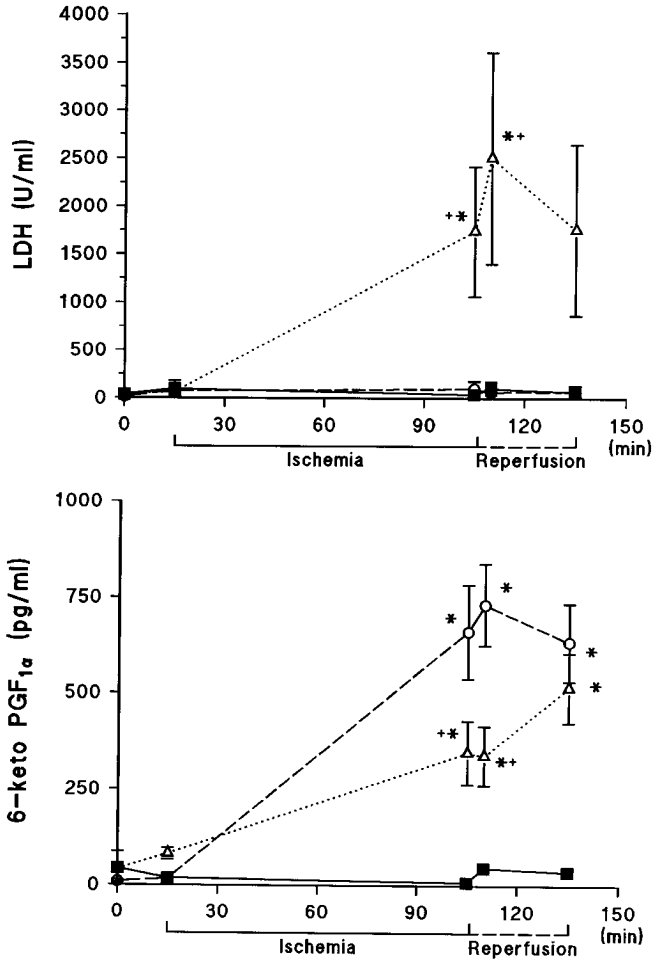

FIG. 4. Profiles of LDH (top) and 6-keto $PGF_{1\alpha}$ (bottom) release in the three experimental groups, when NO was administered or inhibited (n = 10). (—■—) Control; (---○---) Ischemia+NO; (···△···) Preconditioning+L-NAME. (*) $p<0.05$ vs. control; (+) $p<0.05$ vs. ischemia.

process of intestinal preconditioning. Accordingly, when NO donors were administered, no increases were found in LDH release associated with ischemia-reperfusion. This results indicates that NO donors provide significant protection against disfunctions resulting from intestinal ischemia-reperfusion (8).

We do not know the mechanism of action of NO in preconditioning, but an interesting fact is that it appears to be independent of prostacyclin synthesis, since NO inhibition or administration has little effect in the 6-keto $PGF_{1\alpha}$ release. Ischemia induces the release of this metabolite despite NO synthesis inhibition, and preconditioning partially prevents this effect independently of the presence of NO donors (Fig. 2, bottom and Fig. 4 bottom). Accordingly, preconditioning seems to be not related with the known cytoprotective effect of prostaglandins (13).

The generation of tissue NO (measured as nitrite and nitrate levels) showed unchanged levels at all the times measured, except at the end of preconditioning (figure 5). At this moment, increased

FIG. 3. Light microscopy appearance of intestine at the end of the experimental protocol (×10). (a) Control. Normal mucosa. (b) Ischemic group. Massive epithelial lifting, a few denuded and melted villi and hyperaemia. (c) Effect of preconditioning. Moderate epithelial lifting.

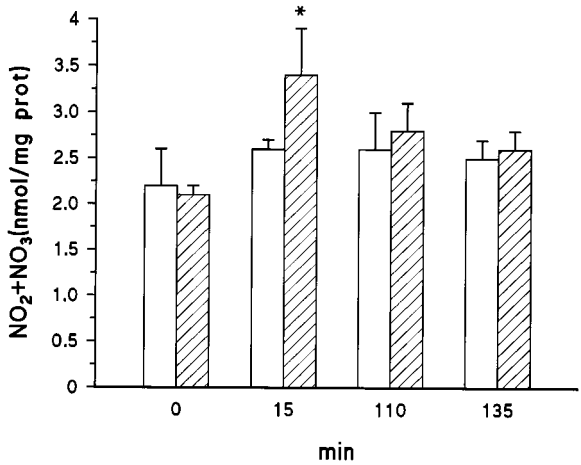

FIG. 5. Tissue levels of NO, measured as nitrite and nitrate levels in ischemia (open bars) or preconditioned groups (hatched bars). Samples were obtained at time 0; 15 min (end of preconditioning); 110 min (end of ischemia); 135 min (end of reperfusion). (*) $p<0.05$ vs. time 0.

levels of NO were detected in the preconditioned group. It could be suggested that a brief period of ischemia induces an increase in intracellular calcium concentration (14). This fact can activate the calcium-dependent constitutive NO synthase (15) resulting in an increased NO generation. Nevertheless, the changes induced by a transient increase in NO, leading to a resistance to the organ in front of the ischemia-reperfusion remain to be elucidated.

In conclusion, this work suggests that intestinal preconditioning is triggered by an initial transient increase in NO synthesis.

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REFERENCES

1. Murry, C. E., Jennings, R. B., and Reimer, K. A. (1986) *Circulation* **74,** 1124–1136.
2. Lloris-Carsi, J. M., Cejalvo, D., Toledo-Pereira, L. H., Calvo, M. A., and Suzuki, S. (1993) *Transplantation Proceedings* **25,** 3303–3304.
3. Richard, V., Kaeffer, N., Tron, C., and Thuillez, C. (1994) *Circulation* **89,** 1254–1261.
4. Wainwright, C. (1992) *TiPS.* **13,** 90–93.
5. Parrat, J. R. (1994) *TiPS.* **15,** 19–25.
6. Kubes, P., Suzuki, M., and Granger, D. N. (1991) *Proc Natl Acad Sci.* **88,** 4651–4657.
7. Kubes, P. (1992) *Am J Physiol.* **262,** G1138–G1144.
8. Villarreal, D., Grisham, M. B., and Granger, D. N. (1995) *Transplantation.* **59,** 685–689.
9. Wallace, J. L., and Tigley, A. W. (1995) *Aliment Pharmacol Ther.* **9,** 227–235.
10. Beck, P. L., McKnight, W., Lee, S. S., and Wallace, J. L. (1993) *Am J Physiol.* **265,** G453–G458.
11. Powell, W. S. (1982) Methods in Enzymology, pp. 467–477, Academic Press, New York.
12. Hotter, G., Closa, D., and Pí, F., *et al.* (1995) *Transplantation.* **59,** 417–421.
13. Wallace, J. L. (1993) *Can J Physiol Pharmacol.* **71,** 98–102.
14. Pierce, C. N., and Czubryt, M. P. (1995) *J Mol Cell Cardiol.* **27,** 53–63.
15. Kanwar, S., Tepperman, B. L., Payne, D., Sutherland, L. R., and Kubes, P. (1994) *Circ Shock.* **42,** 135–140.