Fructose-1,6-biphosphate in rat intestinal preconditioning: involvement of nitric oxide

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Abstract

Background and aims—Inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by nitric oxide (NO) in intestinal preconditioning could modify the rate of formation of glycolytic intermediates. Fructose-1,6-biphosphate (F16BP) is a glycolytic intermediate that protects tissue from ischaemia/reperfusion injury. We evaluated if F16BP may be endogenously accumulated as a consequence of GAPDH inhibition by NO during intestinal preconditioning in rats.

Methods—We assessed: (1) effect of preconditioning on F16BP content; (2) effect of NO on GAPDH activity before and during sustained ischaemia; and (3) protective effect of F16BP in control, ischaemic, and preconditioned animals with or without administration of N-nitro-l-arginine methyl ester (l-NAME), NO donor, or F16BP.

Results—Preconditioned rats showed a significant transient decrease in GAPDH activity and also maintained basal F16BP levels longer than ischaemic rats. l-NAME administration to preconditioned rats reversed these effects. F16BP administration to ischaemic rats decreased protein release in the perfusate. Administration of F16BP to l-NAME treated rats attenuated the harmful effect of l-NAME.

Conclusions—Our study indicates that F16BP may be endogenously accumulated in preconditioned rats as a consequence of inhibition of GAPDH by NO, and this may contribute to the protection observed in intestinal preconditioning.

Keywords: fructose-1,6-biphosphate; glyceraldehyde-3-phosphate dehydrogenase; intestinal preconditioning; ischaemia/reperfusion injury; nitric oxide

Ischaemia/reperfusion (I/R) syndrome is one of the most important problems in the primary non-function associated with transplantation procedures. Preconditioning is a phenomenon defined as one or more brief periods of ischaemia with intermittent reperfusion that can protect the organ against the subsequent sustained I/R injury. The protective effect of preconditioning was first proposed in the heart and the technique has since been applied to other tissues such as endothelium, brain, skeletal muscle, skin, liver, and the small intestine. For these reasons, it seems clear that preconditioning could, in the future, be successfully applied in different types of transplantation.

Although preconditioning was first described more than a decade ago, the mechanisms underlying its protective effect are not fully understood. Nitric oxide (NO) is one of the mediators that has been most widely studied in this process. In the small intestine, previous studies have demonstrated an increase in NO synthesis after preconditioning. Blocking of endogenous production of NO may inhibit part of the protection induced by this process. Moreover, other studies have suggested that NO may be an important protective molecule against intestinal I/R injury. These data clearly suggest that NO may be useful as a therapeutic agent in minimising the dysfunction at the onset of I/R.

In addition to its protective role, NO also has a metabolic effect. It can inhibit the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) through a rapid event such as S-nitrosylation of thiol groups present in the molecule or by ADP ribosylation, which changes the number of thiol groups. Our previous studies have shown that this critical step in glycolysis appears to be reversibly inactivated by NO generated during intestinal preconditioning.

Fructose 1,6-biphosphate (F16BP), a high energy glycolytic intermediate, has been shown to be therapeutically effective in shock, ischaemia, and post-ischaemic reperfusion injury in different organs. Several authors have associated the protective role of exogenous administration of F16BP, observed in a wide variety of organ and cell injuries, with the prevention of intracellular ATP depletion. In the case of intestinal I/R, it has been shown that administration of F16BP could enhance energy production from carbohydrates, thereby attenuating ischaemic damage and accelerating regeneration of intestinal villi after reperfusion.

There are several protective effects of F16BP that are independent of the tissue or experimental model used. An exogenous supply of F16BP can inhibit neutrophil free radical production, maintain the correct XDH/XO ratio, prevent changes in intracellular calcium, increase peristaltic activity, and im-
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prove the efficacy of preservation solutions in renal transplantation.20

In the glycolysis pathway, the formation rate of a glycolytic intermediate (among them F16BP) can be modified by inhibition of any glycolytic enzyme. The fact that NO generated during preconditioning inhibits the enzyme GAPDH10 could lead to NO dependent F16BP accumulation. Moreover, this could be a new protective mechanism before I/R injury, possibly due to the action of the known endogenous protector F16BP.

Hence the first objective of this study was to determine if preconditioning can modify the production of F16BP via the glycolysis pathway. The second objective was to determine if this modification was caused by NO dependent GAPDH inhibition produced during preconditioning. The third objective was to assess the protective effect of F16BP on intestinal preconditioning. For this purpose, we determined F16BP, glucose content, and GAPDH activity in the small intestine of rats subjected to intestinal preconditioning with or without NO. The effect of F16BP administration was also evaluated.

Material and methods

The study was performed using male Wistar rats (Ifa Credo, Barcelona, Spain) weighing 250–300 g. Animals were fasted for 12 hours before surgery, anaesthetised with urethane 10% (10 mg/kg intraperitoneally), and placed in the supine position, with body temperature maintained at 36–37°C. The abdominal area was covered with saline soaked gauze at 37°C and a plastic cover to minimise dehydration of exposed tissues.

The experiment was conducted under the supervision of our institution’s research commission and followed EU guidelines for the handling and care of laboratory animals.

INTESTINAL ISAECMIC PRECONDITIONING

To induce intestinal ischaemic preconditioning, a laparotomy was performed and the superior mesenteric artery was exposed and occluded for 10 minutes using an atrumatic arterial clamp followed by 10 minutes of reperfusion.

EXPERIMENTAL GROUPS

Effect of preconditioning on F16BP and glucose content during the ischaemic period.

To evaluate the effect of preconditioning and its relationship with NO on F16BP and glucose content over a range of ischaemic periods, the following groups of animals (n=8 each group) were added to the above groups.

Group I—Control. Animals were subjected to anaesthesia and laparotomy.

Group II—Control+L-NAME (C+NAME). As for group I but with previous administration of L-nitro-L-arginine methyl ester (L-NAME 10 mg/kg) by direct puncture into the inferior cava.

Group III—Ischaemia (I). Four subgroups of eight animals, where each group was subjected to different periods of ischaemia (2, 30, or 90 minutes).

Group IV—Ischaemia+spерmine NONOate (I+NONOS). Animals underwent the same procedure as in group III but with previous intravenous administration of the NO donor spermine NONOate (NONOS 10 mg/kg resuspended in phosphate buffered saline, pH=7.4, 30 minutes before administration) by direct puncture into the inferior cava.21

Group V—Preconditioning (Prec+I). As for group III (ischaemia) but with previous preconditioning, as described above in all animals.

Group VI—Preconditioning+L-NAME (Prec+I+NAME). As for group V but with previous administration of L-NAME (10 mg/kg) by direct puncture into the inferior cava, five minutes before the beginning of the preconditioning process. Previous studies have shown that this dose and the preincubation conditions of the NO inhibitor are effective.21

Effect of NO on GAPDH activity before and during sustained ischaemia

To study the effect of the preconditioning period on GAPDH activity and its relationship with NO generation, the following animals (n=8 each group) were added to the above groups.

Group VII—Preconditioning (P). Animals were subjected to 10 minutes of ischaemia followed by 10 minutes of reperfusion.

Group VIII—Preconditioning+L-NAME (P+NAME). As for group VII but with previous administration of L-NAME (10 mg/kg) by direct puncture into the inferior cava, five minutes before the beginning of the preconditioning process.

Group IX—Preconditioning+iNOS inhibitor (P+1400W). As for group VII but with previous administration of the inducible nitric oxide synthase (iNOS) inhibitor 1400W (N-(3-aminomethyl)benzyl) acetamide) (3 mg/kg subcutaneously, dissolved in 500 μl of saline) 40 minutes before the beginning of the preconditioning process. This dose has been proved to be effective in rats.22

Tissue samples from groups I–IX were obtained, immediately frozen, and maintained at −80°C for biochemical analysis.

Role of F16BP on intestinal preconditioning

To assess the protective effect of F16BP on intestinal preconditioning by measuring protein release in the intestinal lumen, the following protocol was performed. 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. Animals were divided into the following groups (n=8).

Group X—Control. Animals were subjected to anaesthesia and laparotomy for 120 minutes.
Group XI—Ischaemia/reperfusion (I/R). Animals were subjected to 90 minutes of ischaemia followed by 30 minutes of reperfusion.

Group XII—Ischaemia/reperfusion+F16BP (I/R+F16BP). As for group XI but with superfusion over the small intestine of 5 mM of F16BP throughout the process (a dose proved to be effective in previous studies).17

Group XIII—Ischaemia/reperfusion+spermine NONOate (I/R+N+NONOS). Animals underwent the same procedure as group XI but with previous intravenous administration of the NO donor spermine NONOate (10 mg/kg resuspended in phosphate buffered saline, pH=7.4, 30 minutes before administration) by direct puncture into the inferior cava.2

Group XIV—Preconditioning (P+I/R). As for group XI (I/R) but with previous preconditioning, as described above.

Group XV—Preconditioning+L-NAME (P+N+I/R). As for group XIV but with previous administration of L-NAME (10 mg/kg) by direct puncture into the inferior cava, five minutes before the beginning of the preconditioning process.

Group XVI—Preconditioning+L-NAME+F16BP (P+N+I/R+F16BP). As for group XV but with superfusion over the small intestine of 5 mM of F16BP throughout the process.

Samples of intestinal perfusate were collected after 0, 30, and 90 minutes of ischaemia, and at five and 30 minutes after reperfusion.

To directly address F16BP incorporation in the intestine, we performed an additional experiment. We added F16BP (5 mM) by superfusion over the small intestine to a group of animals subjected to 90 minutes of ischaemia. In this case, mesentery as well as intestinal samples were obtained at the end of the protocol to measure F16BP levels.

**BIOCHEMICAL ANALYSIS**

**F16BP concentration**

To evaluate F16BP content, tissue samples were homogenised in ice cold perchloric acid 0.6 mmol/l. The homogenate was centrifuged at 10 000 g for 30 minutes. The supernatant was neutralised with potassium carbonate solution to a pH of 3.5–4.5. The assay mixture consisted of a triethanol amine buffer (pH 7.6), 5 mmol/l NADH, a commercially mixed glycerol-3-phosphate dehydrogenase/triosephosphate isomerase preparation and Aldolase obtained from Boehringer Mannheim (Munich, Germany). The reaction was started by addition of the NADH solution and the enzymatic suspension. Changes in absorbance at 365 nm were monitored for 15 minutes.

**Glucose content**

To assess glucose concentrations, approximately 150 mg of frozen intestinal samples were homogenised in 0.6 ml of ice cold HCl 0.33 mol/l. The tissue homogenates were centrifuged at 10 000 g for five minutes at 4°C. Supernatant was used to measure glucose with a commercial kit from Boehringer Mannheim.

**GAPDH activity**

GAPDH activity was determined in intestinal tissues after homogenisation in ice cold buffer consisting of 100 mM triethanol amine buffer (pH 7.4), 1 mM EDTA, 2 mM dithiothreitol, 0.2 mM phenylmethylsulphonyl fluoride, leupeptin (1 µg/ml), and 10 mM GSH. The homogenate was centrifuged at 10 000 g for 15 minutes. Supernatant was used as the cytosolic fraction for determination of GAPDH activity. Enzyme activity was measured using a mixture containing 82.3 mM triethanol amine buffer (pH 7.6), 1.1 mM ATP, 6.2 mM glyc erate-3-phosphate, 0.2 mM NADH, 0.9 mM EDTA, 2 mM MgSO4, and phosphoglycerate kinase (13 units/ml). The reaction was started by addition of the tissue sample and changes in absorbance at 340 nm were monitored for five minutes.

**NO production**

NO production in intestinal bowel was determined by tissue accumulation of nitrite and nitrate using a modification of a method previously described.18,19 Briefly, frozen tissue specimens were homogenised in 2 ml of phosphate buffered saline (pH 7.4) at 4°C. Homogenates (1 ml) were centrifuged at 100 000 g for 60 minutes, and 200 µl of supernatants were used for subsequent measurement. Briefly, nitrate was reduced to nitrite with 0.5 units of nitrate

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**Figure 1** Intestinal fructose-1,6-biphosphate (F16BP) levels during the different ischaemic periods (0, 2, 30, or 90 minutes) in the control, ischaemia (I), preconditioning followed by sustained ischaemia (Prec+I), and preconditioning followed by sustained ischaemia but with prior addition of 1-NAME (Prec+I+NAME) groups (A) and in the control, control with previous administration of 1-NAME (Control+NAME), ischaemia (I), and ischaemia with previous administration of the NO donor spermine NONOate (I+NONOS) groups (B). *p<0.05 v control; †p<0.05 v I.
reductase in the presence of 50 µM NADPH and 5 µM FAD. Excess NADPH was oxidised in the presence of 0.2 mM pyruvate and 1 µg of lactate dehydrogenase. Nitrite was determined with Greiss reagent by adding 1 mM sulphanilic acid and 100 mM HCl. After five minutes of incubation, tubes were centrifuged and 150 µl of supernatant were transferred to a 96 well microtitre plate. After a first reading of the absorbance at 595 nm, 50 µl of naphthylene-diamine were added. After 15 minutes of incubation, absorbance was compared with the standard NaNO₂.

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

STATISTICAL ANALYSIS
Data are expressed as mean (SEM). Means of different groups were compared using one way analysis of variance. The Student’s t test was performed for evaluation of significant differences between groups. Significant differences were assumed when p<0.05.

Results
EFFECT OF PRECONDITIONING ON F16BP AND GLUCOSE CONTENT DURING THE ISCHAEMIC PERIOD
Figure 1A shows intestinal F16BP levels after various ischaemic periods. F16BP concentrations decreased as a function of sustained ischaemic time. After two minutes of ischaemia, the ischaemic group (I) showed a significant decrease in F16BP content compared with the control group. In contrast, when preconditioning was carried out before ischaemia (Prec+I), no significant decreases were detected. Addition of L-NAME to preconditioning rats (Prec+I+NAME) reversed this effect. After 30 and 90 minutes of sustained ischaemia, all groups showed a significant decrease in F16BP content compared with the control group. However, preconditioned animals maintained a higher F16BP content than the ischaemic group. L-NAME treatment reversed these effects. As shown in fig 1B, NO administration to ischaemic rats (I+NONOS) resulted in a significant increase in F16BP content compared with the ischaemic group for all ischaemic periods studied. However, administration of L-NAME to control rats (Control+NAME) had no effect on F16BP levels.

Figure 2A shows intestinal levels of glucose after the different ischaemic periods. There was a significant decrease in glucose for all
ischaemic periods compared with the control group. The decrease in glucose content was significant after two minutes of ischaemia and persisted for the other sustained ischaemic periods. This effect was apparent in all groups. As shown in fig 2B, there were no variations in this pattern when NO was administered to ischaemic rats (I+NONOS) or L-NAME was administered to control rats (Control+NAME).

**EFFECT OF NO ON GAPDH ACTIVITY BEFORE AND DURING SUSTAINED ISAEMIA**

As shown in fig 3A, preconditioning induced a significant decrease in GAPDH activity compared with the other groups. This decrease was evident at the end of the preconditioning period and was maintained after two minutes of the subsequent sustained ischaemia. L-NAME administration reversed this inhibition whereas addition of the iNOS inhibitor 1400W did not modify the decrease observed during the preconditioned process. In the next ischaemic periods (30 and 90 minutes), levels of enzyme activity returned to control values, indicating transient GAPDH inhibition in preconditioned animals. Figure 3B shows that administration of NO to ischaemic rats also significantly decreased GAPDH activity compared with the control group. Administration of L-NAME to control rats did not affect GAPDH activity.

Figure 4A shows intestinal generation of NO, evaluated as nitrite and nitrate produc-
was eliminated, showing a large increase in protein release at the end of ischaemia and after reperfusion. Addition of F16BP to this group (P+N+I/R+F16BP) decreased protein release, increasing it to an I/R group. This indicates that F16BP protects against the deleterious effect of iNOS.

In the same way, addition of F16BP to the I/R group (I/R+F16BP) (fig 5B) also had a protective effect, similar to that of preconditioning treatment. This was reflected by a similar pattern of protein release. These findings indicate that F16BP exerts a protective effect against I/R injury. Administration of NO to the ischaemic group (I/R+NOS) also had a beneficial effect, showing a protein release pattern similar to the I/R+F16BP group.

Figure 6 shows incorporation of F16BP superfused in the intestine and mesentery. F16BP levels were significantly higher than those in the non-treated group at the end of sustained ischaemia in the intestine as well as in the mesentery.

Discussion
In contrast with other solid organ transplants, progress in intestinal transplantation from an experimental level to clinical practice has been slow. But intestinal transplantation is possible and is now, for some conditions, the most suitable therapeutic option. The small intestine is one of the most sensitive tissues to the I/R syndrome, and hence is one of the leading factors in small bowel rejection. Any mechanism that could protect against I/R injury would have a beneficial role in small bowel transplantation.

This is the case with ischaemia preconditioning in which the tissue is rendered resistant to the deleterious effects of prolonged ischaemia and reperfusion by previous exposure to brief periods of vascular occlusion. As shown in fig 5, preconditioning prevented the increase in protein release in the perfusate (used here as a marker of tissue injury) compared with the ischaemic group, indicating the effectiveness of preconditioning in preventing intestinal ischaemia/reperfusion damage. When NO generation was inhibited prior to preconditioning, the protective effect was eliminated. In contrast, when the NO donor was administered to I/R animals, the protective effect reappeared; the rate of protein release was significantly lower than that in the I/R group. These results confirm our previously reported data which suggested that part of the protective effect of preconditioning is dependent on NO generation.

Weisbrodt and colleagues reported the presence of both the inducible and constitutive forms of NOS in the mouse and rat ileum but only the constitutive form in the jejunum. In this organ, NO has been shown to modulate jejunal motility, blood flow, oxygen uptake, nutrient induced intestinal hyperaemia, epithelial permeability, leucocyte adhesion to post-capillary venules, and to maintain vascular integrity in endotoxin induced acute intestinal damage in the rat.

Previous studies have demonstrated that NO can inhibit GAPDH, a critical enzyme in the glycolytic pathway, indicating that NO may be able to modulate anaerobic glycolysis by inhibiting an important step in the pathway. We recently demonstrated that NO generated during preconditioning inhibits this enzyme in the jejunum. In the same study we also reported that iNOS was not involved in this effect. Our study confirms this finding. As shown in fig 3A, administration of the specific iNOS inhibitor 1400W did not modify inhibited GAPDH activity after preconditioning. In contrast, L-NAME reversed this inhibition.

The profile of NO generation in the tissue fits well with these results (fig 4A). NO generated during the preconditioning process decreased with addition of L-NAME. The presence of 1400W could not inhibit NO generation, indicating that iNOS is not involved in NO generation. Nitrite and nitrate tissue levels returned to basal levels during sustained ischaemia (fig 4B), indicating that NO release is not maintained, as previously described.

Therefore, this transient NO generation can also inhibit transient GAPDH activity. As shown in fig 3B, GAPDH inhibition is still patient at two minutes of preconditioning but disappears in the other groups and throughout the other ischaemic periods. Direct participation of NO was also confirmed when NO was administered to the ischaemic group; in this case, it maintained inhibition throughout the ischaemic process.

Alteration of GAPDH activity during glycolysis may modify the formation rate of glycolytic intermediates. One important glycolytic intermediate that has a recognised effect as a tissue protector is F16BP, which acts by facilitating metabolic recovery during hypoxia and ischaemia. Our study has described a mechanism by which this recognised endogenous protector may be accumulated as a consequence of transient GAPDH inhibition.

When preconditioning was carried out before ischaemia (Prec+I/R), there were no significant modifications in F16BP content compared with control levels until 30 minutes of ischaemia (fig 1). In contrast, in non-preconditioned rats, significant decreases in F16BP were already detected after just two minutes of ischaemia. This suggests that preconditioning can maintain basal F16BP levels for longer. Moreover, F16BP modifications as a consequence of preconditioning are dependent on NO generated in the precondi-
tioning period. This is supported by the fact that administration of 1-NAME to the preconditioned groups resulted in F16BP levels similar to those obtained in the ischaemic group at 30 and 90 minutes. The direct relationship between NO presence and F16BP accumulation was also confirmed when NO was administered to ischaemic animals; in this case, F16BP levels remained similar to controls in spite of the ischaemic insult. The increase in F16BP content in the preconditioned group at the end of ischaemia may facilitate metabolic recovery in the following reperfusion period, possibly due to normal activity of GAPDH at the end of ischaemia (see fig 3).

Figure 2 shows glucose content. The anoxia period presented a stress that led to a rapid decrease in glucose content in the first minutes of ischaemia. Probably for this reason no differences were detected in any of the groups. Therefore, part of the protective effect of preconditioning is not due to an increase in glucose content but may well be due to accumulation of F16BP.

Another interesting finding was the lack of effect of 1-NAME administration to control animals (see figs 1–4). It is true that control animals have physiological NO levels but these basal levels are probably too low to affect GAPDH inhibition, and consequently F16BP accumulation.

F16BP has a wide variety of protective effects independent of its action as a glycolytic intermediate. It increases peristaltic activity,16 it may inhibit neutrophil free radical production,17 it maintains the correct XDH/XO ratio,18 it stabilises intracellular calcium in the hypoxic rat brain,19 and it has been used to improve preservation solutions in renal transplantation.20 In addition, the protective effects of exogenously administered F16BP have also been observed in intestinal ischaemia21 and in ileum muscle.14

To elucidate the effect of F16BP under our experimental conditions, we tested if F16BP superfusion in ischaemic animals had any effect in the prevention of tissue injury. As shown in fig 5A and 5B, I/R animals administered F16BP behaved in much the same way as those subjected to preconditioning; hence F16BP can diminish the damage caused by I/R injury. Moreover, administration of F16BP to the P+N+I/R group attenuated the deleterious effect of 1-NAME treatment. In this last group, F16BP improved damage but did not prevent it completely. This could be explained as follows: F16BP only helps to minimise the damage, and in this group the absence of NO obstructed complete improvement. This suggests that irreversible NO inhibition by 1-NAME probably stops essential cell functions and has harmful consequences, independent of its effect on glycolysis.22,23 This damage is probably irrecoverable, even with the beneficial effect of F16BP.

A number of reports have suggested a protective role for exogenously applied F16BP in tissues but many questions remain regarding the ability of fructose to cross the cell membrane. This is because F16BP has a neutral pH by virtue of its two phosphates. However, recent studies by Hardin and colleagues17 have demonstrated that F16BP can cross the cell membrane of vascular smooth muscle. They proposed possible mechanisms, such as the use of a membrane carrier, an increase in lipid solubility by binding with other enzymes such as aldolase, passive permeability of the membrane to F16BP, or endocytosis of extracellular fluid containing F16BP.

This question was not specifically addressed in our study. We propose a mechanism by which F16BP is accumulated endogenously in preconditioned rats. If preconditioning is carried out before ischaemia, the intestine can maintain high levels of this well known protector. This mechanism of protection from I/R injury depends on NO synthesis. Nevertheless, NO could offer protection from I/R injury via some other mechanism.

In summary, our study indicates that NO generated during preconditioning inhibits the glycolytic enzyme GAPDH, leading to NO dependent accumulation of F16BP that exerts a protective effect. These data confirm that preconditioning is an alternative protective mechanism against I/R injury.

This work was supported by grant FIS 98/003901. A Sola was supported by a grant from Institut d’Investigacions Biomèdiques August Pi i Sunyer.