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Fructose 1,6-Bisphosphate Prevents Oxidative Stress in the Isolated and Perfused Rat Heart

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Rat hearts were perfused with the Langendorff technique at constant flux in the presence of the oxidizing agents hydrogen peroxide and diamide. Fructose 1,6-bisphosphate strongly prevented the decline of heart contractility due to the infusion of these oxidizing agents. On the other hand, fructose 1,6-bisphosphate had no effect on the release of total glutathione into the perfusate but prevented the loss of lactate dehydrogenase indicating a protective effect on cell membranes. Comparing the cytosolic and mitochondrial loss of glutathione, fructose 1,6-bisphosphate exerted a beneficial action only on the mitochondrial fraction. Several mechanisms of action have been considered to explain the protective action of fructose 1,6-bisphosphate. In our experimental conditions fructose 1,6-bisphosphate might stimulate its own production giving rise to dihydroxyacetone phosphate, that, after reduction to glycerol 3-phosphate, can permeate the mitochondrial membrane with the final production of energy.

KEY WORDS—Fructose 1,6-bisphosphate; heart perfusion; glutathione; oxidative stress; thiol groups.

ABBREVIATIONS—BSA, bovine serum albumin; diamide, diazenedicarboxylic acid bis (*N*,*N*-dimethylamide); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; FDP, fructose 1,6-bisphosphate; LVP, left ventricular pressure; SDS, sodium dodecyl sulfate.

INTRODUCTION

Fructose 1,6-bisphosphate is an intermediate of the glycolytic process which has also been used therapeutically.[1,2] Previous research has demonstrated that FDP induces modifications of the ionic permeability of cell membranes by favouring the uptake of K^+ ions[3] and release of protons;[4] in addition, it regulates the flux of Ca^{2+} by reducing its binding to cardiac tissue.[5,6] In the isolated and perfused rat heart, exogenous FDP was shown to preserve high energy metabolites during anoxia and to restore myocardial metabolism and contractility during reperfusion,[7] at variance with fructose which is not utilized for the energy demand of the heart.[7] According to some studies[8,9] the preservation of high energy metabolites during anoxia prevents both the irreversible transformation of xanthine dehydrogenase into xanthine oxidase and the formation of thiobarbituric acid reactive material.[8,9] A protective effect of FDP is also apparent in liver intoxicated with carbon tetrachloride.[10]

A certain amount of FDP appears to penetrate through the membranes of the myocardial cells and is then metabolized through the glycolytic pathway.[7,11,12–14] Nevertheless, several problems connected with its action at cellular and molecular level still need to be clarified.

In the present paper the protective effect of FDP on oxidative stress of perfused heart induced by oxidizing agents (diamide and hydrogen peroxide) is reported. The effect of FDP in terms of a direct or indirect intervention in the oxidative stress is discussed.

MATERIALS AND METHODS

Fructose 1,6-bisphosphate was a gift of Biomedica Foscama, Ferentino, Italy. All the other reagents were of analytical grade.

The animals (Wistar albino rats weighing about

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300 g) were anaesthetized with ethyl ether and, after decapitation, the hearts were removed rapidly and placed into an ice-cold medium containing 115 mM NaCl, 25 mM $NaHCO_3$, 4·7 mM KCl; 0·9 mM KH_2PO_4, 1·2 mM $MgSO_4$, 1·5 mM $CaCl_2$ and 11 mM glucose at pH 7·4. When added, FDP was 0·2 mM. The hearts were perfused retrogradely with the same medium in a non-recirculating Langendorff apparatus[15] with a constant flux of 12 ml min^{-1}. The solution was kept at 37°C and saturated with 95 per cent O_2 and 5 per cent CO_2. Before any experiments, the hearts were perfused for about 20 min to allow metabolic stabilization of the preparation. Left ventricular pressure was measured after inserting a fluid-filled balloon in the left ventricular cavity via the atrium; the balloon was connected to a pressure transducer for determining the pressure. Fractions of the effluent were collected for the determination of released glutathione and lactate dehydrogenase. At the end of the perfusion experiment, each heart was removed rapidly and homogenized with an Ultra Turrax in 10 ml of 0·180 M KCl, 5 g l^{-1} BSA, 3 mM EDTA, 1 mM EGTA, buffered with 5 mM Hepes/10 mM Tris (pH 7·4). One ml of the homogenate was used for the estimation of protein, glutathione and total sulfhydryl groups, while the remaining volume was utilized for the preparation of mitochondria according to the method of Lindenmayer *et al.*[16]

Total sulfhydryl groups of the homogenate and mitochondria were titrated by using the Ellman's reagent DTNB.[17] Homogenate or mitochondria (0·5 mg protein) were incubated in 0·2 M Tris–HCl buffer (pH 8·1) containing 5 mM EDTA and 0·8 per cent SDS to a final volume of 2·5 ml. The reaction was started by adding DTNB to a final concentration of 2 mM and the increase in absorbance was followed at 412 nm until a constant value was obtained. The concentration of sulfhydryl groups was calculated using $\epsilon_M = 13\,600$.

Glutathione was measured essentially by the procedure of Tietze,[18] modified for the determination of oxidized glutathione (GSSG), as described by Anderson.[19]

Lactate dehydrogenase in the coronary effluent was determined at 25°C by following NADH oxidation according to Bergmeyer and Bernt.[20]

Protein was determined by the biuret method.[21]

The reported results are expressed as mean ± S.E. of at least five experiments, each one representing an individual perfusion experiment. Statistical evaluation of multiple group differences was performed using a one-factor analysis of variance (ANOVA)-repeated measures program. When significant differences ($P < 0·05$) were evident, comparisons between individual groups were made with the Scheffé's test.[22]

RESULTS

Fructose 1,6-bisphosphate in isolated rat heart, perfused in the presence of glucose with the Langendorff technique at constant flow, in concentrations ranging from 0·05 to 1·5 mM initially increased the contractile force from about 70 mmHg to about 120 mmHg (Figure 1) in agreement with other reports,[5,23,24] while concentrations higher than 0·5 mM induced a negative inotropic and chronotropic effect[5,6,25] probably related to a direct chelation of calcium ions.[5]

The infusion of 0·1 mM hydrogen peroxide during normoxic perfusion caused serious alterations of the contractile activity reflected by an increase in the resting tension that led, in about 1 h, to a complete arrest of the heart function in hypercontraction (Figure 2 B). A similar result was obtained with diamide (Figure 2 D). The inclusion of 0·2 mM FDP in the perfusion medium relieved the toxic effect of diamide (Figure 2 E) and especially that of hydrogen peroxide (Figure 2 C) by

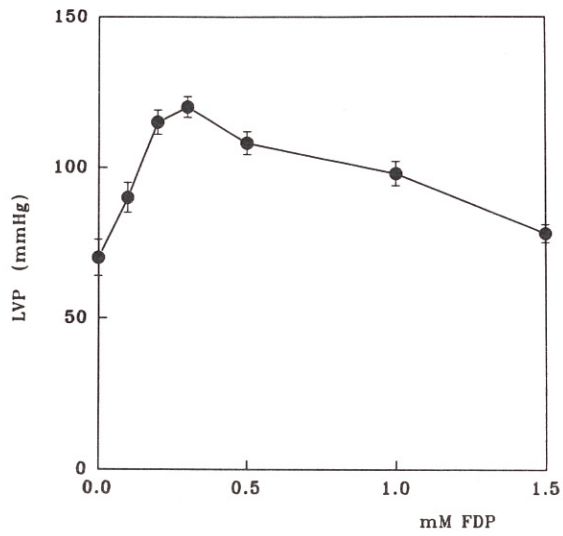

Figure 1. The correlation between left ventricular pressure and increasing concentrations of FDP on the isolated and perfused rat heart. After equilibration, the heart was perfused for 15 min and, at this point, the left ventricular pressure (LVP) value of separate experiments was recorded.

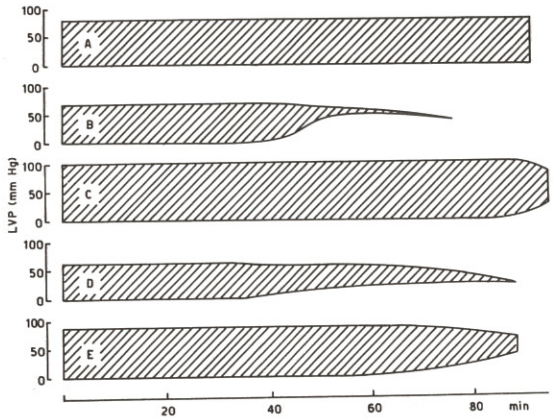

Figure 2. The protective effect of FDP on the time course of left ventricular pressure (LVP) of the isolated rat heart during perfusion in the presence of hydrogen peroxide or diamide. Hearts were perfused in the presence of 11 mM glucose (A) and 0·1 mM H_2O_2 (B and C) or 0·1 mM diamide (D and E). In C and E, 0·2 mM FDP was also present.

prolonging the duration of the contractile activity which, in the presence of hydrogen peroxide was almost doubled (compare Figure 2 B and C). In the absence of glucose, the decrease of the contractile activity induced by H_2O_2 was far more evident (not shown).

As reported in Figure 3 A and B, treatment with hydrogen peroxide or diamide induced a release, into the perfusate, of lactate dehydrogenase that indicates damage at the level of the cell membrane. The presence of 0·2 mM FDP had no protective effect on the release of total glutathione, but in the experiments with hydrogen peroxide it completely prevented the release of lactate dehydrogenase.

As reported in Table 1, after 60 min of perfusion in the presence of 0·1 mM hydrogen peroxide or diamide, a large decrease of total cytosolic glutathione was observed. The fraction of cytosolic total glutathione in the oxidized form remaining in the heart was about 40 per cent and 30 per cent in the presence of hydrogen peroxide and diamide, respectively. In the absence of glucose, the fraction of the oxidized form found in the presence of hydrogen peroxide was even higher (about 55 per cent). FDP did not prevent the decrease of cytosolic glutathione after treatment with hydrogen peroxide or diamide, but shifted the ratio of the remaining glutathione towards the reduced form. The mitochondrial glutathione also decreased by 50 per cent and 65 per cent in the presence of hydrogen peroxide and diamide respectively but, at variance with the cytosol, FDP elicited a significant protective effect.

Total sulfhydryl groups appeared to be extensively oxidized after treatment with hydrogen peroxide or diamide both in cytosol (23 per cent and 36 per cent respectively) and mitochondria (22 per cent and 39 per cent respectively); the inclusion of 0·2 mM FDP in the perfusion medium significantly reduced their oxidation (Table 1). The same experiments reported in Table 1 were performed for 90 min instead of 60 min and no substantial differences were found.

DISCUSSION

When perfused hearts, maintained in ischemic conditions for 1 h, are reperfused with normoxic medium, a massive efflux of glutathione together with protein, lactate and lactate dehydrogenase is observed.[26,27] Similarly, a large efflux of glutathione, that rapidly reaches a maximum and subsequently decreases, can be obtained with thiol oxidizing agents such as diamide and hydrogen peroxide.[26] Diamide is a well known diazocompound able to oxidize glutathione to its disulfide directly while hydrogen peroxide acts through the enzyme glutathione peroxidase.

The results reported here indicate that FDP exerts a specific action at the level of cardiac muscle that is not observable with fructose or fructose 6-phosphate (not shown). Its action on the oxidative stress seems to be linked to a potentiation of the glycolytic pathway. Usually hexose phosphates are poorly transported across the cell membrane, but, in studies with rat diaphragm, Beloff-Chain *et al.*[28] demonstrated that, at sufficiently high extracellular concentrations, hexose phosphates enter the cell. There is evidence that exogenous fructose 1,6-bisphosphate is able to cross the cellular membrane and, once in the cytosol, it undergoes further metabolism with the production of ATP and metabolites that favour the conservation of cellular energy.[7,8,12–14]

It is interesting to note that, in our experimental conditions, FDP was used at a relatively low concentration (0·2 mM) at variance with other experiments where it was employed at a concentration of 5 mM,[6–8] with the low concentrations of FDP, the direct interaction with calcium ions is negligible.

As far as the mechanism of action of FDP is concerned, there are several potential modes of action reported in the literature[2–6] and described in the introduction. An indirect protective action of

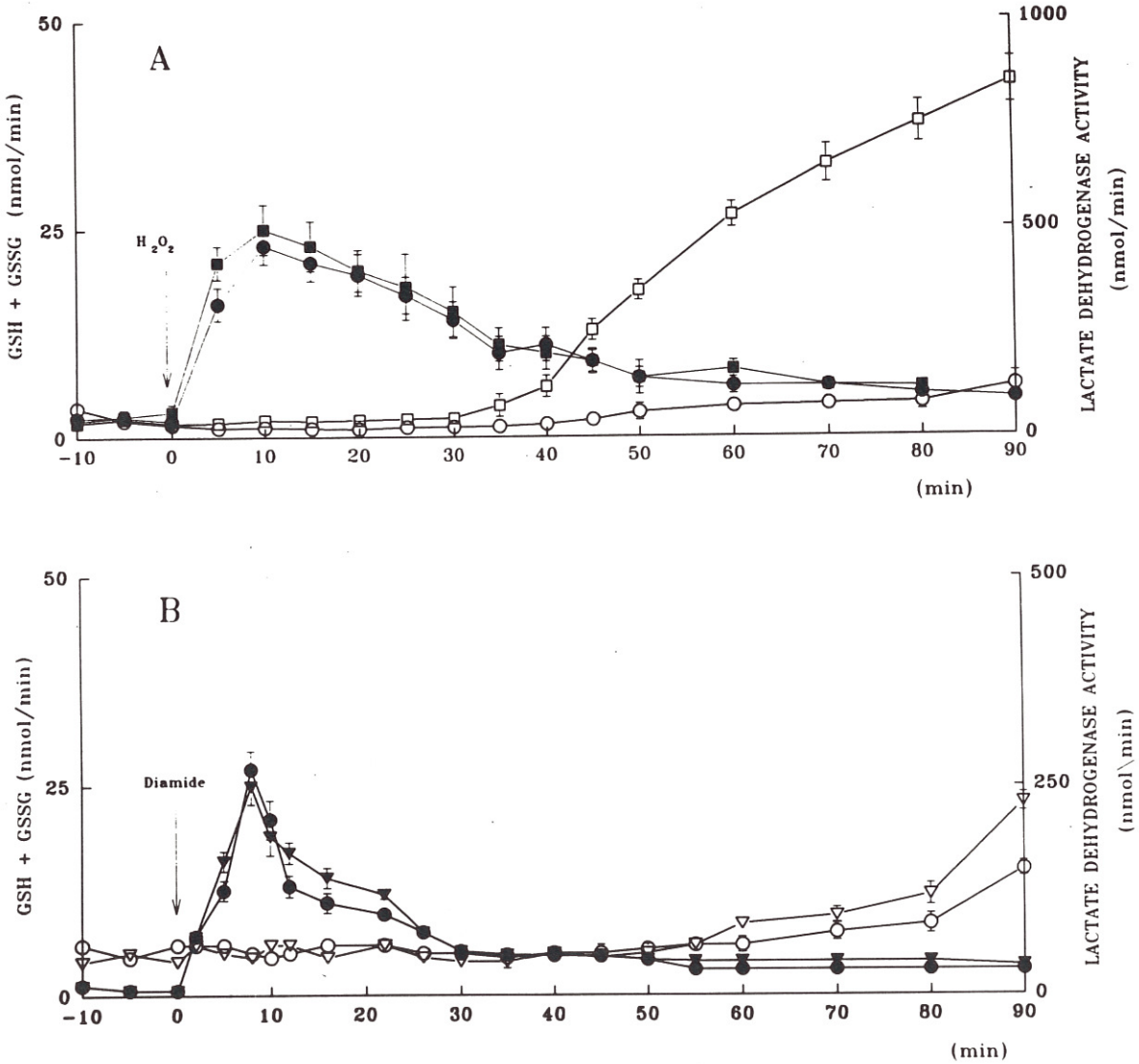

Figure 3. Time course of the release of total glutathione and lactate dehydrogenase from rat heart perfused in the presence of 0·1 mM hydrogen peroxide (A) or 0·1 mM diamide (B). Filled symbols: total glutathione; open symbols: lactate dehydrogenase. ●, ○: + 0·2 Mм FDP; ■, □; ▼, ▽: − FDP.

FDP against oxidative stress was attributed to inhibition that depended on the chelation of calcium, of the conversion of xanthine dehydrogenase to xanthine oxidase.[8,9] The hydrogen peroxide and diamide-dependent oxidative stress is linked to depletion of thiols and particularly to loss of glutathione; FDP might act intracellularly by preventing the release of glutathione from the mitochondrial compartment which is important in maintaining the viability of the cell.[29] Antioxidant agents are extremely effective in preventing peroxidations occurring during the oxidative challenge.[30] We tested if the protective effect of FDP can be linked to a direct antioxidant action but FDP does not show any inhibition of the lipid peroxidation elicited by NADPH/Fe^{2+}/ADP (not reported) indicating that it does not act as a free-radical scavenger or iron chelator. The anti-alcohol effect of FDP indicates that it modifies altered NAD^+/NADH

Table I. The protective effect of FDP on the depletion from the rat heart of cytosolic and mitochondrial glutathione and sulfhydryl groups induced by hydrogen peroxide or diamide.

	Cytosol			Mitochondria		
	GSH + GSSG (nmol mg^{-1} prot)	GSSG (%)	SH (nmol mg^{-1} prot)	GSH + GSSG (nmol mg^{-1} prot)	GSSG (%)	SH (nmol mg^{-1} prot)
Normoxic control	$11{\cdot}09 \pm 0{\cdot}52$	$3{\cdot}0 \pm 0{\cdot}07$	$94 \pm 0{\cdot}73$	$2{\cdot}24 \pm 0{\cdot}04$	$2{\cdot}3 \pm 0{\cdot}14$	$72 \pm 0{\cdot}55$
Hydrogen peroxide	$4{\cdot}14 \pm 0{\cdot}11^{*}$	$43 \pm 1{\cdot}21^{*}$	$72 \pm 2{\cdot}51^{*}$	$1{\cdot}12 \pm 0{\cdot}09^{*}$	$6{\cdot}0 \pm 0{\cdot}68^{*}$	$56 \pm 0{\cdot}58^{*}$
Hydrogen peroxide + FDP	$4{\cdot}11 \pm 0{\cdot}67^{*}$	$36 \pm 1{\cdot}01^{*,\dagger}$	$81 \pm 1{\cdot}06^{*,\dagger}$	$1{\cdot}70 \pm 0{\cdot}03^{*,\dagger}$	$2{\cdot}2 \pm 0{\cdot}51^{\dagger}$	$65 \pm 0{\cdot}37^{*,\dagger}$
Hydrogen peroxide − glucose	$4{\cdot}16 \pm 0{\cdot}10^{*}$	$55 \pm 2{\cdot}59^{*,\dagger}$	$76 \pm 1{\cdot}53^{*}$	$0{\cdot}95 \pm 0{\cdot}09^{*}$	$12 \pm 0{\cdot}60^{*,\dagger}$	$51 \pm 0{\cdot}39^{*}$
Hydrogen peroxide − glucose + FDP	$3{\cdot}95 \pm 0{\cdot}09^{*}$	$46 \pm 1{\cdot}22^{*,\ddagger}$	$74 \pm 0{\cdot}86^{*}$	$0{\cdot}94 \pm 0{\cdot}02^{*}$	$11 \pm 0{\cdot}35^{*,\ddagger}$	$55 \pm 0{\cdot}51^{*,\ddagger}$
Diamide	$2{\cdot}12 \pm 0{\cdot}08^{*}$	$28 \pm 0{\cdot}70^{*}$	$60 \pm 1{\cdot}61^{*}$	$0{\cdot}78 \pm 0{\cdot}02^{*}$	$13 \pm 1{\cdot}15^{*}$	$44 \pm 1{\cdot}32^{*}$
Diamide + FDP	$2{\cdot}36 \pm 0{\cdot}14^{*}$	$13 \pm 1{\cdot}35^{*,\S}$	$70 \pm 2{\cdot}02^{*,\S}$	$1{\cdot}05 \pm 0{\cdot}03^{*,\S}$	$5{\cdot}3 \pm 0{\cdot}53^{*,\S}$	$46 \pm 0{\cdot}71^{*}$

Perfusion experiments were performed, for 60 min, in the presence of 11 mM glucose and, when indicated, 0·1 mM H_2O_2, 0·1 mM diamide and 0·2 mM FDP. Values significant at confidence level of 95 per cent are compared in the following way: * versus normoxic control; † versus hydrogen peroxide; ‡ versus hydrogen peroxide + FDP; § versus diamide

ratios and this could be reflected in the oxido-reduction of glutathione.[31]

An hypothesis that can be drawn from our results takes into account the control mechanisms of phosphofructokinase by FDP. This enzyme is the major site of regulation of the glycolytic process[32] and it is inhibited by ischemia and intracellular acidosis.[33] Fructose 2,6-bisphosphate is the physiological regulator of this enzyme[34,35] but, as pointed out by Leblanc *et al.*[36] FDP, because of its structural similarity to fructose 2,6-bisphosphate, is able to stimulate phosphofructokinase; nevertheless, for eliciting this effect, the concentration of FDP must be at least 10 to 100 times higher than that of fructose 2,6-bisphosphate. FDP appears to act as a regulator of phosphofructokinase by acting both as an activator and deinhibitor of this enzyme.[37] The treatment with oxidizing agents leads to the inactivation of sulfhydryl-sensitive enzymes such as 3-phosphoglyceraldehyde dehydrogenase[38] and the pyruvate dehydrogenase complex.[39] The metabolic flow through 3-phosphoglyceraldehyde dehydrogenase is reduced significantly,[38] while pyruvate dehydrogenase appears to be strongly inhibited[39] and consequently an increase of lactate (acidosis) occurs. In this context FDP would deinhibit phosphofructokinase thereby producing more FDP, that, after being transformed by aldolase to dihydroxyacetone phosphate is in turn reduced to glycerol 3-phosphate; the latter can permeate the mitochondrial membrane and feed reducing equivalents to the respiratory chain with the final formation of energy. In this way the inhibition of the phosphoglyceraldehyde dehydrogenase and pyruvate dehydrogenase complex is bypassed and the energetic needs could be met, at least partially.

In conclusion, in the heart, there appears to be a biphasic effect of FDP: at high concentrations (higher than 1 mM and around 5 mM) it can act either as a substrate[7] replenishing the energy stores particularly after anoxia or as a calcium-antagonist;[40] at lower concentrations (around 0·2 mM) it increases the performance of the heart by preserving the intramitochondrial content of ATP thereby increasing the energetic potentialities of the cell.

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