Mechanism of the effect of exogenous fructose 1,6-bisphosphate on myocardial energy metabolism.
I E Hassinen, E M Nuutinen, K Ito, S Nioka, G Lazzarino, B Giardina and B Chance

_Circulation_. 1991;83:584-593
doi: 10.1161/01.CIR.83.2.584
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/83/2/584

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/
Mechanism of the Effect of Exogenous Fructose 1,6-Bisphosphate on Myocardial Energy Metabolism

Ilmo E. Hassinen, MD, PhD; E. Matti Nuutinen, MD, PhD; Kinji Ito, MD, PhD; Shoko Nioka, MD, PhD; Giuseppe Lazzarino, PhD; Bruno Giardina, PhD; and Britton Chance, PhD

The effects of fructose 1,6-bisphosphate (F-1,6-P$_2$) on the isolated Langendorff-perfused heart were studied by monitoring flavoprotein fluorescence, oxygen consumption (MV$_{O2}$), coronary flow (F$_C$), systolic intraventricular pressure (P$_{sys}$), diastolic intraventricular pressure, and contraction frequency. The cellular energy state and cytosolic pH were determined by means of $^{31}$P nuclear magnetic resonance. Infusion of 5 mM F-1,6-P$_2$ caused a rapid shift toward reduction in the flavoprotein redox state and initial 50% and 44% decreases in P$_{sys}$ and MV$_{O2}$, respectively. After a partial recovery, these measures remained 11% and 25% below the basal value. Concomitantly, after an initial transient increase of 13%, F$_C$ remained 17% lower than in the basal state. When the F-1,6-P$_2$ concentration was subsequently increased to 10 mM, P$_{sys}$ and MV$_{O2}$ dropped temporarily to 31% and 29% of the basal value and then remained at 50% and 53%, respectively. Simultaneously, a brief increase was observed in F$_C$, which then fell 34% below the basal value. Rapid reoxidation of the flavoproteins and increases in MV$_{O2}$, P$_{sys}$, and F$_C$ occurred on discontinuation of the F-1,6-P$_2$ infusion. $^{31}$P nuclear magnetic resonance during infusions of both 5 and 10 mM F-1,6-P$_2$ revealed a decrease in cytosolic inorganic phosphate and a tendency to increase creatine phosphate, suggesting elevation in the cellular energy state. No changes in intracellular pH occurred as estimated from the chemical shift of the nuclear magnetic resonance of inorganic phosphate. F-1,6-P$_2$ (5 mM and 10 mM) lowered the free Ca$^{2+}$ concentration in the Krebs-Henseleit bicarbonate buffer (by 32% and 47%, respectively). This probably explains the effects of F-1,6-P$_2$ on mechanical work performance and cellular respiration. A direct metabolic effect also exists, however, because flavoprotein reduction by F-1,6-P$_2$ could be observed in the K$^+$-arrested heart, where its effects on MV$_{O2}$ were minimal. This redox effect may not be caused by changes in free Ca$^{2+}$ concentration because it could not be reproduced by infusion of EGTA. (Circulation 1991;83:584–593)

Experimental data concerning the possible protective effects of fructose 1,6-bisphosphate (F-1,6-P$_2$) against ischemic damage have been disputed.1,2 According to Markov et al.,1 F-1,6-P$_2$ restores the depressed glycolytic activity in the ischemic myocardium and intervenes in the glycolytic pathway not only as a metabolic regulator but also as a substrate. The former should occur due to the known activation of 6-phosphofructo-1-kinase (phosphofructokinase) by F-1,6-P$_2$. Similarly, it has been reported3 that F-1,6-P$_2$ has a protective effect in cases of acute myocardial infarction and that it prolongs the time before hypoxemic cardiac arrest occurs in rabbits and increases immediate salvage from cardiac arrest.4 Although these findings and their explanations have been challenged,2 mainly on the basis that sugar phosphates cannot cross the cell membrane,5 many reports have been published concerning positive effects of exogenous F-1,6-P$_2$ under different experimental conditions in protecting cultured astrocytes from hypoxic damage6 and delaying ST segment depression and angina in patients suffering from stable exertional angina.7
The work of Pfaffmann et al. has shown that F-1,6-P2 has a positive inotropic effect on the isolated perfused heart, but the tracings clearly show an acute negative inotropy at the dosages used. Unfortunately, only bolus injections of F-1,6-P2 into the aortic cannula were performed, so that the concentrations remain unknown.

Phosphofructokinase is the main regulator of glycolysis under physiological conditions and also becomes inhibited in ischemia, although rate limitation by glyceraldehyde phosphate dehydrogenase, the first phosphorylating reaction of glycolysis in the ischemic myocardium, has also been found.

Because of the clinical importance of ischemic heart disease, all possibilities for rational treatment should be tested and used. A number of reports of preclinical and clinical trials with F-1,6-P2 have been published, although a solid theoretical background is still lacking. If F-1,6-P2 were to prove as beneficial in alleviating the sequelae of myocardial ischemia, a rationale for its mechanism of action would have to be found. The present experiments were undertaken to reveal the possible effects of F-1,6-P2 on the cellular energy state and metabolism. Myocardial energization and a decrease in mechanical work load were found during F-1,6-P2 infusion and were linked to binding of extracellular Ca2+ by F-1,6-P2. A direct effect on the cellular redox state not related to changes on mechanical work was also revealed in the K+-arrested heart.

Methods

Chemicals

Routine chemicals were obtained from E. Merck AG, Darmstadt, FRG. F-1,6-P2 was from Biomedica Foscama, Rome, and Sigma Chemical Co., St. Louis.

Designs of 31P Nuclear Magnetic Resonance Experiments

Male Sprague-Dawley rats weighing 355±47 g (mean±SD) were anesthetized with intra-peritoneal pentobarbital (100 mg/kg body wt), the heart was isolated, and aortic perfusion was performed by the Langendorff procedure. The perfusion fluid was a phosphate-free Krebs-Henseleit bicarbonate buffer containing 10 mM glucose and equilibrated with 95% O2-5% CO2. The pulmonary artery was cannulated, and samples were taken constantly by suction to prevent exposure of the effluent fluid to air. A stock solution of F-1,6-P2 was injected at variable rates into the constant main flow of perfusate with an adjustable precision syringe pump; a constant perfusion pressure of 60 mm Hg was maintained by means of an overflow head. The 5-m tubing connecting the heart to the supportive perfusion equipment were covered entirely with a water mantle kept at 37°C.

Left ventricular pressure was monitored by a pressure transducer (Gould, Inc., Cupertino, Calif.) connected to a Teflon catheter inserted in the left ventricle through the left anterior wall. Oxygen concentrations in the aortic and pulmonary arterial perfusate were monitored with miniature Clark electrodes (model MI-730, Microelectrodes, Inc., Londonderry, N.H.) connected to a dual oxygen monitor (University of Pennsylvania Biomedical Instrumentation Group, Philadelphia). Coronary flow was measured by collecting the combined effluents from the pulmonary artery drain and the nuclear magnetic resonance (NMR) tube outflow.

Experiments on Mechanical Performance, Oxygen Consumption, Coronary Regulation, and the Oxidation-Reduction State of the Flavoproteins of the Heart Muscle

Because of the extensive length of catheter needed in the NMR experiments, the frequency response of the pressure monitoring system deteriorated so that more precise measurements of the mechanical performance of the heart were performed in separate experiments optimized for pressure, oxygen concentration, coronary flow, and epicardial surface fluorescence determinations. Sprague-Dawley rats were anesthetized with pentobarbital and 500 IU heparin injected into the inferior caval vein before excision of the heart. After beginning retrograde aortic perfusion, a polyethylene cannula was inserted through the left ventricular wall and connected to a model P23 ID transducer and an SP 1400 pressure monitor (Statham). The minimum and maximum pressures and the phasic pressure output connected to a digital frequency counter were recorded continuously on a strip chart. The thermostated heart compartment was equipped with fiber optics to measure the epicardial fluorescence of the tissue. Coronary flow was recorded by a drop counter with an analog output, and the venous oxygen concentration was determined with an oxygen probe (model YSI 5331, Yellow Springs Instrument Co., Yellow Springs, Ohio). A 0.5-M stock solution of F-1,6-P2, adjusted to pH 7.4 with NaOH, was infused with a calibrated Microperpex peristaltic pump (LKB, Bromma, Sweden) to a constant main perfusate flow against an overflow head (60 mm Hg). Other details were practically similar to those used in the NMR experiments. In the experiment assessing the effects of specific Ca2+ binding by EGTA, pH 7.4 was infused into the perfusate with a Microperpex pump to give a final concentration of 0.65 or 0.96 mM. These concentrations were selected to give the same free Ca2+ concentration ([Ca2+]) as the infusion of 5 or 10 mM F-1,6-P2 (see below).

Spectroscopic Methods

Nuclear magnetic resonance. The 31P NMR spectra were recorded on a pulsed–Fourier transform NMR spectrometer (model AM-500, Bruker Instruments, Wilmington, Del.) equipped with a superconducting magnet having a field strength of 11.8 T (phosphorus frequency, 202.5 MHz). The heart was placed in a 16-mm diameter NMR sample tube, which was inserted into a laboratory-built phosphorus probe de-
Figure 1. $^{31}$P nuclear magnetic resonance spectra of a perfused rat heart in the absence (panel A) and presence (panel B) of fructose 1,6-bisphosphate (FDP). In panel A, the heart was perfused with phosphate-free Krebs-Henseleit bicarbonate solution containing 10 mM glucose. In panel B, FDP was infused to obtain a final concentration of 5 mM. The spectra were constructed from 128 free-induction decays. The peak assignments in panels A and B are as follows: FDP, 6.9 ppm; extracellular inorganic phosphate [$P_{\text{i,el}}$, 5.2 ppm; cytosolic inorganic phosphate [$P_{\text{i,c}}$, 5.0 ppm; creatine phosphate (CrP), 0 ppm; $\gamma$-ATP, −2.4 ppm; $\alpha$-ATP, −7.4 ppm; $\beta$-ATP, −16.1 ppm.

Figure 2. Graphs showing effects of fructose 1,6-bisphosphate (FDP) on mechanical work performance (panel A), coronary flow and oxygen consumption (panel B), and the oxidation-reduction state of mitochondrial fluorescent flavoproteins (panel C) in isolated perfused rat heart. The flavoprotein fluorescence is an indicator of the redox state of the mitochondrial free NADH/NAD. The tracings in panels A, B, and C are representative original recordings obtained simultaneously from the same heart. For details, see “Methods” and Figure 1. During the time period indicated by $N_2$, the perfusion medium was in equilibrium at 95% $N_2$-5% CO$_2$.

signed for small, isolated tissue volumes. The field was shimmed with the aid of a phantom of the approximate dimensions of a rat heart and containing phosphate solution in water. A 0.03-ppm line width was obtained for the water proton resonance peak.

The spectra were accumulated using a pulse width of 40 μsec, resulting in a 60° tilting angle, a pulse interval of 3.6 seconds, and an acquisition time of 0.2 second. A line broadening of 40 Hz was imposed. The saturation factor was determined by measuring control spectra at 10-second pulse intervals and the same pulse width of 40 μsec. No saturation was observed with the 3.6-second repetition rate. Two 8-minute sets of 128 spectra were collected for each metabolic steady state of 16 minutes. The results were presented as averages of 128 spectra.

The phosphorus moieties of F-1,6-P$_2$ appeared in a single peak at 6.9 ppm by reference to creatine phosphate (CrP). The position of the cytosolic inorganic phosphate ($P_{\text{i,c}}$) peak at physiological pH was at 4.9 ppm (Figure 1). This allowed $P_{\text{i,c}}$ to be estimated in the presence of 5–10 mM F-1,6-P$_2$ with reasonable accuracy.

Optical methods. Flavoprotein fluorescence was recorded from the tissue surface with a custom-built three-channel fluorometer/spectrophotometer. The excitation wavelength was 465 nm, and fluorescence emission above 510 nm was recorded. Myoglobin oxygenation grade in some experiments was monitored as organ surface reflectance changes at 582 and 630 nm simultaneously with the flavin fluorescence.

Ionized calcium. Free [Ca$^{2+}$] in the perfusion medium was determined with an ISA calcium electrode (Radiometer, Copenhagen).

Statistical Analysis

The analysis of variance for repeated measurements was used for the analysis of the NMR data.

Results

Systolic and Diastolic Pressures and Heart Rate

Infusion of F-1,6-P$_2$ at a concentration of 5 mM in the perfusate resulted in a rapid decrease in peak systolic pressure and an increase in diastolic pressure (Figure 2A). The initial decrease in systolic pressure was partially reversible; after a few minutes, it re-
gained 90% of its basal value, although the diastolic pressure remained elevated. When the perfusate F-1,6-P₂ concentration was increased by a further step to 10 mM, a decrease in the peak systolic pressure again occurred, and although there was a partial recovery, the peak pressure remained at 50% of the control value, and a further increase in the diastolic pressure was recorded. On discontinuation of the F-1,6-P₂ infusion, a temporary overshoot beyond the initial value of systolic pressure was observed. Because the rate-pressure product has been shown to be in excellent correlation with the oxygen consumption and hence with the energy expenditure of the isolated perfused heart, the mechanical work load is here described as the rate-pressure product (Figures 2, 4, and 5).

Oxygen Consumption and Coronary Flow

A rapid, partly reversible increase in the venous oxygen concentration and a decrease in oxygen consumption were found at each step of F-1,6-P₂ infusion (Figure 2B); the time course of the phenomenon was very similar to the changes in the work performance (rate-pressure product) of the heart muscle (Figure 2B). A simultaneous transient increase in the coronary flow was observed, but the relative effect was small, and in the new steady state that was reached during F-1,6-P₂ infusion, the coronary flow was smaller than the basal value. The net effect was a diminution in the oxygen consumption. On discontinuation of the F-1,6-P₂ infusion, the oxygen consumption and coronary flow rate rapidly returned to their original values; also, a transient overshoot was observed in the oxygen consumption, and its timing matched that of the changes in contractility.

Cellular Redox State

Fluorescence was used as a measure of changes in the oxidation-reduction state of flavoproteins, since their fluorescence was quenched upon reduction. It has been shown that mitochondrial fluorescent flavoproteins are in equilibrium with the mitochondrial NADH/NAD pool, that the main fluorescent component is lipoamide dehydrogenase, and that in intact tissue the flavoprotein fluorescence originates from mitochondria.

The commencement of a constant infusion of 5 mM F-1,6-P₂ caused a shift in the redox state of the flavoproteins toward reduction after a rapid initial spike of oxidation (Figure 2C). The increase of the F-1,6-P₂ concentration to 10 mM caused a further reduction, with a similar initial oxidation spike. Proper functioning of the fluorescence readout was ascertained with a 6-minute pulse of anoxia effected by perfusion with a medium equilibrated with 95% N₂−5% CO₂ (Figure 2C, right).

Cellular Energy State

The cellular energy state was estimated by means of ³¹P NMR. The resonance peak area ratio of CrP to Pₐ gives a good impression of the CrP/creatine ratio, because under physiological conditions the concent-

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Graphs showing effect of fructose 1,6-bisphosphate (FDP) on the cellular energy state and cytosolic pH (pHᵣ) in isolated perfused rat hearts. The data were compiled from ³¹P nuclear magnetic resonance experiments and represent mean±SEM from four to six accumulations of 128 free induction decays in each of four hearts. pHᵣ was calculated from the chemical shift of inorganic phosphate (Pᵢ) in reference to creatine phosphate (CrP). For details, see “Methods.”

Brain tissue the changes in the oxidation-reduction state is ascertained with the chemical shift of inorganic phosphate (Pᵢ) in reference to creatine phosphate (CrP). For details, see “Methods.” The net effect was a diminution in the oxygen consumption. On discontinuation of the F-1,6-P₂ infusion, the oxygen consumption and coronary flow rate rapidly returned to their original values; also, a transient overshoot was observed in the oxygen consumption, and its timing matched that of the changes in contractility.

**Cellular Energy State**

The cellular energy state was estimated by means of ³¹P NMR. The ratio of CrP to Pᵢ gives a good impression of the CrP/creatine ratio, because under physiological conditions the concentration changes in Pᵢ and creatine are practically equal but opposite in sign. Moreover, due to the near-equilibrium of the creatine kinase reaction, the CrP/creatine ratio is an indicator of the free ATP/free ADP ratio, and thus the CrP/Pᵢ ratio can be used for qualitative monitoring of the cellular energy state.

Typical NMR spectra in the presence and absence of F-1,6-P₂ are shown in Figure 1. The very large peak area of F-1,6-P₂ is due to the fact that the present experimental setup inevitably led to bathing the perfused heart in several milliliters of perfusion medium in the NMR sample tube.

It was observed that F-1,6-P₂ infusion caused a tendency to increase the CrP/Pᵢ ratio (Figure 3B), mainly due to a decrease in the concentration of Pᵢ, the most sensitive indicator of the cellular energy state of intact tissues observed with phosphorus NMR. The Pᵢ values in Figure 3B are expressed as peak areas in the NMR spectrum, but by reference to the β-phosphorus resonance of ATP and a value of 5.55 mM for ATP, an initial intracellular Pᵢ concentration of 1.3±0.7 mM can be calculated. Because of experimental variation, the changes in CrP/Pᵢ did not reach statistical significance (p=0.212), whereas the changes in Pᵢ were almost significant (p=0.052).

It was of interest to test the possible changes in cytosolic pH. If the F-1,6-P₂ infusion resulted in the stimulation of anaerobic glycolysis, as postulated by previous researchers, a disproportionate activation of anaerobic glycolysis (compared with mitochondrial oxidation of extramitochondrial NADH) would result in the production of lactic acid leading to a pH change.
The chemical shift of $P_1$ in NMR can be used to determine cytosolic pH according to the equation:

$$\text{pH} = 6.77 + \log \left( \frac{\delta P_1 - 3.29}{5.68 - \delta P_1} \right)$$

where $\delta P_1$ is the chemical shift (in parts per million) of $P_1$ by reference to CrP.\(^{19}\) As can be seen from Figure 3A, there were no statistically significant changes in the cytosolic pH (Figure 3). This also corroborates the rather efficient buffering of cytosolic pH in the isolated heart perfused with Krebs-Henseleit bicarbonate solution.

**Extracellular Free Calcium**

It would be tempting to suggest that the pronounced negative inotropic effect of F-1,6-P\(_2\) was caused by perturbations of calcium homeostasis, possibly due to an effect on the transmembrane calcium fluxes or simply on the extracellular free $[\text{Ca}^{2+}]$. Because of the known low solubility of calcium phosphates, a low dissociation constant could be anticipated for the calcium salt of F-1,6-P\(_2\). To test the latter possibility, free $[\text{Ca}^{2+}]$ was measured with a calcium electrode. This concentration was found to be 2.03 mM in the Krebs-Henseleit bicarbonate solution with a total $[\text{Ca}^{2+}]$ of 2.5 mM and in equilibration with 5% CO\(_2\). Addition of 5 mM F-1,6-P\(_2\) lowered free $[\text{Ca}^{2+}]$ to 1.38 mM and 10 mM F-1,6-P\(_2\) to 1.07 mM. These data suggest a value of 10 mM for the dissociation constant ($K_d$) of the monocalcium salt of F-1,6-P\(_2\). Prompted by these observations, experiments were conducted by titrating 10 mM F-1,6-P\(_2\) with 0–10 mM CaCl\(_2\) in 5 mM Tris-Cl at pH 7.4 (Figure 4). These experiments yielded 2.76–3.03 mM as the apparent $K_d$ of the monocalcium salt of F-1,6-P\(_2\), depending on the principle used for graphical data reduction.\(^{21}\) A typical experiment is shown in Figure 4. As expected, the binding is dependent on the ionic strength demonstrated by the different $K_d$ values in the Krebs-Henseleit solution and 5 mM Tris-HCl at pH 7.4.

**Comparison of the F-1,6-P\(_2\) Effects With Those of Extracellular Calcium Transients**

Since the calcium-lowering effect could be merely coincidental, experiments were conducted to observe the effects of manipulations of extracellular free $[\text{Ca}^{2+}]$ by other means. Total $[\text{Ca}^{2+}]$ was kept constant, and free $[\text{Ca}^{2+}]$ was lowered by addition of the calcium chelator EGTA to the perfusion medium.

The inhibition of oxygen consumption by F-1,6-P\(_2\) was reproduced by EGTA (Figure 5), although the biphasic nature of the response was less pronounced than with F-1,6-P\(_2\). However, there was an important difference in the pattern of the redox changes: EGTA caused an oxidation of the flavoproteins (Figure 5C), whereas F-1,6-P\(_2\) caused a reduction. This EGTA effect has been observed previously.\(^{22}\) There were differences also in the temporal pattern and direction of the effects of F-1,6-P\(_2\) and EGTA on the coronary flow: the F-1,6-P\(_2\) effects showed a pronounced biphasic pattern, whereas EGTA caused a monotonous increase. The same was true with the mechanical work load (Figure 5), which was reduced more in the steady state by EGTA than by F-1,6-P\(_2\), which gave only a sharp and pronounced transient decrease at a 5 mM concentration and a subsequent permanent decrease at 10 mM.

These results indicate the involvement of extracellular calcium but cannot exclude specific effects of EGTA. Therefore, experiments were conducted also with lowering of only the total extracellular $[\text{Ca}^{2+}]$ (Figures 6 and 7). It was found that step decreases in total $[\text{Ca}^{2+}]$ caused transient reduction of the flavoproteins without significant simultaneous myoglobin deoxygenation. On returning total $[\text{Ca}^{2+}]$ to the original value of 2.5 mM, an oxidation of myoglobin occurred. The oxygen consumption was concomitantly sharply increased, and a transient myoglobin deoxygenation occurred, probably due to a momentary imbalance of the coronary vasoregulation on commencement of the normal mechanical work. The oxygen consumption changes closely followed those of the mechanical work load measured as the rate–pressure product (Figure 6).

It was also of interest to test which changes were secondary to alterations in the mechanical work load. When the contractility was eliminated by diastolic arrest with 18 mM KCl, there were still effects on the mitochondrial redox state as indicated by the flavoprotein fluorescence (Figure 8C) and the coronary flow (Figure 8B), but the effects on oxygen consumption were small. Both F-1,6-P\(_2\) and EGTA increased the coronary flow, but as in the beating heart, the responses of the redox state to F-1,6-P\(_2\) and EGTA were opposite each other; that is, F-1,6-P\(_2\) caused flavin reduction, whereas EGTA caused flavin oxidation.

The experiments on the KCl-arrested heart were repeated with lowering only of total $[\text{Ca}^{2+}]$. Hardly any changes were observed in the redox state or in oxygen consumption or myoglobin oxygenation grade during total $[\text{Ca}^{2+}]$ transients in the quiescent heart (Figure 7).
Discussion

Although the mechanism of ischemic damage is complicated, the paradigms of many approaches for its alleviation are based on the involvement of the cellular energy state. It has also been indicated that the tissue damage may be more related to the accumulation of glycolytic end products than to any decrease in ATP, and in this light the rationale of treatment of ischemia with F-1,6-P2 is interesting. Phosphorylation of sugars has been generally regarded as a mechanism for trapping them into cells because of their low penetration through biological membranes. Even P translocation across the plasma membrane is slow. The concentration of free F-1,6-P2 in the cytosol, calculated from the equilibrium of the aldolase reaction, is only 1.2 μM, so that infusion of F-1,6-P2 in the millimolar concentration range results in a more than thousandfold gradient across the plasma membrane. It remains to be shown whether F-1,6-P2 crosses the membrane at all, let alone in substrate quantities. The intravenous total doses of F-1,6-P2 in clinical use vary up to 250 mg (0.6 mmol) trisodium salt/kg body wt, and the peak concentration achieved in the blood depends on the infusion rate. Five minutes after an infusion of 250 mg trisodium salt of F-1,6-P2/kg body wt, its concentration in blood plasma is 2.26 mM; thereafter, it disappears, in accordance with exponential kinetics and a half-life of 9.5 minutes.

The present results show that extracellular F-1,6-P2 in the heart has effects compatible with its original intended use; namely, it has a tendency to increase the CrP/Pi ratio (Figure 2C), the changes of which reflect short-term changes in the ATP/ADP-Pi concentration ratio, but the mechanism of the action of F-1,6-P2 on this parameter and also the mechanism of the postulated protective action are more complex than previously believed. Because the effects on oxygen consumption in the K+-arrested heart were small, the observed tendency to increase the energy state of the beating heart must be due to the drastic changes in the mechanical work, which in turn are most probably linked to primary changes in the concentration of extracellular free calcium. The free [Ca2+] decrease in the Krebs-Henseleit bicarbonate solution in the presence of 5 mM F-1,6-P2 was 32%, and the decrease achieved with 10 mM F-1,6-P2 was 47%. The extent of the free [Ca2+] decrease in vivo remains to be established. It is less probable that the increase in the CrP/Pi ratio would be due to alleviating a relative hypoxia, because the effects of F-1,6-P2 on coronary flow are still within the range of effective autoregulation, as shown in Figures 2 and 4. Although steep oxygen gradients can be easily dem-
The matrix of the isolated rat heart is perfused with Krebs-Henseleit bicarbonate solution, which maintains the redox state of flavoproteins and the oxygenation grade of myoglobin. The experiment depicted in Figure 6 also argues against a major role of hypoxia as a reason for the metabolic changes observed during F-1,6-P2 infusion. The adequacy of oxygen supply in the crystalloid-perfused isolated rat heart has been discussed previously.

The reason for the partial recovery of the contractility of the heart from the depression that occurs with stepwise increases in F-1,6-P2 concentration remains to be shown. The phenomenon is reminiscent of the effects of lowering free [Ca2+] by the infusion of 1.25 mM EGTA into isolated hearts perfused with Krebs-Henseleit bicarbonate solution, with a notable difference in the direction of the redox change in the flavoproteins, indicators of the redox state of the free NADH/NAD system in the mitochondrial matrix. The oxidation of the mitochondrial matrix NADH when extracellular calcium decreases can be explained by a diminution in the thermodynamic driving force of NAD reduction brought about by the calcium-regulated nonequilibrium enzyme pyruvate dehydrogenase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase, but the change that occurs with F-1,6-P2 indicates that, in this case, the respiratory inhibition outweighs the potential effects at the level of mitochondrial Ca2+-dependent dehydrogenases. The possible direct metabolic effects of F-1,6-P2 on carbohydrate utilization are complicated by its effect on free [Ca2+], for it has been shown that the activation state of phosphofructokinase in the myocardium is dependent on extracellular free [Ca2+]. This counteracts the direct effect of F-1,6-P2 on phosphofructokinase. A major direct effect of external F-1,6-P2 on glycolysis has been recently ruled out (E.M. Nuutinen, G. Lazzarino, B. Giardina, and I.E. Hassinen, unpublished observations). The experiments with total [Ca2+] lowering alone indicate that the difference between F-1,6-P2 and EGTA effects on the flavoprotein redox state might be explainable by some additional cellular effects of EGTA causing more prominent Ca2+ intracellular transients than F-1,6-P2, which more closely mimics the actions of the lowering of extracellular Ca2+.

Some reported effects of F-1,6-P2 in human volunteers are compatible with the suggestion that the cardiac effects may be due to a lowering of calcium. Ripari and Pieralisi report that 15 g F-1,6-P2 infused within 10 minutes lowered the heart rate and oxygen consumption increments in an ergometer exercise.
although a simultaneous increase in the total work accumulated until exhaustion is difficult to interpret only in terms of changes in calcium homeostasis.

F-1,6-P_2 (tested up to 7.5 mM) prevents anoxic damage of cultured astrocytes in an atmosphere of 95% N_2-5% CO_2 without having any effect on the accumulation of lactate. Near-maximum protection is achieved with 6 mM F-1,6-P_2, but this is dependent on the simultaneous presence of glucose, which indicates that external F-1,6-P_2 is not used as a glycolytic substrate. This concentration range fits with the present results, which indicate that extracellular free [Ca^{2+}] must have been greatly diminished under those conditions, which should result in protection against the hypoxic cell damage in which Ca^{2+} has been shown to be implicated (for references, see Opie and Khandoudi et al). Direct observation of cytosolic free [Ca^{2+}] by using a fluorescent intracellular calcium indicator have demonstrated an increase in cytosolic free [Ca^{2+}] during ischemia, and the same approach has corroborated the influence of extracellular calcium on intracellular free [Ca^{2+}]. There is also evidence that lowering of extracellular [Ca^{2+}] offers protection against reperfusion injury. One observation that is at variance with the notion that phosphorylated sugars are not able to penetrate the plasma membrane is that astrocytes have been found capable of taking up the ^14C label from extracellular [^14C]F-1,6-P_2 at a rate of 2.3 nmol/min during the first 5 minutes.

**Figure 7.** Graphs showing effects of perfusate total Ca^{2+} concentration ([Ca^{2+}]) changes on oxygen consumption and coronary flow (panel A) and the redox state of flavoproteins and oxygenation grade of myoglobin (panel B) in a K⁺-arrested isolated rat heart. KCl (18 mM) was present throughout the perfusion time period shown, and the NaCl concentration was reduced to keep the osmotic pressure constant. Upward deflections of the flavin fluorescence and myoglobin reflectance tracings indicate a shift toward oxidation and an increase in oxygenation grade, respectively. Panels A and B represent simultaneous original recordings from the same heart.

**Figure 8.** Graphs comparing the effects of fructose 1,6-bisphosphate (FDP) and EGTA on the energy metabolism of a K⁺-arrested isolated perfused rat heart. Panel A: Work load and heart rate. Panel B: Oxygen consumption and coronary flow. Panel C: Flavoprotein fluorescence. The infusion periods and final concentrations of the substances administered are shown by the rectangles on the lower edge of panel C. The tracings in panels A, B, and C are representative original recordings obtained simultaneously from the same heart. Upward deflection in the flavin fluorescence tracing indicates a shift toward oxidation.
The negative inotropic effect observed in the present experiments cannot be due to contaminating Pi in the F-1,6-P2 preparation or Pi, accumulation due to the extracellular metabolism at F-1,6-P2. A second P1 resonance peak with a δ of 5.2 ppm appeared in the 31P NMR spectrum during F-1,6-P2 infusion, but this δ value indicates a pH value of 7.4; thus, the resonance must originate from extracellular Pi. One can estimate from the peak area ratios that the Pi contamination of F-1,6-P2 is not more than 2.5% (in agreement with the manufacturer's specifications); thus, the perfusate Pi concentration would be 0.12 or 0.25 mM in the presence of 5 mM or 10 mM F-1,6-P2, respectively, compared with a zero concentration of Pi in the absence of F-1,6-P2. These values are low compared with the 1.2 mM Pi of normal Krebs-Henseleit bicarbonate solution. Although Pi is an inhibitor of the force production of the actomyosin ATPase, its transport through the plasma membrane is extremely slow, so that extracellular Pi does not have metabolic effects on an isolated perfused heart.

In conclusion, F-1,6-P2 at concentrations comparable to those reported as being used to treat humans has pronounced effects on the mechanical performance and cellular energy balance of an isolated perfused rat heart. The present results enable the underlying mechanism of F-1,6-P2 action at this concentration range to be traced to perturbation of the homeostasis of calcium by the binding of extracellular Ca2+ to F-1,6-P2. This phenomenon must be taken into account when using high dosages in vivo.

References

---

**KEY WORDS**  •  fructose 1,6-bisphosphate  •  cell redox state  •  inotropic agents  •  nuclear magnetic resonance spectroscopy