Fructose prevents hypoxic cell death in liver

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ANUNDI, IRENE, JAYMIE KING, DAWN A. OWEN, HEINZ SCHNEIDER, JOHN J. LEMASTERS, AND RONALD G. THURMAN. *Fructose prevents hypoxic cell death in liver.* Am. J. Physiol. 253 (Gastrointest. Liver Physiol. 16): G390–G396, 1987.—Perfusion of livers from fasted rats with nitrogen-saturated buffer caused hepatocellular damage within 30 min. Lactate dehydrogenase (LDH) was released at maximal rates of ~300 $U \cdot g^{-1} \cdot h^{-1}$ under these conditions, and virtually all cells in periportal and pericentral regions of the liver lobule were stained with trypan blue. Infusion of glucose, xylitol, sorbitol, or mannitol (20 mM) did not appreciably change the time course or extent of damage due to perfusion with nitrogen-saturated perfusate. However, fructose (20 mM) completely prevented damage assessed by LDH release, trypan blue uptake, and ultrastructural changes for at least 2 h of perfusion. Neither glucose, xylitol, sorbitol, nor mannitol (20 mM) increased lactate formation above basal levels during hypoxia. On the other hand, fructose (0.4–20 mM) caused a concentration-dependent increase in lactate formation that reached maximal rates of ~180 $\mu mol \cdot g^{-1} \cdot h^{-1}$. The dose-dependent increase in glycolytic lactate production from fructose correlated well with cellular protection reflected by decreases in LDH release. ATP:ADP ratios were also increased from 0.4 to 1.8 in a dose-dependent manner by fructose. The results indicate that fructose protects the liver against hypoxic cell death by the glycolytic production of ATP in the absence of oxygen.

protection; adenine nucleotides; perfused liver

ISOLATED ORGAN SYSTEMS such as the perfused liver are suitable models in which to assess hypoxic damage (4, 13). Oxygen tension may be manipulated easily, and damage can be assessed by lactate dehydrogenase (LDH) release and electron microscopy. In addition, zone-specific injury can be evaluated by trypan blue uptake. In a recent report from this laboratory, three hypoxic models were characterized; one produced by nitrogen, one with low flow followed by reflow, and one with total ischemia (4). Dependence of hypoxic damage on nutritional status was also demonstrated. In livers from fed rats, damage was not observed, possibly due to the high capacity for glycolytic ATP production from endogenous glycogen. On the other hand, hypoxic injury was demonstrated readily in livers from fasted rats that do not produce ATP glycolytically due to lack of glycogen.

Mechanisms involved in hypoxic damage are not clear. It has been proposed that hypoxic injury may be due to deleterious effects of reduced oxygen species (25). Also, calcium channel blockers and calmodulin antagonists have been shown to protect the liver and the kidney against damage due to hypoxia, suggesting the involvement of calcium (19, 21, 23, 26). Adenine nucleotides may be linked to both of these proposed mechanisms. A rapid decline in ATP levels under hypoxic conditions may cause an increase in calcium influx into the cytosol due to inhibition of the calcium pumps in the plasma membrane, mitochondria, and endoplasmic reticulum. Alternatively, ATP may be metabolized to hypoxanthine, a substrate for superoxide anion formation (18).

Human livers can only be stored for 8–10 h (2) prior to transplantation, most likely because of hypoxic damage. Thus an understanding of pharmacological and nutritional strategies that protect the liver against hypoxic damage may be important clinically. Because the toxic effects of hypoxia in the fasted state may be related to lack of glycolytic substrate, we have evaluated the protective effect of various carbohydrates in the hypoxic perfused rat liver model. The results demonstrate that fructose and not other sugars prevents liver cell death due to hypoxia completely. A preliminary account of this work has appeared elsewhere (14).

MATERIALS AND METHODS

Liver perfusion. Rats (female, Sprague-Dawley, 180–220 g) were fasted 24 h prior to experiments. Livers were perfused with Krebs-Henseleit bicarbonate buffer (pH 7.4, 37°C) saturated with 95% O_2-5% CO_2, or 95% N_2-5% CO_2 in a non-recirculating system as described previously (22). The buffer was pumped into livers via the portal vein at rates of 3.5–4.5 $ml \cdot g^{-1} \cdot min^{-1}$ and flowed past a Teflon-shielded, Clark-type oxygen electrode allowing oxygen concentration to be monitored continuously.

Samples of effluent perfusate were collected for the determination of glucose, lactate, pyruvate, and LDH activity as described in detail elsewhere (3). Rates were calculated from influent minus effluent concentration or activity differences, the flow rate, and the liver wet weight.

Histological procedures. Trypan blue (0.2 mM; Sigma) was infused into livers for 10 min at the end of any given experimental protocol (1). After removal of excess dye by perfusion for an additional 10 min, livers were perfused with 1% paraformaldehyde for 10 min, and the fixed tissue was embedded in paraffin and processed for light microscopy. Sections were stained only with eosin,

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a cytoplasmic stain, so that trypan blue could be identified readily in the nuclei of damaged cells.

The percentage of stained nuclei was established as follows. First, a periportal or pericentral region was identified microscopically based on the presence or absence of a hepatic artery and bile duct near a venous lumen. All nuclei in a zone radiating five cells from the vessel in either periportal or pericentral regions were identified as trypan blue negative or positive. The percent staining was calculated from the number of stained nuclei divided by the total number of cells in any given region. Because there were no zonal differences in cell death in this study, data from periportal and pericentral regions were averaged.

Electron microscopy. Livers were fixed for scanning electron microscopy by changing to a perfusion medium containing 2% glutaraldehyde and 2% paraformaldehyde in Krebs-Henseleit buffer. After 3–5 min of perfusion, the fixed tissue was cut into 1-cm cubes and placed in cold secondary fixative containing 2% glutaraldehyde, 0.1 M $NaPO_4$ buffer, pH 7.4. Subsequently, the tissue was washed in water, dehydrated in 2,2-dimethoxypropane, and dried in carbon dioxide (critical point). The dried tissue was fractured with a razor blade, mounted, and sputter-coated with Au-Pd for viewing with a JEOL 35C scanning electron microscope (13).

Assay of adenine nucleotides. Livers were freeze clamped with aluminum Wollenberger tongs, cooled in liquid nitrogen, and were stored at −70°C until assayed for adenine nucleotides. Acid extracts of frozen tissue were prepared at −40°C. Approximately 100 mg of frozen liver was placed on 0.4 ml of frozen 3.0 M $HClO_4$ in a centrifuge tube and powdered at −40°C with a flat-bottomed glass rod. Samples were then thawed and diluted with 1 mM ethylenediaminetetraacetic acid at 4°C, centrifuged, and neutralized with potassium carbonate as described elsewhere (12). Adenine nucleotides in neutralized extracts were determined by isocratic reverse-phase high-performance liquid chromatography modified from Hoffman and Liao (9). The chromatography system consisted of a series 10 pump, a model LC-95-UV-visible spectrophotometric detector operating at 260 nm, and a model LCI-100 laborabory computer intergrator (Perkin-Elmer, Norwalk, CT). A Zorbax BDS column (Dupont, Wilmington, DE) was eluted with a mobile phase containing 1 mM tetrabutylammonium phosphate, 220 mM potassium phosphate buffer, pH 7.8, at a flow rate of 1 ml/min.

RESULTS

Protection of hypoxic cell death by fructose. A model based on perfusion of livers with nitrogen-saturated buffer was used in this study to produce hypoxia. Livers from fasted rats were perfused for 20 min with oxygen-saturated buffer and subsequently for up to 2 h with nitrogen-saturated buffer. Under these conditions, maximal LDH release of ~300 $U \cdot g^{-1} \cdot h^{-1}$ was observed within 40 min of onset of perfusion with nitrogen-saturated perfusate (Table 1, Fig. 1). Rates of LDH release declined subsequently to values ~40 $U \cdot g^{-1} \cdot h^{-1}$ after 2 h (Fig. 1). LDH release was minimal from livers perfused with oxygen-saturated buffer for the same time period (Table 1). When fructose (20 mM) was infused beginning 8 min prior to initiation of nitrogen-saturated perfusion, LDH release was nearly undetectable during the first hour and only reached ~40 $U \cdot g^{-1} \cdot h^{-1}$ during the second hour of perfusion (Fig. 1).

Perfusion of livers for 2 h in the presence of oxygen did not alter cell structure nor was trypan blue taken up. In contrast, irreversible liver damage, indicated by trypan blue uptake, was produced by perfusion with nitrogen in virtually all cells of the liver lobule (Fig. 2*A*, Table 1). In the presence of fructose, however, nitrogen did not cause trypan blue uptake (Fig. 2*B*). In addition, infusion of fructose (20 mM) prevented ultrastructural alterations due to hypoxic damage (Fig. 3). After 15 min of perfusion with nitrogen-saturated perfusate (Fig. 3*A*), hepatocytes were covered with numerous small surface blebs similar to those observed previously during the early stages of

TABLE 1. *Effect of carbohydrates on cell death due to hypoxia*

	Addition	mM	Maximal LDH Release, $U \cdot g^{-1} h^{-1}$	Percent Cells Stained with Trypan Blue	Lactate Production, $\mu mol \cdot g^{-1} \cdot h^{-1}$
Normoxia	None		<4	ND	
Hypoxia	None (6)		324±40	100	6±3
	Fructose (4)	0.4	368±38	97	6
	Fructose (4)	2	350±84	100	21
	Fructose (4)	4	174±26	42±24	32
	Fructose (4)	10	136±44	ND	135
	Fructose (4)	15	36±18	ND	171
	Fructose (5)	20	35±15	ND	182
	Xylitol (3)	20	398±26	100	14
	Mannitol (3)	20	310±35	50	5
	Glucose (3)	20	426±41	100±1	7
	Sorbitol (3)	20	398±56	100	8

Values are means ± SE. Values for lactate production and trypan blue staining represent data from 2 to 4 individual expts. Nos. in parentheses refer to no. of livers employed to assay lactate dehydrogenase (LDH) release. ND, not detectable. Livers from fasted rats were perfused for 120 min with either oxygen-saturated buffer or 20 min with normoxic buffer followed by 120 min with nitrogen-saturated buffer. Carbohydrates were added 8 min prior to initiation of hypoxic perfusion. Effluent perfusate was collected every 10 min for determination of LDH activity. At the end of the experiment, trypan blue was infused for 10 min and livers were processed for light microscopy. Staining with trypan blue was determined as described in MATERIALS AND METHODS. Lactate was assayed enzymatically in the effluent perfusate.

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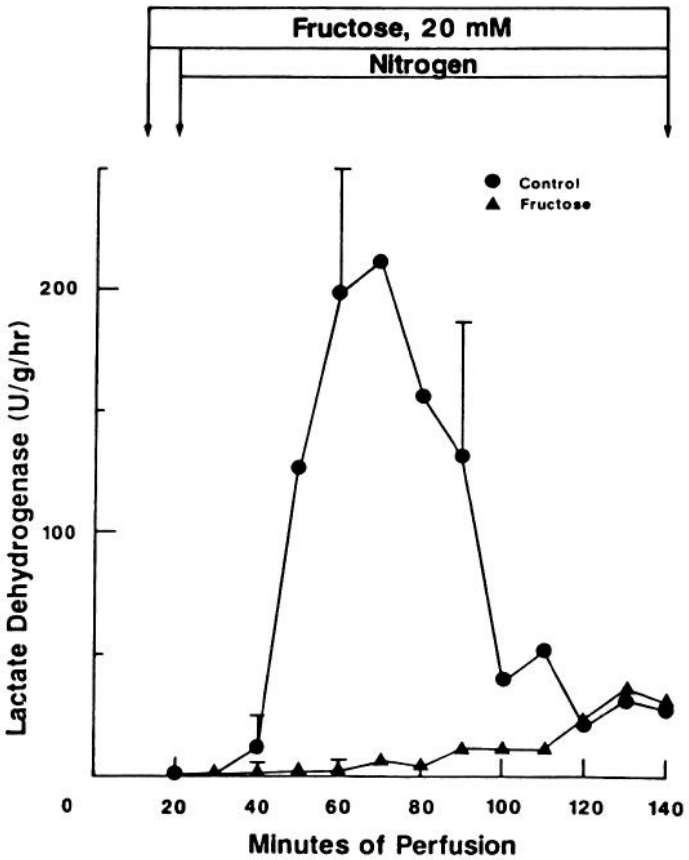

FIG. 1. Time course of lactate dehydrogenase (LDH) release during hypoxia in presence or absence of fructose. Livers were perfused with Krebs-Henseleit buffer saturated with 95% N_2-5% CO_2 (*closed circles*). At 10-min intervals, effluent perfusate was collected for determination of LDH. Values were averaged at each time point. In parallel experiments, fructose (20 mM; *closed triangles*) was infused 8 min prior to initiation of N_2-saturated perfusate. Values are representative means ± SE of 3–4 livers/group.

hypoxic injury (13). After 15 min of anoxia, livers perfused with fructose showed normal cellular architecture (e.g., bleb formation was prevented totally with fructose, Fig. 3*B*). After 30 min of perfusion with hypoxic buffer (Fig. 3*C*), hepatocytes displayed large blebs or were fragmented, indicative of irreversible injury. Fructose also prevented these changes and maintained normal ultrastructure for a full 30 min of anoxia (Fig. 3*D*).

Several other carbohydrates were also evaluated in the perfused liver. Glucose, xylitol, mannitol, or sorbitol (20 mM) were infused beginning 8 min prior to the initiation of nitrogen-saturated perfusate. None of these carbohydrates prevented LDH release and trypan blue uptake by the liver (Table 1).

Metabolism of carbohydrates during hypoxia. At the highest concentration of fructose studied (20 mM), maximal rates of lactate formation were 160–180 $\mu mol \cdot g^{-1} \cdot h^{-1}$, and glucose and pyruvate were formed at rates ~15 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (Fig. 4). With increasing concentrations of fructose (0.4–20 mM), lactate production increased from basal values of 5 $\mu mol \cdot g^{-1} \cdot h^{-1}$ to ~180 $\mu mol \cdot g^{-1} \cdot h^{-1}$ in a dose-dependent manner (Fig. 5*A*). An increase in lactate formation correlated well with a decrease in maximal LDH release. With 15 and 20 mM fructose, lactate production was maximal and the liver was protected completely as indicated by perfusate LDH values in the normal range (Fig. 5*A*). The concentration of fructose required for half-maximal inhibition of LDH release was between 2–3 mM. In contrast to fructose, glucose infusion (20 mM) did not increase lactate formation during hypoxia (Table 1). Lactate production was also minimal with xylitol, sorbitol, and mannitol. When fructose (20 mM) was infused 10 min after the initiation of perfusion with nitrogen-saturated buffer, lactate was formed at lower rates (70 $\mu mol \cdot g^{-1} \cdot h^{-1}$) than when fructose was infused 8 min prior to nitrogen. Under these conditions, LDH release was delayed 10–20 min (data not shown).

Effect of fructose on adenine nucleotides during hypoxia. If lactate formation from the glycolytic metabolism of fructose under hypoxic conditions is related to its protective effect, then ATP levels should increase with fructose in the absence of oxygen. Accordingly, adenine nucleotides were assayed in livers perfused with nitrogen-

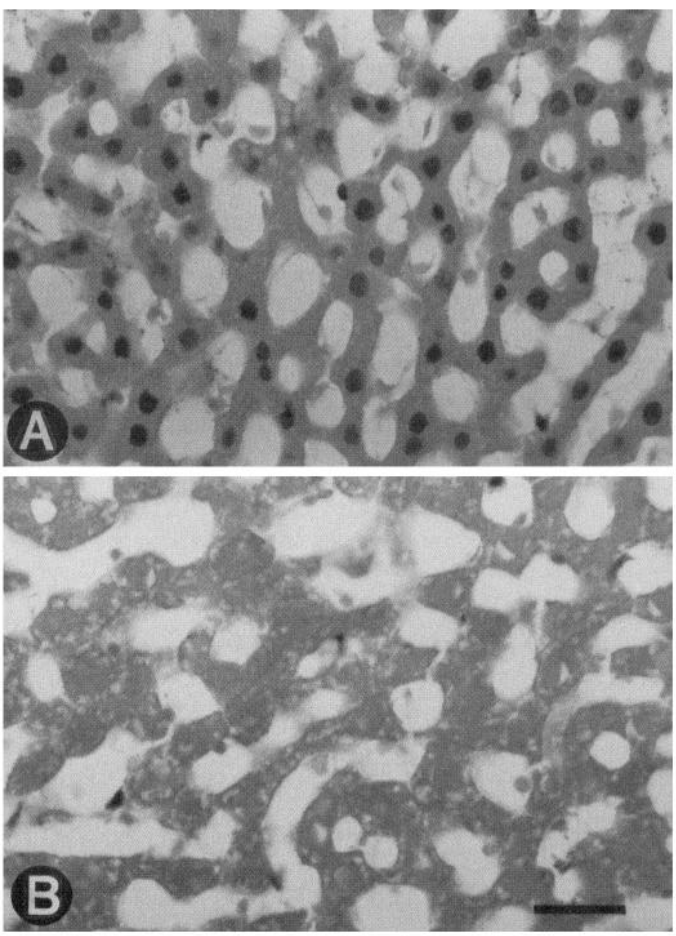

FIG. 2. Trypan blue uptake in livers perfused with N_2-saturated perfusate in presence or absence of fructose. *A*: livers were perfused with N_2-saturated perfusate for 120 min followed by trypan blue for 10 min. Excess trypan blue was removed by perfusion for 10 min, and livers were fixed by infusion of 1% paraformaldehyde for 10 min. Sections of left lateral lobe were embedded and processed for light microscopy. Sections were stained with eosin only. Typical experiment. Magnification ×600. *B*: conditions as in *A* except that fructose (20 mM) was infused 8 min prior to initiation of N_2-saturated buffer and was continued for an additional 120 min. Typical experiment. *Bar* is 25 μm.

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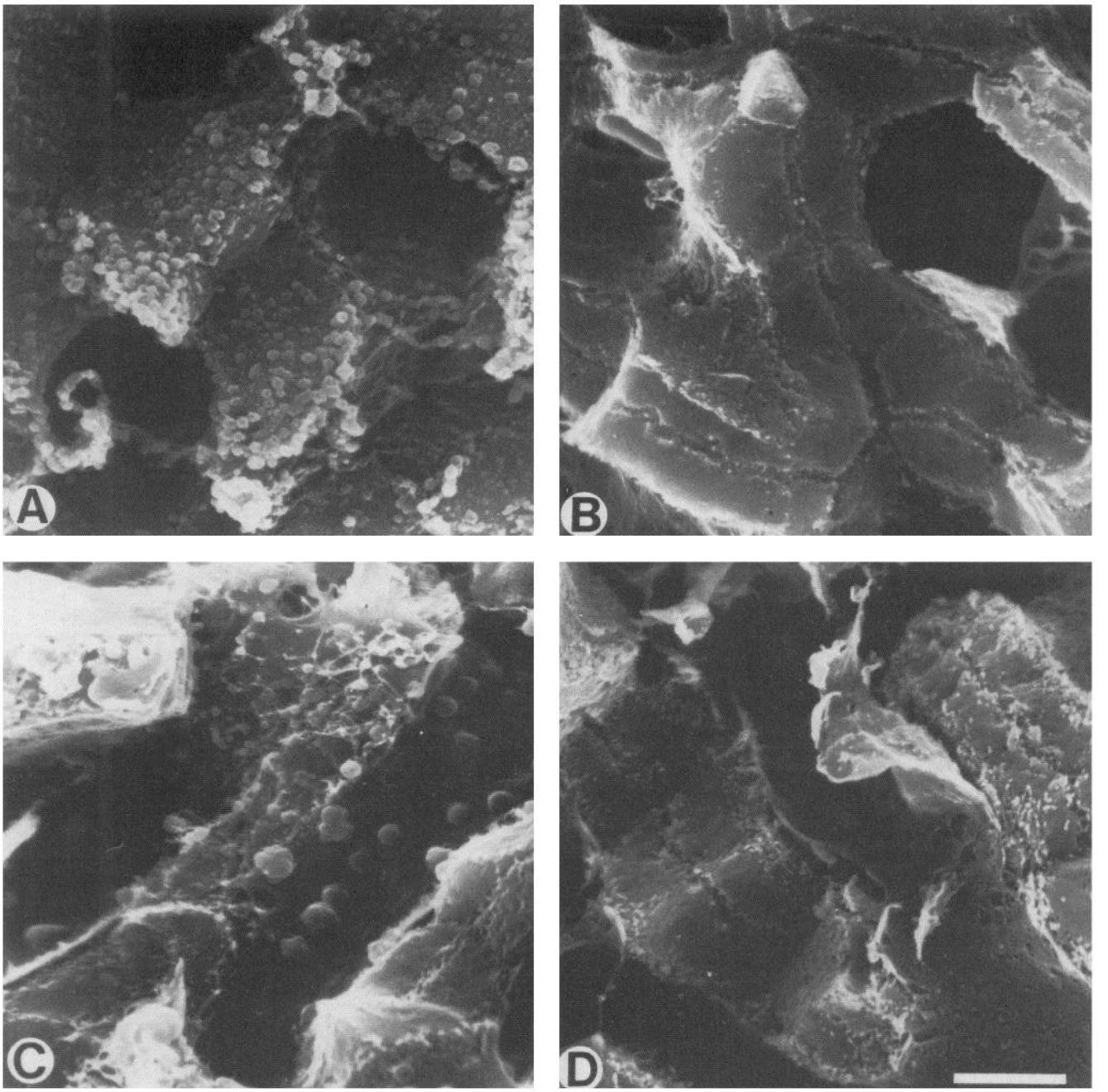

FIG. 3. Effect of fructose on hepatic ultrastructure. Livers were perfused for 30 min and prepared for scanning electron microscopy as described in MATERIALS AND METHODS. *A*: scanning electron micrograph of liver perfused for 20 min, equilibrated with 95% O_2-5% CO_2, and subsequently for 15 min with 95% N_2-5% CO_2. Note numerous small surface blebs. *B*: scanning electron micrograph of liver perfused as in *A* except that fructose (20 mM) was infused starting 8 min prior to initiation of N_2-saturated perfusate. Blebs are absent. *C*: scanning electron micrograph of liver perfused as in *A* except after 30 min of N_2. Note large and small blebs as well as fragmented cell (*top left*). *D*: scanning electron micrograph of liver perfused as in *C* except that fructose (20 mM) was infused beginning 8 min prior to the initiation of N_2-saturated perfusate. Structure is normal. *Bar* is 5 μm.

saturated perfusate in the presence of various concentrations of fructose. After 30 min of perfusion with nitrogen-saturated perfusate alone, ATP:ADP ratios declined from ~4 to <0.5 (Fig. 5*B*), whereas AMP levels were increased dramatically (data not shown). In the presence of fructose, ATP and ADP were elevated, whereas AMP was diminished markedly compared with perfusions with nitrogen-saturated buffer alone. Thus fructose prevented the decline in ATP:ADP ratios under hypoxic conditions (Fig. 5*B*). Ratios were maintained at 1.8 with fructose concentrations >4 mM (half-maximal effect = 2–3 mM fructose).

DISCUSSION

Of 1,500 autopsies performed on patients with heart failure, 13% had pericentral liver damage (7). Because pericentral regions of the liver lobule have lower oxygen

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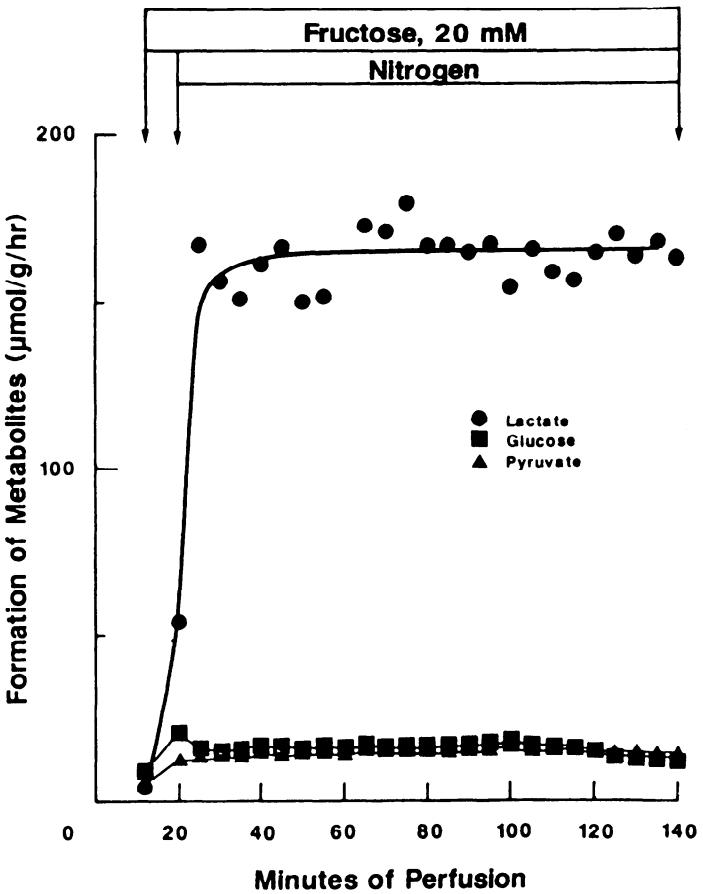

FIG. 4. Lactate, pyruvate, and glucose production during hypoxia in presence of fructose. Conditions as in Fig. 1. Fructose (20 mM) was infused as indicated by *horizontal bars* and *vertical arrows*. Perfusion with N_2-saturated perfusate was initiated 8 min later. Effluent perfusate was collected at 5-min intervals and assayed for lactate (*closed circles*), pyruvate (*closed triangles*), and glucose (*closed squares*) enzymatically. Typical experiment.

tension, this observation is indicative of hypoxic damage due to diminished hepatic blood flow. Several reports also suggest that hypoxia plays an important role in alcohol-induced liver damage (11, 31). In addition, hypoxia may be particularly important in organ transplantation. Transplantation surgery is becoming an accepted therapy for patients with irreversible liver disease; now ~500 human orthotopic liver transplantations are performed each year. For the transplantation procedure to be successful, the preservation of the organ during storage and transportation is vital. At present, simple cold storage of liver in artificial intracellular medium (Euro-Collins solution) is used, and preservation time is limited to ~8 h. Thus the development of strategies that would improve organ storage could be important clinically.

Perfusion of livers with nitrogen-saturated perfusate is a convenient and useful model to study hypoxic damage. Moreover, damage has been shown to depend on the nutritional status. In the fasted state characterized by an absence of glycogen and glycolytic substrates, hepatocytes are more vulnerable to hypoxic injury than in the fed state (4). Carbohydrates such as xylitol and mannitol had little protective effect against hypoxic damage (Table 1). Sorbitol is converted to fructose via sorbitol dehydrogenase; however, this conversion is slower than the activation of fructose via fructokinase, and therefore sorbitol provides less intermediate for glycolytic ATP production than fructose. Despite this drawback, sorbitol did delay maximal LDH release by ~10 min. Glucose failed to produce lactate or prevent LDH release in the perfused liver during hypoxia (Fig. 2, Table 1). Thus glucose is a poor substrate for glycolysis in livers from fasted rats during anoxia probably because of the high K_m of hepatic glucokinase for glucose (24). Fructose, on the other hand, is a good glycolytic substrate and produces lactate at high rates anaerobically (27, 29), most likely due to the low K_m of fructokinase for fructose. None of the sugars that failed to protect the liver produced lactate at appreciable rates (Table 1). Lactate per se was not involved in the mechanism of protection by fructose, since addition of lactate alone (1 mM) did not alleviate hypoxic liver damage (I. Anundi and R. G.

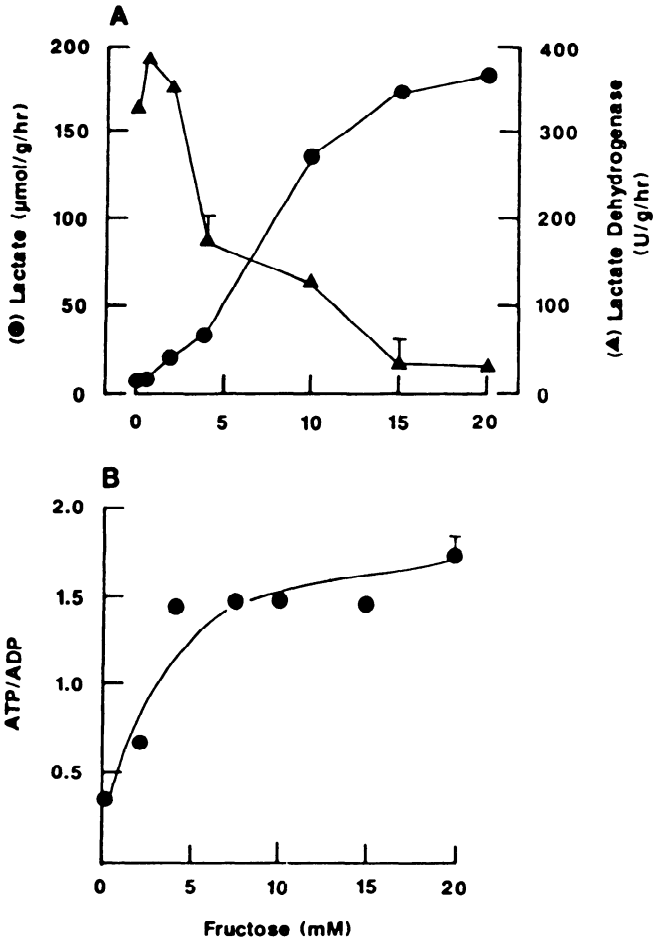

FIG. 5. Effect of various concentrations of fructose on lactate formation, maximal lactate dehydrogenase (LDH) release, and ATP:ADP ratios. *A*: fructose (0.4–20 mM) was added 8 min prior to initiation of N_2-saturated perfusate and perfusion was continued for 2 h as in Fig. 1. Lactate and LDH were measured in effluent perfusate as described in MATERIALS AND METHODS. Values are representative means of 2–4 livers/*point*. *B*: ATP:ADP ratios determined in freeze-clamped livers perfused as in *A* as described in MATERIALS AND METHODS. Values represent means of 2–5 livers/*point*. ATP values ranged from 2.6 to 3.5 μmol/g for livers perfused with oxygenated buffer and from 0.1 to 0.2 μmol/g for livers perfused with N_2-saturated buffer.

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Thurman, unpublished observations).

Fructose protected completely against hypoxic damage assessed by dye uptake, enzyme release (Fig. 1, Table 1), and ultrastructural alterations (Fig. 3). The mechanism of protection by fructose is most likely related to glycolytic ATP production. Indeed, ATP:ADP ratios increased in parallel with decreases in LDH release (half-maximal concentration of fructose = 2–3 mM) when fructose was infused (Fig. 5). Studies on ischemia-induced myocardial injury also indicated that a decline in ATP levels was a critical event in the development of cell damage (16). The decline in ATP levels during hypoxia may be a common event linked to the mechanism of damage. Low ATP levels may lead to inactivation of energy-requiring calcium pumps and intracellular calcium may rise. Indeed, in studies with cultured kidney cells, a correlation between a decline in ATP levels and an increase in intracellular calcium was observed (23). The increase in intracellular calcium may secondarily activate phospholipases (5, 8) and cause breakdown of membrane lipids. However, some studies indicate that control of cytosolic free calcium may not be dependent on metabolic energy (6, 15). Furthermore, catabolism of ATP due to hypoxia may produce hypoxanthine and lead to superoxide anion production via xanthine oxidase when oxygen is restored or via residual oxygen present during hypoxia (18). These data are also consistent with the observation that allopurinol, an inhibitor of xanthine dehydrogenase, protects against hypoxic damage in rat liver (17). In addition, fructose protected in a model of reoxygenation injury where oxygen radicals may be important (I. Anundi, J. J. Lemasters, and R. G. Thurman, unpublished observations). Maintenance of ATP levels during hypoxia may also be important for other critical cellular functions such as those involving the cytoskeleton.

These results were unexpected given the extensive literature on adverse effects due to fructose in the presence of oxygen. Fructose lowers cellular adenine nucleotides and may produce lactic acidosis (20, 28). Although the decline in ATP levels due to fructose is transient, this decrease may be particularly dangerous in individuals with fructose intolerance limiting its use in the clinic (30). On the other hand, fructose is hepatoprotective in the absence of oxygen. Because fructose prevented damage for up to 2 h at 37°C under hypoxic conditions (Fig. 1), it is possible that it may be useful in preventing hypoxic damage during preservation of organs such as liver for transplantation.

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