Glucose Loading During Primary Culture Has Opposite Effects on the Viability of Hepatocytes Exposed to Potassium Cyanide and to Iodoacetic Acid

Kazuhisa Shiroyama, Katsuyuki Moriwaki, Shinji Kusunoki, Noboru Saeki, and Osafumi Yuge

Whether or not to apply nutritional pretreatment and how to do so are controversial issues with respect to the liver about to undergo aggressive intervention. We studied the effects of glucose loading on the viability of hepatocytes that were subsequently exposed to the inhibitors of carbohydrate metabolism, potassium cyanide (KCN) and iodoacetic acid (IAA). After rat hepatocytes were cultured for 24 hours in Leibovitz's L-15 medium containing 0, 10, 20, and 30 mmol/L glucose, the medium was replaced with modified Hanks-HEPES buffer with or without 2.5 mmol/L KCN or 0.5 mmol/L IAA. Lactate dehydrogenase (LDH) activity, lactate concentration, and pH of the supernatant were measured after 0, 2, 4, and 6 hours of exposure to KCN and after 0, 20, 40, and 60 minutes of exposure to IAA. Glycogen and adenosine triphosphate (ATP) contents in the hepatocytes were measured simultaneously. Hepatocytes cultured with various concentrations of glucose for 24 hours stored levels of glycogen in proportion to the glucose concentration in the culture medium without any significant difference in viability. The hepatocytes cultured with higher glucose concentrations maintained a higher ATP content and released less LDH and more lactate, and the pH decreased in the supernatant during exposure to KCN. Conversely, hepatocytes cultured with lower glucose concentrations maintained a higher ATP content and released less LDH during exposure to IAA. In conclusion, prior glucose loading appears to be beneficial for hepatocytes if oxidative phosphorylation is to be inhibited, whereas withholding glucose appears to be beneficial if glycolysis is to be inhibited.

THE NUTRITIONAL status of the liver is closely related to its tolerance to hypoxia and hepatotoxic agents. The viability of hepatocytes during hypoxia is dependent on nutritional status both in the perfused rat liver[1] and in isolated rat hepatocytes.[2,3] Glycogen-rich hepatocytes tolerate menadione-induced cytotoxicity in isolated rat hepatocytes.[4] These reports indicate that strategies that enhance glycogen storage have cytoprotective effects on the liver undergoing aggressive intervention such as exposure to hypoxia and hepatotoxic agents.

However, the effect of the nutritional status of the liver on the outcome of liver transplantation is controversial. High levels of glycogen in the donor liver supposedly improve the outcome of transplantation in animal experiments.[5-7] On the contrary, livers from fasted rats acquire resistance to warm or cold ischemia.[8] Furthermore, the essential metabolic functions of glycogen-depleted human liver grafts are better restored during the postreperfusion period than those of glycogen-rich grafts after liver transplantation.[9]

During liver transplantation, donor livers undergo hypoxia during preservation.[10] Donor livers are also exposed to acidosis and high concentrations of stress hormones after reperfusion.[11,12] Hypoxia inhibits oxidative phosphorylation,[13,14] whereas acidosis and stress hormones inhibit glycolysis.[15-17] Therefore, we speculated that inhibition of glycolysis and oxidative phosphorylation would differently affect the viability of hepatocytes in the donor livers under various nutritional conditions.

The present study uses potassium cyanide (KCN) and iodoacetic acid (IAA) to inhibit mitochondrial oxidative phosphorylation and glycolysis, respectively. We determined the effect of extant nutritional conditions on the viability of hepatocytes in which carbohydrate metabolism was inhibited in oxidative phosphorylation and in glycolysis. To prepare hepatocytes with different nutritional carbohydrate conditions, hepatocytes were cultured in medium containing four concentrations of glucose.

MATERIALS AND METHODS

Materials

Leibovitz's L-15 medium was purchased from Life Technologies, Inc, Grand Island, NY. *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), collagenase, IAA, and bicinchoninic acid were purchased from Sigma Chemical Co, St Louis, MO. Insulin, dexamethasone, and KCN were purchased from Katayama Chemical Co, Osaka, Japan. Amyloglucosidase, glucose oxidase, and peroxidase were purchased from Boehringer Mannheim Co, Mannheim, Germany.

Isolation and Primary Culture of Rat Hepatocytes

With the approval of our institutional animal care committee, adult male Wistar rats weighing approximately 200 g were fed with laboratory chow and given ad libitum access to water. Parenchymal hepatocytes were isolated from the rats by a modification of the procedure described by Seglen.[18] Cell viability was measured by the Trypan blue exclusion test, and only cells that were more than 90% viable were primary cultured in Leibovitz's L-15 medium (pH 7.4) supplemented with 20 mmol/L HEPES, 4 mmol/L sodium bicarbonate, 6% fetal bovine serum, 10^{-7} mol/L insulin, 10^{-7} mol/L dexamethasone, and 0 to 30 mmol/L glucose. Cells (2.5×10^5/0.5 mL) were seeded in 24-well collagen-coated plates and cultured as monolayers in a humidified incubator at 37°C under 30% O_2 and 70% N_2. The isolated hepatocytes were cultured in medium containing with 0, 10, 20, and 30 mmol/L glucose and labeled G0, G10, G20, and G30, respectively. The media were renewed 4 hours later, and the cells were cultured for another 20 hours.

From the Department of Anesthesiology and Critical Care Medicine, Hiroshima University School of Medicine, Hiroshima, Japan.

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Address reprint requests to Kazuhisa Shiroyama, MD, Department of Anesthesiology, Akane Foundation Tsuchiya General Hospital, Nakashima-chou 3-30, Naka-ku, Hiroshima 730-8655, Japan.

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Table 1. Extracellular and Intracellular LDH Activities of Cultured Rat Hepatocytes After 24 Hours in Culture

	Extracellular LDH Activity (mU/mg protein)	Intracellular LDH Activity (mU/mg protein)
G0 group	320 ± 30	4,990 ± 130
G10 group	330 ± 20	4,980 ± 160
G20 group	340 ± 20	4,900 ± 260
G30 group	350 ± 50	4,900 ± 90

NOTE. Hepatocytes were cultured for 24 hours in medium containing 0, 10, 20, or 30 mmol/L glucose. LDH activity was measured in supernatant and in hepatocytes dissolved in 1% Triton X. Data are expressed as means ± SD for five cultured hepatocytes preparations. The four groups of hepatocytes did not differ significantly in terms of either extracellular or intracellular LDH activities.

Viability of Hepatocytes After the Initial Culture

Extracellular and intracellular lactate dehydrogenase (LDH) activities were measured to evaluate the viability of hepatocytes after 24 hours in primary culture. The medium was sampled then hepatocytes were washed with modified Hanks-HEPES buffer and dissolved in 1% Triton X to determine intracellular LDH activity.

Exposure to KCN and IAA and Sampling Protocol

After the initial culture, the medium was replaced with modified Hanks-HEPES buffer (Na, 140 mmol/L; K, 4 mmol/L; Ca, 1.3 mmol/L; Mg, 0.9 mmol/L; P, 0.8 mmol/L; Cl, 144 mmol/L; glucose, 5 mmol/L; HEPES, 20 mmol/L) containing 2.5 mmol/L KCN or 0.5 mmol/L IAA. The effect of these inhibitor concentrations is maximal cell killing.[19] The initial pH of the modified Hanks-HEPES buffer before incubation with KCN or IAA was maintained at 7.4 in all plates. Cells were placed in a humidified incubator at 37°C under 20% O_2 and 80% N_2 for 0, 2, 4, and 6 hours in the presence of KCN and for 0, 20, 40, and 60 minutes in the presence of IAA. At the end of each incubation period, LDH activity, lactate concentration, and pH were measured in the supernatant, and the hepatocytes were quickly washed with modified Hanks-HEPES buffer and dissolved in 1% Triton X to determine protein content or in 3% $HClO_4$ to measure glycogen and adenosine triphosphate (ATP) contents. Samples from the hepatocytes cultured in the absence of KCN or IAA at each sampling point served as controls.

Biochemical Assays

LDH activity was determined using a colorimetric assay (Cytotoxicity Detection Kit; Boehringer Mannheim Co). The viability of cultured hepatocytes was evaluated by extracellular LDH activity, which directly reflects the amount of LDH released into the supernatant. Lactate concentration of the supernatant was measured using an enzyme electrode (YSI MODEL 2300 STAT plus glucose & lactate analyzer, Yellow Springs, OH), and the pH was measured using a blood gas analyzer ABL520 (Radiometer Co., Copenhagen, Denmark). Glycogen content in the hepatocytes was determined enzymatically using amyloglucosidase[20] and by a colorimetric determination of glucose using glucose oxidase–peroxidase.[21] The ATP content of hepatocytes was quantified using the luciferin–luciferase assay (ATP Bioluminescence Assay CLS II kit; Boehringer Mannheim Co), and the protein content was measured using bicinchoninic acid.[22] All data were standardized by dividing the values by the protein content of the hepatocytes.

Statistical Analysis

Data are expressed as means ± SD. Groups of data were analyzed using one-way or two-way ANOVA. When statistically significant,

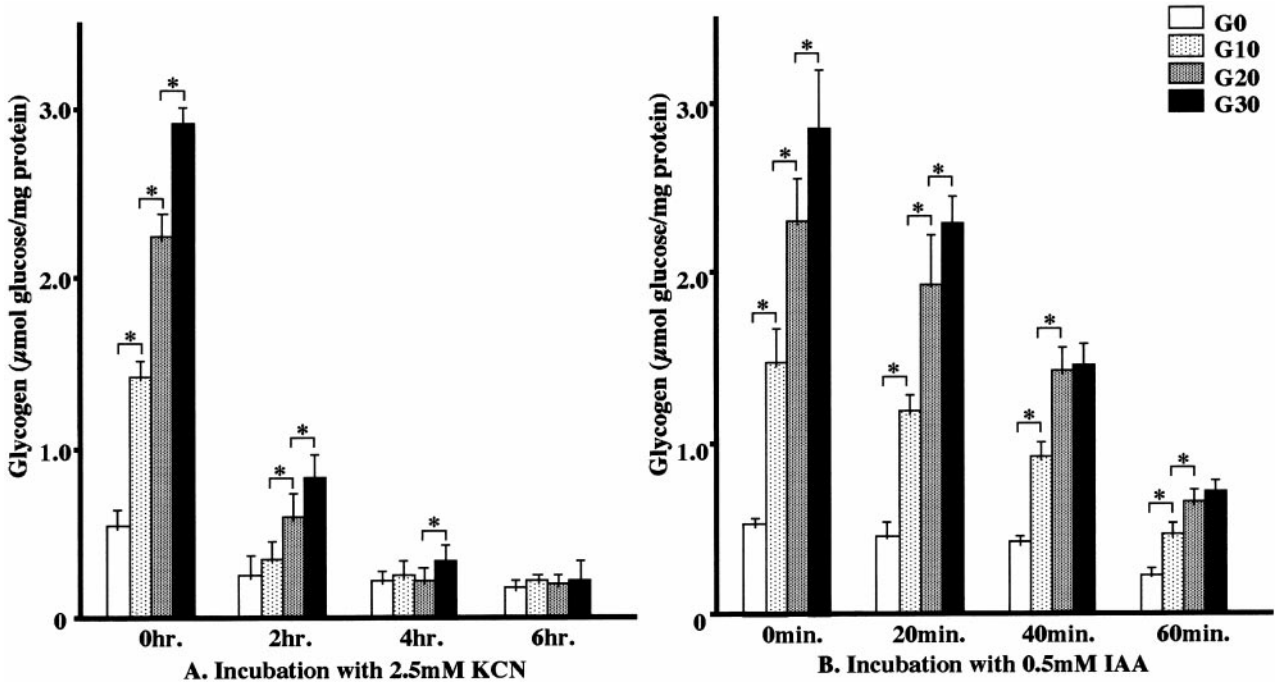

Fig 1. Effects of KCN and IAA on intracellular glycogen. Cultured hepatocytes were incubated with (A) 2.5 mmol/L KCN or (B) 0.5 mmol/L IAA in modified Hanks-HEPES buffer in a humidified incubator at 37°C under 20% O_2 and 80% N_2. At the end of each period, hepatocytes were dissolved in 3% $HClO_4$ to determine glycogen content. Data are expressed as means ± SD for 5 cultured hepatocytes preparations. Glycogen content of each group of hepatocytes decreased significantly with increasing periods of exposure to both KCN and IAA. * Comparison between groups, $P < .05$.

data groups were compared post hoc using Fisher's PLSD. A *P* value of <5% was considered statistically significant.

RESULTS

Hepatocellular Viability and Nutritional Condition After Initial Culture

The extracellular and intracellular LDH activities among the four hepatocyte groups (G0, G10, G20, and G30) at 24 hours of initial culture did not statistically differ (Table 1). The glycogen contents in the four groups increased according to the glucose concentration in the medium and the ATP content was not significantly different among the four groups before exposure to KCN or to IAA (at 0 hours or 0 minutes in Figs 1 and 2).

Findings After Exposure to KCN or IAA

LDH release. Exposure to either KCN or IAA significantly increased the level of LDH released by the hepatocytes over time (Fig 3A and B) compared with the absence of these agents for up to 6 hours (Fig 3C). Less LDH was released by hepatocytes cultured with higher glucose concentrations after 2, 4 or 6 hours of exposure to KCN (Fig 3A). Conversely, more LDH was released by hepatocytes cultured with higher glucose concentrations after 20, 40, or 60 minutes of exposure to IAA (Fig 3B).

Intracellular glycogen content. The glycogen content of each group of hepatocytes decreased after exposure to both KCN and IAA for increasing lengths of time (Fig 1).

Intracellular ATP content. The intracellular ATP levels in the four groups of hepatocytes did not significantly differ before exposure to the inhibitors but decreased significantly for up to 4 hours in the presence of KCN and for up to 60 minutes in the presence of IAA. Hepatocytes cultured with higher glucose concentrations after 2- and 4-hour incubations with KCN maintained higher levels of ATP (Fig 2A). Conversely, the ATP content was maintained at higher levels in hepatocytes cultured with lower glucose concentrations after 20- and 40-minute incubations with IAA (Fig 2B).

Lactate release. The amount of lactate released by all groups of hepatocytes significantly increased with the length of exposure to KCN. Cells cultured in the medium with higher glucose concentrations released more lactate into the supernatant after 2, 4, or 6 hours of exposure to KCN (Fig 4A). On the other hand, less than 0.15 μmol of lactate/mg protein was released after exposure to IAA, and no significant differences were identified among the groups with regard to the effect of the exposure period (Fig 4B).

Extracellular pH. The pH in the supernatant of each group of hepatocytes significantly decreased depending on the duration of exposure to KCN. The extracellular pH in the groups of hepatocytes cultured in the medium with higher glucose concentrations was lower than that in those that were cultured with lower glucose concentrations after 2, 4, or 6 hours of exposure to KCN (Table 2). The pH of the supernatant did not differ

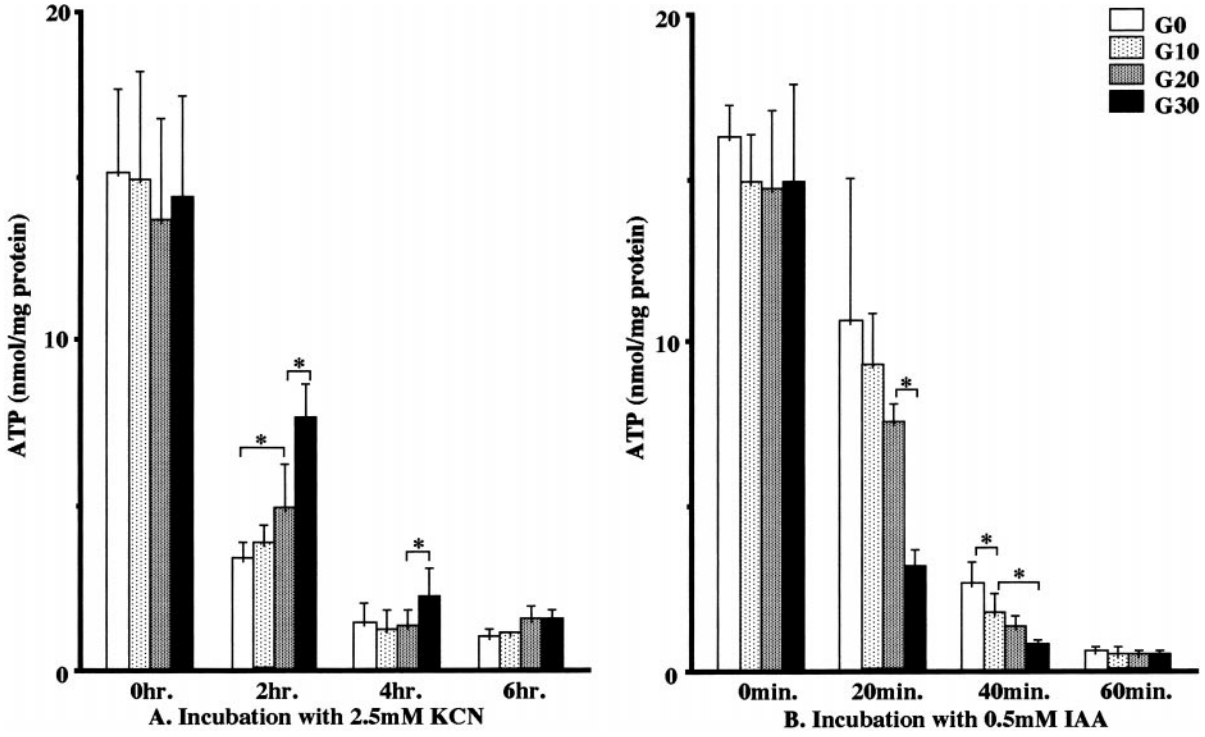

Fig 2. Effects of KCN and IAA on intracellular ATP content. Cultured hepatocytes were incubated with (A) KCN or (B) IAA as described in the legend to Fig 1. At the end of each incubation period, hepatocytes were dissolved in 3% $HClO_4$ to measure ATP contents. Data are expressed as means ± SD for 5 cultured hepatocyte preparations. ATP levels in each group significantly decreased (A) for up to 4 hours of exposure to KCN and (B) for up to 60 minutes of exposure to IAA. * Comparison between groups, *P* < .05.

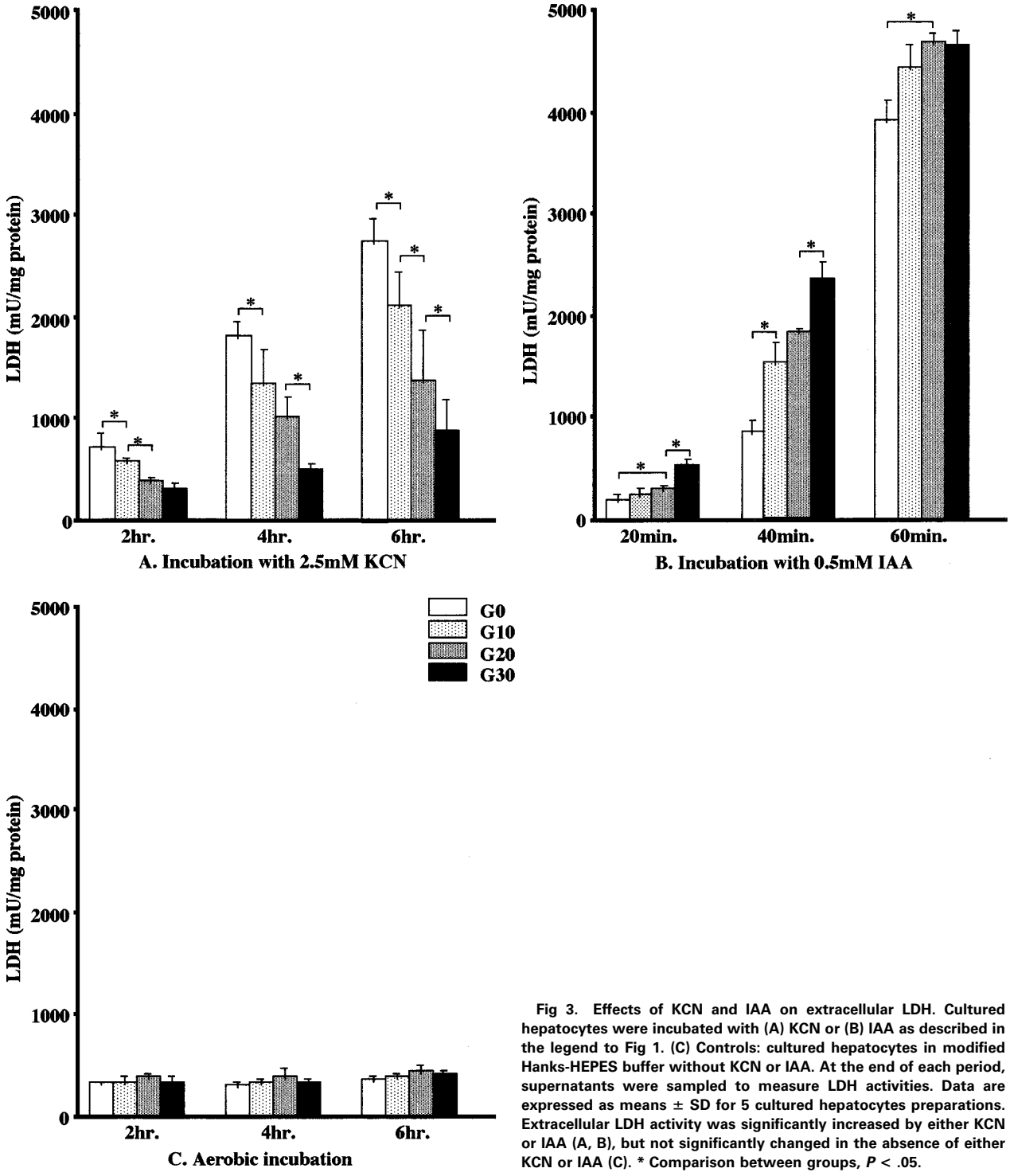

Fig 3. Effects of KCN and IAA on extracellular LDH. Cultured hepatocytes were incubated with (A) KCN or (B) IAA as described in the legend to Fig 1. (C) Controls: cultured hepatocytes in modified Hanks-HEPES buffer without KCN or IAA. At the end of each period, supernatants were sampled to measure LDH activities. Data are expressed as means ± SD for 5 cultured hepatocytes preparations. Extracellular LDH activity was significantly increased by either KCN or IAA (A, B), but not significantly changed in the absence of either KCN or IAA (C). * Comparison between groups, $P < .05$.

significantly among the four groups during exposure to IAA (Table 3).

DISCUSSION

The present study examined the effects of glucose loading on the viability of hepatocytes subsequently exposed to KCN (inhibitor of mitochondrial oxidative phosphorylation) or to IAA (inhibitor of glycolysis). The results show that glucose loading had opposite effects on the type of hepatocyte injury caused by two inhibitors of carbohydrate metabolism. To define these effects, we cultured hepatocytes in medium containing four concentrations of glucose; all other conditions remained

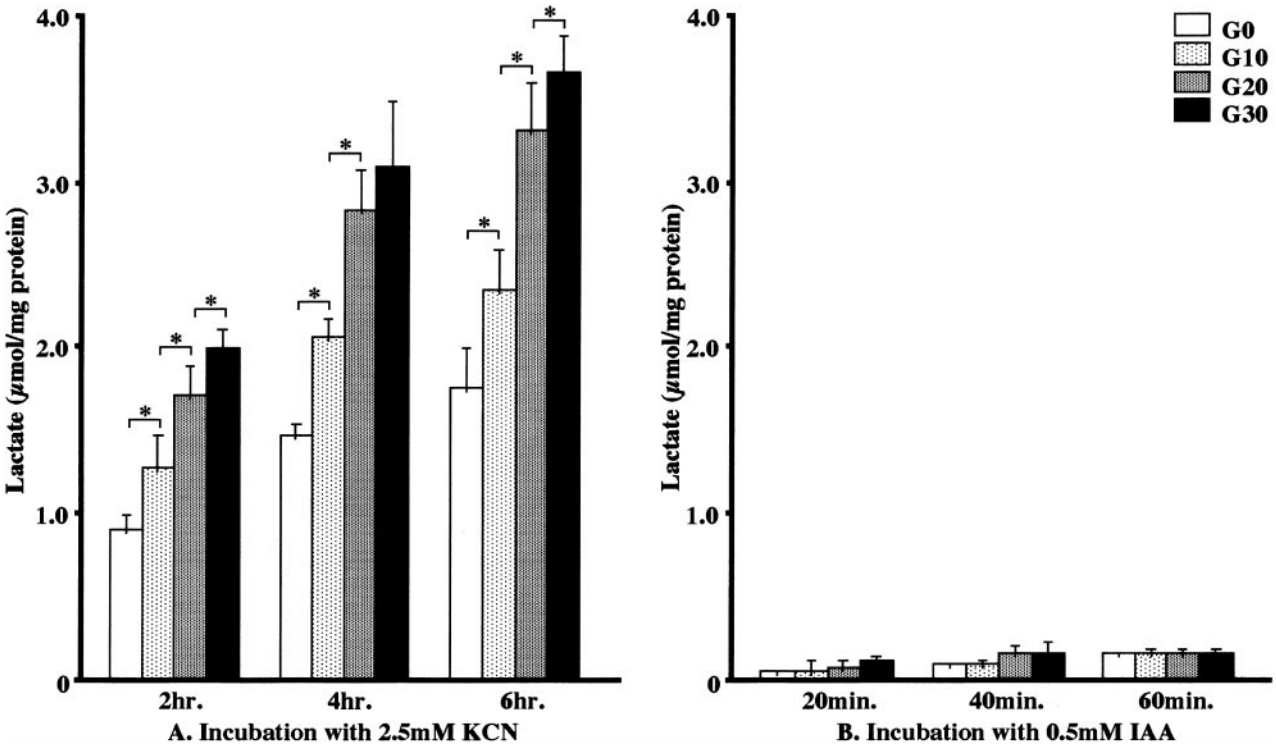

Fig 4. Effects KCN and IAA on lactate release. Cultured hepatocytes were incubated with (A) KCN or (B) IAA as described in the legend to Fig 1. At the end of each incubation period, supernatant lactate levels were determined. Data are expressed as means ± SD for 5 cultured hepatocytes preparations. Lactate release from the hepatocytes in each group significantly increased as the period of KCN exposure increased (A), but was reduced to less than 0.15 μmol/mg protein during exposure to IAA. * Comparison between groups, $P < .05$.

identical. We found that the glycogen contents increased in direct proportion to the amount of glucose contained in the medium (approximately from 0.5 to 3.0 μmol glucose/mg protein; Fig 1A and B at 0 hours or 0 minutes). These four groups of hepatocytes (G0, G10, G20, and G30) did not differ in terms of either hepatocellular viability or ATP content at the end of the initial culture period without inhibitors (Table 1 and Fig 2).

We found that hepatocytes initially cultured in medium with higher glucose concentrations released less LDH upon exposure to KCN (Fig 3A). In other words, hepatocytes cultured under these conditions developed more tolerance to injury caused by the subsequent inhibition of oxidative phosphorylation. In contrast, hepatocytes cultured in medium with lower glucose concentrations released less LDH when exposed to IAA (Fig 3B). Hepatocytes cultured under these conditions developed higher tolerance to injury caused by the inhibition of glycolysis. These findings may explain the conflicting effects of various nutritional conditions (glycogen storage) on hepatocyte viability found during clinical and experimental liver transplantation.[5-9]

Two scenarios can explain our findings of increased tolerance by glucose-loaded hepatocytes to KCN-inhibited oxidative phosphorylation. One is that higher levels of intracellular

Table 2. Effects of Incubation With 2.5 mmol/L KCN on Extracellular pH

	2 Hours	4 Hours	6 Hours
G0 group	7.35 ± 0.01	7.32 ± 0.01	7.31 ± 0.02
G10 group	7.33 ± 0.01*	7.30 ± 0.01*	7.28 ± 0.01*
G20 group	7.32 ± 0.01†	7.28 ± 0.01†	7.25 ± 0.01†
G30 group	7.31 ± 0.01‡	7.26 ± 0.02‡	7.23 ± 0.01‡

NOTE. Cultured hepatocytes were incubated with KCN as described in Fig 1. At the end of each incubation period, supernatant pH values were measured. pH values in all supernatants decreased significantly with increasing periods of exposure to KCN. Data are expressed as means ± SD for five cultured hepatocyte preparations.

* $P < .05$ *v* G0 group.

† $P < .05$ *v* G10 group.

‡ $P < .05$ *v* G20 group.

Table 3. Effects of Incubation With 0.5 mmol/L IAA on Extracellular pH

	20 Minutes	40 Minutes	60 Minutes
G0 group	7.40 ± 0.01	7.39 ± 0.01	7.39 ± 0.01
G10 group	7.39 ± 0.01	7.38 ± 0.01	7.38 ± 0.01
G20 group	7.39 ± 0.01	7.39 ± 0.01	7.39 ± 0.01
G30 group	7.39 ± 0.01	7.39 ± 0.01	7.38 ± 0.01

NOTE. Cultured hepatocytes were incubated with IAA as described in Fig 1. At the end of each incubation period, supernatant pH values were measured. Exposure to IAA did not affect the pH values of any group. Data are expressed as means ± SD for five cultured hepatocyte preparations.

ATP (Fig 2A) can be maintained for longer periods by glycolysis in glycogen-rich hepatocytes. The depletion of ATP in hepatocytes causes membrane blebs, and their eventual rupture results in lethal cell injury.[23,24] The other is the decreased pH (Table 2), which was probably caused by both the liberation of protons by ATP hydrolysis and by glycolytic lactate production. Decreased extracellular and intracellular pH delays the death of hepatocytes during ATP depletion.[19,25,26] High levels of nonlysosomal protease and phospholipase A_2 activities contribute to lethal hepatocellular injury during ATP depletion. Acidosis inhibits both nonlysosomal protease and phospholipase A_2 activities and exerts cytoprotective effects during ATP depletion.[27-29] Prior glucose loading appears to be beneficial for hepatocytes if oxidative phosphorylation is to be inhibited because of the improved ATP maintenance and decreased pH, both of which are derived from rich glycogen storage.

In contrast, glucose loading during primary culture was rather hazardous to hepatocytes when glycolysis was inhibited by IAA (Fig 3B). Intracellular glycogen and extracellular glucose are not used as energy substrates when glycolysis is inhibited by IAA. Lactate production was suppressed by IAA (Fig 4B), and the pH of the extracellular medium did not subsequently decrease (Table 3). Thus, low pH cannot exert a cytoprotective effect during exposure to IAA. Furthermore, hepatocytes cultured in the presence of lower glucose concentrations, with a result of storing less glycogen, maintained higher ATP levels upon exposure to IAA (Fig 1B). The fact that glucose-loaded hepatocytes did not maintain high ATP levels explains the decreased tolerance of IAA-inhibited glycolysis caused by glucose loading. Although our present study did not aim to clarify the cause of this mechanism, the following two metabolic changes might explain it. First, hepatocytes initially cultured in medium with higher glucose concentrations synthesized less ATP during the glycolytic inhibition. During primary culture, they probably accumulated fewer amino acids and/or fatty acids to be used as substrates for ATP through oxidative phosphorylation. Second, hepatocytes initially cultured in medium with higher glucose concentrations consumed more ATP during the glycolytic inhibition. Additional investigations are needed to identify the types of metabolic changes that would occur during initial primary culture.

In conclusion, the present study determined that glucose loading of cultured hepatocytes produces opposite effects on the type of hepatocellular injuries caused by KCN and IAA. Glucose loading during initial culture is desirable if oxidative phosphorylation is to be inhibited, whereas withholding glucose is desirable if glycolysis is to be inhibited. Whether or not to apply nutritional pretreatment to the liver and how should be determined with respect to the metabolic conditions that the liver may undergo.

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