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A SIMPLIFIED MODEL OF HYPOXIC INJURY IN PRIMARY CULTURED RAT HEPATOCYTES

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SUMMARY

The Anaeropack system for cell culture, which was originally designed for the growth of anaerobic bacteria, was used to produce a hypoxic atmosphere for cultured hepatocytes. We measured changes in the oxygen and carbon dioxide concentrations and the atmospheric temperature in an airtight jar. We also measured changes in the pH of the medium during hypoxia to assess the accuracy of this system. Moreover, we used three durations (2, 3, and 4 h) of hypoxia and 8 h of reoxygenation in cultured rat hepatocytes, and then measured the lactate dehydrogenase (LDH), ketone body concentration (acetoacetate + β-hydroxybutyrate), and the ketone body ratio (KBR: acetoacetate/β-hydroxybutyrate) in the medium in order to assess the suitability of this system as a model for reperfusion following liver ischemia. The oxygen concentration dropped to 1% or less within 1 h. The concentration of carbon dioxide rose to about 5% at 30 min after the induction of the hypoxic conditions, and was maintained at this level for 5 h. No effect of the reaction heat produced by the oxygen absorbent in the jar was recognized. The extent of cell injury produced by changing the hypoxic parameters was satisfactorily reflected by the KBR, the ketone body concentration, and the LDH activity released into the medium. Because this model can duplicate the conditions of the hepatocytes during revascularization following ischemic liver, and the Anaeropack system for cell culture is easy to manipulate, it seems suitable for the experimental study of hypoxic injury and revascularization *in vitro*.

Key words: hypoxia; reoxygenation; liver; ketone bodies; enzyme release.

INTRODUCTION

Revascularization following hepatic ischemia is one of the most important events in the surgery and transplantation of the liver. Although the occurrence of revascularization injury has been widely accepted, the mechanisms remain unclear. To clarify this mechanism, both ischemic liver injury *in vivo* and anoxic hepatocyte injury *in vitro* have been characterized by the morphologic, metabolic, and functional changes in liver cells following reperfusion and reoxygenation (6,17,19,20). Primary cultured hepatocytes are very useful for the study of liver function *in vitro* (2,7,18). However, the conventional method to produce a hypoxic atmosphere in culture requires placing the culture dishes in an airtight pressure bottle. The air is then evacuated from the bottle, and is replaced by a gas mixture of 95% nitrogen and 5% carbon dioxide (23). This method is tedious, and there is a limit to the number of examination dishes that can be tested in this manner.

In the present study, we used the Anaeropack system for cell culture (Trial Products, Mitsubishi Gas Chemical Co., Tokyo, Japan) to create a hypoxic atmosphere for use within a jar. The Anaeropack system for cell culture was made by modifying an Anaeropack-anaero® designed to support the growth of anaerobes. This system provides a hypoxic atmosphere with about 5% carbon dioxide without the need for evacuation and replacement with an anaerobic atmosphere. It is easy to manipulate, and seems suitable as a model for hypoxic injury, provided that it can function as an experimental model and can reflect the conditions of the hepatocytes during liver ischemia and revascularization.

To assess the utility of this system, we measured changes in the oxygen and carbon dioxide concentrations and the atmospheric temperature in the jar during hypoxia. Because ischemia/revascularization injury results in structural deterioration (15) and metabolic dysfunction (3), we measured the lactate dehydrogenase (LDH) activity in the medium as an indicator of the structural damage, and the concentration of ketone bodies in the medium as the metabolic indicator, respectively.

The Anaeropack for cell culture was made by modifying the Anaeropack-anaero® for 20% carbon dioxide atmospheric concentration, which was designed to support the growth of anaerobes. The carbon dioxide concentration is known to be an important factor in primary tissue culture. The optimum concentration of carbon dioxide is believed to be 5% for cultured hepatocytes. Therefore, the Anaeropack for cell culture was modified to produce a carbon dioxide concentration of about 5%. To assess the importance of the carbon dioxide concentration, we manufactured another system that provided a hypoxic atmosphere of 10% carbon dioxide [Anaeropack for

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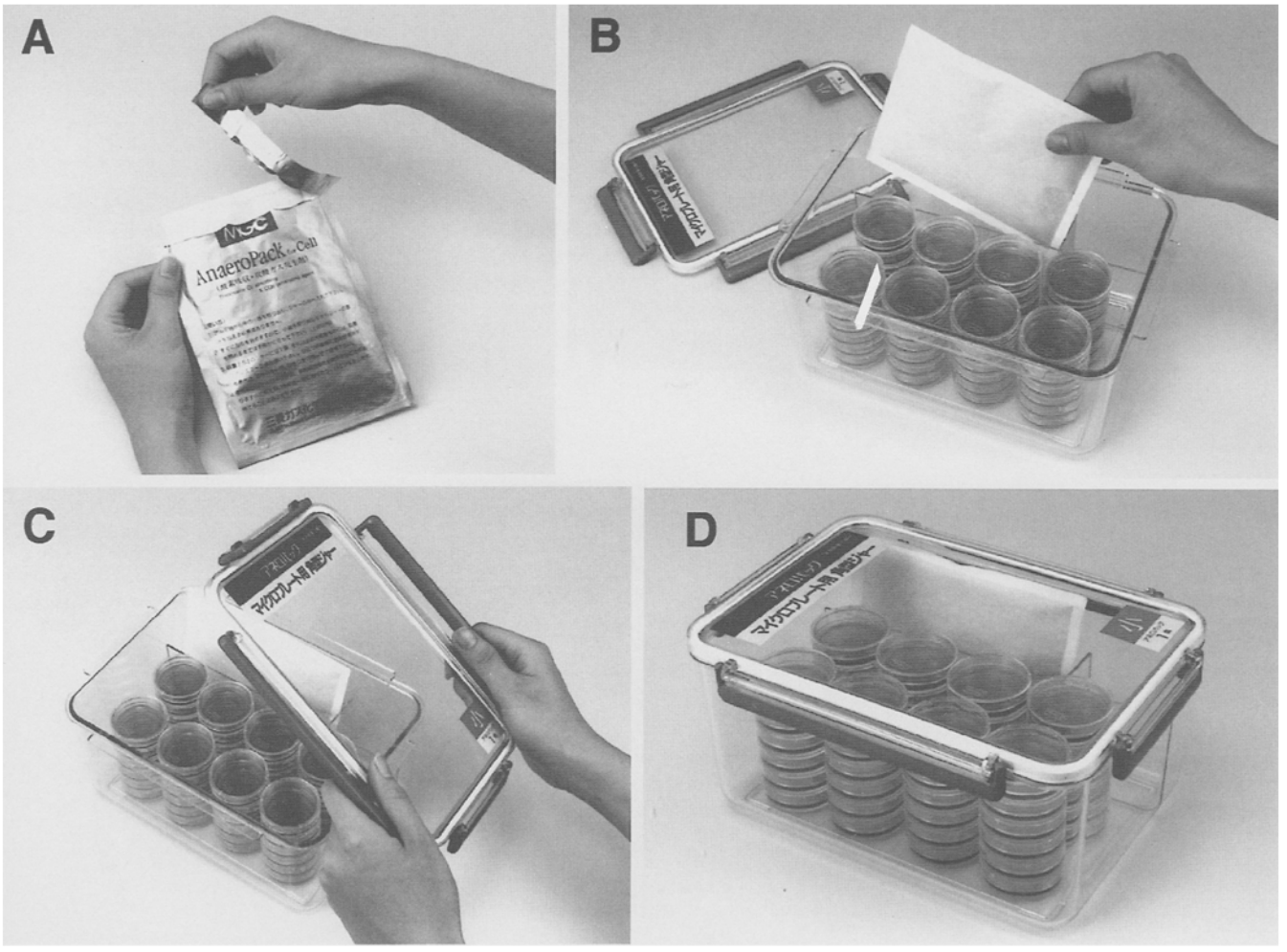

FIG. 1. Use of the Anaeropack system for cell culture.

cell culture (10%)], and examined the effects of a 10% carbon dioxide atmosphere on the pH value in the medium. This system was also supplied by Mitsubishi Gas Chemical.

The purpose of this study was to elucidate the suitability of the Anaeropack for cell culture in primary cultured hepatocytes as a model for revascularization following normothermic ischemia in the liver.

MATERIALS AND METHODS

Primary Culture of Rat Hepatocytes

Hepatocytes were obtained from adult male Wistar rats (6–7 wk old, 180–240 g) fed ad libitum. The cells were isolated by perfusing the liver with collagenase under sterile conditions, as previously described (16). The isolated hepatocytes were suspended in culture medium at a density of 0.5–0.6 $\times$ 10^6 cells/ml, and were inoculated into plastic culture dishes (2 ml per well, 35 mm $\times$ 10 mm, Falcon Plastic, Oxnard, CA) and cultured as monolayers in a CO_2 incubator (humidified 5% CO_2 in air) at 37° C. Unless otherwise specified, the hepatocytes were maintained at a concentration of 0.8–1.0 $\times$ 10^6 cells per well in Williams' medium E supplemented with 10% fetal bovine serum (FBS), [2-[4-(2-Hydroxyethyl)-1-piperazinyl-]ethanesulfonic Acid] (HEPES) (5 m*M*), penicillin (100 U/ml), streptomycin (0.1 mg/ml), dexamethasone (10^{-8} *M*), insulin (10^{-8} *M*), and glucagon (10^{-9} *M*). After 2 h, the medium was replaced by a serum-free medium (1.5 ml per dish), and the cell medium was changed daily thereafter. After 2 d of culture, the cell medium was changed to a new medium and then used in the experiments.

Preparation of Hypoxic Conditions

Anaeropack for cell culture. The Anaeropack for cell culture is the gas concentration controlling reagent for the hypoxic atmosphere. This reagent contains sodium ascorbate as the principal ingredient, which absorbs oxygen and generates carbon dioxide by oxidative degradation. Another reagent is used as a carbon dioxide absorber to scavenge the carbon dioxide. These reagents were packed in a paper sachet that had good gas permeability, and the paper sachet was packed in foil packaging before use (Fig. 1 *A*). The culture dishes were placed into an airtight jar with a paper sachet (Fig. 1 *B*), and the lid was closed immediately (Fig. 1 *C*). The inner paper sachet became activated upon contact with air, and the hypoxia was then initiated (Fig. 1 *D*). As a result, the oxygen concentration decreased to less than 1% within 1 h, and the carbon dioxide concentration was maintained at about 5% when the sachet was placed into a 1.6 l airtight jar (Mitsubishi Gas Chemical). The airtight jar was then incubated in a CO_2 incubator at 37° C. After a designated time period, the hypoxia was terminated by opening the jar and starting reoxygenation.

Anaeropack for cell culture (10%). The components of the Anaeropack for cell culture (10%) were the same as the Anaeropack for cell culture, except for the quantity of carbon dioxide absorber.

Experimental Protocol

Experiment A: Assessment of the experimental conditions. 1. Oxygen and carbon dioxide concentrations in the jar during hypoxia using the Anaeropack for cell culture. Immediately after a sachet was placed into a 1.6 l airtight jar, the concentrations of oxygen and carbon dioxide in the jar were measured by an Oxygen Analyzer Model LC700F (Toray Engineering Co., Ltd., Tokyo, Japan), and an Infrared Gas Analyzer Model UR-025S (Komyo Rikagaku Kogyo Co., Ltd., Kawasaki, Japan), respectively. These measurements were taken every 30 min until 2 h after the induction of hypoxia, and were taken again at 3 and 5 h after the induction of hypoxia.

2. Temperature of the paper sachet surface and atmospheric temperature in the jar during hypoxia by using the Anaeropack for cell culture. Immediately after a sachet was placed into a 1.6 l airtight jar, the jar was placed into a constant temperature bath set at 37° C, and both temperatures were measured. The measurements were taken every 10 min until 80 min after the induction of hypoxia by a Digital Record Thermometer Model TNA-120 (Tasco Japan Co., Ltd., Tokyo, Japan).

3. Comparison of the Anaeropack for cell culture (5%) and Anaeropack for cell culture (10%) on the pH value of the medium during hypoxia and reoxygenation. The cells were incubated under a hypoxic atmosphere for 3 h using the Anaeropack for cell culture (5%), with a subsequent 6 h of reoxygenation (humidified 5% CO_2 in air). This experiment serves as a time-matched control for the hypoxia/reoxygenation experiment. The control incubation was performed under normoxic conditions (humidified 5% CO_2 in air) for the entire experiment. Immediately upon removal of the culture dish from the jar at various time points, the culture medium was collected into a plastic test tube and the pH value was measured immediately by a pH Meter F-11 (Horiba, Ltd., Kyoto, Japan). The measurements were taken every hour during the hypoxia for 3 h, and for 6 h following the reoxygenation.

The same procedure was performed using Anaeropack for cell culture (10%) with time-matched controls.

Experiment B: Assessment of the cell conditions during hypoxia/reoxygenation. 1. LDH activity in the medium. We used three durations of hypoxia (2, 3, and 4 h) and 8 h of reoxygenation in cultured rat hepatocytes, and then measured the LDH activity in the medium. This experiment also serves as a time-matched control for the hypoxia/reoxygenation experiment. The control incubation was performed under normoxic conditions (humidified 5% CO_2 in air) for the entire experiment. Immediately upon removal of the dish from the jar, the culture medium was collected into a plastic test tube. The LDH activity in the cell supernatant and lysate were then determined according to the methods of Bergmeyer (1). The percentage of LDH released was calculated as a ratio of the LDH released into medium over the total amount of LDH present in the cells, as determined by lysis of the cells with 0.1% Triton X-100 (8). This percentage of LDH activity was proportional to the percentage of dead cells. The measurements were taken every hour during the three time periods of hypoxia (2, 3, and 4 h), and during reoxygenation for the first 4 h, and again at 6 and 8 h after starting the reoxygenation.

2. Ketone body ratio and ketone body concentration in the medium. Using the same experimental procedure described above for the assessment of the LDH activity, the concentration of acetoacetate and β-hydroxybutyrate in the medium was measured to assess mitochondrial function. Immediately upon removal from the jar, the culture dish was placed on crushed ice and the culture medium was collected into a cold glass test tube. The ketone body concentration (acetoacetate and β-hydroxybutyrate) of the medium was measured using a Ketolex kit (Sanwa Kagaku Co., Ltd., Nagoya, Japan) (21). The ketone body ratio (KBR) was calculated as the ratio of acetoacetate to β-hydroxybutyrate. The measurement time points were the same as those used in the LDH measurements.

Statistics

All of the data are expressed as the means of triplicate samples at each time point. The *error bars* denote the standard error of the mean. Significant differences were analyzed by the unpaired Student's t test, and a $P < 0.05$ was considered to be significant. The *error bars* were omitted when they were within the size of the graphic symbol.

Results

Assessment of the Experimental Conditions (Experiment A)

The concentration of oxygen in the jar dropped to less than 1% within 1 h after the induction of hypoxia, and reached its lowest level

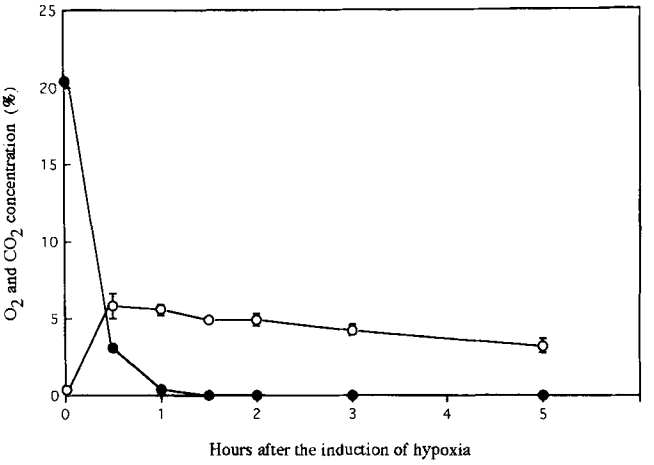

FIG. 2. Changes in the oxygen and carbon dioxide concentrations in the jar during hypoxia using the Anaeropack for cell culture. *Solid circles* = oxygen concentration; *open circles* = carbon dioxide concentration.

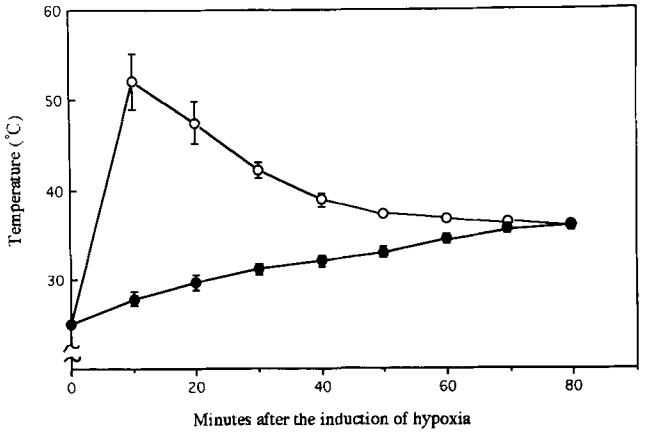

FIG. 3. Changes in the temperature of the paper sachet surface and atmospheric temperature in the jar during hypoxia using the Anaeropack for cell culture. *Solid circles* = atmospheric temperature; *open circles* = the temperature of the paper sachet surface.

(less than 0.1%) after 3 h. The concentration of carbon dioxide in the jar rose to about 5% at 30 min after the induction of hypoxia. Both oxygen and carbon dioxide concentrations were maintained until 5 h after starting the hypoxia (Fig. 2).

The surface temperature of the oxygen absorbent rose to a peak of 52° C at 10 min after the induction of hypoxia, but fell gradually with the passage of time and returned to the temperature of the bath at 80 min after the induction of hypoxia. The temperature inside the anaerobic jar reached 27° C at 10 min after the induction of hypoxia, and rose gradually with the passage of time. It finally reached the temperature of the bath at 80 min after the induction of hypoxia (Fig. 3).

Fig. 4 shows the changes in the pH value of the medium using the Anaeropack for cell culture (5%) and the Anaeropack for cell culture (10%). When the Anaeropack for cell culture (5%) was used, there was no detectable difference in the pH value of the medium as com-

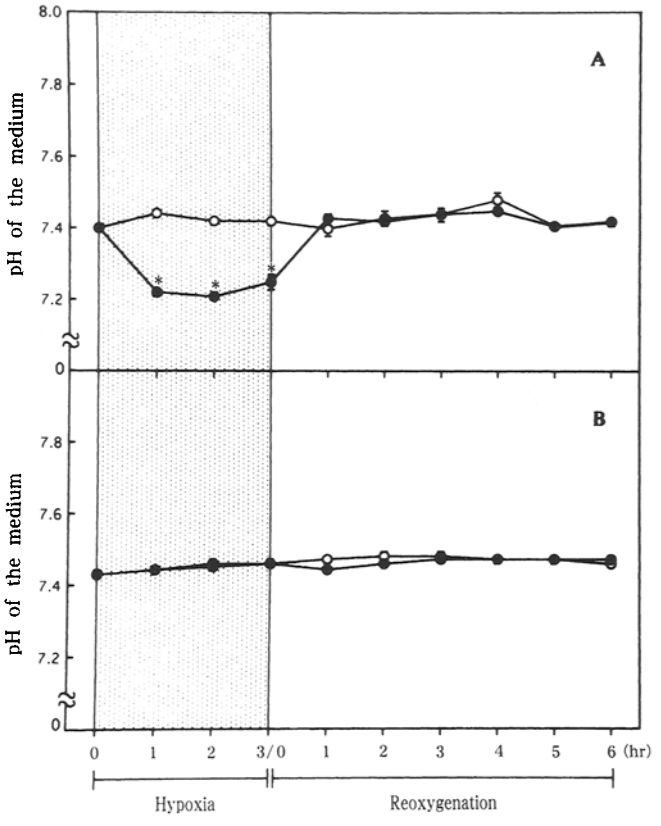

FIG. 4. Effects of the carbon dioxide concentration on the pH of the medium. *A*, Changes in the pH of the medium when the Anaeropack for cell culture (10%) was used as the gas concentration controlling reagent for hypoxia. *Solid circles* = Anaeropack for cell culture (10%); *open circles* = controls. *B*, The Anaeropack for cell culture (5%) was used to control the hypoxia. *Solid circles* = Anaeropack for cell culture (5%); *open circles* = controls, respectively. An *asterisk* indicates a significant difference from the control group ($P < 0.05$). The *dotted area* indicates the hypoxic period.

pared to the controls (Fig. 4 *B*). When the Anaeropack for cell culture (10%) was used (Fig. 4 *A*), the pH value of the medium decreased significantly by about 0.2 units during the hypoxia, as compared to the controls.

Assessment of the Cell Conditions During Hypoxia/Reoxygenation (Experiment B)

No increased LDH activity released into the medium from the hypoxia/reoxygenation hepatocytes was observed at any time point, as compared with the controls for the experimental hypoxia of 2 h duration (Fig. 5 *A*). A significant increase in LDH activity released into the medium was recognized at the start of reoxygenation following ischemia for 3 h, as compared with the controls, but not during the hypoxia. The LDH activity increased gradually with time (Fig. 5 *B*). At 4 h after the induction of hypoxia, the LDH activity released into the medium from the hepatocytes had increased significantly as compared to the controls, and increased rapidly following the reoxygenation period (Fig. 5 *C*). The percentage of LDH leakage into the medium at 8 h after reoxygenation was as follows: 6.4 ± 0.0% (hypoxia for 2 h), 20.0 ± 1.2% (hypoxia for 3 h), and 75.2 ± 7.1% (hypoxia for 4 h), respectively.

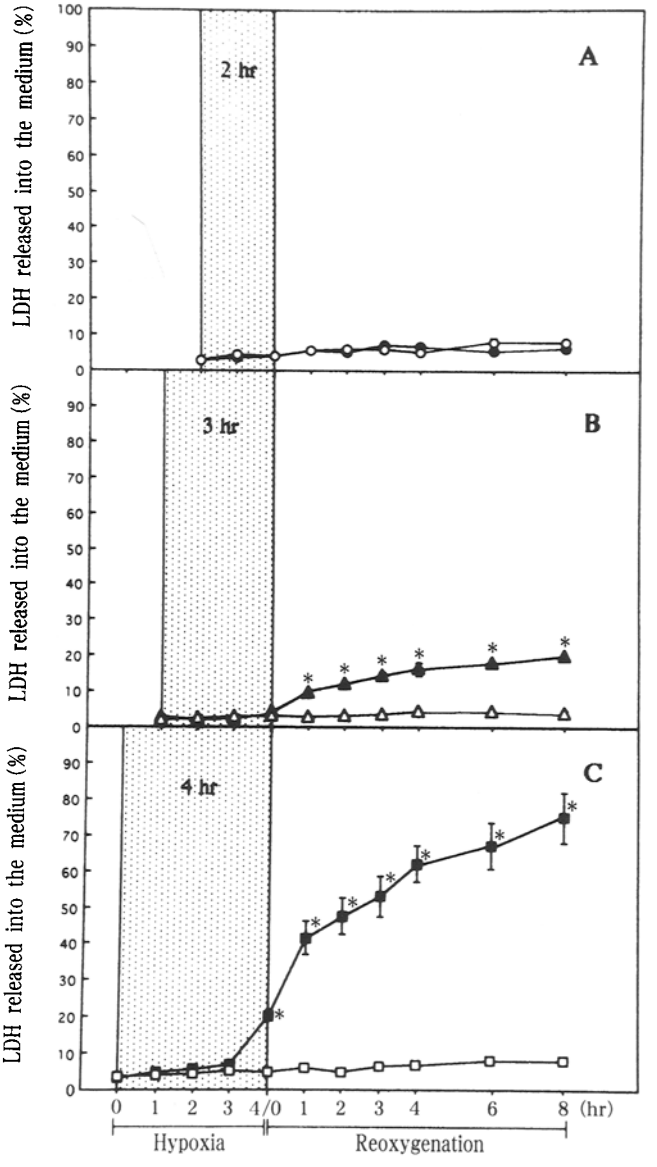

FIG. 5. Effects of the duration of the hypoxia on the lactate dehydrogenase (LDH) activity released into the medium. *A*, Hypoxia for 2 h; *B*, hypoxia for 3 h; *C*, hypoxia for 4 h. *Solid circles* = hypoxia for 2 h; *solid triangles* = hypoxia for 3 h; *solid squares* = hypoxia for 4 h; *open symbol* = controls, respectively. An *asterisk* indicates a significant difference from the control group ($P < 0.05$). The *dotted area* indicates the hypoxic period.

The KBR of the medium decreased rapidly, and remained significantly lower as compared to the controls, during the three different hypoxic conditions. However, it recovered rapidly during the reoxygenation in the 2 h hypoxic experiment, and the KBR was significantly higher than in the controls at 8 h after starting the reoxygenation (Fig. 6 *A*). During the reoxygenation following hypoxia for 3 h, the KBR in the medium recovered gradually, but the value remained significantly lower than the controls (Fig. 6 *B*). The KBR during the reoxygenation following hypoxia for 4 h showed little progress towards recovery (Fig. 6 *C*). The KBR of the medium at 8 h after the reoxygenation were as follows: 3.01 ± 0.02 (hypoxia for 2 h), 1.48

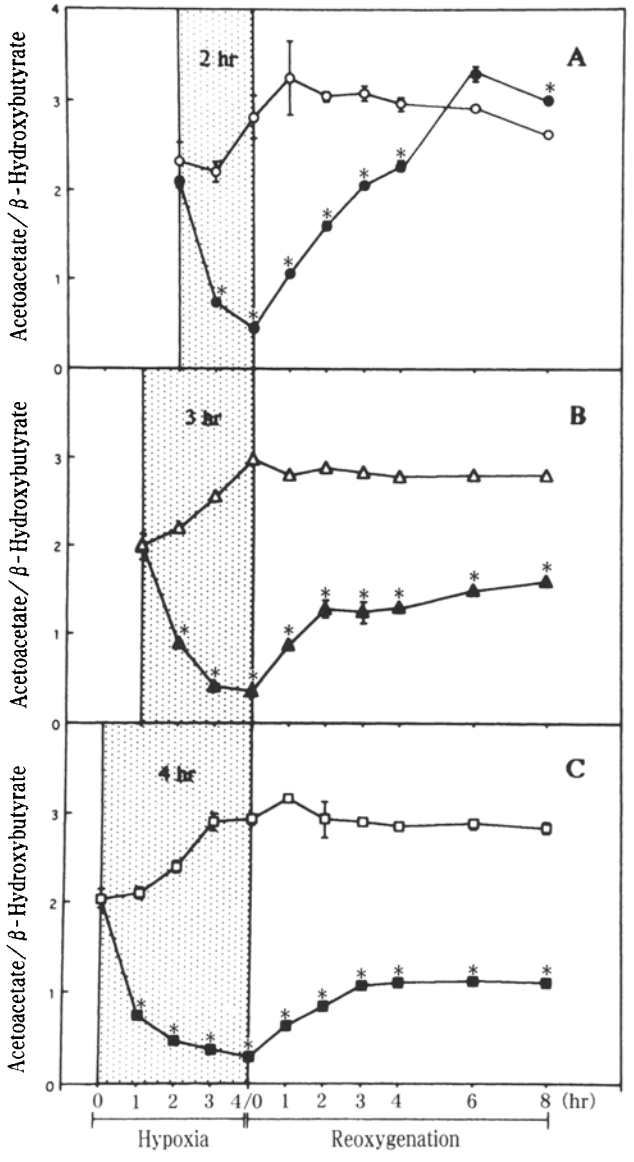

FIG. 6. Effects of the duration of the hypoxia on the ketone body ratio (KBR) of the medium. *A*, Hypoxia for 2 h; *B*, hypoxia for 3 h; *C*, hypoxia for 4 h. *Solid circles* = hypoxia for 2 h; *solid triangles* = hypoxia for 3 h; *solid squares* = hypoxia for 4 h; *open symbol* = controls, respectively. An *asterisk* indicates a significant difference from the control group ($P < 0.05$). The *dotted area* indicates the hypoxic period.

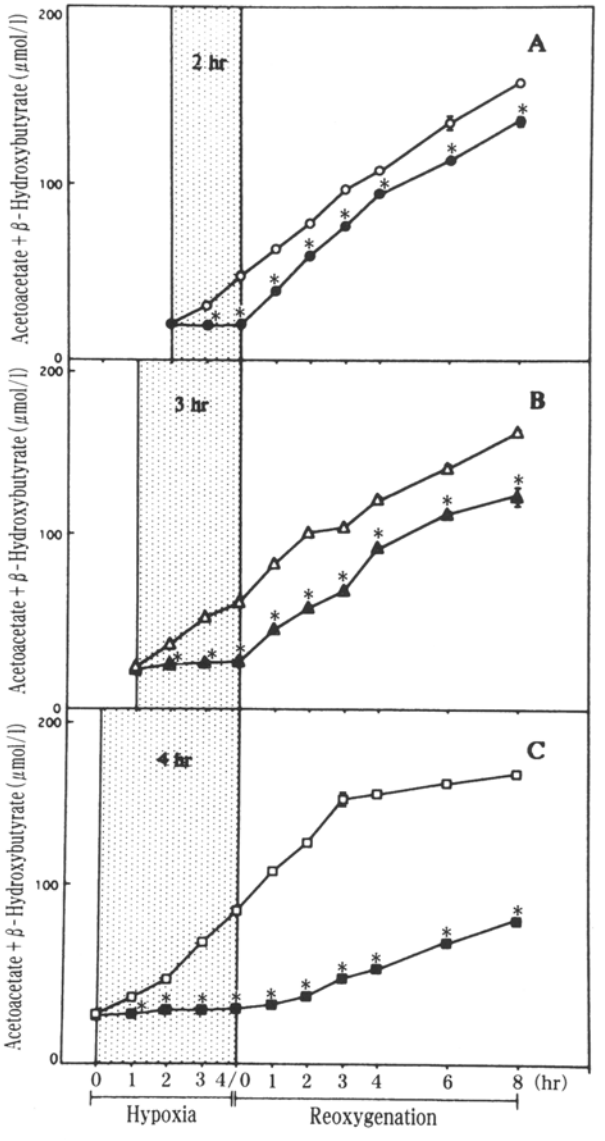

FIG. 7. Effects of the duration of the hypoxia on the ketone body concentration of the medium. *A*, Hypoxia for 2 h; *B*, hypoxia for 3 h; *C*, hypoxia for 4 h. *Solid circles* = hypoxia for 2 h; *solid triangles* = hypoxia for 3 h; *solid squares* = hypoxia for 4 h; *open symbol* = controls, respectively. An *asterisk* indicates a significant difference from the control group ($P < 0.05$). The *dotted area* indicates the hypoxic period.

± 0.03 (hypoxia for 3 h), and 1.11 ± 0.03 (hypoxia for 4 h), respectively.

Increases in the ketone body concentrations of the medium were barely detectable during the three different hypoxic conditions. However, the capacity for ketone body production recovered quickly during the reoxygenation following hypoxia for 2 h as compared to the controls (Fig. 7 *A*). However, during the reoxygenation following hypoxia for 3 h, the ketone body production of the medium had a tendency to be depressed, as compared to the 2-h hypoxia group (Fig. 7 *B*). More remarkable depression was recognized in the 4-h hypoxia group (Fig. 7 *C*). The ketone body concentrations of the medium at 8 h after reoxygenation were as follows: 141 ± 2.9 μ mol/l (hypoxia for 2 h), 130 ± 5.4 μ mol/l (hypoxia for 3 h), and 87 ± 2.0 μ mol/l (hypoxia for 4 h), respectively.

DISCUSSION

In the present study, the oxygen concentration decreased rapidly and dropped to 1% or less within 1 h. The reaction heat produced by the oxygen absorbent did not have a detectable effect on the atmospheric temperature in the jar in this experiment.

The carbon dioxide concentration is known to be an important factor in primary tissue culture. The optimum concentration of carbon

dioxide is believed to be about 5% for cultured hepatocytes, and this was easily obtained with the Anaeropack system for cell culture. There was also no effect of the carbon dioxide concentration on the pH value of the medium. In the experiment in which the Anaeropack system for cell culture (10%) was used, which can generate a 10% carbon dioxide concentration, the pH value of the medium had decreased by 0.2 units at 1 h after the induction of hypoxia, and was maintained at this value during the hypoxia. The high concentration of carbon dioxide dissolved in the medium and the pH value both decreased during the hypoxia. The Anaeropack-anaero® for 20% carbon dioxide atmospheric concentration needed significant modification, since the metabolism of the hepatocytes can be affected by decreases in the pH value of the medium. The Anaeropack system for cell culture solved this problem. These findings demonstrated the suitability of the Anaeropack system for cell culture as an experimental model for hypoxia in cultured hepatocytes.

To assess the effect of reoxygenation following hypoxia on the hepatocytes using the Anaeropack system for cell culture, we measured the LDH activity and ketone body concentrations released into the medium. Because one important consequence of the absence of available oxygen is the loss of mitochondrial oxidative phosphorylation, we calculated the KBR. The KBR is an index of the liver mitochondrial redox state (NAD^+/NADH) (11,25), and the mitochondrial NAD^+/NADH ratio is closely related to oxidative phosphorylation (22). LDH activity was also measured as an indicator of cell viability.

In the hypoxic injury for the 2-h experiment, there was no significant difference in the LDH activity released into the medium, as compared to the control group, at all time points; however, the KBR did decrease rapidly during the hypoxia. During reoxygenation, the KBR recovered with the passage of time, and attained a significantly higher value as compared to the controls. This mitochondrial enhancement of oxidative phosphorylation is in agreement with the previous report using reversible liver ischemia in dogs (12). These results demonstrate that marked depression in mitochondrial function evolved during the 2 h of hypoxia, but that early reoxygenation results in a complete reversal of this process and can maintain plasma membrane integrity. In the hypoxic injury for the 3-h experiment, the percentage of LDH activity released into the medium increased significantly at the start of reoxygenation, as compared to the control group, and reached 20% at 8 h after reoxygenation. In contrast, the KBR value remained low. This suggests that the cell damage caused was an irreversible loss of plasma membrane integrity in 20% of the hepatocytes. In the hypoxic injury for the 4-h experiment, a progressively larger proportion of hepatocytes was recognized to have cell membrane rupture, until 75% was reached. The depression of ketone body production during the hypoxia indicates the termination of β-oxidation in the hepatic mitochondria. Immediately after starting reoxygenation, the ketone body production resumed, and the production of ketone bodies correlated with the duration of the hypoxia. The extent of cell injury caused by changing the duration of the hypoxia was reflected well by the KBR, the concentration of ketone bodies, and the LDH activity in the medium. In the present study, no leakage of LDH activity into the medium was detected during the hypoxia for 3 h; however, LDH leakage was recognized after starting the reoxygenation. This severe injury produced by reoxygenation following hypoxia also agrees with previous reports (4,5,10,13,24).

In this study, we used Williams' medium E as the culture medium. We have published previous studies using hepatocytes cultured in Williams' medium E (9,14), and we believe that this medium is suitable for an experiment on energy metabolism.

This model fulfills all of the criteria for an accurate experimental model: potential reversibility, reproducibility, and a good simulation of the conditions of the hepatocytes during revascularization following liver ischemia. The Anaeropack system for cell culture is easy to manipulate, economical (the list price for the Anaeropack for cell culture is $4 per piece of paper), and seems to be suitable for daily experimental tests. Moreover, revascularization following normothermic ischemia is a very important event in various clinical fields, such as cardiovascular disease, cerebral vascular disease, and peripheral vascular disease. Therefore, we conclude that the Anaeropack for cell culture is very useful for studying revascularization following normothermic ischemia.

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