Korean J Physiol Pharmacol
Vol 11: 45-55, April, 2007

Effects of pH, Buffer System and Lactate on the Simulated Ischemia-reperfusion Injury of H9c2 Cardiac Myocytes

Jun Whee Lee[1], Hye Kyung Lee[1], Hae Won Kim[1], and Young-Hoon Kim[1,2]

[1]Department of Pharmacology, [2]Research Institute for Biomacromolecules, University of Ulsan College of Medicine, Seoul 138-736, Korea

We elucidated the effects of various components of ischemic medium on the outcome of simulated ischemia-reperfusion injury. Hypoxia for up to 12 hours induced neither apoptotic bodies nor LDH release. However, reoxygenation after 6 or 12 hours of hypoxia resulted in a marked LDH release along with morphological changes compatible with oncotic cell death. H9c2 cells were then subjected to 6 hours of simulated ischemia by exposing them to modified hypoxic glucose-free Krebs-Henseleit buffer. Lowered pH (pH 6.4) of simulated-ischemic buffer resulted in the generation of apoptotic bodies during ischemia, with no concomitant LDH release. The degree of reperfusion-induced LDH release was not affected by the pH of ischemic buffer. Removal of sodium bicarbonate from the simulated ischemic buffer markedly increased cellular damages during both the simulated ischemia and reperfusion. Addition of lactate to the simulated ischemic buffer increased apoptotic cell death during the simulated ischemia. Most importantly, concomitant acidosis and high lactate concentration in ischemic buffer augmented the reperfusion-induced oncotic cell death. These results confirmed the influences of acidosis, bicarbonate deprivation and lactate on the progression and outcome of the simulated ischemia-reperfusion, and also demonstrated that concomitant acidosis and high lactate concentration in simulated ischemic buffer contribute to the development of reperfusion injury.

Key Words: H9c2 cell, Simulated ischemia-reperfusion, Serum deprivation, Acidosis, Lactate, Apoptosis, Oncosis

INTRODUCTION

Ischemia of myocardial cells is the major pathophysiologic mechanism in ischemic heart disease and sequence of open heart surgery or cardiac transplantation. During ischemia, increased anaerobic glycolysis results in intracellular accumulation of proton and lactate along with other waste products (King & Opie, 1998). Proton and lactate are transported out of cells with resultant increases of their concentrations in the interstitial space. As these alterations progress, irreversible cellular damage is manifested (Jennings & Reimer, 1989). Therefore, prompt reperfusion of jeopardized myocardial cells is required to salvage the ischemic myocardium. However, reperfusion after a significant duration of ischemia is accompanied by a paradoxical acceleration of cell death, which has been termed "reperfusion injury" (Hearse, 1977). Reperfusion injury has been suggested to be caused by multiple factors, such as sudden reintroduction of oxygen and abrupt normalization of interstitial pH and osmolarity, subsequently resulting in oxidative stress and increase of intracellular sodium and calcium ion concentrations.

One of the most useful experimental models for studies on ischemia-reperfusion injury is the simulated ischemia-reperfusion model, in which cultured myocardial cells are exposed to a defined medium whose composition is similar to the extracellular environment during *in vivo* ischemia-reperfusion. These models provide opportunities to apply advanced cell biological methodology, which is not possible with *in vivo* regional ischemia or perfused heart model. For simulated ischemia-reperfusion, cells cultured in standard medium conditions are incubated in the simulated ischemic buffer for a certain period, and the buffer is then switched to the former standard medium. In most previous studies using simulated ischemia-reperfusion models, the basic conditions of simulated ischemic buffer included hypoxia and glucose deprivation. However, the composition of other constituents has been varied in each experimental model. For example, some investigators set the initial pH of simulated ischemic buffer to lower than 7.0 (Vanden Hoek et al, 1996; Taimor et al, 1999), while others used ischemic buffer of normal pH. Among the studies using lowered pH of ischemic buffer, HEPES or phosphate buffer system was used in some experiments to achieve the desired pH (Altschuld et al, 1981; Piper et al, 1984), while others used conventional bicarbonate buffer system (Bhatti et al, 1989; Vanden Hoek et al, 1996; Taimor et al, 1999). On the other

Corresponding to: Young-Hoon Kim, Department of Pharmacology, University of Ulsan College of Medicine, 388-1, Poongnap-dong, Songpa-gu, Seoul 138-736, Korea. (Tel) +82-2-3010-4298, (Fax) +82-2-3010-4220, (E-mail) kimyh@amc.seoul.kr

ABBREVIATIONS: LDH, lactate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; KH, Krebs-Henseleit buffer

hand, some investigators included high concentration of lactate in ischemic buffer (Rakhit et al, 2000), while others did not (Bhatti et al, 1989; Bond et al, 1991; Vanden et al, 1996; Taimor et al, 1999). Another confounding factor which could possibly influence the outcome of simulated ischemia- reperfusion is the effects of serum deprivation. Most investigators deprived the simulated ischemic buffer of the serum (Piper et al, 1984; Bhatti et al, 1989; Taimor et al, 1999). However, as generally appreciated, abrupt decrease of serum concentration *per se* would induce cellular damages (Bonavita et al, 2003), thereby confounding the ischemic-reperfusion injury with alterations caused by serum deprivation.

The various ischemic conditions mentioned above result in unequivocal outcome of simulated ischemia-reperfusion. The threshold duration of ischemia to induce cell death is widely different depending on the experimental conditions, and there exists a controversy about the resultant pattern of cell death: some reports indicate the apoptosis as the major pattern of cell death during simulated ischemia (Elsässer et al, 2000; Kato et al, 2001; Tatsumi et al, 2003), whereas others show oncosis* as the sole cell death pattern (Vanden Hoek et al, 1996; Taimor et al, 1999). Controversy also exists in regard to the occurrence of cell death during simulated reperfusion. Several studies failed to observe cell death during reperfusion which is invariably accompanied in *in vivo* regional ischemia-reperfusion or isolated ischemic- reperfused heart models (Altschuld et al, 1981; Piper et al, 1984).

Therefore, it seems to be important to elucidate the effects of varied simulated-ischemic conditions on the cellular injury during ischemia and reperfusion. In the present study, we attempted to demonstrate the influences of serum concentration, pH, buffer systems, and lactate during simulated ischemia by evaluating changes in cell morphology and leakage of lactate dehydrogenase (LDH).

METHODS

Materials

H9c2 cell line was purchased from ATCC (Rockville, MD, USA). Cell culture media and fetal bovine serum were from GIBCO BRL Life Technologies (Grand Island, NY, USA). GasPak pouch system from BBL Microbiology System (Cockeysville, MD) was used to maintain cultured cells in hypoxic environment. All other reagents were obtained from Sigma Chemical (St. Louis, MO, USA).

Cell culture

H9c2 cells, derived from cardiac myoblasts of rat embryo, have been characterized as a suitable model of mycocardial cells (Hescheler et al, 1991). These cells were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B. The medium contained (mg/l) glucose 1,000, sodium pyruvate 110, glutamine 584, along with other common components. Cells were grown under an atmosphere of 5% CO_2 in air at 37°C. The medium was replaced by fresh medium every 2 or 3 days. A stock of cells was grown in a 75 cm^2 culture flask and split before confluence. Cells cultured in 35 mm culture dish were used in experiments when they reached about 90% confluence, i.e. $1.5 \sim 2 \times 10^4/cm^2$ density.

Experimental protocol

Cellular injury by serum deprivation: First, we attempted to clarify whether the serum deprivation by itself could induce cell death. Therefore, parameters of cell damage were evaluated after deprivation of FBS and compared with those of cells maintained in 10% or 1% FBS. On 6, 12 or 24 hours after serum deprivation, morphologic alterations were observed and aliquots of media were analyzed for the release of lactate dehydrogenase (LDH).

Cellular injury by hypoxia-reoxygenation (Fig. 1A): One day before experiment, FBS concentration in DMEM was changed to 1% to adapt cells to lowered serum concentration. Hypoxia was induced by changing the medium with 1 ml DMEM (containing 1% FBS) equilibrated with 95% N_2-5% CO_2 and incubating the culture dish in GasPak pouch, in which the oxygen tension was decreased to less than 2% and CO_2 tension was maintained at approximately 5% (Seip & Evans, 1980). After exposure to hypoxia for 6 or 12 hours, reoxygenation was induced by changing the medium with DMEM (containing 1% FBS) equilibrated

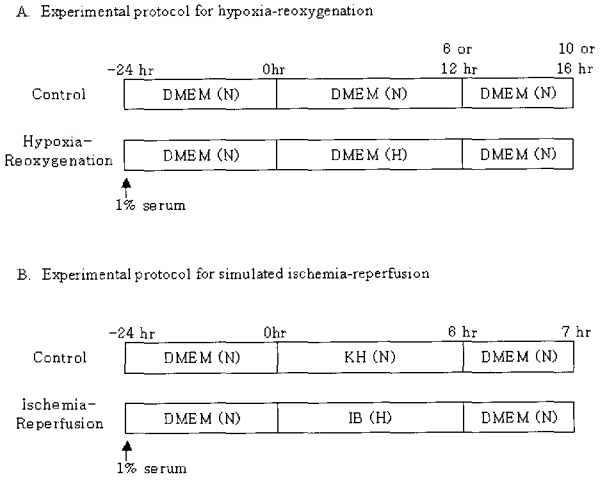

Fig. 1. Experimental protocol for hypoxia-reoxygenation or simulated ischemia-reperfusion. (A) Cellular injury due to hypoxia-reoxygenation was measured by LDH release and morphological changes. (B) Cells were incubated under various conditions of simulated ischemia followed by reperfusion. Cellular injury was measured by LDH release and morphologic changes. DMEM: Dulbecco's modified Eagle's medium; N: normoxia, H: hypoxia, KH: Krebs-Henseleit buffer, IB: simulated ischemic buffer.

*The nomenclature of cell death patterns followed the definition of Majno and Joris (Majno & Joris, 1995). According to this nomenclature, oncosis refers to an accidental cell death characterized by sarcolemmal damages, while apoptosis, as generally accepted, is a suicidal cell death characterized by preservation of mitochondrial and sarcolemmal integrity. The term "necrosis" is reserved for the changes that occur after cell death regardless of the pathway by which the cell died. This nomenclature was accepted by the Society of Toxicologic Pathologists in 1999 (Levine et al, 1999).

with 95% air-5% CO_2. In control cultures, normoxic DMEM was substituted for hypoxic DMEM. In all experiments, media were pre-warmed to 37°C before addition to cells. During hypoxia and reoxygenation, morphologic alterations were examined, and aliquots of media were analyzed for the release of lactate dehydrogenase (LDH).

Effects of lowered pH and $NaHCO_3$ withdrawal on the simulated ischemia-reperfusion injury (Fig. 1B): Similar to the hypoxia-reoxygenation experiments, FBS concentration of DMEM was changed to 1% on the day before experiment. Ischemic buffer used for simulated ischemia was modified glucose-free Krebs-Henseleit buffer containing (mM) 118 NaCl, 4.7 KCl, 1.64 $MgSO_4$, 24.88 $NaHCO_3$, 1.88 KH_2PO_4, 0.5 sodium pyruvate, 10 HEPES, 2.5 $CaCl_2$ and 1% FBS. The pH of ischemic buffer was adjusted to 7.4 or 6.4 while equilibrating with 95% N_2-5% CO_2. Simulated ischemia was induced by changing the medium with 1 ml of ischemic buffer and incubating the culture dish in GasPak pouch. After 6 hours of ischemia, reperfusion was carried out as mentioned above. In control cells, standard normoxic Krebs-Henseleit buffer, containing (mM) 118 NaCl, 4.7 KCl, 1.64 $MgSO_4$, 24.88 $NaHCO_3$, 1.88 KH_2PO_4, 0.5 sodium pyruvate, 5.5 glucose, 2.5 $CaCl_2$ and 1% FBS, was used.

Regardless of the pH of ischemic buffer, the absence of bicarbonate has been suggested to affect the survival during simulated ischemia and reperfusion (Grace et al, 1993; Vandenberg et al, 1993). Therefore, we attempted to elucidate the effects of $NaHCO_3$ withdrawal during simulated ischemia in either normal (pH 7.4) or lowered (pH 6.4) pH conditions. For $NaHCO_3$ withdrawal groups, $NaHCO_3$ in ischemic buffer was substituted with equimolar NaCl to avoid changes in osmolarity.

Effects of lactate on the simulated ischemia-reperfusion injury (Fig. 1B): The presence of high concentration of extracellular lactate was shown to affect the intracellular metabolic state and proton concentration (Tokuno et al, 1999), and these changes might profoundly influence the outcome of simulated ischemia and reperfusion (Vanden Hoek et al, 1996; Tatsumi et al, 2003). Therefore, we attempted to elucidate the effects of lactate by addition of 20 mM sodium lactate during simulated ischemia in either normal (pH 7.4) or lowered (pH 6.4) pH conditions. For lactate addition groups, equimolar NaCl was substituted with sodium lactate.

Quantitation of LDH release

The activity of LDH released into the medium was assayed to evaluate oncosis, i.e. the damage of membrane integrity. LDH assay was performed according to the method of Bergmeyer (Bergmeyer & Bernt, 1974). Samples of media were added to the reaction buffer (50 mM sodium phosphate, 0.6 mM sodium pyruvate, pH 7.5) to a final volume of 990 μl, and 10 μl of 1.8 mM NADH was added to start the reaction. Changes in absorbance for 1 minute were monitored with spectrophotometer at 340 nm. The activity of LDH to convert 1 μmole of pyruvate to lactate in 1 minute was defined as 1 unit.

Morphologic studies

Cell morphology was routinely monitored by inverted phase contrast microscope. For confirmation of apoptosis, Hoechst 33258 nuclear staining or annexin-V-FITC/propidium iodide double staining was conducted with Zeiss Axiovert fluorescence microscope and Chroma filter sets.

Hoechst 33258 staining: Medium was removed from culture dish, and 1 ml of phosphate buffered saline was added, followed by addition of 100 μl of Hoechst 33258 (100 μg/ml) and incubation for 15 minutes in dark. Nuclear morphologies were observed through fluorescence microscope with 365 nm excitation wavelength and 480 nm emission wavelength. Nuclear margination, condensation or fragmentation was regarded as the hallmark of apoptosis (Foglieni et al, 2001).

Annexin-V-FITC/propidium iodide staining: Staining solution was prepared by adding 20 μl of annexin-V-FITC (50 μg/ml) and 20 μl of propidium idodide (50 μg/ml) to 500 μl of staining buffer (10 mM HEPES, 5 mM $CaCl_2$, 5 mM KCl, 140 mM NaCl, pH 7.4). This solution was added to culture dish after removal of media, and they were incubated for 10 minutes in dark. Cellular morphologies were examined through fluorescence microscope with 450~500 nm excitation wavelength and 515~580 nm emission wavelength. Cells stained with annexin-V-FITC, but not stained with prodidium iodide, were regarded as apoptotic (Allen et al, 1997).

Statistical analysis

Values in each experiment were expressed as mean±SEM. Statistical significance between two groups was assessed by unpaired Student's t-test. A p-value less than 0.05 was defined as statistically significant.

RESULTS

Cellular injury by serum deprivation

When the normal culture medium (DMEM with 10% FBS) was replaced with serum-free DMEM, some cells underwent morphologic changes in several hours; from normal spindle shape to condensed spherical shape (Fig. 2A). In most of these cells, nuclei stained with Hoechst 33258 indicated smaller condensed morphology with occasional fragmentation. Moreover, these cells were stained with annexin-V-FITC, but not stained with propidium iodide (Fig. 2B), thus confirming the morphologic changes compatible with apoptotic process.

The frequency of apoptotic bodies was increased during the 12-hour period after serum deprivation, but decreased thereafter. In 24 hours after serum deprivation, hematoxylin & eosin staining revealed that some of the apoptotic bodies were engulfed by neighboring cells (data not shown), indicating that the disappearance of apoptotic bodies was, at least in part, due to phagocytosis. On the other hand, cells incubated in DMEM with 1% FBS also showed occasional apoptotic bodies, but the frequency was markedly less than with the serum deprivation group (Fig. 2A).

After serum deprivation, LDH released into medium was significantly higher than that of control group (Fig. 3). These results implicated that serum deprivation induced oncotic cell damage along with apoptotic degeneration. In cells incubated in DMEM with 1% FBS, LDH release was significantly lower, although not abolished, than the serum deprivation group (Fig. 3).

Based on these results, it was presumed that serum

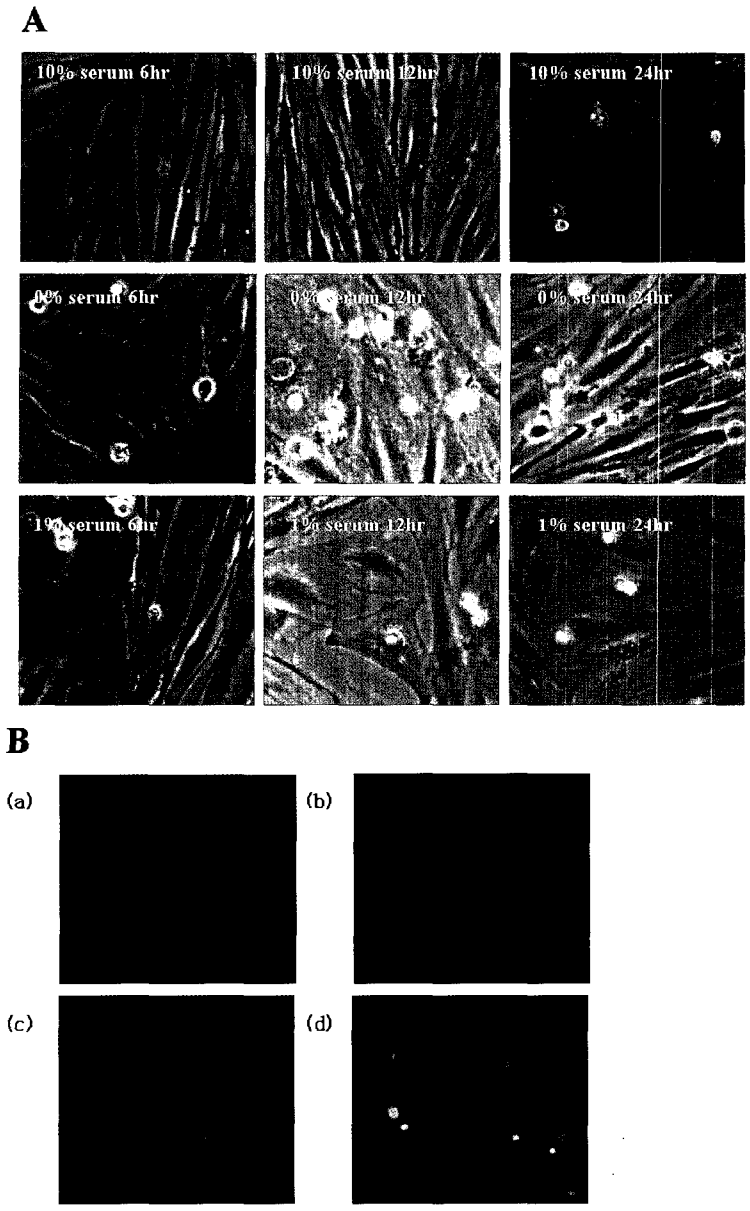

Fig. 2. Morphological changes during serum-deprivation. H9c2 cells were incubated in DMEM containing 10% serum, no serum, or 1% serum. (A) Cells were observed after 6, 12 or 24 hours of incubation using light microscopy. (B) Phase contrast (a, c) and fluorescence microscopic pictures (b, d) of the cells stained with annexin-V-FITC/propidium iodide (a, b), and the cells stained with Hoechst 33258 (c, d). Experiments were conducted after incubation for 24 hours in DMEM containing no serum.

deprivation during simulated ischemia would induce cellular damages including both apoptosis and oncosis, thus confounding the ischemic-reperfusion injury. However, inclusion of high concentration of serum in ischemic buffer would hamper the manipulation of ischemic environment. Therefore, subsequent experiments were performed with 1% FBS included during hypoxia or simulated ischemia, and cells were preincubated in DMEM containing 1% FBS one day before the experiment to adapt the cells to lowered serum concentration.

Cellular injury by hypoxia-reoxygenation: In the first series of experiments, cells were subjected to 12 hours of hypoxia and 4 hours of reoxygenation. As shown in Fig. 4B, formation of apoptotic bodies was not observed at any time point during hypoxia, and LDH release was not significantly increased compared to the control culture. However, reoxygenation for 4 hours induced marked morphologic changes, a significant portion of cells showing irregular deformation and partial detachment from culture dish. Trypan blue staining revealed damaged membrane integrity in these cells (Fig. 4A). LDH release during reoxygenation period correspondingly increased (Fig. 4B), confirming the occurrence of oncosis by reoxygenation following hypoxia. Sixteen hours of hypoxia without reoxygenation did not induce these changes at all (data not shown), thus confirming the conclusion that the oncotic changes occurred during the reoxygenation. Likewise, hypoxia for 6 hours did not result in any morphologic changes or increased LDH release. However, reoxygenation induced increased LDH release, indicating the occurrence of oncotic cell death (Fig. 5).

These results led us to conclude that hypoxia up to 12 hours did not induce apoptotic or oncotic cell loss, but insults during reoxygenation triggered the oncotic process.

Oncosis by reoxygenation was evident after hypoxic duration as short as 6 hours, and increased as reoxygenation was delayed. In another series of experiments, the time course of LDH release was monitored with 1-hour interval during reoxygenation, and significant difference was observed as early as 1 hour after reoxygenation compared to control group (data not shown). Therefore, subsequent experiments were conducted according to the protocol of 6-hour ischemia followed by 1-hour reperfusion.

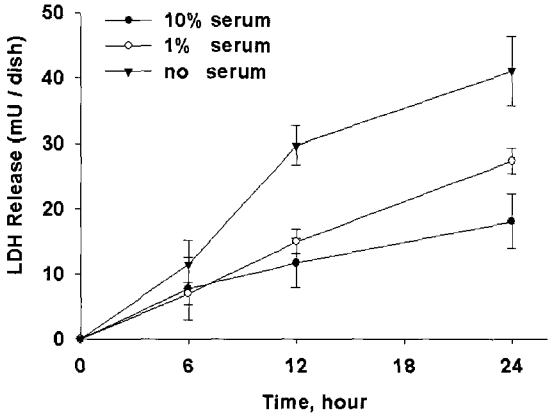

Fig. 3. LDH release during serum deprivation. H9c2 cells were incubated in standard DMEM containing 10% FBS, no serum, or 1% serum. Cellular injury caused by serum deprivation was measured by LDH release after 6, 12 or 24 hours of incubation, respectively. Data are means±S.E.M (●: n=4, ○: n=7, ▼: n=4).

Effects of lowered pH on the simulated ischemia-reperfusion injury: Simulated ischemia, consisting of substrate deprivation in addition to hypoxia, was induced in either normal (pH 7.4) or acidic (pH 6.4) condition. Simulated ischemia in pH 7.4, similar to hypoxia alone, resulted in no noticeable morphologic changes. However, lowered pH during simulated ischemia induced increased formation of apoptotic bodies (Fig. 6A), which was confirmed with annexin-V-FITC/propidium iodide staining. However, LDH release in either normal or acidic ischemia groups was not significantly increased during simulated ischemia (Fig. 6B).

In normal pH conditions, LDH release during simulated reperfusion was increased, which was comparable to the hypoxia-reoxygenation group, and lowered pH during ischemia did not affect the outcome of reoxygenation (Fig. 6B). Therefore, lowered pH of ischemic buffer appeared to induce apoptosis during ischemic period without affecting the oncotic death during reperfusion.

Effects of $NaHCO_3$ withdrawal on the simulated ischemia-reperfusion injury: Simulated ischemia in

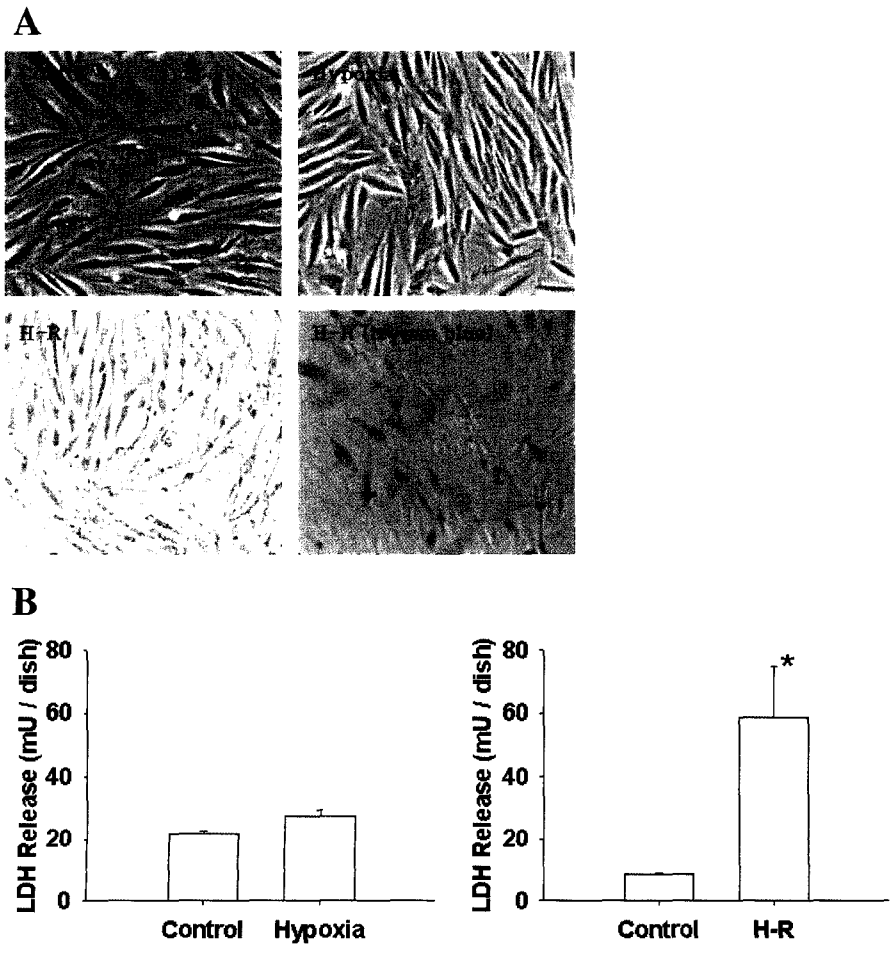

Fig. 4. Cellular injuries caused by 12 hr hypoxia followed by reoxygenation. Cells were exposed to 12 hours of hypoxia followed by 4 hours of reoxygenation. (A) Light microscopic pictures showing morphological changes after hypoxia and reoxygenation. (B) Cellular injury was measured by LDH release into the medium during hypoxia (left) or reoxygenation (right), respectively. H-R: reoxygenation after hypoxia. Data are means ±S.E.M (n=6). *: $p<0.05$ vs. control.

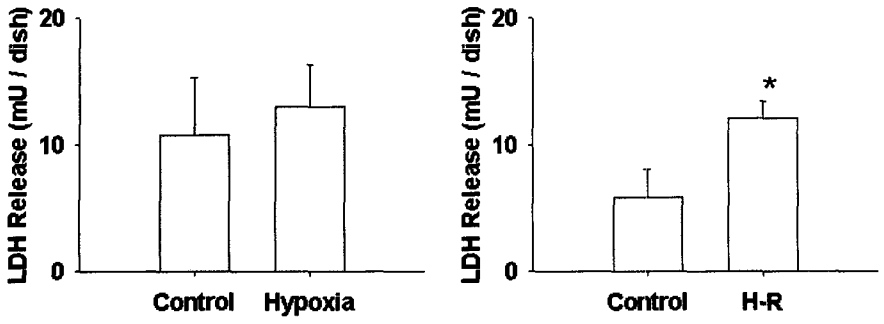

Fig. 5. Cellular injuries caused by 6 hr hypoxia followed by reoxygenation. Cells were exposed to 6 hours of hypoxia followed by 4 hours of reoxygenation in DMEM (1% FBS). Cellular injury was measured by LDH release into the medium during hypoxia (left) or reoxygenation (right). H-R: reoxygenation after hypoxia. Data are means±S.E.M (n=6). *: $p<0.05$ vs. control.

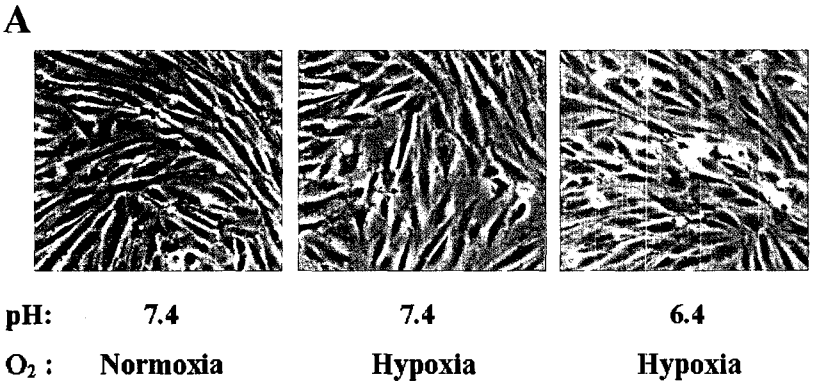

Fig. 6. Effects of pH on simulated ischemia-reperfusion injuries. Cells were exposed to 6 hours of simulated ischemia (pH 7.4 or pH 6.4) followed by 1 hour of reperfusion in DMEM (1% FBS). (A) Light microscopic pictures showing morphological changes after simulated ischemia. (B) Cellular injury was measured by LDH release into the medium during simulated ischemia (left) or reperfusion (right). Data are means±S.E.M (n=7). *: $p<0.05$ vs. control.

ischemic buffer containing no $NaHCO_3$ was performed in pH 7.4 or pH 6.4 conditions and results were compared with those of control ischemic procedures with 25 mM $NaHCO_3$. Although the pH was normal (7.4), $NaHCO_3$ withdrawal during 6-hour simulated ischemia induced increased formation of apoptotic bodies (Fig. 7A), which was similar to the morphologic changes observed during simulated ischemia in acidic pH. LDH release during ischemia was also increased significantly compared to control ischemia group. In addition, LDH release during simulated reperfusion was further increased when compared to control ischemia group (Fig. 7B). These results indicated that $NaHCO_3$ withdrawal during ischemia induces profound alterations, including apoptosis and oncosis during ischemia and amplification of oncosis during subsequent reperfusion.

The effects of $NaHCO_3$ withdrawal were more pronounced in acidic pH condition. Both apoptosis and LDH release during simulated ischemia and LDH release during reperfusion was increased higher when the pH of ischemic buffer was adjusted to 6.4 (Fig. 8), implicating that the deleterious effects of $NaHCO_3$ withdrawal was enhanced by acidic pH of ischemic buffer.

Effects of lactate on the simulated ischemia-reperfusion injury: Simulated ischemia in ischemic buffer containing 20 mM lactate was performed in pH 7.4 or pH 6.4 conditions, and results were compared with those of control ischemic procedures. In normal pH (7.4), high concentration of lactate during 6-hour simulated ischemia induced increased formation of apoptotic bodies (Fig. 9A), which was similar to the morphologic changes found during simulated ischemia in acidic pH. However, LDH release

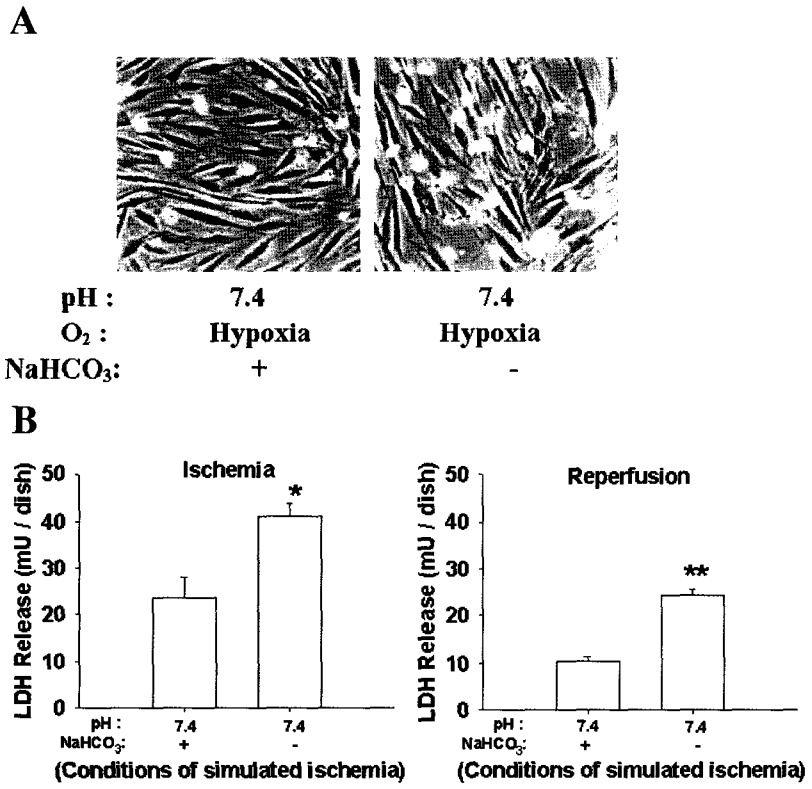

Fig. 7. Effects of the removal of $NaHCO_3$ on simulated ischemia- reperfusion at pH 7.4. Cells were exposed to 6 hours of simulated ischemia (pH 7.4, with or without $NaHCO_3$) followed by 1 hour of reperfusion in DMEM (1% FBS). (A) Light microscopic pictures showing morphological changes after simulated ischemia. (B) Cellular injury was measured by LDH release into the medium during simulated ischemia (left) or reperfusion (right). Data are means±S.E.M (n=7). *: $p<0.05$, **: $p<0.01$ vs. $NaHCO_3$(+) group.

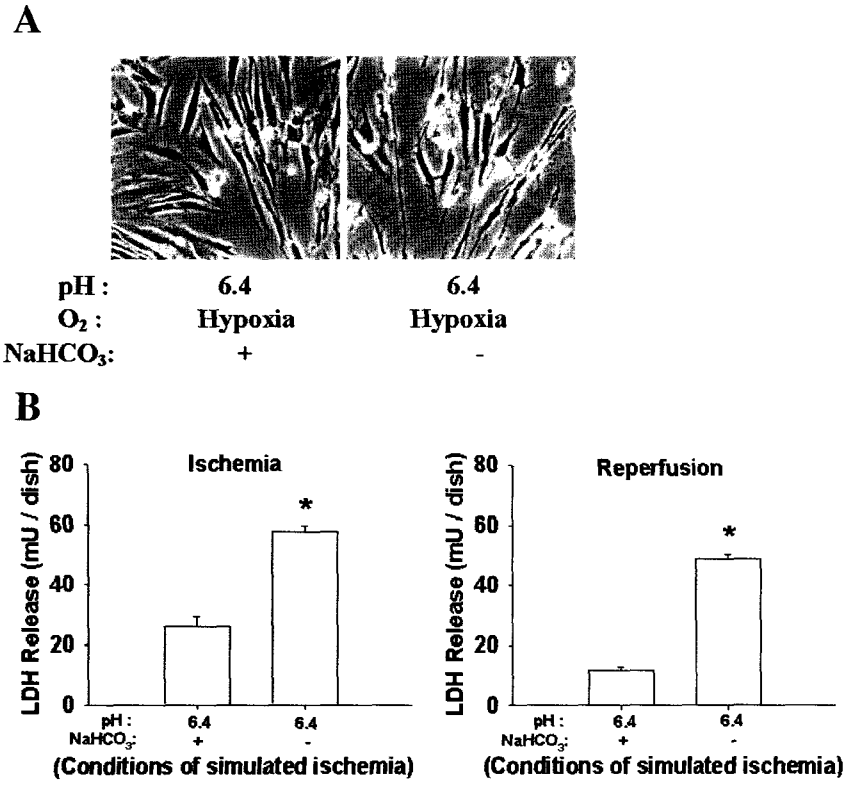

Fig. 8. Effects of the removal of $NaHCO_3$ on simulated ischemia- reperfusion at pH 6.4. Cells were exposed to 6 hours of simulated ischemia (pH 6.4, with or without $NaHCO_3$) followed by 1 hour of reperfusion in DMEM (1% FBS). (A) Light microscopic pictures showing morphological changes observed after simulated ischemia. (B) Cellular injury was measured by LDH release into the medium during simulated ischemia (left) or reperfusion (right). Data are means±S.E.M (n=7). *: $p<0.01$ vs. $NaHCO_3$(+) group.

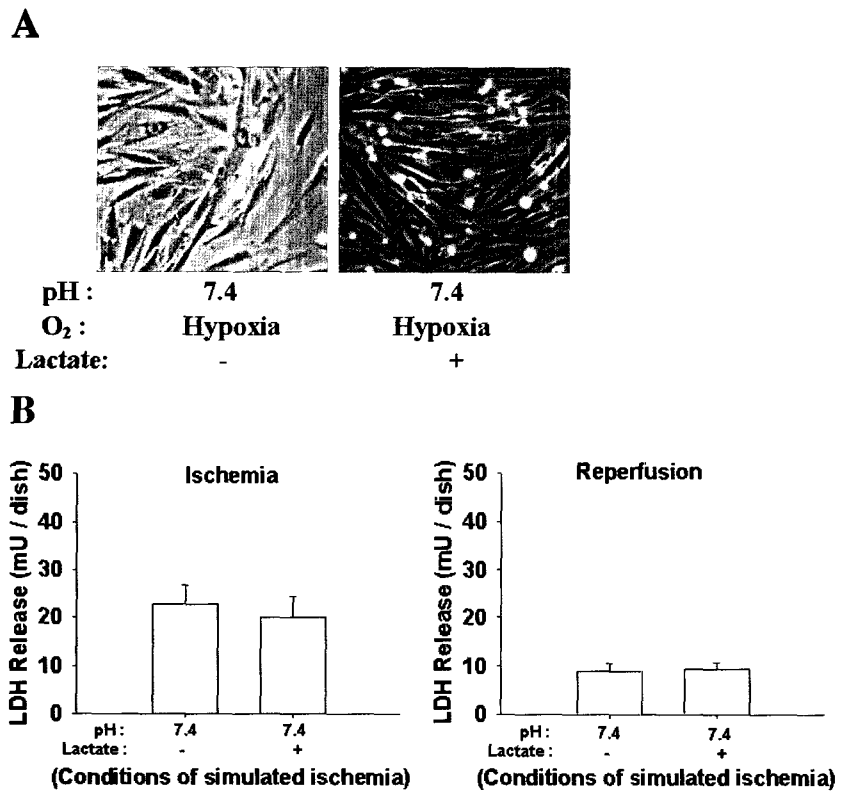

Fig. 9. Effects of the addition of lactate on simulated ischemia- reperfusion at pH 7.4. Cells were exposed to 6 hours of simulated ischemia (pH 7.4, without or with lactate) followed by 1 hour of reperfusion in DMEM (1% FBS). (A) Microscopic pictures showing morphological changes observed after simulated ischemia. (B) Cellular injury was measured by LDH release into the medium during simulated ischemia (left) or reoxygenation (right). Data are means±S.E.M (n=6).

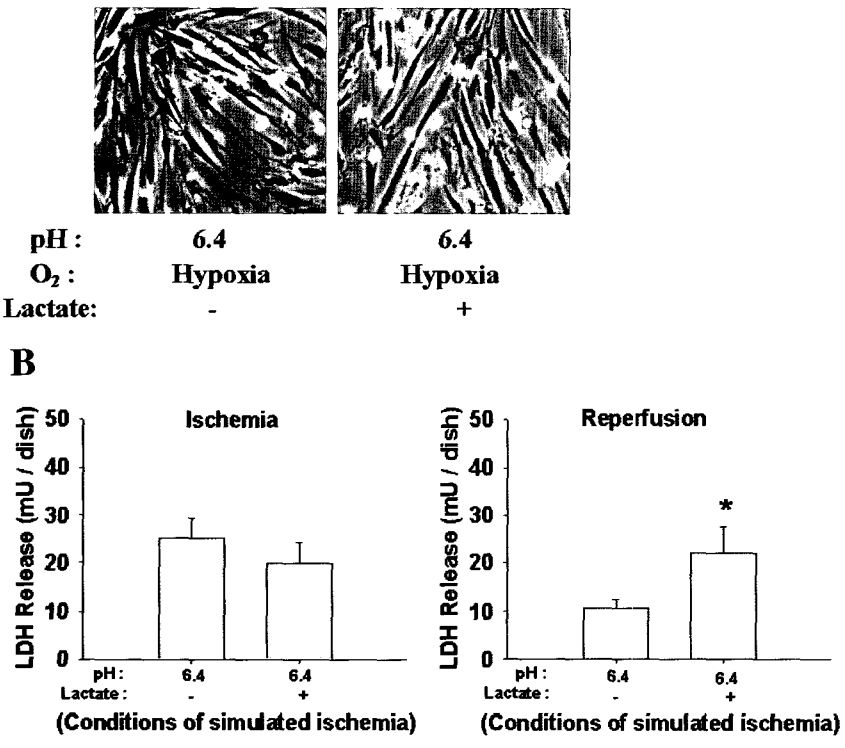

Fig. 10. Effects of the addition of lactate on simulated ischemia- reperfusion at pH 6.4. Cells were exposed to 6 hours of simulated ischemia (pH 7.4, without or with lactate) followed by 1 hour of reperfusion in DMEM (1% FBS). (A) Microscopic pictures showing morphological changes observed after simulated ischemia. (B) Cellular injury was measured by LDH release into the medium during simulated ischemia (left) or reoxygenation (right). Data are means±S.E.M (n=7). *: $p < 0.01$ vs. lactate(−) group.

during simulated ischemia or reperfusion showed no difference between lactate addition group and control ischemia-reperfusion group (Fig. 9B).

The addition of lactate during simulated ischemia under acidic pH condition also induced increased formation of apoptotic bodies during ischemic period (Fig. 10A) without changes in LDH release. However, lactate along with low pH during ischemia affected the outcome of subsequent reperfusion, showing amplified LDH release during reperfusion period (Fig. 10B).

DISCUSSION

Reviewing earlier studies on simulated ischemia, we first noticed that most studies excluded serum from the ischemic buffer, disregarding the possible deleterious effects of abrupt change of serum concentration. The results of the present study demonstrated that serum deprivation induced both apoptotic and oncotic cell death of H9c2 cardiac myocytes in a relatively short time. Therefore, simulated ischemia with concomitant serum deprivation would inevitably lead to a mixture of cell injuries caused by ischemia and by serum deprivation. To prevent the effects of serum deprivation while minimizing the contamination of ischemic buffer by serum components, we decided to include 1% FBS in culture medium during the experiments. After changing the serum concentration to 1%, formation of apoptotic bodies and LDH release were markedly suppressed compared to complete serum deprivation group, although not completely abolished.

Hypoxia alone did not induce cell death up to 12 hours, which is in agreement with the previous report showing that rapidly contracting cardiac myocytes remained fully viable and contractile during culture under severe hypoxia for up to 6 days (Webster & Bishopric, 1992). However, reoxygenation following hypoxia evoked oncotic cell death, which was evident after hypoxic period as short as 6 hours. As generally known, this "reoxygenation injury" is most likely the major contributing factor of reperfusion injury, and our data support the theory that reoxygenation following sublethal duration of hypoxia could induce sufficient cell damage to manifest oncotic cell death. Vanden et al. also reported that reoxygenation following sublethal hypoxia induced marked oncosis, which could be prevented by suppression of reactive oxygen metabolite production (Vanden Hoek et al, 1996).

While hypoxia was conducted in cells bathed in DMEM with physiologic concentrations of glucose and amino acids, the protocols of simulated ischemia consisted of hypoxia with substrate deprivation. The present results of simulated ischemia-reperfusion without further manipulations were comparable to those of hypoxia-reoxygenation, implying that the depletion of extracellular energy substrate during ischemia does not have significant influences on the outcome of simulated ischemia or reperfusion. These results together with those from hypoxia-reoxygenation experiments suggest that the lack of oxygen and substrate is not sufficient to cause the cell death during ischemia, whereas the "reoxygenation injury" invariably contributes to the cell death during reperfusion.

In subsequent experiments, we attempted to manipulate other extracellular environmental factors during ischemia in order to compare with simple ischemic protocol. One of the most pronounced alterations in the interstitial space during *in vivo* ischemia is acidosis. However, in culture conditions where the proton ions exported from hypoxic cells are diluted and buffered in the extracellular fluid of a relatively large amount, extracellular acidosis develops much slowly (Webster et al, 1999). Therefore, many investigators simulated ischemic conditions with low pH of ischemic buffer (Vanden Hoek et al, 1996; Taimor et al, 1999). We compared the results of simulated ischemia-reperfusion with physiological or acidic initial pH of ischemic buffer. Initial acidification of ischemic buffer to pH 6.4 induced apoptosis, which was not evident in simple ischemia group. Recent reports from other laboratories also support the critical role of acidification in the development of apoptosis (Hirpara et al, 2001; Jeong et al, 2001). On the other hand, acidosis during ischemic period has been proposed to contribute to the development of reperfusion injury (Piper et al, 1996, 1998). According to this hypothesis, a large amount of intracellular proton ions are extruded during reperfusion through Na^+/H^+ exchanger, as extracellular pH is normalized, thus generating a large concentration gradient of proton ion. Resultant intracellular accumulation of Na^+ leads to deleterious effects including calcium overload. Therefore, it was predicted that simulated ischemia with low pH would amplify the cell injury during subsequent reperfusion. However, the present results showed that the degree of reperfusion injury after simulated ischemia in pH 6.4 is comparable to that with normal pH. We speculate, therefore, that the "pH paradox" mechanism described above does not seem to play a major role in reperfusion injury under these situations.

Standard cell culture procedure employs CO_2-HCO_3^- buffer system similar to the *in vivo* extracellular fluid. However, some researchers have used HEPES or phosphate buffer system with $NaHCO_3$ excluded from the ischemic buffer, because unusually high CO_2 tension is accompanied by the maintenance of low pH with normal bicarbonate concentration. Furthermore, low HCO_3^- concentration in the extracellular fluid causes alterations in ion transport mechanisms linked with HCO_3^-, causing various alterations including intracellular acidification (Jeong et al, 2001). As expected, the present results demonstrated that the absence of $NaHCO_3$ in ischemic buffer even at normal physiological induced significant cell damage, manifested as both apoptosis and oncosis. These damages were further aggravated by extracellular acidosis, indicating the involvement of pH dysregulation. These results suggest that the absence of HCO_3^- in ischemic buffer, which is not physiologic, would lead to erroneous exaggeration of cell damage by simulated ischemia-reperfusion.

Lactic acid is the end product of anaerobic metabolism, which accumulates in intracellular and interstitial space during ischemia. Accumulation of lactic acid and subsequent pathophysiological alterations including intracellular acidosis and increased osmolarity has been suggested to play a critical role in the progression of ischemic and reperfusion injury. However, simulated ischemia of cultured cells is not accompanied by a large increase of extracellular lactate concentration, due to the dilution of lactate in a relatively large volume of extracellular space. The extracellular lactate concentration measured in preliminary experiments indicated an increase by approximately 2 mM after 12 hours of hypoxia in 1 ml medium for confluent adherent cultures (data now shown), contrasting with the previously reported value of higher than 10 mM measured in 35-minute ischemic rabbit heart preparations

(Marzouk et al, 2002). Since lactate accumulation rather than ATP depletion during ischemic period has been suggested to correlate with the lethal myocardial injury (Vogt et al, 2001), we simulated the ischemic condition by adding high concentration of lactate and compared the results with those of the simple ischemic protocol in which the effects of lactate accumulation should mostly be abolished. Addition of lactate under normal pH condition of ischemic buffer was accompanied by increased apoptosis during simulated ischemia. Since lactate addition in normoxic condition failed to induce apoptosis in these cells (data now shown), it was concluded that high extracellular lactate concentration amplified the deleterious effects of anaerobic metabolism, while having no significant effects on normoxic cells undergoing oxidative energy metabolism. It is highly likely that impaired lactic acid efflux due to high extracellular lactate concentration resulted in accelerated intracellular lactic acid accumulation and enhanced intracellular acidification. This hypothesis is supported by the previous report showing that intracellular pH of cultured cells is lowered by adding lactate in culture medium (Matsuda et al, 1995).

Although lactate induced apoptosis during ischemia, LDHrelease was not evident in hypoxic cells exposed to high lactate concentrations. Likewise, LDH release during reperfusion was not enhanced by lactate addition during ischemia in normal pH, as illustrated by no statistical difference in LDH release during reperfusion between lactate addition group and simple simulated ischemia group. However, lactate addition under low pH condition of simulated ischemic buffer resulted in enhanced oncotic cell death during simulated reperfusion. These results demonstrate that the deleterious effects of lactate accumulation which leads to oncotic cell death do not manifest until reperfusion, and that acidosis and concomitant lactate accumulation act synergistically for the development of reperfusion-induced oncosis.

The precise mechanism by which acidosis concomitantly with lactate accumulation exerts its lethal effects during reperfusion is unclear. Low pH along with high lactate concentration in the ischemic buffer would induce a profound acid load within ischemic cells, and this would amplify or unravel the "pH paradox" mechanism, which does not manifest by either low pH or high lactate alone. Alternatively, the osmotic stress induced by intracellular lactate accumulation could be augmented by extracellular acidosis, since acidosis inhibits the lactate efflux (Poole & Halestrap, 1993) and indirectly imposes osmotic load (O'Neill, 1999).

In summary, the present study illustrated differential involvement of serum deprivation, hypoxia, acidosis, bicarbonate withdrawal, and lactate accumulation in the progression of apoptosis and oncosis during simulated ischemia and reperfusion. In regards to the rational protocol of simulated ischemia, we concluded that serum deprivation or bicarbonate depletion should be avoided during simulated ischemia, because these conditions are not expected to occur in *in vivo* ischemic tissues and they may cause harmful effects unrelated with ischemia. The present results obtained under appropriate simulated ischemia protocols led us to conclude that extracellular accumulation of lactate and proton during ischemia, at least in part, contributes to the development of reperfusion injury.

ACKNOWLEDGEMENT

This work was supported by a grant from the Asan Institute for Life Sciences (2003).

REFERENCES

Allen RT, Hunter WJ 3rd, Agrawal DK. Morphological and biochemical characterization and analysis of apoptosis. *J Pharmacol Toxicol Methods* 37: 215-228, 1997

Altschuld RA, Hosterler JR, Brierley GP. Response of isolated rat heart cells to hypoxia, re-oxygenation, and acidosis. *Circ Res* 49: 307-316, 1981

Bergmeyer HU, Bernt E. UV assay of lactate dehydrogenase activity with pyruvate and NADH. In: Bergmeyer HU, ed. *Methods of Enzymatic Analysis.* 2nd ed. Academic Press., New York, p 118-126, 1974

Bhatti S, Zimmer G, Jurgen BH. Enzyme release from chick myocytes during hypoxia and reoxygenation: dependence on pH. *J Mol Cell Cardiol* 21: 995-1008, 1989

Bonavita F, Stefanelli C, Giordano E, Columbaro M, Facchini A, Francesca B,Caldarera CM, Guarnieri C. H9c2 cardiac myoblasts undergo apoptosis in model of ischemia consisting of serum deprivation and hypoxia: inhibition by PMA. *FEBS Lett* 536: 85-91, 2003

Bond JM, Herman B, Lemasters JJ. Protection by acidotic pH against anoxia/reoxygenation injury to rat neonatal cardiac myocytes. *Biochem Biophys Res Commun* 179: 798-803, 1991

Elsasser A, Suzuki K, Schaper J. Unresolved issues regarding the of apoptosis in the pathogenesis of ischemic injury and heart failue. *J Mol Cell Cardiol* 32: 711-724, 2000

Foglieni C, Meoni C, Davalli AM. Fluorescent dyes for cell viability: an application on prefixed conditions. *Histochem Cell Biol* 115: 223-229, 2001

Go LO, Murry CE, Richard VJ, Weischedel GR, Jennings RB, Reimer KA. Myocardial neutrophil accumulation during reperfusion after reversible or irreversible ischemic injury. *Am J Physiol* 255: H1188-H1198, 1988

Grace AA, Kirschenlohr HL, Metcalfe JC, Smith GA, Weissberg PL, Cragoe EJ, Vandenberg JI. Regulation of intracellular pH in the perfused heart by external HCO_3 and Na^+/H^+ exchange. *Am J Physiol* 265: H289-H298, 1993

Hearse DJ. Reperfusion of ischemic myocardium. *J Mol Cell Cardiol* 9: 605-616, 1977

Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W, Schultz G. Morphological, biochemical and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. *Circ Res* 69: 1476-1486, 1991

Hirpara JL, Clement M, Pervaiz S. Intracellular acidification triggered by mitochondrial-derived hydrogen peroxide is an effector mechanism for drug-induced apoptosis in tumor cells. *J Biol Chem* 276: 514-521, 2001

Jennings RB, Reimer KA. Pathobiology of acute myocardial ischemia. *Hosp Pract* 24; 89-96, 1989

Jeong D, Kim TS, Lee JW, Kim KT, Kim HJ, Kim I, Kim IY. Blocking of acidosis-mediated apoptosis by a reduction of lactate dehydrogenase activity throuht antisense mRNA expression. *Biochem Biophys Res Commun* 289; 1141-1149, 2001

Kato S, Takemura G, Maruyama R, Aoyama T, Hayakawa K, Koda M, Kawase Y, Li Y, Minatoguchi S, Fujiwara T, Fujiwara H. Apoptosis, rather than oncosis, is the predominant mode of spontaneous death of isolated adult rat cariac myocytes in culture. *Jpn Circ J* 65: 743-748, 2001

King LM, Opie LH. Glucose and glycogen utilisation in myocardial ischemia-changes in metabolism and consequences for the myocyte. *Mol Cel Biochem* 180: 3-26, 1998

Levine S, Bucci TJ, Cohen SM, Fix AS, Hardisty JF, LeGrand EK, Maronpot RR, Trump BF. The nomenclature of cell death:

Recommendations of an ad hoc committee of the Society of Toxicologic Pathologists. *Toxicol Pathol* 27: 484–490, 1999
Majno G, Joris I. Apoptosis, oncosis and necrosis. *Am J Pathol* 146: 3–15, 1995
Marzouk SAM, Buck RP, Dunlap LA, Johnson TA, Cascio WE. Measurement of extracellular pH, K^+, and lactate in ischemic heart. *Anal Biochem* 308: 52–60, 2002
Matsuda N, Mori T, Nakamura H, Shigekawa M. Mechanisms of reoxygenation-induced calcium overload in cardiac myocytes: dependence on pHi. *J Surg Res* 59: 712–718, 1995
O'Neill WC. Physiological significance of volume-regulatory transporters. *Am J Physiol* 276, C995-C1011, 1999
Piper HM, Balser C, Ladilov YV., 1996. The role of Na^+/H^+ exchange in ischemia-reperfusion. *Basic Res Cardiol* 91: 191–202, 1996
Piper HM, Garcia-Dorado D, Ovize M. A fresh look at reperfusion injury. *Cardiovas Res* 38: 291–300, 1998
Piper HM, Schwartz P, Hutter JF, Spieckermann PG. Energy metabolism and enzyme release of cultured adult rat heart muscle cells during anoxia. *J Mol Cell Cardiol* 16: 995–1007, 1984
Poole RC, Halestrap AP. Transport of lactate and other monocarboxylates acress mammalian plasma membranes. *Am J Physiol* 264: C761–C782, 1993
Rakhit RD, Edwards RJ, Mockridge JW, Baydoun AR, Wyatt AW, Mann GE, Marber MS. Nitric oxide-induced cardioprotection in cultured rat ventricular myocytes. *Am J Physiol* 278: H1211–H1217, 2000
Seip WF, Evans GL. Atmospheric analysis and redox potentials of culture media in the gaspak system. *J Clin Microbiol* 11: 226–233, 1980
Taimor G, Lorenz H, Hofstaetter B, Schluter KD, Piper HM. Induction of necrosis but not apoptosis after anoxia and reoxygenation in isolated adult cardiomyocytes of rat. *Cardio Res* 41: 147–156, 1999
Tatsumi T, Shiraishi J, Keira N, Akashi K, Mano A, Yamanaka S, Matoba S, Fushiki S, Fliss H, Nakagawa M. Intracellular ATP is required for mitochondrial apoptotic pathways in isolated hypoxic rat cardiac myocytes. *Cardiovas Res* 59: 428–440, 2003
Tokuno T, Watanabe M, Imaizumi Y. Effects of lactate on intracellular pH and hypercontracture during simulated ischemia and reperfusion in cardiac ventricular myocytes of the guinea pig. *Jpn J Pharmacol* 80: 343–350, 1999
Vanden Hoek TL, Shao Z, Li C, Zak R, Schumacker PT, Becker LB. Reperfusion injury in cardiac myocytes after simulated ischemia. *Am J Physiol* 270: H1334–H1341, 1996
Vandenberg JI, Metcalfe JC, Grace A. Mechanisms of pHi recovery after global ischemia in the perfused heart. *Circ Res* 72: 993–1003, 1993
Vogt AM, Ackermann C, Yildiz M, Scheols W, Kuebler W. Lactate accumulation rather than ATP depletion predicts ischemic myocardial necrosis: implications for the development of lethal myocardial injury. *Biochim Biophy Acta* 1586: 219–226, 2001
Webster, KA, Bishopric NH. Molecular regulation of cardiac myocyte adaptation to chronic hypoxia. *J Mol Cell Cardiol* 24: 741–751, 1992
Webster KA, Discher DJ, Kaiser S, Hernadez O, Sato B, Bishopric NH. Hypoxia-activated apoptosis of cardiac myocytes requires reoxygenation or a pH shift and independent of p53. *J Clin Invest* 104: 239–252, 1999