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Mechanism of glutathione depletion during simulated ischemia-reperfusion of H9c2 cardiac myocytes

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Abstract

It has been reported that myocardial glutathione content is decreased during ischemia-reperfusion, but the mechanism of glutathione depletion has remained unclear. The present study tested whether osmotic stress is involved in the glutathione depletion during ischemia. Six hours of hypoxic acidosis with either high CO_2 tension or low HCO_3^- concentration, which simulates the ischemic condition, resulted in a significant decrease of glutathione content and the glutathione depletion was prevented by hyperosmolarity. High-CO_2 acidosis alone without hypoxia induced a similar degree of glutathione depletion. Intracellular pH was lowered by high-CO_2 acidosis to 6.41 ± 0.03 in 15 min. Meanwhile, the cell size gradually increased and reached ~110% in 10 min and the increased cell size was maintained for at least 30 min, which was also prevented by hyperosmolarity. Subsequent experiments observed the effects of simulated reperfusion on the glutathione content. Measured in 1 h after the hypoxic acidotic reperfusion, the glutathione content was further decreased compared to the level at the end of ischemia, which was not suppressed by increasing the osmolarity of reperfusion solution. The degree of glutathione depletion during hypoxic reperfusion with normal pH was similar to the hypoxic acidotic reperfusion group. On the other hand, normoxic reperfusion was not accompanied by further depletion of glutathione content. Based on these results, it was concluded that ischemia induces the glutathione depletion via osmotic stress, which results from intracellular acidification, and the glutathione content is further decreased during reperfusion through a mechanism other than oxygen toxicity.

Keywords: *Ischemia, osmotic stress, glutathione, acidosis, reperfusion*

Introduction

Myocardial ischemic injury is a complex phenomenon, resulting from depletion of high-energy phosphates and accumulation of various waste products during the ischemic period [1]. Moreover, reperfusion of ischemic myocardium hastens the cell damage, which has been termed 'reperfusion injury' [2]. Reperfusion injury is known to be triggered by multiple factors, including sudden reintroduction of oxygen and abrupt normalization of extracellular pH and osmolarity, consequently resulting in oxidative stress and increase of intracellular calcium ion concentration [3].

Much of the experimental efforts to alleviate the ischemia-reperfusion injury has focused on the oxidative stress during the reperfusion period [4]. Oxidative stress is defined as an imbalance between the generation of reactive oxygen metabolites (ROM) and the defense capacity of the endogenous antioxidant system. It is well known that reperfusion of ischemic myocardium induces increased generation of ROMs and these ROMs play a causal role in the reperfusion injury [5,6]. On the other hand, attenuation of endogenous antioxidant capacity may, at least in part, contribute to the oxidative damage during reperfusion [7]. In fact, many researchers have documented the

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changes in endogenous antioxidants during ischemia or reperfusion. While the activities of endogenous antioxidant enzymes were not altered during ischemia or reperfusion, glutathione content was shown to be lower than the pre-ischemic level after ischemia-reperfusion [8,9]. In other studies it was reported that the glutathione depletion was already evident before the onset of reperfusion [5,10,11], implying that the decrease of glutathione content should be regarded not only as a result but also as one of the causal factors of oxidative stress during the reperfusion period. Considering the report that only 27% of GSH depletion eventually resulted in 50% of cell loss even without additional oxidative insults [12], it is highly probable that significant glutathione depletion during the ischemic period may play a critical causal role in the oxidative damage during the reperfusion period. A previous report from our laboratory also confirmed the critical role of GSH in the defense of cardiomyocytes against oxidative stress [13]. In this regard, Robin et al. [5] recently showed that GSH level was decreased during ischemia and supplementation of antioxidants during the ischemic period abrogated cell death during reperfusion. Therefore, the depletion of glutathione during ischemia is an attractive target in the manoeuvre for the prevention of oxidative stress during reperfusion. However, the mechanism of glutathione depletion during ischemia has gained little attention.

Meanwhile, we recently discovered that osmotic stress of H9c2 cardiomyocytes induces depletion of glutathione and consequently increases the sensitivity to H_2O_2 [14]. These observations led us to hypothesize that the glutathione depletion during ischemia is caused by osmotic stress, which is an inevitable complication under ischemic conditions. During ischemia, intracellular osmolarity is increased by enhanced anaerobic glycolysis and breakdown of high-energy phosphates, culminating in the accumulation of lactate and inorganic phosphates, respectively. The calculated increase of the osmolarity of intracellular fluid over the osmolarity of extracellular fluid exceeded 40 mOsmol/L [15]. In addition, anaerobic glycolysis and breakdown of high-energy phosphates both results in the generation of protons, which may induce cell swelling through a mechanism independent on the accumulation of counteranions such as lactates and phosphates [16]. The detailed mechanism of cell swelling by intracellular acidification is still unclear, but decreased K^+ conductance [17] and activation of Na^+/H^+ exchangers [18] have been suggested to contribute to the total osmole gain. Therefore, the first objective of our experiment was to test whether the glutathione depletion during ischemia was associated with osmotic stress and, at the same time, evaluate the relative importance of hypoxic metabolism and acidosis in the glutathione depletion.

Reperfusion after ischemia of moderate duration, which is insufficient to induce cell death, restores the aerobic metabolism and eventually normalizes the high-energy phosphate level. However, as the extracellular fluid containing extra osmolytes such as lactate is rapidly replaced with isosmotic fluid, cells are subjected to a transient osmotic stress. Moreover, normalization of extracellular pH, which has been lowered by efflux of lactic acid and Na^+/H^+ exchange, is accompanied by an abrupt increase in H^+ gradient across the membrane, causing an explosive Na^+/H^+ exchange [3]. These factors, independently on the glutathione consumption due to ROMs generated during reperfusion, may cause further glutathione depletion through osmotic stress and pre-dispose the myocardium to the following oxidative insults. However, most researchers attributed the glutathione depletion during reperfusion to the increase of ROMs and, so far as we are aware of, there have been no efforts to explore other possible causes of glutathione loss during reperfusion. Therefore, the second objective of our experiment was to explore the mechanism of glutathione depletion during simulated reperfusion. For this purpose, we attempted to evaluate the influences of changes in extracellular osmolarity, pH and oxygen tension on the glutathione content during reperfusion.

Methods

Materials

The H9c2 cell line was purchased from ATCC (Rockville, MD). Cell culture media and foetal bovine serum were from GIBCO BRL Life Technologies (Grand Island, NY). All other reagents were obtained from Sigma Chemical (St. Louis, MO).

Cell culture

H9c2 cells, a permanent cell line derived from cardiac myoblasts of rat embryo, have been characterized as a suitable model of mycocardial cells [19]. H9c2 cells were initially cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum. Cells were grown under an atmosphere of 5% CO_2 in air at 37°C. The medium was replaced with fresh DMEM every 2 or 3 days. A stock of cells was grown in a 75 cm^2 culture flask and split before confluence, at a sub-cultivation ratio of 1:10. Cells used in experiments were cultured in 60 mm culture dishes for 5–6 days, when they reached ~70–80% confluence.

We have previously established an experimental model of simulated ischemia-reperfusion using H9c2 cardiomyocytes [20]. Experiments were conducted with cells incubated in modified Krebs-Henseleit

physiologic solution (MKH), containing (mM) 118 NaCl, 4.7 KCl, 1.64 $MgSO_4$, 24.88 $NaHCO_3$, 1.18 KH_2PO_4, 0.5 sodium pyruvate, 5.5 glucose, 0.4 cysteine, 1.8 $CaCl_2$ and 10% FBS. Cysteine was routinely included in the physiologic solution because glutathione homeostasis of these cells was affected by the lack of extracellular cysteine/cystine and FBS concentration was also maintained because an abrupt decrease of FBS concentration caused significant cell damages. For stabilization of the culture condition, medium was replaced with MKH 1 day before the experiment.

Hypoxia was induced by subjecting the culture to hypoxic environment by replacing the medium with pre-warmed hypoxic solution (containing the same constituents as MKH, unless otherwise specified) equilibrated with 95% N_2–5% CO_2 gas. The culture dishes of these cells were immediately fixed onto the bottom of a gas-tight plastic chamber and the chamber was flushed with the hypoxic gas for 5 min and placed in a 37°C incubator. We had confirmed that the initial gas tension was not significantly altered during the overnight incubation with this method.

Measurement of glutathione content

After simulated ischemia or reperfusion, cells were rapidly washed with 3 ml of cold phosphate-buffered saline (PBS) and harvested with a scraper after adding 1 ml of cold PBS. To this suspension, 0.2 ml of 30% perchloric acid in 6 mM EDTA solution was immediately added to minimize the alteration of glutathione [21]. The cell lysate was centrifuged at 8000 g for 5 min and the supernatant and pellet was used for measurements of glutathione concentration and protein amount, respectively.

For glutathione assay, the sample was neutralized with ~0.1 ml of 5 M K_2CO_3 and the potassium perchlorate salt was removed by centrifugation. Two hundred microlitres of the neutralized sample was added to a total 1 ml of reaction mixture containing a final 0.3 mM NADPH, 0.6 mM DTNB (5,5'-Dithiobis (2-nitrobenzoic acid)), 0.5 unit/ml glutathione reductase, 100 mM sodium phosphate (pH 7.5) and 5 mM EDTA. Glutathione concentration was measured from the rate of linear increase in OD_{412} [22]. Values were normalized with protein content measured by Bradford method after dissolving the pellet in 100 µl of 2 M NaOH by incubating at 95°C for 10 min and cooling at room temperature.

Determination of intracellular pH

Intracellular pH was measured by fluorometry with cells pre-loaded with BCECF (2', 7'-bis(carboxyethyl) -5',6'-carboxyfluorescein), according to the method of Jakubovicz and Klip [23] with modifications. Cells were trypsinized and suspended in MKH medium which was equilibrated with 5% CO_2 in air at 37°C ($5–6 \times 10^5$ cells/ml), incubated for 30 min with 5 µg/ml of BCECF acetoxymethylester (from a 1 mg/ml solution in DMSO) and then washed twice with 1 ml MKH. An aliquot of these cells ($1–2 \times 10^5$ cells) was suspended in 2 ml of MKH or other experimental solutions in the fluorescence cuvette at 37°C. The gas phase in the cuvette was continuously flushed with the indicated gas mixture which was humidified by passing through a warmed water reservoir. Fluorescence was monitored at an emission wavelength of 530 nm and an excitation wavelength of 440 or 500 nm. It is known that the fluorescence intensity at 440 nm excitation and 530 nm emission is dependent on the concentration of BCECF, while the fluorescence intensity at 500 nm excitation and 530 nm emission is dependent on pH and therefore the intracellular pH is indicated by the 500/440 ratio [24]. In separate experiments, calibration of the fluorescence was performed by clamping the intracellular pH with nigericin (1 µg/ml from 1 mg/ml stock solution in ethanol) in KCl solution (140 mM KCl, 10 mM glucose, 1 mM $CaCl_2$ and 20 mM Tris-MES), of which the pH was varied by adding 1 M Tris or MES. The pH vs 500/440 ratio showed a linear relationship in the pH range from 6.4–7.8 ($pH = 0.235 \times (500/440 \text{ ratio}) + 5.6$, $R^2 = 0.959$, results of five independent experiments).

Estimation of cell swelling

For the estimation of cell swelling, cells were trypsinized and suspended in MKH medium which was equilibrated with 5% CO_2 in air at 37°C. The suspension was divided into aliquots and rapidly centrifuged. The supernatant was aspirated and subsequently the cell pellets were suspended in the indicated experimental medium. These suspensions were loaded onto the haemocytometer and photographs were taken 2, 5, 10, 20, 30 and 40 min after the addition of medium. The cell density was controlled so that each high power field ($\times 200$ magnification) contains ~30–40 cells. Most of the cells exhibited spherical shape and a small portion of cells were oval-shaped. The long diameter and the short diameter of each cell was measured using Scion Image software and the product of these two values were calculated and used as the parameter of cell size [14].

Measurement of lactate content

Concentrations of lactate were assayed using conventional enzymatic analysis [25]. Two hundred microlitres of neutralized cell homogenate sample was added to 876 µL of reaction buffer (116 mM sodium glutamate, pH 8.9, 0.93 mM NAD) and the reaction was initiated by adding 17 µL of 80 units/ml alanine aminotransferase and 7 µL of 4 units/ml

lactate dehydrogenase. Changes in OD at 340 nm were measured after incubation for 60 min at room temperature. Standard solutions of sodium lactate were used for calculation of concentrations.

Statistical analysis

Values were expressed as the 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

Glutathione depletion during simulated ischemia

Due to the inherent limitations of the cell culture model in the simulation of *in vivo* ischemic conditions, simple hypoxia is often inappropriate for the observation of the alterations during ischemia [20]. While the extracellular pH is markedly lowered in the course of ischemia *in vivo*, hypoxia of cultured cells is usually not accompanied by significant acidosis because lactic acid or proton generated in hypoxic cultured cells is exported out of cells and diluted in the relatively large volume of extracellular space. Therefore, we simulated the ischemic condition by hypoxia combined with extracellular acidosis. As the acidosis in *in vivo* ischemic tissue may manifest with higher CO_2 tension, lower HCO_3^- concentration or both, we achieved the acidosis of hypoxic solution either by reducing the HCO_3^- concentration to 2.4 mM or by increasing the CO_2 tension to 45%. Measured after the equilibration with the corresponding gas phase, the pH values of both acidotic solutions were within the range from 6.4–6.5.

Six hours of hypoxic acidosis with high CO_2 tension resulted in a marked decrease of glutathione content to 31.3 ± 3.3% of the basal level measured before the medium replacement (Figure 1A). Compared to the control group subjected to medium replacement with standard MKH, the glutathione content was lowered by 52.2%. Hypoxic acidosis with low HCO_3^- concentration also induced significant glutathione depletion, but the degree of depletion was relatively lower. To test if the glutathione depletion was related with osmotic stress, we observed the effects of increased extracellular osmolarity achieved by adding 40 mM NaCl. The glutathione depletion by hypoxic acidosis was effectively prevented by this manipulation, regardless of the mode of acidosis. Moreover, substitution of NaCl with equivalent osmolarity of sucrose or mannitol was as effective as the former manipulation, corroborating the involvement of osmotic stress (Figure 1B). Control cells showed a moderate decrease of glutathione content, which was presumably attributable to the mild osmotic stress caused by the medium replacement [14].

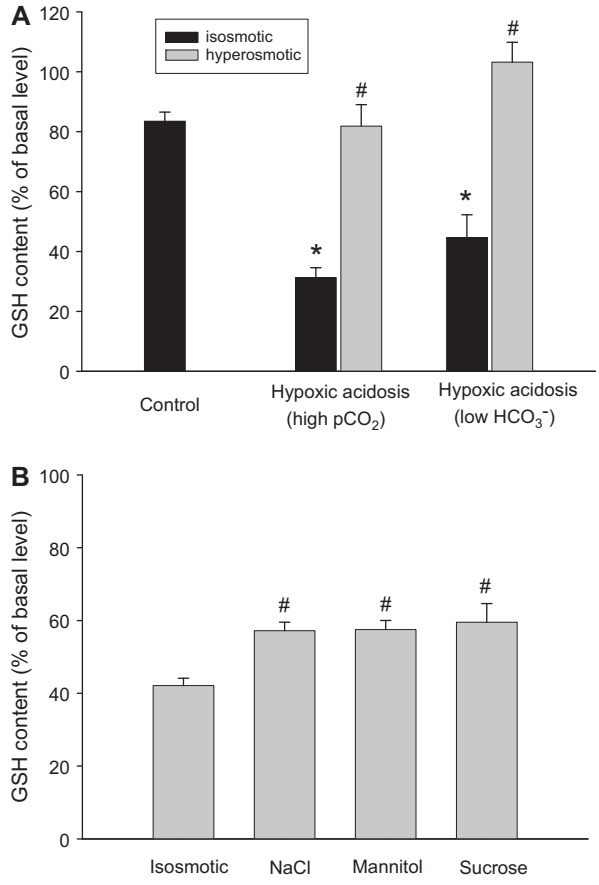

Figure 1. (A) Changes in glutathione content after exposure to hypoxic acidosis. H9c2 cells were exposed to hypoxic acidosis for 6 h and glutathione contents were measured in cell homogenates. Acidosis was induced either by raising the CO_2 tension to 45% or by lowering the HCO_3^- concentration to 2.4 mM. Control cells were exposed to medium replacement with normal MKH. In the hyperosmotic group, the osmolarity of medium was raised by adding 40 mM NaCl during the hypoxic acidosis. (B) Changes in glutathione content after exposure to hypoxic acidosis with or without various osmotic agents. Additional experiments were conducted to compare the effects of 80 mM sucrose or mannitol to those of 40 mM NaCl. Values were expressed as the percentage of basal level measured before hypoxic acidosis. Results of at least four experiments. $^*p < 0.05$ vs control, $^\#p < 0.05$ vs corresponding isosmotic group.

In the next step, we attempted to discriminate the effects of hypoxia and acidosis. Glutathione content of cells subjected to acidosis by increasing the CO_2 tension to 45% was 59.6% lower than the control group. However, simple hypoxia or acidosis by reducing the HCO_3^- concentration to 2.4 mM was insufficient to induce significant glutathione depletion by itself (Figure 2). The glutathione depletion by high-CO_2 acidosis was also effectively suppressed by increasing the osmolarity of the medium.

In separate experiments, intracellular pH was measured after exposure to acidosis. The intracellular pH of control cells suspended in MKH was 6.93 ± 0.02 and was not altered during pH measurement for up to 1 h. Exposure to 45% CO_2 induced a rapid intracellular acidification to 6.41 ± 0.03 in 15 min.

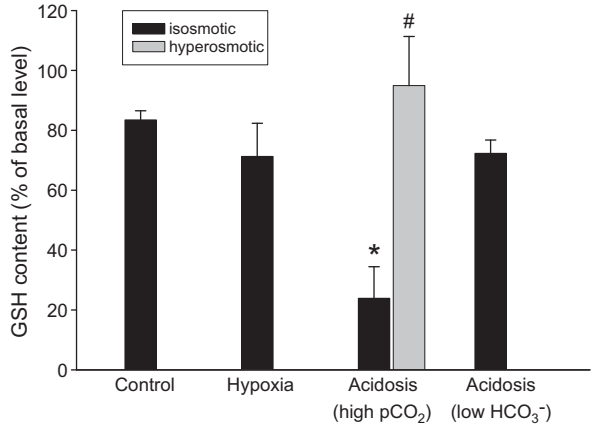

Figure 2. Changes in glutathione content after exposure to hypoxia or acidosis. Cells were exposed to hypoxia or acidosis for 6 h and glutathione contents were measured in cell homogenates. Acidosis was induced either by raising the CO_2 tension to 45% or by lowering the HCO_3^- concentration to 2.4 mM. Control cells were exposed to medium replacement with normal MKH. In the hyperosmotic group, the osmolarity of medium was raised by adding 40 mM NaCl during the hypoxia or acidosis. Values were expressed as the percentage of basal level measured before hypoxic acidosis. Results of at least four experiments. $^*p < 0.05$ vs control, $^\#p < 0.05$ vs corresponding isosmotic group.

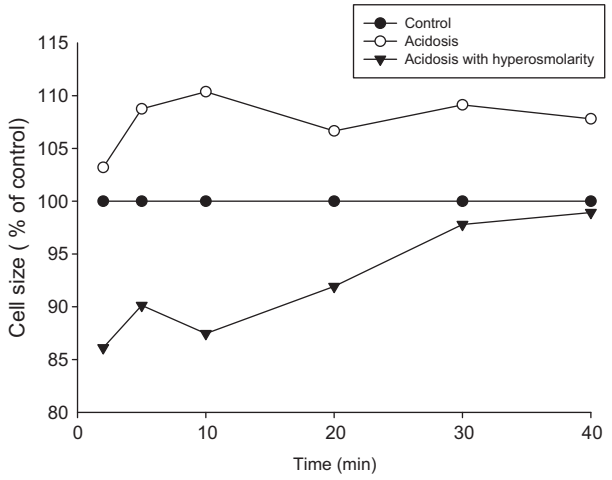

Figure 3. Changes in cell size after exposure to acidosis. Cells were exposed to high-CO_2 acidoisis and photographs were taken 2, 5, 10, 20, 30 and 40 min after the exposure. The cell density was controlled so that each high power (×200 magnification) field contains ~30 cells. The long and short diameters of these cells were measured using Scion Image software and the product of these two values were used as the index of cell size. Hyperosmolarity was induced by adding 40 mM NaCl during the acidosis. Results of at least three independent experiments.

Meanwhile, exposure to 2.4 mM HCO_3^- also induced a significant intracellular acidification, but the pH drop was much lower (6.73 ± 0.06 in 15 min). Intracellular pH was not recovered during the observation period for up to 40 min.

To confirm the association of cell swelling with glutathione depletion, we observed the changes in cell volume after exposure to high-CO_2 acidosis. After the exposure to the acidic medium, the cell size gradually increased and reached 110% of that of control cells in 10 min and was continuously higher than that of control cells at least for 30 min. Addition of 40 mM NaCl in the high-CO_2 acidic MKH induced an immediate cell shrinkage and the cell size was lower than the control level during the 40 min of observation (Figure 3).

Glutathione depletion during simulated reperfusion

Presumably, the osmotic stress during *in vivo* reperfusion of ischemic myocardium is caused, at least in part, by the washout of lactate from the interstitial fluid of the ischemic region. However, as mentioned above, lactic acid exported from hypoxic cells are diluted in the medium and, therefore, reintroduction of normal MKH solution after hypoxic acidosis might not be accompanied by the osmotic stress, which is inevitable in cases of *in vivo* reperfusion. In fact, lactate content of cells subjected to hypoxic acidosis was not increased (data not shown). Therefore, in an attempt to simulate the *in vivo* reperfusion, we modified the conditions of simulated ischemia by adding 40 mM sodium lactate in the hypoxic acidotic medium. In this condition, the glutathione depletion by simulated ischemia was less prominent (16.8% depletion compared to control), presumably due to the suppression of cell swelling by high osmolarity.

As mentioned in the introduction, reperfusion of ischemic cells imposes a multitude of alterations, each of which may affect the glutathione homeostasis. Among these, reoxygenation and extracellular pH normalization might induce glutathione depletion through the generation of ROMs and the Na^+/H^+ exchange, respectively. Therefore, we first intended to observe the effects of reperfusion on the glutathione content while maintaining the hypoxic acidosis, thereby excluding the effects of both reoxygenation and pH normalization. For this purpose, the culture dish was fixed to the bottom of an hypoxic chamber at the beginning of the experiment and, after 6 h of simulated ischemia, the simulated ischemic solution was decanted by inverting the chamber. Subsequently, MKH solution equilibrated with 55% N_2–45% CO_2 gas was introduced to the cells through a tube that has an inlet outside the chamber. We hypothesized that the abrupt washout of ischemic solution containing high concentration of lactate might induce an osmotic stress and, thereby, deplete glutathione by itself.

Measured in 1 h after the hypoxic acidotic reperfusion, the glutathione content was further decreased by 11.0% compared to the level before the onset of reperfusion (Figure 4). However, the glutathione depletion during reperfusion was not suppressed by increasing the osmolarity of reperfusion solution, indicating that the glutathione depletion during the simulated reperfusion was not caused by the abrupt

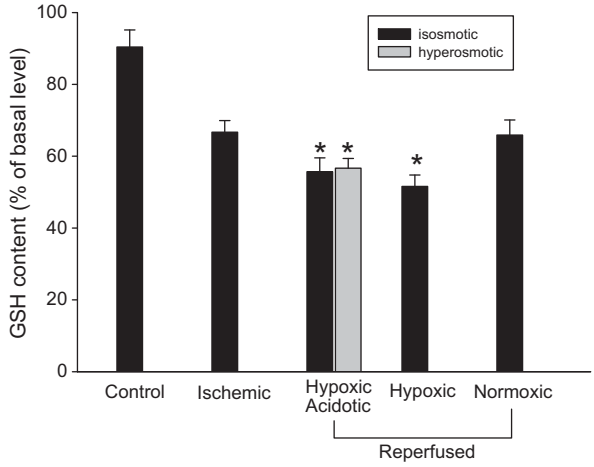

Figure 4. Changes in glutathione content after simulated ischemia and reperfusion. After exposure to simulated ischemia (hypoxic acidosis with 40 mM sodium lactate) for 6 h, the medium was replaced with either hypoxic acidotic, hypoxic or normoxic MKH. Glutathione contents were measured after simulated ischemia or in 1 h after the simulated reperfusion. Control cells were subjected to 2-times of medium replacement at the corresponding time points. In the hyperosmotic group, the osmolarity of medium was raised by adding 40 mM NaCl during the simulated reperfusion. Values were expressed as the percentage of basal level measured before hypoxic acidosis. Results of 12 independent experiments. $^{*}p < 0.05$ vs ischemic.

change in the osmolarity of medium. Consistently with this conclusion, the high lactate content of cells, which had been induced by simulated ischemia with 40 mM sodium lactate, was rapidly lowered during the reperfusion for 1 h (Figure 5), showing that lactic acid was efficiently exported out of cells and the transmembrane osmotic gradient due to the difference of lactate concentrations was rapidly dissipated.

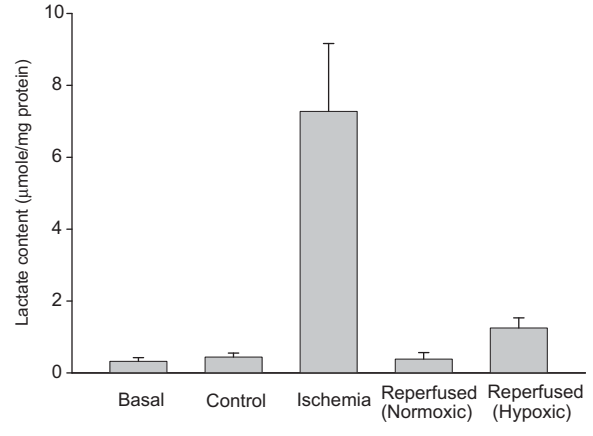

Figure 5. Cellular lactate contents after simulated ischemia and reperfusion. After exposure to simulated ischemia (hypoxic acidosis with 40 mM sodium lactate) for 6 h, the medium was replaced with either hypoxic or normoxic MKH. Lactate contents were measured after simulated ischemia or 1 h after the simulated reperfusion. Basal level represents the lactate content measured in cells not exposed to simulated ischemia. Control cells were subjected to 2-times of medium replacement at the corresponding time. Results of six experiments. $^{*}p < 0.05$ vs control.

In other cells of the same series of experiments, the pH of reperfusion medium was normalized while the lactate was washed out and hypoxia was maintained. In these cells, the glutathione depletion during reperfusion was comparable to the hypoxic acidotic reperfusion group, indicating that the influence of pH normalization on the glutathione content is not significant (Figure 4). On the other hand, glutathione content was not significantly decreased during reperfusion in the normoxic reperfusion group.

Discussion

Our results indicate that the intracellular glutathione content is decreased during simulated ischemia of cardiomyocytes and the glutathione depletion is related with osmotic stress. Many scientists have documented, although based on indirect measurements, the occurrence of osmotic cell swelling during myocardial ischemia. However, the exact nature of transmembrane osmotic gradient during ischemia has remained uncertain. It is well known that a large amount of lactate is produced in ischemic myocardium, but intracellular accumulation of lactate is not probable to be the major cause of osmotic stress, as the capacity of lactic acid transport across the plasma membrane is usually very high and therefore extracellular lactate concentration would rapidly increase to the equilibrium [26]. On the other hand, inorganic phosphates generated from breakdown of high energy phosphates are trapped in the cell, potentially generating the transmembrane osmotic gradient. Jennings et al. [27] have reported that the calculated intracellular phosphate concentration increased from basal level of 2.8 mM to 28.6 mM after only 15 min of ischemia. However, the rapid breakdown of high energy phosphates is dependent on the residual electrical and contractile activity during the initial period of ischemia and/or insufficient anaerobic energy production, so a significant accumulation of inorganic phosphates is, provided that anaerobic energy metabolism is adequately maintained, not expected. In this regard, Webster et al. [28] reported that contracting myocytes under severe hypoxia for 24 h maintained ~75% intracellular ATP levels of aerobic control cells.

Another important factor possibly associated with osmotic stress during ischemia is intracellular acidification, which is caused by the protons liberated mainly from lactic acid. Cell swelling was observed in all the known modes of acidosis [16,23]. The present study showed that simulation of *in vivo* ischemic conditions in cultured H9c2 cardiomyocytes by lowering the oxygen tension and pH induced a marked depletion of glutathione. In subsequent experiments, however, simple hypoxia failed to induce significant glutathione depletion, while high-CO_2 acidosis by itself induced glutathione depletion to a comparable

extent to the combination of hypoxia and acidosis. The glutathione depletion during hypoxic acidosis or simple acidosis was efficiently prevented by extracellular hyperosmolarity. Meanwhile, the cell swelling was evident in 10 min of high-CO_2 acidosis and the increased cell size was not normalized within 40 min, which was also prevented by hyperosmolarity. Taken together, these results indicated that acidosis during ischemia induces glutathione depletion through osmotic stress. So far as we are aware of, this is the first study revealing the mechanism of glutathione depletion during ischemia.

Although the glutathione depletion during hypoxic acidosis was evident also when the extracellular acidosis was induced by lowering the HCO_3- concentration, low-HCO_3- acidosis by itself was not sufficient to induce significant glutathione depletion. In fact, the intracellular pH drop during acidosis was much lower in cells exposed to low-HCO_3- acidosis, although the extracellular pH was comparable to high-CO_2 acidosis group. It is speculated that the osmotic stress might be dependent on the severity of intracellular acidification. Otherwise, it is also possible that the osmotic stress might be dependent on the mode of acidosis rather than the severity of acidosis. In support of this hypothesis, Sullivan et al. [29] have demonstrated that high-CO_2 acidosis and low-HCO_3- acidosis caused the cell volume to increase by 8.6% and 5.7%, respectively, even though the intracellular pH was lower in cells exposed to low-HCO_3- acidosis. One of the major differences between high-CO_2 acidosis and low-HCO_3- acidosis was the intracellular osmole gain by HCO_3- ions. While CO_2 diffused into the cells and liberated a large amount of HCO_3- ions during high-CO2 acidosis, intracellular HCO_3- concentration was decreased during low-HCO_3- acidosis. Therefore, it is reasonably expected that not only the intracellular pH but also the total amount of carbogens would influence the cell swelling and glutathione depletion during ischemia. If we assume that the total amount of carbogens is not altered and pH is lowered to 6.4 in ischemic tissue, it is calculated from the Henderson-Hasselbalch equation that intracellular CO_2 tension and HCO_3- concentration are 38% and 17.5 mM, respectively. This composition falls between the two modes of acidosis adopted in our experiments. However, if we assume that CO_2 in ischemic tissue partly diffuses out, the final status of ischemic tissue would be more similar to the low-HCO_3- acidosis in our experiments. On the other hand, if we assume that mitochondrial CO_2 production is partially maintained during the initial phase of ischemia, total carbogen amount will be increased and the final status of ischemic tissue will be more similar to the high-CO_2 acidosis condition in our experiments.

One of the unique features of the cell swelling due to intracellular acidification is that the cell volume regulation mechanism, normally active enough to rapidly compensate the osmotic gradient in hypoosmotic conditions, does not seem to work effectively during the cell swelling by acidosis [16]. Sahlin et al. [30] have reported that exposure of isolated skeletal muscle to 30% CO_2, which lowered the intracellular pH to 6.67, showed increased water content still after 95 min. Sullivan et al. [29] also observed that cell swelling by acidosis was not followed by efficient cell volume regulation. Moreover, acidosis with 15% CO_2 reduced the maximum fractional rate of cell volume recovery when exposed to hypoosmolarity. Therefore, it is presumed that even a small osmole gain might lead to a sustained cell swelling under acidic conditions.

Glutathione content was further decreased by simulated reperfusion with hypoxic acidotic medium. As hypoxia and acidosis were maintained during this protocol of simulated reperfusion, the only change in extracellular environmental factors was the abrupt washout of sodium lactate. Therefore, we initially speculated that the osmotic gradient due to high intracellular lactate concentration might have induced the glutathione depletion. However, the glutathione depletion was not suppressed by extracellular hyperosmolarity, implying that a mechanism other than osmotic stress underlies the glutathione depletion. In support of this conclusion, intracellular lactate content was rapidly normalized within 1 h of washout period. The mechanism of glutathione depletion during this simulated reperfusion is left unknown, but we speculate that the rapid washout of lactate from cells, rather than the transient osmotic gradient due to the residual intracellular lactate, might be responsible to the glutathione depletion. This implies that lactate accumulated in ischemic cells might play a role in suppressing the glutathione depletion. In fact, the glutathione depletion during simulated reperfusion was partially prevented by maintaining the high concentration of sodium lactate (data not shown). These results were contradictory to our expectation because lactic acid is generally known to cause serious detrimental effects during ischemia and reperfusion. However, there have been some reports showing that lactate did not enhance ischemia/reperfusion damage [31,32]. Moreover, it was revealed in several recent studies that lactate added to the medium during ischemia and reperfusion reduced the cell injury under these conditions [33,34]. More importantly, de Groot et al. [35] have reported that 5 mM lactate prevented the glutathione depletion during ischemia-reperfusion in isolated hearts. Further studies are required to confirm the preventive role of lactate against the glutathione depletion during ischemia-reperfusion.

Another unexpected result from simulated reperfusion experiments was that the reintroduction of oxygen after hypoxic acidosis suppressed the glutathione depletion. Most researchers regard the reoxygenation

as the most important triggering factor of oxidative stress and therefore the most probable candidate as the cause of glutathione depletion during reperfusion. However, reoxygenation would also restore the aerobic energy metabolism and the improved energy state may help the cells replenish the glutathione pool, as the *de novo* synthesis of glutathione is an energy-requiring process. In addition, reoxygenation-induced oxidative stress, if not severe enough, can result in an overall increase in glutathione level by activation or induction of synthetic enzymes [13,36]. From this viewpoint, it might be speculated that reoxygenation has two opposite effects on glutathione level, depleting glutathione via generation of hydrogen peroxide and other ROMs and at the same time restoring the glutathione pool via improvement of the energy status and stimulation of glutathione synthetic enzymes. Under our experimental conditions, the detrimental effect of reoxygenation on glutathione level was probably overwhelmed by the beneficial effects.

Based on these results, we concluded that ischemia induces the glutathione depletion via osmotic stress resulting from intracellular acidification and the glutathione content is further decreased during reperfusion by an unknown mechanism other than the increased oxygen tension. It is supposed that the decrease of antioxidant capacity during ischemia, at least in part, plays a causal role in the oxidative stress during reperfusion.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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