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Osmotic stress induces loss of glutathione and increases the sensitivity to oxidative stress in H9c2 cardiac myocytes

JUN WHEE LEE[1], YOUNG EUN KO[1,2], IN HYE LEE[1], HYE KYUNG LEE[1], HAE WON KIM[1], & YOUNG-HOON KIM[1,2]

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

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Abstract

It has been observed that H9c2 cardiac cells cultured in physiologic solutions exhibit delayed cell death after repeated medium replacements, of which the cause was the relatively mild osmotic challenges during the renewal of the culture medium. Interestingly, the cell damage was associated with altered intracellular GSH homeostasis. Therefore, this study attempted to elucidate the effects of osmotic stress on GSH metabolism. In cells subjected to osmotic stress by lowering the NaCl concentration of the medium, the cell swelling was rapidly counterbalanced, but the intracellular GSH content was significantly lower in 3 h. Meanwhile, the ratio of GSH-to-GSSG was not affected. As expected, osmotic stress also increased the sensitivity to H_2O_2, which was attributable to the decrease of GSH content. The decrease of GSH content was similarly evident when the synthetic pathways of GSH were blocked by BSO or acivicin. It was concluded that osmotic stress induced the decrease of intracellular GSH content by increased consumption and this loss of GSH rendered the cells susceptible to a subsequent oxidative stress.

Keywords: *Osmotic stress, glutathione, oxidative stress, BSO, hydrogen peroxide*

Introduction

Osmotic stress, occurring after exposure of cells to hypoosmotic extracellular environment or accumulation of active osmolytes in cytosol, accompanies many important physiologic and pathologic changes. For example, hyponatremia constitutes one of the most common disturbances of fluid and electrolyte balance in clinical medicine [1]. On the other hand, intracellular accumulation of osmolytes results from numerous pathologic alterations including acidosis and energy depletion [2]. In these situations, cell swelling, which might cause membrane damage and perturbation of intracellular metabolic rates, are normally counterbalanced by active volume-regulatory mechanisms [3]. Therefore, most cells tolerate a wide range of osmotic stress without acute injuries.

However, we have observed that cultured cells, in certain situations, exhibit profound cell death after relatively mild osmotic challenges occurring with renewal of culture medium. As described in the results of preliminary experiments, unexpected cell damage developed gradually after repeated medium replacements and resulted in complete cell loss through oncotic cell death within 24 h, while cells cultured without medium replacements maintained viability. While searching for the causal factor of this cell damage, we found that the cell death was prevented by increasing the osmolarity of the replaced culture medium, which suggested that the cause of damage was the osmotic shock during the medium replacement. However, the cell death could not be attributed to acute cell swelling and membrane rupture, as the cell volume was normalized within

Correspondence: Young-Hoon Kim, PhD, Department of Pharmacology, University of Ulsan College of Medicine, 388-1 Poongnap-dong, Songpa-ku, Seoul, 138-736, South Korea. Tel: +82-2-3010-4298. Fax: +82-2-3010-2941. Email: kimyh@amc.seoul.kr

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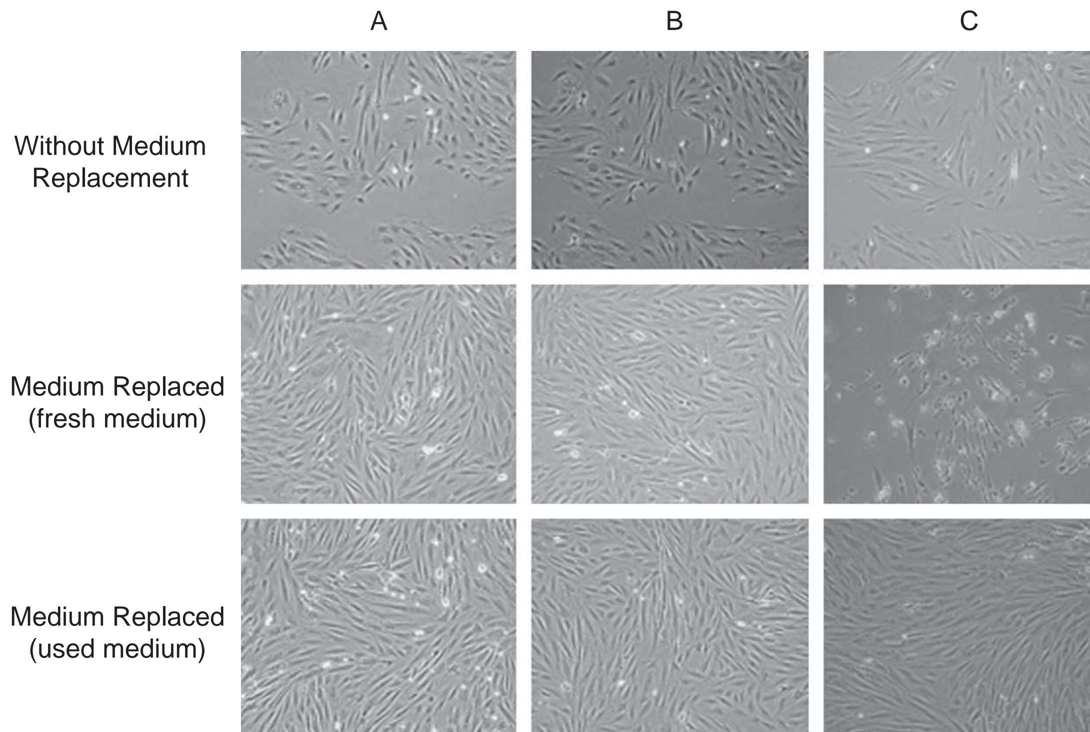

Figure 1. Cell damage by medium replacements. H9c2 cells incubated in KH solutions were challenged with two episodes of medium replacements (middle panel). The second episode was conducted 3 h later than the first episode. In the used medium group (bottom panel), medium replacement was conducted with used medium from parallel cultures. Control cells (top panel) were maintained without medium replacements. Cell morphology was observed before the first medium replacement (A), at the time of the second medium replacement (B) and 20 h after the second medium replacement (C).

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1 h while cell death began to develop after a considerable interval.

The background which led us to the above discovery is the theory that the conventional culture techniques inevitably include the osmotic stress during the replacement of culture medium. As widely known, cellular metabolism results in the intracellular accumulation of lactate and other waste products, which impose an additional osmotic burden. Especially, transformed cell lines, including H9c2 cells used in our experiment, depend largely on anaerobic glycolysis for glucose metabolism and therefore produce more than one molecule of lactate per each glucose molecule consumed [4]. More importantly, humidification of culture incubator cannot completely prevent the evaporation of culture medium and concomitant hyperosmolarity of extracellular fluid, which results in a significant osmotic stress at the time of medium replacements. It was reported that the osmolarity of the medium in standard culture dishes, kept in a humidified incubator, increased 10.5 mOsm/day [5]. Therefore, although mostly not appreciated, osmotic stress inevitably occurs when the medium is replaced.

Interestingly, we also observed that the cell damage by osmotic stress was accelerated in cells with low intracellular reduced glutathione (GSH) content and prevented in cells in which the GSH content was higher than normal, indicating the involvement of GSH homeostasis. GSH is the most important cellular antioxidant and protects the cell from the reactive oxygen metabolites which are continuously produced during metabolic processes of aerobic cells. In support of the critical role of GSH in the maintenance of cell viability, Michiels et al. [6] reported that only 27% of GSH depletion eventually resulted in 50% of cell loss. They also demonstrated that inhibition of glutathione peroxidase was similarly effective to induce cell death, indicating that the detoxification of hydrogen peroxide and other peroxides, using GSH as substrate and glutathione peroxidase as catalyst, is the critical reaction. A recent report from our laboratory confirmed the role of GSH in the defense against endogenous and exogenous oxidative stress and also demonstrated the relative importance of GSH-dependent antioxidant mechanism in comparison with catalase-mediated detoxification [7]. We also demonstrated that intracellular GSH content was increased after sublethal oxidative stimulus by hydrogen peroxide treatment with concomitant tolerance to lethal oxidative stress.

Occurrence of delayed cell death by osmotic stress and the correlation of this cell damage with intracellular GSH content suggest an unknown mechanism by which osmotic stress induces cell damage through alteration of the intracellular antioxidant system. However, the preliminary findings had been obtained in an unusual culture condition in which simple

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physiologic salt solution was used instead of standard culture medium and intracellular GSH homeostasis was severely affected by low cysteine availability. Moreover, the degree and nature of osmotic stress by medium replacement was mostly uncontrollable. Therefore, we attempted to elucidate the detail of this mechanism in a well-defined osmotic stress condition. For this purpose, cysteine-containing modified Krebs-Henseleit (KH) physiologic solution with varying NaCl concentration was used to expose cells to normal (300 mOsm) or low (200 mOsm) osmolarity and the time-dependent changes in the GSH content was monitored. At the same time, sensitivity to hydrogen peroxide was evaluated to gain the insight into the physiological significance of GSH alteration. In addition, the redox status of GSH was monitored and the involvement of GSH synthetic pathways was tested to elucidate the downstream mechanism of GSH alteration.

Experimental procedures

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). H_2O_2 was freshly prepared in each experiment and the concentration of stock solution was validated by spectrophotometry. L-buthionine sulphoximine (BSO) and acivicin were dissolved in normal saline at 100-fold of desired concentration and directly added to the culture medium.

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 [8]. 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 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, at a sub-cultivation ratio of 1:6. Cells used in experiments were cultured in 60 mm culture dishes for 5–6 days, when they reached ~70–80% confluence.

Experiments were conducted with cells incubated in modified KH physiologic solution, 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, 2.5 $CaCl_2$ and 10% FBS. Pyruvate was routinely included because its concentration is an important variable in the assessment of cell damage by H_2O_2, as described in our previous study [7]. For stabilization of the culture condition, medium was replaced 1 day before the experiment with modified KH physiologic solution. All experiments except estimation of cell swelling were conducted with adherent cells.

Estimation of cell swelling

For the estimation of cell swelling by osmotic stress, cells were trypsinized and suspended in KH 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 normal or hypoosmotic KH 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 (× 200 magnification) contains ~30–40 cells. Most cells exhibited spherical shape and a small portion of cells were oval-shaped. Assuming that all cells were oval, 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.

High performance liquid chromatography analysis for GSH and related metabolites

Cells were trypsinized, washed with phosphate buffered saline and suspended in 100 μl homogenation buffer (10 mM potassium phosphate, pH 7.2, 1 mM EDTA, 30 mM KCl). Triton X-100 was added to a final concentration of 0.2% and the lysate was centrifuged at 8000 g for 5 min. The resulting supernatants were used for high performance liquid chromatography (HPLC) analysis for GSH and related metabolites. Values were normalized with protein concentration measured by Bradford method.

GSH and oxidized glutathione (GSSG) concentration was measured by HPLC method [9] with minor modifications. Fifty microlitres of cell lysate was treated by adding 5 μl of γ-glutamyl glutamate (3.75 mM, internal standard), 5 μl bathophenanthrolinedisulphonic acid (15 mM) and 10 μl perchloric acid solution (70%). After centrifugation, 50 μl supernatant was treated with 5 μl iodoacetic acid (100 mM) and brought to pH 8–9 by addition of 48 μl of KOH (2 M)-$KHCO_3$ (2.4 M) mixture. Next, 100 μl fluorodinitrobenzene (1% in ethanol) was added for derivatization. After derivatization reaction for at least 2 h at room temperature, the sample was centrifuged and subjected to ion-exchange HPLC with an amine column (Waters Spherisorb 831115) and UV detector (365 nm).

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Evaluation of H_2O_2-induced injury

The percentage of LDH release (LDH activity released into the media/total cellular LDH activity) was quantitated as an index of cellular injury. For measurement of LDH activity in the medium, 200 μl of medium sample was added to 800 μl of the reaction mixture containing 50 mM potassium phosphate (pH 7.4), 0.6 mM pyruvate and 0.18 mM NADH and the rate of NADH decomposition was measured at 340 nm [10].

For measurement of total cellular LDH activity, parallel cells not subjected to H_2O_2 stress were lysed by switching the culture medium with the same volume of phosphate-buffered saline and subsequently adding Triton X-100 to the final concentration of 0.2% and the lysate sample was used for LDH assay.

Statistical analysis

Values in each experiment 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

Preliminary observations

H9c2 cells, incubated in modified KH physiologic solution containing 10% FBS and stabilized overnight, was challenged with two episodes of medium replacements with a 3 h interval. While control cells maintained without medium replacements showed no significant alterations, cells subjected to medium replacements showed severe morphologic changes and detachment from the dish 20 h after the second medium replacement (see Figure 1). The morphologic changes were accompanied by a marked LDH release (data not shown), confirming the occurrence of cell death. However, these changes were completely prevented when medium was replaced with the used medium from parallel cultures instead of the fresh medium, showing that the cause of cell death is one of the changes of medium composition, rather than the procedure of medium replacements.

While searching for the causal factor in the medium, we hypothesized that the changes in pH or oxygen tension of the medium might play a role and tested whether lowering the pH or oxygen tension of replaced medium could prevent or delay the cell damage by repeated medium replacements, but failed to observe any influence (data not shown). Meanwhile, we found that the osmolarity of the used medium is higher than the fresh medium by ~10 mOsm (292 mOsm in used medium vs 283 mOsm in fresh medium, results of two independent measurements) and therefore tested whether the cell death could be prevented by increasing the osmolarity of the fresh medium. As shown in Figure 2, addition of 20 mM NaCl to the replacing medium completely abolished the cell death, suggesting that the decrease of the medium osmolarity at the time of medium replacement is the main causal factor of the cell death.

In another series of experiments, intracellular GSH content was manipulated either by 0.1 mM BSO (a specific inhibitor of *de novo* GSH synthesis, which lowers the GSH content to an undetectable level by overnight incubation) or 100 μM H_2O_2 pre-treatment (which increases intracellular GSH content by ~100%). The effects of these manipulations on the GSH content had been previously confirmed [7]. As

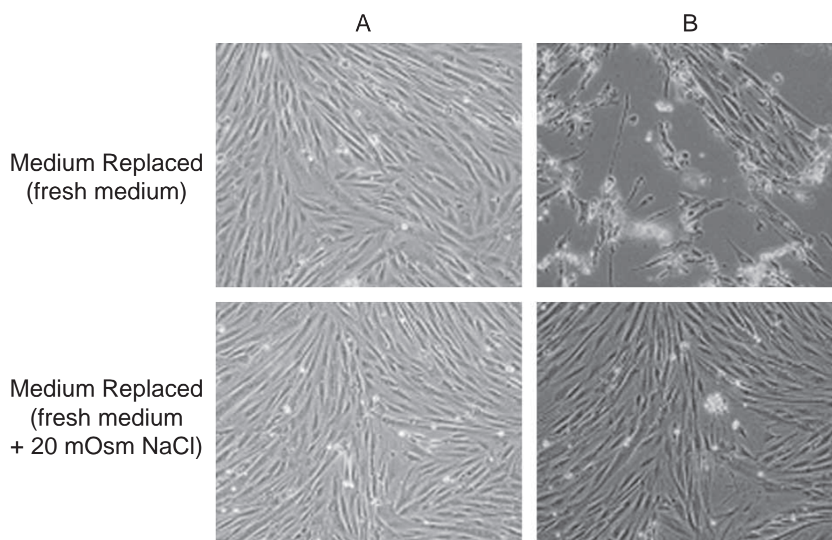

Figure 2. Effects of higher osmolarity on the cell damage by medium replacements. H9c2 cells incubated in KH physiologic solutions were challenged with two episodes of medium replacement (top panel). The second episode was conducted 3 h later than the first episode. In the NaCl addition group (bottom panels), medium replacement was conducted with fresh medium to which 20 mM NaCl was added. Cell morphology was observed before the first medium replacement (A) and 20 h after the second medium replacement (B).

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shown in Figure 3, BSO-treated group showed severe cell damage as early as 6 h after the medium replacements, while the alteration was not yet evident in control cells. In contrast, the cell damage was prevented in the H_2O_2 pre-treatment group. The LDH release was increased or decreased in parallel with the morphologic alterations (data not shown).

However, the cell damage after medium replacements was not manifested at all when experiments were conducted with conventional Dulbecco's modified Eagle's medium (DMEM). Based on the relation with GSH, we speculated that the deficiency of cysteine (or cystine) in KH medium, which lowers the basal GSH level and renders the cells more susceptible to the depletion of GSH, is the essential condition for the development of cell damage after medium replacements. In fact, intracellular GSH content measured after overnight stabilization in KH medium was markedly lower than that of the parallel cells incubated in DMEM (7.3 nmole/mg protein in KH-incubated cells vs 31.2 nmole/mg protein in DMEM-incubated cells, results of two independent measurements). As expected, the partial depletion of GSH during overnight incubation in KH medium was prevented by adding 0.4 mM cysteine to KH medium (33.9 nmole/mg protein, result of three independent measurements) and the cell death after medium replacements was also prevented by this regimen of culture medium. Subsequent experiments were conducted with cysteine-containing KH medium.

Cell swelling by exposure to hypoosmolarity

We sought to establish the effects of osmotic stress on the GSH content in a defined osmotic stress condition in which extracellular osmolarity is lowered. For this purpose, NaCl concentration of KH physiologic solution was lowered by 50 mM and this hypoosmotic medium was challenged to the cells. Cells exposed to hypoosmotic medium exhibited maximal swelling within 2 min and the cell size was decreased thereafter. In 40 min, cell size was normalized to 109% of control, while the cell size at 2 min after exposure to hypoosmotic medium was 136% of control (see Figure 4). Meanwhile, there was no evidence of morphological derangements or LDH release (data not shown).

Effects of osmotic stress on glutathione homeostasis

GSH contents were measured 1, 3 and 5 h after the exposure of adherent cells to hypoosmotic KH medium. In the control group, culture medium was replaced with normal fresh KH medium. Intracellular GSH content significantly decreased by osmotic stress, showing $74.4 \pm 2.6\%$ of basal level in 3 h. GSH level was gradually recovered thereafter. For

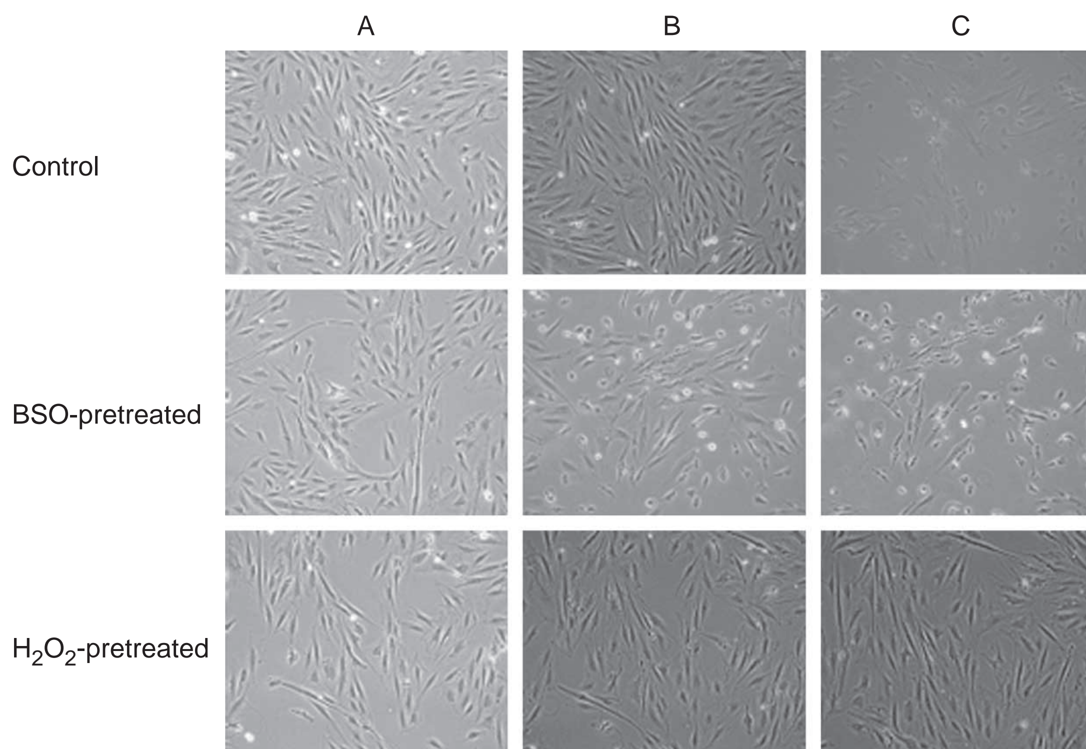

Figure 3. Dependence of cell damage by medium replacements on the glutathione content. Prior to the experiments, intracellular GSH content was manipulated either by 0.1 mM BSO (middle panels) or 100 μM H_2O_2 (bottom panels) pre-treatment. On the next day, cells were challenged with two episodes of medium replacements. Control cells (top panels) were subjected to medium replacements without prior manipulations. Cell morphology was observed before the first medium replacement (A) and in 6 h (B) or 20 h (C) after the second medium replacement.

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comparison, we also measured the changes of GSH content in parallel cells treated with hyperosmotic medium which contained an additional 50 mM NaCl. Hyperosmolarity did not affect the GSH content (see Figure 5A).

At the same time, the ratio of GSH-to-GSSG was calculated. The basal value of the ratio measured in cells before the medium replacement was 8.1 ± 1.8 and it was not significantly affected by medium replacement with either normal KH medium or hypoosmotic KH medium (see Figure 5B). These results indicated that the redox potential of the GSH/GSSG couple was not altered by osmotic stress, while GSH level was decreased.

Effects of osmotic stress on the sensitivity to oxidative stress

Cell damage by oxidative stress was evaluated by measuring LDH release for 2 h after the treatment of H_2O_2 to cultured cells. As specified in our previous study [7], LDH release by exogenous H_2O_2 in H9c2 cells was maximal in the concentration range above 400 μM.

First, we sought to establish the dependence of H_2O_2-induced cell damage on GSH content. To manipulate the GSH content, cells were treated with 0.1 mM BSO for 1, 3 or 5 h. At each time point, GSH content and the dose-dependent LDH release by H_2O_2 was evaluated. GSH content was time-dependently lowered by BSO treatment to 61, 43 and 28% of basal level at 1, 3 and 5 h, respectively. As shown in Figure 6A, LDH release by lower concentrations of H_2O_2 was increasingly higher in BSO-treated cells in correlation with the duration of the BSO treatment. Meanwhile, the maximal LDH release evoked by 400 μM H_2O_2 was not affected. Plotted against the GSH content, LDH release by 100 μM H_2O_2 linearly increased as the GSH content decreases (see Figure 6B).

As expected, osmotic stress not only decreased the GSH content but also increased the sensitivity to H_2O_2. LDH release by 100 and 200 μM H_2O_2 was significantly higher than the control group, while the maximal release was unaffected (see Figure 7). Overall, the concentration-response curve of LDH release by H_2O_2 in cells exposed to hypoosmotic medium was regarded to be compatible with the increase of the sensitivity by the decrease of GSH content.

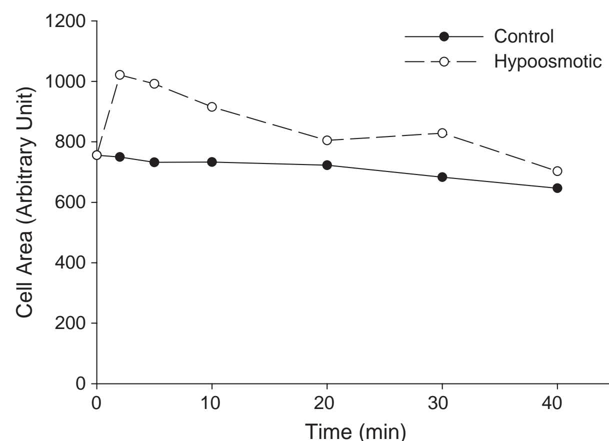

Figure 4. Cell swelling by hypoosmolarity. Cells were exposed to normal or hypoosmotic KH medium and photographs were taken 2, 5, 10, 20, 30 and 40 min after the exposure. The cell density was controlled so that each high power (×200 magnification) field contains ~30–40 cells. The long diameter and the short diameter of these cells were measured using Scion Image software and the product of these two values were calculated and used as the parameter of cell area. Results of two independent experiments were averaged and plotted. The initial cell area was extrapolated from control plots.

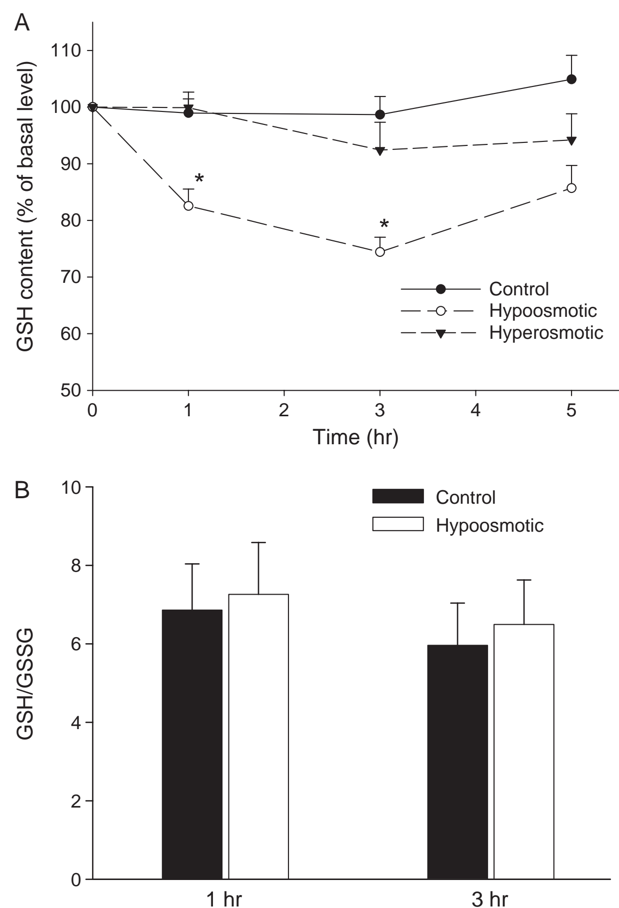

Figure 5. Changes in the GSH level and GSH/GSSG ratio after the exposure to hypoosmotic medium. (A) Glutathione contents were measured in cell homogenate at 1, 3 and 5 h after the exposure to hypoosmotic or hyperosmotic KH medium. In the control group, culture medium was replaced with normal fresh KH medium. Values were expressed as the percentage of basal level measured before the medium replacement. (B) Changes in GSH/GSSG ratio after the exposure to hypoosmotic medium. GSH and GSSG concentrations were measured in cell homogenate by HPLC and GSH/GSSG ratio was calculated. Illustrated are the results of measurements at 1 and 3 h after the medium replacement, at which time points the GSH level was significantly lower in the hypoosmotic medium group. Results of at least seven independent experiments. *$p < 0.05$ vs control.

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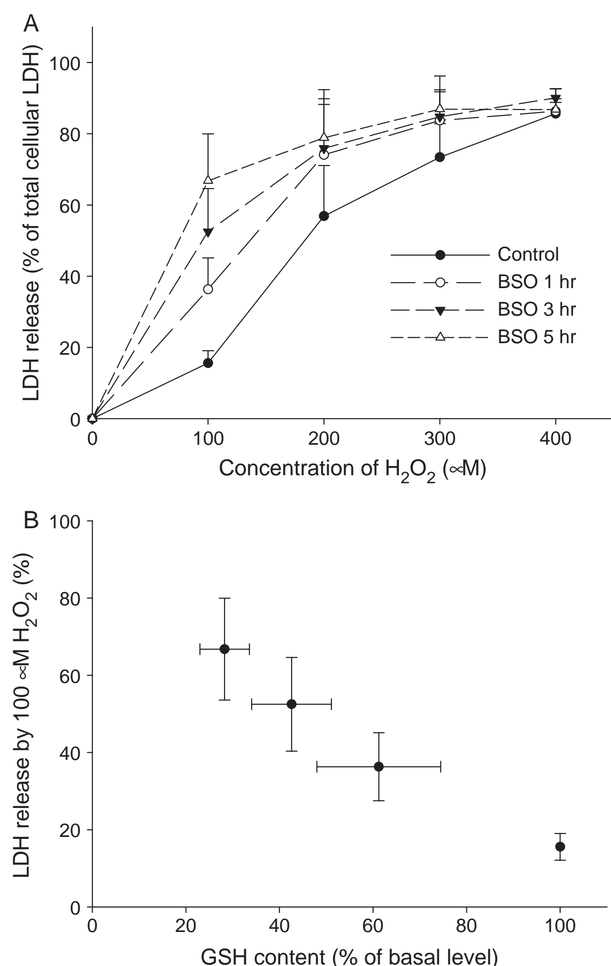

Figure 6. Dependence of H_2O_2-induced cell damage on GSH content. Cells were treated with 0.1 mM BSO for 1, 3 or 5 h and at each time point, GSH content and the concentration-dependent LDH release by H_2O_2 was evaluated. (A) Cell damage by oxidative stress was evaluated by measuring LDH release for 2 h after the treatment of H_2O_2 to cultured cells. (B) LDH release by 100 µM H_2O_2 was plotted against the GSH content measured at each time point. Results of five independent experiments.

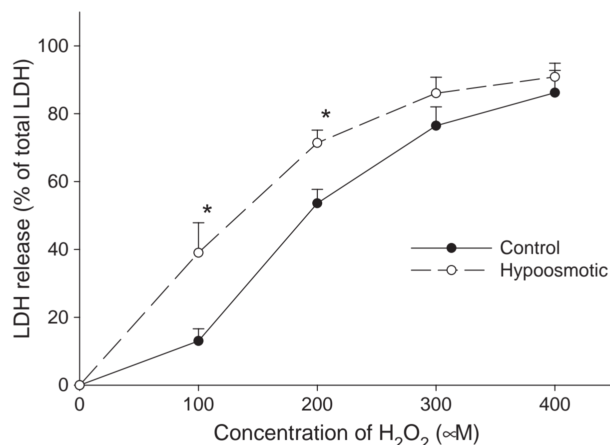

Figure 7. H_2O_2-induced cell damage in cells exposed to hypoosmotic medium. Cells were treated with hypoosmotic medium for 3 h and the concentration-dependent LDH release by H_2O_2 was evaluated as described in Figure 6. Results of seven independent experiments. *$p < 0.05$ vs control.

Contribution of GSH synthetic pathways to the decrease of GSH content by osmotic stress

Intracellular GSH content is maintained at a steady state in which loss of GSH is counterbalanced by synthetic pathways. As mentioned above, the redox potential of GSH/GSSG couple was not affected during the decrease of GSH by osmotic stress, suggesting that the GSH alteration is not attributable to increased oxidation to GSSG or protein-GSH mixed disulphide. Therefore, we hypothesized that the synthesis of GSH might be suppressed by osmotic stress. The *de novo* synthesis of GSH is known to be completely blocked by BSO in cultured cells [11]. If we assume that the basal rate of the *de novo* synthesis is lowered by osmotic stress, the difference of GSH content between control group and osmotic stress group will disappear by BSO treatment in both groups.

Therefore, we first observed the time course of GSH loss in the basal condition after the blocking of *de novo* synthesis. GSH level decreased time-dependently during the 9-h period after the administration of BSO. The pattern of GSH loss was compatible with the first-order kinetics (see Figure 8A), suggesting that the basal consumption rate of GSH is proportional to the intracellular concentration of GSH. The half-life of intracellular GSH in the BSO-treated cells was estimated to be 3.72 h. The effect of BSO was not further enhanced by higher concentrations (data not shown), indicating that the concentration used in our experiment was sufficient to induce the complete blocking of *de novo* GSH synthesis. In this condition, the GSH loss in cells exposed to hypoosmotic medium was compared with the control group. As shown in Figure 8B, osmotic stress caused a significant decrease of GSH content also in BSO-treated cells. These results indicated that the decrease of GSH content by osmotic stress was not associated with impaired *de novo* synthesis.

Besides the well-known *de novo* synthesis pathway of GSH, it has been reported that an alternative pathway exists for the intracellular synthesis of GSH. Formation of γ-glutamylcysteine, the rate-limiting step of GSH synthesis, can be alternatively achieved by uptake of extracellular γ-glutamylcystine, which is formed by transpeptidation between GSH and cystine in the extracellular space by an ectoenzyme γ-glutamyltranspetidase [12,13]. Therefore, we tested whether the inhibition of γ-glutamyltranspetidase by acivicin abolished the effect of osmotic stress on GSH content. As shown in Figure 9, osmotic stress caused a significant decrease of GSH content also in the acivicin-treated cells. Combined with the finding in BSO-treated cells, these results indicated that both the conventional and the alternative pathway of GSH synthesis were not involved in the effects of osmotic

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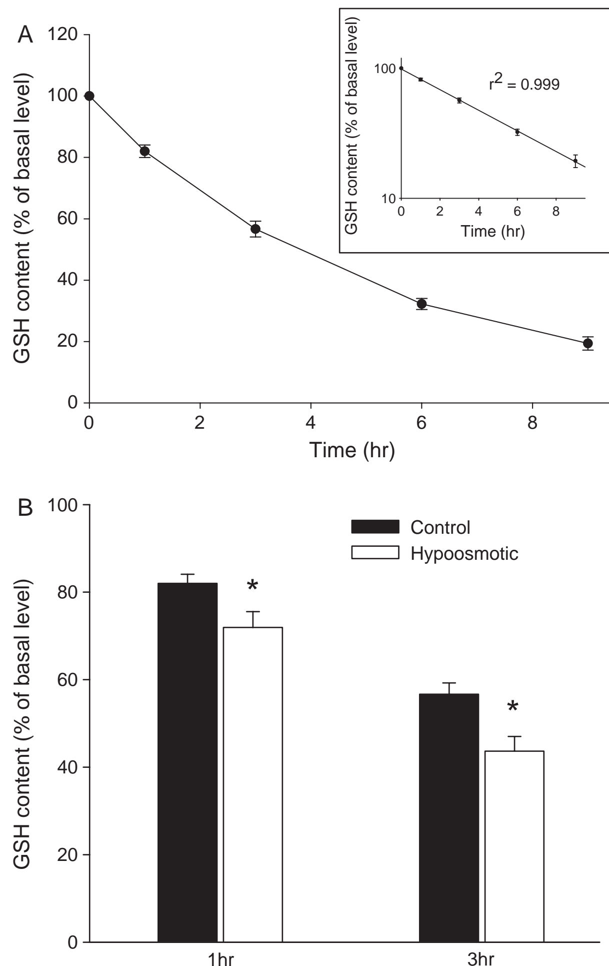

Figure 8. (A) Time course of GSH loss in the basal condition after blocking *de novo* synthesis. GSH contents were measured in cell homogenate at 1, 3, 6 and 9 h after the administration of 0.1 mM BSO. Values were expressed as the percentage of basal level measured before the treatment. Inset: Values were plotted in a semi-log scale to reveal the first-order kinetics of GSH loss. Results of at least four independent experiments. (B) Changes in GSH level after the exposure to hypoosmotic medium in BSO-treated cells. Glutathione contents were measured in cell homogenate at 1 and 3 h after the exposure to hypoosmotic KH medium. In the control group, culture medium was replaced with normal fresh KH medium. Values were expressed as the percentage of basal level measured before the medium replacement. Results of at least seven independent experiments. *$p < 0.05$ vs control.

stress and the decrease of GSH content was caused by accelerated loss of GSH.

Discussion

The results of the present study demonstrate that the osmotic stress causes a decrease of intracellular GSH content which amounts to 26% at 3 h after the exposure. Considering the vital function of GSH in the antioxidant system, this alteration presumably results in a significant risk in the maintenance of cell viability, although no cell death occurs immediately. When subjected to a mild oxidative stress with 100 μM H_2O_2, which normally induces only a minimal cell injury in our experimental condition, the cells conditioned with prior osmotic stress showed ~3-fold increase in LDH release compared to control. The increased sensitivity to oxidative stress was attributed to the decrease of GSH content, as the direct effect of osmotic stress on the cell volume had already been counterbalanced within 1 h after the exposure to osmotic stress by volume regulatory mechanisms and the increased sensitivity to H_2O_2 fell into the range predicted from the correlation of GSH content and the LDH release by 100 μM H_2O_2.

The findings described in preliminary observations further reveal the physiological significance of GSH alteration by osmotic stress. In these experiments, the concentration of cysteine equivalents in the medium was presumably ~1/10 of normal blood level, as the foetal bovine serum included in the medium was the only source of them. The GSH content in this culture condition was ~23% of normal steady state level. When cells were subjected to relatively mild osmotic stress in this condition, delayed cell death occurred with a complete loss of viability within 20 h. Therefore, it is implied that the limited capacity for GSH synthesis renders the cells highly susceptible to a relatively mild degree of osmotic stress, which might be otherwise sufficiently compensated by increased supply of GSH. In support of this conclusion, further suppression of GSH synthesis with BSO accelerated the cell death by osmotic stress, while prior enhancement of GSH content through adaptation mechanism prevented the cell death.

The decrease of GSH content by osmotic stress may be attributable either to the decreased synthesis rate or to the increased loss. From the results of experiments conducted after blocking GSH synthetic

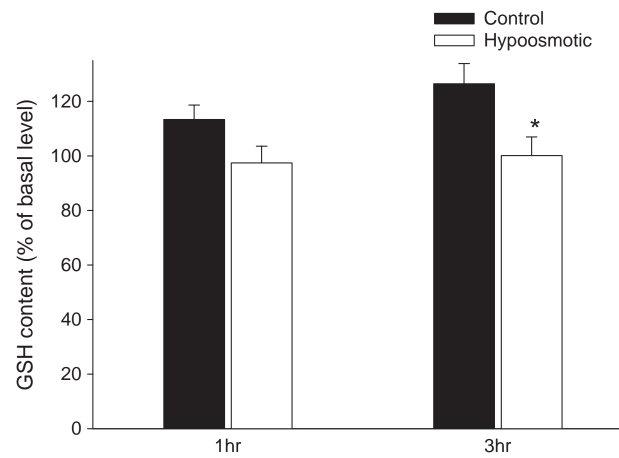

Figure 9. Changes in GSH level after the exposure to hypoosmotic medium in acivicin (0.2 mM)-treated cells. Glutathione contents were measured in cell homogenate at 1 and 3 h after the exposure to hypoosmotic KH medium. In the control group, culture medium was replaced with normal fresh KH medium. Values were expressed as the percentage of basal level measured before the medium replacement. Results of six independent experiments. *$p < 0.05$ vs control.

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pathways with BSO or acivicin, the hypothesis that osmotic stress inhibits the synthesis of GSH was rejected. Therefore, the decrease of GSH content is rationally supposed to be caused by increased consumption. The most well-known pathway of intracellular GSH consumption is the two electron transfer to oxidizing agents, e.g. hydrogen peroxide, linked with highly negative redox potential of GSH/GSSG couple [14]. However, increased oxidation to GSSG is the least probable as the mechanism of increased consumption, because GSH/GSSG ratio was not affected in cells subjected to osmotic stress. For the same reason, we excluded the possibility that the loss of GSH is associated with increased formation of mixed disulphide with protein-incorporated thiol group. In conclusion, increased GSH consumption was attributed to a flux of GSH into a still unidentified pathway other than disulphide formation.

It is widely accepted that the loss of intracellular GSH occurs by oxidation and/or export into extracellular space. The oxidation reactions of GSH include (1) the disulphide formations with another GSH molecule or protein-incorporated cysteine residues, accompanying the two-electron transfer as mentioned above and (2) one-electron transfer and resultant conjugation with various electrophilic agents. The conjugation reaction, catalysed by glutathione S-transferase, is utilized for the detoxification of exogenous electrophilic agents and also for the metabolism of endogenous electrophiles [15]. Endogenous electrophiles metabolized through conjugation with GSH include 4-hydroxynonenal, a major product of lipid peroxidation [16]. In addition, some carbonyl compounds such as acetaldehyde and pyruvate react non-enzymatically with GSH [17]. Therefore, it is possible that one or more of these conjugation reactions are involved in the GSH loss after osmotic stress.

Reaction products of GSH with various electrophiles, if not recycled, are transported out of cells [15]. Moreover, the GSH itself is also subject to the transport, although the exact nature and identity of the transporter is not yet known [18]. Therefore, it is also possible that the GSH loss after osmotic stress is associated with increased efflux rate of unconjugated GSH. In this regard, it was reported that, in astroglial cell cultures, GSH is released into the extracellular space with a rate of 3.1 nmole/h/mg protein and this efflux is significantly suppressed by a inhibitor of multi-drug resistance protein 1 (Mrp1), one of the transporter proteins known to mediate cellular export of GSSG and GSH conjugates [19]. However, the efflux of GSH is probably mediated by more than one transporter which differs in dependence on the cell type [18]. For example, the efflux of GSH from airway epithelial cells has been reported to be mediated by the cystic fibrosis transmembrane conductance regulator (CFTR) [20]. To our experience, conventional inhibitors of Mrp1 or CFTR did not affect the basal decrease of GSH content in BSO-treated cells nor the loss of GSH after the osmotic stress (unpublished data). However, definite conclusions could not be attained in these experiments due to the lack of specific inhibitors.

Although the detailed mechanism of GSH loss after osmotic stress is still unclear, it is speculated that this phenomenon might play a critical role in several major pathologic processes. Osmotic stress by decreased extracellular osmolarity occurs *in vivo* as a result of systemic hyponatremia. This pathologic condition might potentially affect the GSH homeostasis and induce tissue damage by concomitant oxidative stress. In fact, it has been reported that the brain is depleted of GSH in response to hyponatremia [21], although the depletion was organ-specific and dependent on the cell type. More importantly, it is highly probable that cellular damages during the development of diabetic complications and ischemia-reperfusion injury are associated with the GSH loss by osmotic stress, in which the osmotic stress is caused by intracellular accumulation of osmolytes. In the diabetic state, osmotic stress may occur as a result of the intracellular accumulation of glucose metabolites such as lactate and sorbitol. On the other hand, oxidative stress is a common feature of various diabetic complications. Therefore, the loss of GSH by osmotic stress may play an important pivotal role in the pathogenesis of diabetic complications. In accordance with this hypothesis, Obrosova et al. [22] reported that augmented osmotic stress by inhibition of sorbitol metabolism resulted in an increased oxidative damage in diabetic lens. During ischemia and the early phase of reperfusion, lactate and inorganic phosphate accumulation impose a severe osmotic stress [23]. In this context, reactive oxygen species are generated during reperfusion [24]. We speculate that the osmotic stress during the ischemic period and the initial reperfusion period lowers the GSH content and thus plays a critical role in the cell death induced by oxidative stress during the reperfusion. In fact, numerous reports have documented the decrease of GSH content during ischemia-reperfusion [25,26]. It is also known that the osmotic load by lactate accumulation rather than ATP depletion determines the development of lethal myocardial injury [27]. However, in so far as we are aware of, the mechanistic link between osmotic stress and oxidative damage during ischemia-reperfusion has never been appreciated.

It is also noticeable that the cell damage by osmolyte accumulation was prevented by increasing the medium osmolarity with additional 20 mM NaCl (Figure 2). This finding implicates that the GSH loss and subsequent oxidative damage in various pathologic processes mentioned above may be prevented by transient hyperosmolarity. In fact, it was proved in many experiments that increased extracellular osmolarity during reperfusion have beneficial effects to the

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ischemic-reperfused myocardium [24]. Similarly, we observed that the GSH loss during organic acidosis, which induces cell swelling presumably through an increase in the intracellular osmolarity, was prevented by additional NaCl in the medium (unpublished data). Moreover, the protective effect was comparable when the hyperosmolarity was achieved with sodium lactate instead of NaCl. Therefore, preservation of GSH by increased extracellular osmolarity would possibly be an effective strategy to prevent the oxidative damage in many pathologic conditions.

In summary, we discovered that osmotic stress induces the decrease of intracellular GSH content by increased consumption and this loss of GSH renders the cells susceptible to a subsequent oxidative stress. In addition, it was shown that the loss of GSH by osmotic stress may by itself result in cell death when the normal GSH synthesis mechanism fails to compensate the loss due to e.g. low cysteine availability. It was speculated that this phenomenon presumably plays a critical role in the development of many major pathophysiological processes.

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