Oxidative stress affects responsiveness to hypotonicity of renal cells

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Abstract

Oxidative stress plays a critical role in the pathophysiology of several kidney diseases and is the consequence of alterations like ischemic events. The regulatory volume decrease (RVD) is an homeostatic response essential to many cells, including renal cells, to counteract changes in the osmolarity of the external medium. The aim of the present work is to verify whether oxidative stress affects RVD in a model of renal cells (human embryonic kidney cells, HEK 293 Phoenix). To accomplish this aim, the experimental procedure consisted in: i) cell culture preparation and treatment with 200 μM H_2O_2; and ii) measurement of cell volume changes in isotonic conditions or following hypotonic stress. H_2O_2 added to the extracellular isotonic solution induced a significant reduction in cell volume, and added to the extracellular hypotonic solution dramatically impaired the expected osmotic cell swelling. Pre-incubation of cells in an extracellular isotonic solution containing H_2O_2 prevented cell from swelling after hypotonic stress application. In conclusion, H_2O_2 leads to cell shrinkage in isotonic conditions, inhibits the hypotonicity-induced cell swelling and consequently prevents RVD, hypothetically due to an activation of transport pathways determining ion loss and, in turn, water efflux. Cell shrinkage in isotonic conditions is a hallmark of apoptosis and is known as the apoptotic volume decrease.

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Introduction

Kidney plays a major role in the control of salt and water homeostasis in all mammals, and hence determines the extracellular volume and solute composition of the organism. Alterations in water and ion fluxes at the level of the kidney may be often associated with diseases, like the hereditary disorders nephrogenic diabetes insipidus and Liddle's syndrome.[1,2] Renal tubule cells are continuously exposed to hyposmotic and hyperosmotic conditions during the process of urine production, therefore, adaptive mechanisms to regulate the cellular volume are critical to maintain their viability and functionality.[3,4]

As a general feature, cell volume homeostasis is common to all animals and plants and can be influenced by different physiological and pathological factors including diet and environment.[3,5-7] Trans-membrane water movement follows the osmotic gradient and may be achieved by simple diffusion through the lipid bilayer or through water channels, referred to as aquaporins (AQPs),[8,9] or both. Osmotically driven water movement, along with ion fluxes, underlies the physiological mechanisms regulating volume and fluid homeostasis in most cells.[4,10]

Volume regulation under anisosmotic conditions is essential to cell survival and counteracts changes in the osmolarity of the extracellular medium. In particular, when exposed to a hyposmotic medium, cells undergo to osmotic swelling, known as osmotic phase (OP). Cell swelling can disrupt the cell membrane if not opportunely counterbalanced. Depending on the species and cell type, cell volume returns either immediately or slowly to control values because of the adaptive mechanism of the regulatory volume decrease (RVD). This homeostatic response has been mostly investigated in mammalian cells such as astrocytes,[11] erythrocytes from different sources,[12] lymphocytes[13] and sperm,[14] but also in cells of lower vertebrates and invertebrates.[15-18]

The series of events leading to RVD has been at least partially defined and consists in loss of intracellular osmolytes followed by a concomitant rapid efflux of intracellular water. Thus, the pivotal counterparts of RVD are: i) ion conducting pathways, mainly permeable to K^+, Cl^- and organic anions, allowing for the flux of osmolytes out of the cell; and ii) water transporting proteins (AQPs), allowing for the efflux of water along the osmotic gradient.[4,19,20]

Oxidative stress has a critical role in the pathophysiology of several kidney diseases. Several systemic diseases such as hypertension and diabetes mellitus, environmental and occupational chemicals, smoking, as well as alcohol consumption induce renal oxidative stress.[21] Oxidative stress also occurs during ischemia/reperfusion (I/R) injury.[22]

Since the kidney is an organ highly vulnerable to damage caused by oxidative stress and adaptive mechanisms to face changes in the

osmolarity of the extracellular medium are critical for viability and function of renal cells,[20] we investigated if the oxidative stress may alter the RVD response. To this aim, the time course of cell volume changes in HEK 293 Phoenix cells has been monitored isotonic and hypotonic solutions in the presence or absence of 200 μM H_2O_2.

Materials and Methods

Human renal HEK 293 Phoenix cells[23] were cultured in minimum essential eagle medium [(MEM); Sigma Aldrich, St. Louis, MO, USA] supplemented with 10% fetal bovine serum [(FBS); Cambrex Bio Science, East Rutherford, NJ, USA], 2 mM l-glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin and 1 mM pyruvic acid (sodium salt). The cells were maintained at 37°C, 5% CO_2, 95% air and 100% humidity. Subcultures were routinely established every second to third day by seeding the cells into 100 mm diameter Petri dishes following trypsin/ethylene diamine tetraacetic acid treatment.

Regulatory volume decrease tests

To perform RVD tests, HEK 293 Phoenix cells were grown on 18×18 mm coverslips successively placed upside down on a glass slide. A make-shift perfusion chamber was assembled by placing double sided tape between the glass slide and the coverslip with cells. The extracellular medium was completely and rapidly exchanged by adding either an isosmotic or hyposmotic solution to one side of the coverslip and removing it at the opposite side with strips of filter paper, according to the experimental plans described below.

Control tests

With regard to control RVD tests, cells were exposed to: 1st step, isosmotic solution for 5 min; 2nd step, hyposmotic solution (~15% reduction of osmolality) for 30 min; 3rd step, isosmotic solution for 5 min. In addition, volume of cells was monitored in isosmotic solution for 30 min.

H_2O_2 treatment

With regard to the RVD tests in 200 μM H_2O_2-containing medium, the following experimental protocols were used: i) 1st step, isosmotic solution with or without 200 μM H_2O_2 for 30 min; 2nd step, hyposmotic solution for 15 min; ii) 1st step, isosmotic solution for 5 min; 2nd step, hyposmotic solution with or without 200 μM H_2O_2 for 30 min; 3rd step, isosmotic solution for 5 min.

In addition, volume of cells was monitored in isosmotic solution with or without 200 μM H_2O_2 for 30 min. Cell volume measurements were taken from cells demonstrating strong adhesion to the coverslip after checking with a microscope (Leica DMLS, 400× magnification; Leica Microsystems GmbH, Wetzlar, Germany) during continuous perfusion. For each experiment, about 30 images/cell (1 image/min) were recorded with a phase contrast microscope (Leica DMLS, 400× magnification; Leica Microsystems GmbH) connected to a video camera (Digital CCD camera) and a computer equipped with suitable software (Movie Maker; Microsoft Co., Redmond, WA, USA). Cell diameter was measured for each recorded image and, assuming the cell as a sphere, cell volume was successively calculated and expressed as V/V_0, where V_0 was the initial volume in isotonic solution.

Experimental solutions and reagents

Isotonic solution: $MgCl_2$, 2.5 mM; $CaCl_2$, 2.5 mM; HEPES, 10 mM; NaCl, 125 mM; mannitol, 50 mM; pH 7.4; osmotic pressure 314.33 mOsm/kg_{H2O}. Hyposmotic solution (~15% reduction of osmolality): $MgCl_2$, 2.5 mM; $CaCl_2$, 2.5 mM; HEPES, 10 mM; NaCl, 125 mM; pH 7.4; osmotic pressure 263 mOsm/kg_{H2O}. pH measurements were taken with an Orion pH-meter and osmolality was measured by a Fiske osmometer.

Statistics

Data are shown as mean values±standard error of the mean. Each data set is derived from five individual experiments. Significance of the differences between data was tested using one- or two-way analysis of variance (ANOVA), followed by Dunnet's or Bonferroni's *post-hoc* test, as appropriated. $P<0.05$ was considered as statistically significant.

Results

Control tests

Cells were bathed in isotonic solution for 5 min (1st step; Figure 1A) and then exposed to an extracellular hypotonic solution for 30 min (2nd step; Figure 1A). Upon hyposmotic shock, an increase in the cell V/V_0 ratio was observed, indicating a substantial cell swelling corresponding to OP. V/V_0 reached a peak value of 1.294±0.025 within 5 min, a value significantly higher than the one observed in isotonic solution ($P<0.001$). After the OP, the V/V_0 ratio gradually fell to 1.031±0.016, a

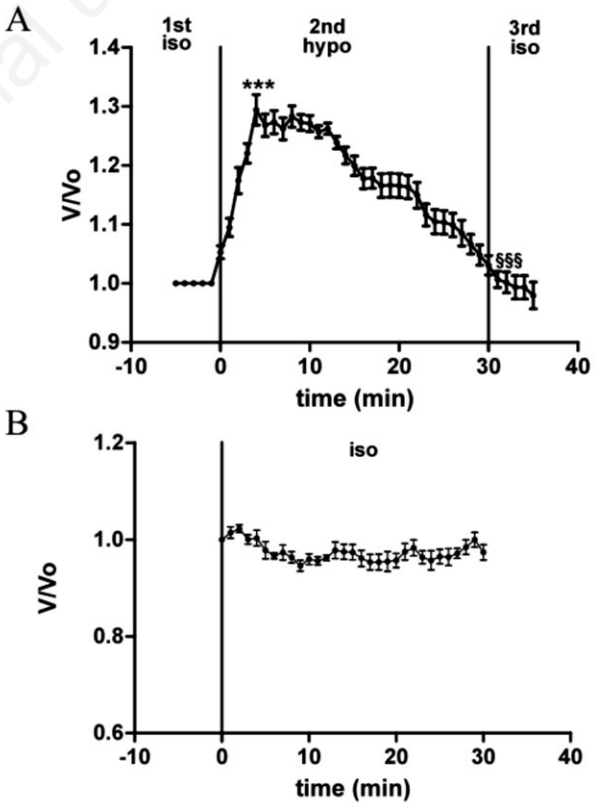

Figure 1. Cells bathed in isotonic solution. A) Cell volume (V/V_0) measured as a function of time after exposure of cells to isosmotic (1st and 3rd step) and hyposmotic (2nd step) solution. The peak V/V_0 value in hypotonic solution was significantly higher than that measured in isosmotic solution (*$P<0.001$). At the end of the 2nd step V/V_0 was significantly different from the peak value (§§§$P<0.001$). B) V/V_0 determined during a 30 min observation in isosmotic condition. No significant volume changes were observed.**

value significantly lower respect to the peak value (P<0.001), corresponding to the RVD phase. After returning to the isosmotic medium (3rd step), cell volume was comparable to the one observed during the 1st step (0.979±0.022), without a post-RVD regulatory volume increase (RVI). Post-RVD RVI has not been seen in these experiments, probably because of the short time of observation after the replacement of the hyposmotic medium with the isosmotic one.

In addition, cells were maintained in isotonic solution for 30 min and their volume was monitored (Figure 1B). At the beginning of the incubation is isotonic solution V/V_0 was 1±0.025, with no significant changes during the whole experiment.

H_2O_2 treatment

The exposure of cells to 200 μM H_2O_2 in isotonic solution (Figure 2) induced a gradual and significant decrease in V/V_0, starting after 10 min of treatment. V/V_0 reached a value of 0.656±0.023 after 30 min. This value was significantly lower respect to that observed in untreated cells (P<0.001).

In a further experimental protocol, cells were first bathed in isotonic solution with or without (control) 200 μM H_2O_2 (Figure 3; 1st step), and then exposed to the hypotonic solution (Figure 3; 2nd step). Interestingly, pre-incubation of cells in isotonic medium plus 200 μM H_2O_2 prevented cell swelling, being V/V_0 after 5 min of hypotonic shock (0.712±0.018) comparable, from one hand, to the value observed at the end of the 1st period (0.734±0.011) and, from the other hand, significantly lower than the corresponding value of untreated cells (1.290±0.011).

Treatment of cells with a hyposmotic solution containing 200 μM H_2O_2 (Figure 4) significantly impaired the cell swelling normally observed during the osmotic phase. During the 2nd step, V/V_0 reached a peak value of 1.046±0.023, comparable to that observed in isotonic conditions (1st step) and significantly lower than that of untreated cells (1.290±0.011; P<0.001). At the end of the hyposmotic challenge (2nd step), V/V_0 decreased to a value of 0.753±0.040, significantly lower with respect to the peak value of both treated and untreated cells (P<0.001). Cell volume further decreased when the hyposmotic solution was substituted with the isotonic one (V/V_0=0.645±0.053; end of the 3rd step).

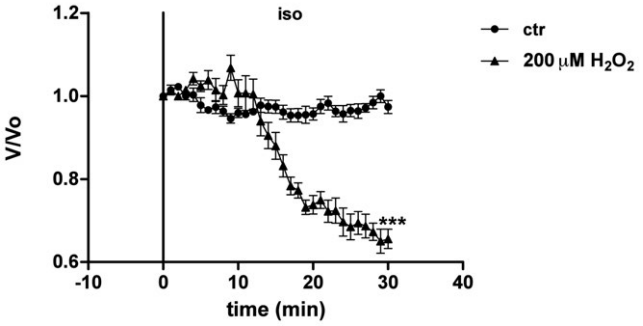

Figure 2. Volume (V/V_0) of cells exposed to an isosmotic solution containing 200 μM H_2O_2; comparison with untreated cells [control (ctr)]. Cell volume of treated cells reached a value significantly lower than that observed in control cells (*P<0.001).**

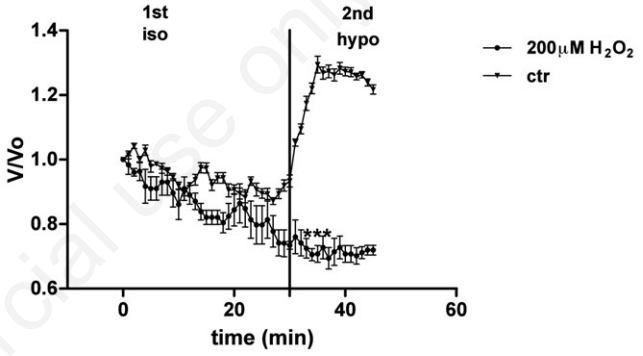

Figure 3. Volume (V/V_0) of cells exposed to 200 μM H_2O_2 in isotonic medium (1st step) and then submitted to a hyposmotic challenge (2nd step); comparison with untreated cells [control (ctr)]. H_2O_2 was present only during the 1st step. After 5 min of hypotonic stress, V/V_0 of treated cells was significantly lower than that observed in control cells (*P<0.001).**

Discussion

The results obtained in the present work confirm that HEK 293 Phoenix cells, submitted to a ~15% hyposmotic stress, exhibit the expected significant cell swelling within 5 min of hyposmotic shock application and progressively regulate their volume, completely restoring the initial V/V_0 values within 30 min of hyposmotic challenge (Figure 1A). Cell volume regulation in renal cells, including HEK 293 Phoenix cells, as well as in many other cell types, including those of invertebrates, has been already studied[24,25] and is recognized as a homeostatic response accounting for cell viability. It is thus reasonable to consider this parameter to monitor cell function in different conditions, such as diseases, exposure to drugs, pollutants and metabolites, with their possible detrimental effects, including oxidative stress events.[26-31] Amongst renal alterations, interesting is the case of renal I/R injury, which causes acute renal failure in several clinical settings like kidney transplantation.[32,33] The mechanisms of renal acute injury induced by I/R is multifactorial and involves hypoperfusion, inflammatory responses, along with free radical induced damage.

On this basis, in an attempt to give more information about the damage deriving from oxidative stress events in renal cells, the present

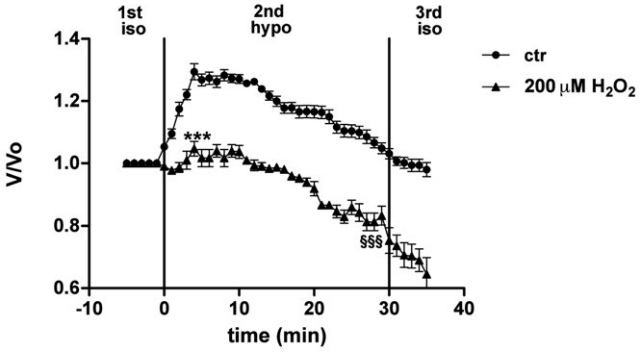

Figure 4. Volume (V/V_0) of cells exposed to a hyposmotic solution containing 200 μM H_2O_2; comparison with untreated cells [control (ctr)]. H_2O_2 was added at the beginning of the 2nd step and remained throughout. The peak V/V_0 value of treated cells was significantly lower than that observed for control cells (*P<0.001). V/V_0 of treated cells at the end of the 2nd step was significantly lower than the peak V/V_0 value and lower than that observed for control cells (§§§P<0.001).**

paper proposes HEK 293 Phoenix cells as an *in vitro* model to ascertain the effect of H_2O_2-induced oxidative stress on a cell response like cell volume regulation capability, with specific regard to RVD.

Our results show that the exposure of HEK 293 Phoenix cells to H_2O_2 in isotonic conditions induced, within 10 min, a notable cell shrinkage (Figure 2), possibly as a consequence of ion and obliged water efflux. Two hundred μM H_2O_2 was effective in altering cell morphology without inducing cell death, since cell viability was assessed after each experiment by the Trypan blue dye exclusion test (data not shown).

Moreover, a 30 min exposure of cells to an isotonic medium containing H_2O_2 determined, in addition to a significant shrinkage, the impairment of the expected cell swelling after hyposmotic challenge (Figure 3). This observation can be explained by oxidative stress events, due to H_2O_2 treatment, provoking cell shrinkage because of ion and water loss, a response associated to an initial stage of apoptosis, as reviewed by Bortner and Cidlowski.[34] It is likely that the application of 15% hyposmotic shock impaired cell swelling since the intracellular ions concentration, namely K^+ and Cl^-, was diminished under H_2O_2 treatment.

A further experimental protocol demonstrated that H_2O_2 treatment during 15% hyposmotic stress dramatically blunted the expected cell swelling and consequently prevented RVD mechanisms. In contrast, pronounced cell shrinkage occurred and was obvious at the end of the hyposmotic challenge (Figure 4).

Reduction of cell volume in isotonic conditions or cell shrinkage is considered as a hallmark of programmed cell death, a process called apoptosis.[34] This phenomenon has been termed apoptotic volume decrease (AVD) and differs from RVD occurring in anisotonic conditions.[35]

The signaling pathways underlying AVD modulate ion fluxes.[35] In this regard, monovalent ions, like intracellular K^+, seem to be specifically involved in controlling the cell death process. Blockage of loss of this ions has been in fact demonstrated to protect cells from apoptosis.[34]

Potassium has received considerable attention being the most abundant, osmotically relevant cation of the intracellular environment. As already stated, K^+ and Cl^- efflux have been shown to play a major role in RVD mechanisms in many cell types, leading to obliged water efflux.[3,4] Bortner and Cidlowski (2007) in their review[34] also reported about the relationship between morphological change and intracellular monovalent ions during apoptosis. According to these and other authors[36] it is reasonable to suggest that, in our model of renal cell, the oxidative stress induced by the exposure of cells to H_2O_2 elicited ion efflux and, hence, water loss in isotonic conditions and cell shrinkage as an early step in the apoptosis process. It is likely that the H_2O_2-induced cell shrinkage masked the cell swelling and the subsequent RVD normally expected after hypotonic challenge. Apoptosis has not been ascertained in the present work.

Conclusions

In conclusion, our results suggest that: i) HEK 293 Phoenix cells are a suitable model to study cell volume regulation; ii) these cells exhibit a RVD response occurring within 30 min of hyposmotic challenge; iii) oxidative stress under H_2O_2 exposure provokes cell shrinkage in isotonicity and unresponsiveness to hyposmotic challenge, probably due to ion and water loss. These observations suggest that ion channels, involved in RVD and playing a role in triggering AVD and apoptosis, may be considered as a possible target for therapeutic strategies to counteract organ and tissue damage deriving from oxidative stress and subsequent apoptotic events, as in the case of I/R injury. Moreover, a precise window of time for a possible modulation of ion fluxes affected by oxidative stress could also be defined.

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