Original Paper

Short-Term Hyperosmolality Pretreatment on Cells Can Reduce the Radiosensitivity via RVI and Akt1 Activation

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Key Words

Ionizing radiation • Regulatory volume increase • Cation channels • Akt1

Abstract

Background/Aims: The ionizing radiation (IR) has been applied in clinical treatment for many years and the radiosensitivity is crucial to the treatment. Radiosensitivity of cells is subjected to many environmental factors, such as hypoxia and temperature. Hyperosmolality as a common environmental factor has been demonstrated to be associated with survival and apoptosis of cells in many studies. Thus we investigated the influence of hyperosmolality on cells radiosensitivity. **Methods:** We examined the viability and surviving fraction of L-O2 cells of irradiated L-O2 cells, and detected the effect on AHH-1 cells by flow cytometry, in order to investigate the effect of short-term hyperosmolality pretreatment on cells radiosensitivity. Comet assay was used to assess the DNA strand breaks. Then the detection of Akt1 by western blot and the process of regulatory volume increase by CYSY-TT were involved in the mechanism. **Result:** We demonstrated that a short-term hyperosmolality pretreatment on cells could reduce their radiosensitivity. Further research indicated that the short-term hypertonic condition could induce regulatory volume increase (RVI), which activated Akt1 and degenerated the I κ B- α . This process was associated with reduced cells radiosensitivity. Finally, we used the flufenamic acid (FFA), a blocker to cation channels (HICCs) to inhabit RVI and consequently inhabit the protective effect of hyperosmolality on irradiated cells. **Conclusion:** a short-term hyperosmolality pretreatment could reduce the cells radiosensitivity by RVI and following activation of Akt1.

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Introduction

Along with the widely use of radiotherapy in oncotherapy, it has also come to light that ionizing radiation (IR) can induce apoptosis of cells. The cells' radiosensitivity is subjected to many factors, such as the hypoxia [1], temperature [2], concentration of NO [3]. In the investigation of possible factors influencing radiosensitivity, previous studies mainly focused on the process of apoptosis and DNA-repair [4]. Several environmental factors can affect the process of apoptosis, and accordingly influence the radiosensitivity. Hyperosmolality, a common environmental factor of life, has been investigated in some recent studies. Hyperosmolality can lead to apoptosis by inhibition of growth factor receptor signaling [5], and p53 activation followed by arrest of cell cycle [6]. Besides, autophagy [7] and Tau phosphorylation [5] were also reported to be induced by hyperosmolality. On the other hand, hyperosmolality, can activate serine-threonine kinase Akt1 [6] and shock transcription factor 1 (HSF1) [8], which further prevent the mild hyperosmolality-induced apoptotic changes in MDCK cells [6] and rescue cells from staurosporine-elicited apoptosis [9]. Since radiosensitivity is correlated with apoptosis, there may be an interesting relationship between cells' radiosensitivity and hyperosmolality. Actually, exposure of cells to high salt is a common fact of life [10], thus investigating the effect of hyperosmolality on cells under IR is very meaningful for clinical radiotherapy, especially for those patients in dehydration.

Materials and Methods

Cell culture conditions and hyperosmotic treatment

Human Lymphocyte AHH-1 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI 1640 (PAA, Pasching, Austria) with 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine at 37°C in a 5% CO₂ humidified chamber. L-O2, A549, HUVEC cells were maintained in DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine at 37°C in a 5% CO₂ humidified chamber. In order to obtain the experimental osmolality, NaCl was added to the RPMI 1640/DMEM and the final concentration of osmolality was measured by using a vapor pressure osmometer (VAPRO5520). Before receiving γ-radiation, cells were cultured in required osmotic solution for required period of time according to the experimental design. After irradiation the hyperosmotic solution was changed to isotonic solution.

Irradiation

Cobalt-60 gamma rays in irradiation center (Faculty of Naval Medicine, Second Military Medical University, China) were used for the irradiation. Cells (with or without hyperosmolality pretreatment) were exposed to different doses of irradiation, depending on the requirement of present study.

Cell viability tests and Clonogenic survival

Cell viability was determined by calorimetric MTT assay (Cell Counting Kit-8; Dojindo, Kumamoto, Japan). Cells were cultured in the 96 wells plate with the cells number of 5×10^3 in each plate, and 24h after irradiation the cells viability was examined using the Cell Counting Kit-8 as the protocol described.

Colony-forming assay was performed as described [11]. Calculated numbers of cells were plated to enable normalization for plating efficiencies. Pretreated cells were then irradiated with 0, 4, or 8 Gy. After being incubated for 14 days, the plates were fixed with 70 % EtOH and stained with 1% methylene blue. Colonies consisting of >50 cells were counted under microscope. The survival fraction was calculated as (number of colonies/number of cells plated) / (number of colonies for corresponding control/number of cells plated).

Apoptosis assays for cultured cells

Apoptosis was examined by Annexin V-APC and propidium iodide staining using apoptosis detection kit (Invitrogen, California, USA). 15 minutes after Annexin V-APC staining and 5minutes propidium iodide staining, treated cells were analyzed by flow cytometry (Beckman Coulter, California, USA).

Comet assay

AHH-1 cells were exposed to 0 or 8Gy of γ -radiation, and 5h after irradiation the DNA strand breaks (DSBs) was determined using single-cell gel electrophoresis (comet assay) based on the method of Gandhi et al. [12].

Western blot analysis

AHH-1 cells were collected after treatment. Then cells were resuspended in PBS and washed for 3 times. Collected in extraction buffer (1% Triton X-100, 0.5% sodium deoxycholate, 20mM Tris-HCl, pH 7.5, 12mM-glycero-phosphate, 150mM NaCl, 5mM EGTA, 10mM NaF, 3mM dithiothreitol, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 20g/ml aprotinin), cells were incubated on ice for 3×10 minutes, and lysate was centrifuged (12,000g for 15 minutes at 4°C) to obtain the supernatant containing protein. Equal amount of proteins was separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. 5% low-fat milk was used to block the membranes for 2 hours then the membranes were washed 3 times with TBST for 10mins. The membranes were probed overnight at 4°C with antibodies recognizing the following antigens: Akt1 (1:1000), p-Akt(308) (1:1000), I κ B- α (1:1000), and all the antibodies were obtained from cell-signal corporation. The antibody-antigen complex was detected using horseradish peroxidase-conjugated secondary antibody. Peroxidase labeling was visualized with enhanced chemiluminescence labeling using an ECL Western blotting detection system (Thermo, USA).

Determination of cell volume

Changes of cells' volumes were detected by the CYSY-TT (Roche, Germany). AHH-1 cells volumes were measured under isotonic or hyperosmotic condition with or without FFA interference at designed time points. Finally, a curve to indicate the change of cells' volumes was performed.

Statistical Analysis

The data, presented as means $\pm$ S.E., were statistically analyzed using analysis of variance followed by Benferroni's test and Student's t test. Differences were considered significant when $p < 0.05$.

Result

Short-term hyperosmolality pretreatment reduces cell's radiosensitivity

In order to investigate the effect of osmotic pressure on cells radiosensitivity, we firstly examined the viability of irradiated L-O₂ cells. After pretreatment of different osmotic concentration, the L-O₂ cells were exposed to 8Gy of γ -radiation, and the viability was examined. As shown in the Fig. 1A, hyperosmolality (400mOsm, 500mOsm) pretreatment in a short period of time (within 60min) reduced the radiosensitivity of L-O₂ cells, but increased the radiosensitivity in 120min (Fig. 1A). A subsequent surviving fraction of L-O₂ cells further confirmed the effect of short-term hyperosmolality pretreatment reduces cell's radiosensitivity (Fig. 1B). For further investigating whether the protective effect of short-term hyperosmolality was universal in other irradiated cells, we then examined the viability of HUVEC and A549 cells which both received 8Gy of γ -radiation with pretreatment of hyperosmolality (500mOsm) for 60 minutes. The results also suggested the protective effects of short-term hyperosmolality pretreatment on other irradiated cells (Table 1). Actually, the lymphocyte population is often subjected to the interruption of osmotic concentration and is sensitive to ionizing radiation. Thus, the effect of hyperosmolality pretreatment on AHH-1 cells was further assessed by flow cytometry. As shown in the Fig. 1C, the apoptosis of irradiated AHH-1 cells with hyperosmolality pretreatment was significantly decreased (Fig. 1C).

Short-term hyperosmolality pretreatment decreases double-strand DNA breaks (DSBs)

One of main causes of apoptosis of irradiated cells is the DNA damage [13]. In present study, the DNA damage was assessed by the Comet assay as an indicator for the radiosensitivity. As shown in Fig. 2, pretreated under hyperosmolality, irradiated AHH-1 cells

Fig. 1. The short-term hyperosmolality reduced cells' radiosensitivity (A) The viability percent of L-O2 cells undergoing 8Gy of γ -radiation exposure with pretreatment of different osmotic concentration for different period of time. L-O2 cells were pretreated with different osmotic concentration produced by adding NaCl (300mOsm is isotonic condition) for different period of time, then cells were exposed to 0Gy or 8Gy of γ -radiation. After irradiation cells were transferred to isotonic condition to culture for 24h and the viability was determined by Cell Counting Kit-8. (B) Surviving fraction of L-O2 cells after 8Gy of γ -radiation exposure with pretreatment of isotonic condition (300mOsm) or hypertonic condition (500mOsm) was determined. (C) Apoptosis percent of AHH-1 cells was determined by flow cytometry. AHH-1 were pretreated under isotonic condition (300mOsm) or hypertonic condition (500mOsm) for 1h then were exposed to 0Gy or 6Gy of γ -radiation, and 24h after radiation the apoptosis was examined using method of flow cytometry. Values are given as mean $\pm$ SEM (n=3), *P<0.05 Vs the Control group.

reached a decreased level of all comet parameters (%DNA in tail, tail length, tail moment, and olive tail moment) in comparison to those irradiated alone. (Fig. 2).

Hyperosmolality activates protein kinase Akt1 of AHH-1 cells

In the results above, a protective effect of hyperosmolality on irradiated cells had been suggested, but which signal transduction mechanism was involved in the process need further

Table 1. Hypertonic decrease ironizing radiosensitivity of three types of cells. Cells were exposed to 8Gy of γ -radiation with pretreatment under isotonic condition (300mOsm) or hypertonic condition (500mOsm) for 1h. 24h after radiation cells viability were measured by Cell Counting Kit-8

Cell types	Cell viability(%)	
	300mOsm+8Gy	500mOsm+8Gy
HUVEC	100 $\pm$ 10.6	113 $\pm$ 7.9
L-O2	75 $\pm$ 2.2	105 $\pm$ 9.7
A549	26 $\pm$ 3.0	58 $\pm$ 4.9

Fig. 2. Short-term hyperosmolality decreased double-strand DNA breaks. 5h after radiation of 0Gy or 6Gy of γ -radiation pretreated cells were collected to assess the DNA damage by single cell gel electrophoresis (comet assay) and damaged DNA appears in the "tails" of the "comets". Values are given as mean $\pm$ SEM (n=3), *P<0.05 Vs the Control group.

investigations. Previous studies have indicated that some stresses including hyperthermia can activate the Protein Kinase Akt1 which can inhibit the stress-induced damage, and a

Fig. 3. Hyperosmolality induced phosphorylation of Akt and degradation of I κ B α . AHH-1 cells were treated under hypertonic condition (500mOsm) for times required then the phosphorylated Akt1 and I κ B- α levels were detected by Western blotting.

Fig. 4. The process of regulatory volume increase (RVI) was associated with the activation of Akt1. (A) The volume response of cells was revealed under isotonic condition (300mOsm), hypertonic condition (500mOsm), and hypertonicity plus 200 μ M flufenamic acid (FFA) interference. (B) With the volume response of cells in different treatment the activation of Akt1 was detected.

rapid hyperosmolality is a stress to cells. In this study, we mainly focused on Akt1, a Ser/Thr protein kinase alternatively termed protein kinase B and the PI3K/Akt pathway, and the expression of Akt1 in AHH-1 cells under hypertonic condition was assessed. With the treatment of hyperosmolality, Akt1 was phosphorylated and reached the peak at 60min (Fig. 3). There was also a time-dependent degradation of I κ B α (Fig. 3). These results were consistent with the canonical activation of NF- κ B (Fig. 3).

Regulatory volume increase (RVI) is associated with Akt1 activation

Since the protective effect of hyperosmolality on irradiated cells was only made within a short period of time, we hypothesized that a rapid regulation may play a role in it. Here we focused on the RVI, and the concomitant activation of Akt1. RVI means that cells' volume recovers after osmotic cell shrinkage, which is important for the intracellular balance of osmolality and metabolism [14]. The volumes of AHH-1 cells were determined while cells

Fig. 5. Inhibition of RVI eliminated the protective effect of hyperosmolality on irradiated cells. The cells were pretreated under isotonic or hypertonic condition, and 200 μ M flufenamic acid (FFA) was applied in these conditions respectively. After receiving 8Gy (L-O2) or 6Gy (AHH-1) of γ -radiation, the viability percent of L-O2 cells were detected by CCK-8 Cell Counting Kit-8 (A) and the Apoptosis percent of AHH-1 cells was examined by flow cytometry (B) Values are given as mean $\pm$ SEM (n=3), *P<0.05 Vs the Control group.

undergoing a hypertonic condition stress (500mOsm) or isotonic conditions (310mOsm). The cells' volumes recovered after a rapid osmotic shrinkage, and the flufenamic acid (FFA) inhibited the recovery of volume (Fig. 4A). To assess the relationship between RVI and the activation of Akt1, the activation of Akt1 under hypertonic condition with or without FFA was also detected. As a result, the FFA eliminated the time-dependent phosphorylation of Akt1 under hyperosmolality (Fig. 4B).

Inhibition of RVI eliminates the protective effect of hyperosmolality on irradiated L-O2 and AHH-1 cells.

We hypothesized that the RVI may be involved in reducing radiosensitivity of cells, and FFA was used to inhibit the RVI in the experiments above, and we further examined whether the FFA could affect the protective effect of hyperosmolality on irradiated AHH-1 cells. Interestingly, the addition of FFA eliminated the protective effect of hyperosmolality on irradiated L-O2 and AHH-1 cells (Fig. 5)

Discussion

It is well known that the radiosensitivity of cells can be influenced by many environmental factors. The environmental factors including hypoxia and temperature have been studied profoundly, and the environmental factors plus radiotherapy is being extensively practiced in clinical treatments of cancer. For example, hyperthermia has been applied as an adjuvant therapy of radiotherapy in the cancer treatment [15]. However, the osmotic pressure, a common influencing factor of the radiosensitivity of cells, receives less attention. Actually the relationship between osmotic pressure, especially hyperosmolality, and apoptosis

of cells has been investigated in many studies, but the effect of osmotic pressure on cells' radiosensitivity is still unclear. Thus we assessed whether osmotic pressure could exert influence on the cells' radiosensitivity.

In our study, we firstly tested the viability of L-O2 cells under different osmotic concentrations (90mOsm, 150mOsm, 300mOsm, 400mOsm, 500mOsm) plus γ -radiation. The results demonstrated that hyperosmolality pretreatment for a long period of time (beyond 60min) increased cells' radiosensitivity, but a reduced radiosensitivity occurred in a short-term hyperosmolality pretreatment (within 60min). Effect of hyperosmolality on cells' damage has been reported in many studies. In this study, we found an interesting phenomenon that a short-term hypertonic stress could reduce radiosensitivity. To verify the effect, we applied the hyperosmolality pretreatment of L-O2 cells to other types of cells (A549, HUVEC, AHH-1) and investigated the cells' viability or apoptosis. The final results further identified the protective effect of hyperosmolality on irradiated cells.

It is clear that hyperosmolality can induce the damage and apoptosis of cells. As is reported, hypertonic-stress induces apoptotic cell death, activates caspase-3 [16], inhibits proliferation [17], leads to DNA damage [18], and even brings about autophagy [7]. In our study, as was shown in Fig. 1A, with hyperosmolality pretreatment for a long period of time (120min), L-O2 cells underwent increased damage from radiation, which was consistent with previous reports. However, a short-term pretreatment within 60min can lead to a protective effect (Fig. 1). We speculated that a transient regulatory mechanism may be involved in the process. Accumulated evidences indicate that the serine-threonine kinase Akt1/protein kinase B (PKB) is crucial in regulating cells' survival and apoptosis [19], and Akt1 pathway is activated while cells undergoing stress such as heat shock and oxidative stress [20-22]. Hyperosmolality stress is also reported to activate Akt1 pathway [6] in renal tubular cells. Accordingly, we investigated the activation of Akt1 under hypertonic condition within 60min, and found the phosphorylation of Akt1 and degraded expression of I κ B- α . These findings indicate that the hypertonic condition can stimulate Akt1 and NF- κ B pathway, and accordingly prevent apoptotic changes. However, the regulatory mechanism between survival and apoptosis in cells is complex and sophisticated. In our hypothesis, under the hypertonic condition for a short period of time, the activation of Akt1 mainly prevents the apoptotic process and rescues cells from radial stress. While under persisting hyperosmolality for a long time, the cells' apoptotic change may prevail, and the radiosensitivity consequently increases. In addition to the activation of NF- κ B, Akt1 can also phosphorylate MDM to inhibit p53 and promote cells' survival [23].

Since short-term hyperosmolality can induce activation of Akt1 and subsequently stimulate NF- κ B to promote cells' survival, we wonder what initially activates the Akt1 under hyperosmolality. Acute hyperosmolality immediately gives rise to osmotic shrinkage, which further triggers RVI. In fact, RVI takes place instantaneously and is followed by rapidly activation of ion channels to uptake in water [24]. Subramanyam M [25] had demonstrated that RVI activated Akt1, and Numata T [9] found that hypertonic stress reduced staurosporine-induced apoptosis. Thereby we speculated that an initial RVI could activate the Akt1 and reduce the cells' radiosensitivity. Firstly, we recorded the changes of cells' volumes under isotonic or hypertonic condition at different time points. The cells volumes began to recover within 1min when undergoing osmotic shrinkage. Along with RVI, the Akt1 was phosphorylated simultaneously. In order to confirm the association, we used FFA, a blocker to cation channels (HICCs), to inhibit the RVI because HICCs had been found to be the main mediators of RVI [26]. The findings suggested that FFA inhibited the RVI, which was associated with the attenuation in activation of Akt1 and decreased viability of irradiated AHH-1 cells pretreated with hyperosmolality. These results above indicate the important role of RVI in Akt1 pathway and apoptosis of cells. However, the specific mechanism that RVI regulates the activation of Akt1 and apoptotic signal is still unclear. In present study, FFA inhibited the stimulation of Akt1 and the rescue of cells from radiation under hyperosmolality. It is consistent with a previous study in which inhibition of the HICCs sensitizes HeLa cells to shrinkage-induced apoptosis [27]. Actually osmotic shrinkage changes the ionic strength

and consequently perturbs the cellular rates of biochemical reactions and the stability as well as conformation of macromolecules [28-30]. Cells take up cations by activating HICCs [26], inducing influent cations (Na^+ , K^+) and finally raise cellular osmolality following up-take of water. Cations are necessary for cellular metabolism as is well known. In rat hepatocytes, hypertonic activation of HICCs was proposed to be involved in the activation of PKC [31], PLC, G proteins, and tyrosine kinases [31, 32]. Therefore the inflow of cations induced by RVI may directly lead to the activation of Akt1 and promotes survival of irradiated cells.

In conclusion, we demonstrate that a short-term hyperosmolality pretreatment can reduce the cells' radiosensitivity. The mechanism is that hypertonic condition induces RVI in which the inflow of cations further activates Akt/NF- κ B pathway to promote the irradiated cells' survival.

Conflict of Interest

The authors have no conflict of interest to disclose.

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