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Original

Effect of Hydrogen Peroxide and High Glucose on the Glucose Metabolism of Lymphoma-derived U937 Cells

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Abstract: Our study aimed to clarify specific oxidative stress and glucose metabolic disorders in hemodialysis patients, by examining hydrogen peroxide (H_2O_2)- and high glucose-induced oxidative stress, glucose transport and the failure of glycolysis. As an *in vitro* blood cell model of end-stage renal disease (ESRD) in patients with diabetes, human monocytic U937 cells of malignant lymphoma origin were exposed to high glucose (28.9 mM) for 6 days, with 5 mM H_2O_2 added on the last day. The generation of intracellular reactive oxygen species (ROS), glucose levels, lactate levels, AMP-activated protein kinase (AMPK) activity and Glut4 levels were examined. Exposure of U937 cells to H_2O_2 resulted in a significant increase in intracellular ROS generation and glucose levels. Under high glucose conditions, treatment with H_2O_2 significantly promoted these actions. In H_2O_2-induced U937 cells, AMPK activity and Glut4 levels were significantly increased, but lactate and pyruvate levels were significantly decreased. Thus, exposure of U937 cells to H_2O_2 and a high glucose load promoted an increase in intracellular ROS, and exposure to H_2O_2 induced increased glucose transport and high intracellular glucose due to reduced glycolytic metabolism. This suggests that reduced glycolytic metabolism might be induced in states of high oxidative stress in hemodialysis patients with diabetes.

Key words: glucose, hydrogen peroxide, lactate, oxidative stress, U937 cells

Introduction

Patients with diabetes mellitus (DM) undergoing hemodialysis are exposed to significant oxidative stress[1-6)]. We previously reported increased Cu / Zn-superoxide dismutase (SOD) in the blood and upregulated Cu / Zn-SOD mRNA in the leukocytes of hemodialysis patients, as well as a correlation between leukocyte membrane lipid peroxidation and expression of Cu / Zn-SOD mRNA in hemodialysis patients; this causes oxidative stress which may also occur due to blood cell contact with the dialysis membrane[7, 8)]. Dialysis-associated oxidative stress is associated with complications such as arteriosclerosis[9-11)], however few studies have investigated the effect of oxidative stress on glucose metabolism in dialysis patients. Sugar transport increases

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in response to depletion of the intracellular ATP supply, shifting to other intracellular glucose metabolic pathways, such as the polyol pathway from the glycolytic system, hexosamine pathway, pentose phosphate pathway, and protein kinase C pathway[12, 13], however many aspects of this shift are not well defined. Strict control of blood glucose is necessary in dialysis patients to improve prognosis and prevent myocardial infarction, cerebral infarction, and lower extremity arterial disease[14–17]. Therefore, given the potential changes in glycolytic metabolism due to the influence of dialysis on blood glucose, it is important to investigate the effect of oxidative stress on glycolytic metabolism.

In this *in vitro* study, we cultured malignant lymphoma-derived U937 cells in a high glucose medium to model end-stage renal disease (ESRD) associated with DM. We then induced oxidative stress by adding hydrogen peroxide (H_2O_2) to these cells and analyzed the effects on reactive oxygen species (ROS), glucose transport, and glucose metabolism.

Methods

Study materials and cell culture

Human monocytes (U937 cells, EC85011440) were purchased from the European Collection of Animal Cell Culture (ECACC, UK). The following reagents were also used in the study: H_2O_2 (Wako Co., Osaka, Japan); fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO); penicillin G sodium (Invitrogen, Carlsbad, CA); streptomycin sulfate (Invitrogen); and 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate (CM-H2DCF-DA; Molecular Probes, Eugene, OR). In order to treat the U937 cells with a high glucose load, cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640) containing 28.9 mM glucose for 5 days (high glucose group), while the normal glucose group consisted of U937 cells cultured in standard RPMI-1640 medium containing 11.1 mM D-glucose. Previous experimental studies showed no dead cells due to apoptosis when the mannitol osmotic pressure was adjusted to equal the osmotic pressure of the glucose (30 mM)[18]. U937 cells were cultured by routine methods with 10% FBS, 100 units / mL of penicillin G sodium, and 100 μg / mL of streptomycin sulfate, at 37°C and 5% CO_2.

Drug treatment

U937 cells in the normal and high glucose groups (two groups each) were cultured for 5 days, and each medium was replaced with or without H_2O_2 (5 mM), and then cells were incubated for 24 h, after that all the parameters were measured. The cultural experiment was carried out under aseptic conditions.

Evaluation of ROS

CM-H2DCFDA was used to evaluate intracellular ROS formation in U937 cells treated with H_2O_2 (5 mM). After cellular uptake, CM-H2DCFDA is first modified to 2′,7′-dichlorofluorescein by intracellular esterases, and then by intracellular ROS to create oxidized fluorescent 2′, 7′-dichlorofluorescein[19]. After dissolving CM-H2DCFDA with dimethyl sulfoxide, 7 μM

CM-H2DCFDA was added to the cells (3×10^6 cells / mL) and incubated for 15 min at 37℃, 5% CO_2. After treatment, the cells were washed with heated phosphate-buffered saline. Fluorescence intensity was measured on a plate reader (Berthold Technologies, Bad Wildbad, Germany) at an excitation wavelength of 488 nm and a measuring wavelength of 525 nm.

Glucose uptake and glycolysis

1. Intracellular glucose

Intracellular glucose concentrations in U937 cells after 5 mM H_2O_2 treatment were measured by high performance liquid chromatography with electrochemical detection (HPLC-ECD), using a separation column ACT-gelPAC (ϕ 4.6 × 150 mm, Act-Science, Tokyo, Japan) and a glucose oxidase-stabilized enzyme-immobilized column GO-ENZ (ϕ 4.0 × 5 mm, Eicom Co., Kyoto, Japan). Endogenous H_2O_2 and glucose-derived H_2O_2 were separated with 100 mM phosphate buffer solution (pH 6.5) in the mobile phase.

2. AMP-activated protein kinase (AMPK)

AMPK was measured with an AMPK Kinase Assay Kit (CycLex, Nagano, Japan). Extracted samples were added to a microplate coated with AMPK substrate consisting of mouse Insulin Receptor substrate-1 (IRS-1) serine 789-peptide, which induced the phosphoric acid reaction. After detection using phosphorylated IRS-1 mouse monoclonal antibody and horseradish peroxidase-labeled anti-mouse IgG, the sample was stained using tetra-methylbenzidine and measured at 450 nm on a plate reader (Berthold Technologies). Fluorescence intensity was measured for each protein.

3. Glut4

Glut4 was measured using an SLC2A4 (Human) ELISA kit (Abnova, Walnut, CA). Extracted samples and Glut4 were treated with specific biotin-conjugated polyclonal antibody and avidin-conjugated horseradish peroxidase, and then placed in a microplate. After incubation, tetra-methylbenzidine substrate was added, and the sample was measured at 450 nm.

4. Intracellular pyruvate

Intracellular pyruvate concentrations were measured with a Pyruvate Assay Kit (BioVision Research Products, Milpitas, CA). Extracted samples were mixed with 50 μL Reaction Mix (Pyruvate Assay Buffer 46 μL, Pyruvate Probe 2 μL, Enzyme Mix 2 μL), incubated in the dark for 30 min, and then measured at 570 nm on a plate reader (Berthold Technologies). The fluorescence intensity was measured for each protein.

5. Intracellular lactic acid

Intracellular lactic acid concentrations were evaluated in a series using an LT-GEL separation column (ϕ 4.6 × 50 mm ; Eicom) and a lactic acid oxidase-immobilized enzyme-immobilized column LT-ENZ (ϕ 4.0 × 5 mm ; Eicom) and measured by HPLC-ECD. Endogenous H_2O_2 and lactic acid-derived H_2O_2 were separated using 100 mM phosphate buffer solution containing 200 mg tetra-n-hexylammonium bromide / L (pH 7.0).

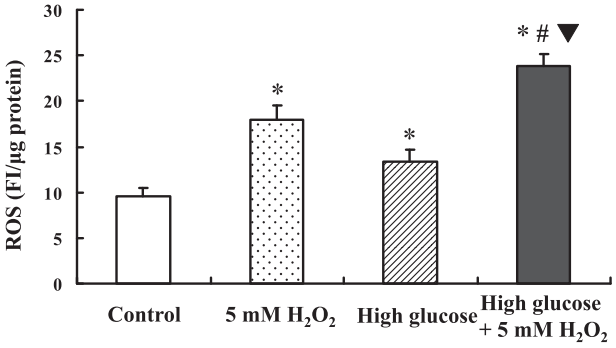

Fig. 1. Effect of hydrogen peroxide (H_2O_2) and high glucose on intracellular reactive oxygen species (ROS) production. U937 cells were cultured for 6 days in RPMI-1640 medium containing either 11.1 mM D-glucose (Control) or 28.9 mM glucose (High glucose). For the last 24 h, 5 mM H_2O_2 was added to the control group (5 mM H_2O_2) and the high glucose group (High glucose +5 mM H_2O_2). $^*P < 0.05$ vs. Control; #$P < 0.05$ vs. 5 mM H_2O_2; ▼ $P < 0.05$ vs. High glucose.

Statistical Analysis

Results are presented as means + standard error. Dunnett's tests were used, and statistical significance was set at $P < 0.05$.

Results

Effect of H_2O_2 and high glucose on intracellular ROS production

Compared with untreated cells, ROS production was significantly higher (2-fold) in the normal and high glucose groups treated with 5 mM H_2O_2. Compared with the normal glucose group, ROS production in the high glucose group was significantly higher (1.4-fold), confirming that H_2O_2 increases ROS production (Fig. 1).

Intracellular glucose uptake (glucose transport mechanism) and glycolysis

1. Effect of H_2O_2 and high glucose on intracellular glucose

Compared with untreated cells, intracellular glucose was significantly higher (8-fold) in H_2O_2-treated cells. Intracellular glucose was 2-fold higher in the high glucose group than in the normal group ($P < 0.05$; Fig. 2a), thus validating the H_2O_2-associated increase in intracellular glucose.

2. Effect of H_2O_2 and high glucose on AMPK production

AMPK production was significantly higher (1.3-fold) in H_2O_2-treated cells than in untreated cells, however there were no significant differences between the normal and high glucose groups (Fig. 2b).

3. Effect of H_2O_2 and high glucose on Glut4 production

Compared with untreated cells, Glut4 production was significantly higher (1.5-fold) in cells from the normal glucose group treated with 5 mM H_2O_2. Glut4 production tended to be higher

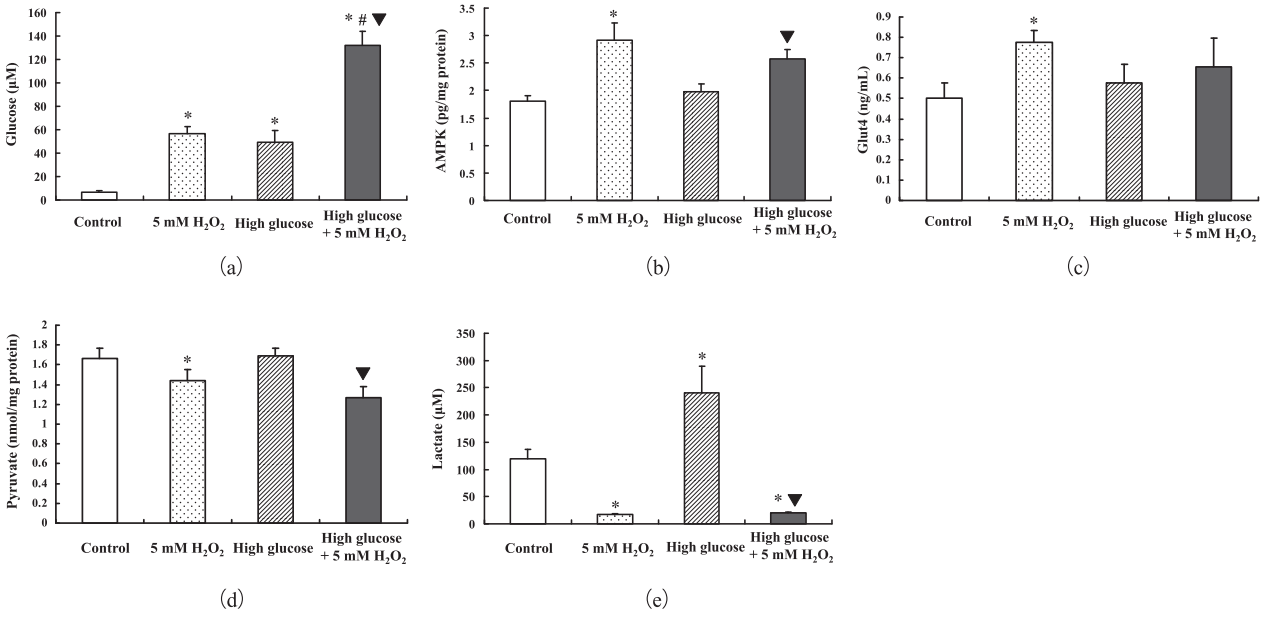

Fig. 2.

(a) Effect of hydrogen peroxide (H_2O_2) and high glucose on intracellular glucose. U937 cells were cultured for 6 days in RPMI-1640 medium containing either 11.1 mM D-glucose (Control) or 28.9 mM glucose (High glucose). For the last 24 h, 5 mM H_2O_2 was added to the control group (5 mM H_2O_2) and the high glucose group (High glucose + 5 mM H_2O_2) . $^*P < 0.05$ vs. Control ; $\#P < 0.05$ vs. 5 mM H_2O_2 ; ▼ $P < 0.05$ vs. High glucose.

(b) Effect of hydrogen peroxide (H_2O_2) and high glucose on AMP-activated protein kinase (AMPK) production. U937 cells were cultured for 6 days in RPMI-1640 medium containing either 11.1 mM D-glucose (Control) or 28.9 mM glucose (High glucose). For the last 24 h, 5 mM H_2O_2 was added to the control group (5 mM H_2O_2) and the high glucose group (High glucose + 5 mM H_2O_2). $^*P < 0.05$ vs. Control ; ▼ $P < 0.05$ vs. High glucose.

(c) Effect of hydrogen peroxide (H_2O_2) and high glucose on Glut4 production. U937 cells were cultured for 6 days in RPMI-1640 medium containing either 11.1 mM D-glucose (Control) or 28.9 mM glucose (High glucose). For the last 24 h, 5 mM H_2O_2 was added to the control group (5 mM H_2O_2) and the high glucose group (High glucose + 5 mM H_2O_2). $^*P < 0.05$ vs. Control.

(d) Effect of hydrogen peroxide (H_2O_2) and high glucose on intracellular pyruvic acid concentration. U937 cells were cultured for 6 days in RPMI-1640 medium containing either 11.1 mM D-glucose (Control) or 28.9 mM glucose (High glucose). For the last 24 h, 5 mM H_2O_2 was added to the control group (5 mM H_2O_2) and the high glucose group (High glucose + 5 mM H_2O_2). $^*P < 0.05$ vs. Control ; ▼ $P < 0.05$ vs. High glucose.

(e) Effect of hydrogen peroxide (H_2O_2) and high glucose on intracellular lactic acid concentration. U937 cells were cultured for 6 days in RPMI-1640 medium containing either 11.1 mM D-glucose (Control) or 28.9 mM glucose (High glucose). For the last 24 h, 5 mM H_2O_2 was added to the control group (5 mM H_2O_2) and the high glucose group (High glucose + 5 mM H_2O_2). $^*P < 0.05$ vs. Control ; ▼ $P < 0.05$ vs. High glucose.

in cells treated with 5 mM H_2O_2, but the change was not significant. Comparison of the normal and high glucose groups revealed no significant changes (Fig. 2c).

4. Effect of H_2O_2 and high glucose on intracellular pyruvic acid

Treatment with 5 mM H_2O_2 significantly reduced intracellular pyruvic acid concentrations in cells from both the normal and high glucose groups, compared to untreated cells. There was no significant difference between groups (Fig. 2d).

5. Effect of H_2O_2 and high glucose on intracellular lactic acid

Compared with untreated cells, the amount of intracellular lactic acid was significantly reduced in cells of the normal and high glucose groups treated with 5 mM H_2O_2. Intracellular lactic acid

was 1.5-fold higher than the control in the high glucose group ($P < 0.05$; Fig. 2e).

Discussion

This *in vitro* study using U937 cells revealed that intracellular ROS production was 2-fold higher after H_2O_2 treatment, whereas high glucose treatment caused a 1.4-fold increase in intracellular ROS, compared to cells treated with normal glucose levels. Previously, Sakai and colleagues[20] cultured human and mouse pancreatic β cells in a high glucose medium (30 mM glucose) for 4 days, and found that intracellular ROS production was 4 times higher than in cells cultured with 5 mM glucose. In this study, increased ROS production due to H_2O_2 was augmented by a high glucose load. Malignant lymphoma-derived U937 cells provide an *in vitro* model of blood cells for DM-associated hemodialysis, raising the possibility that high oxidative stress during treatment is worsened in patients with high blood glucose. This *in vitro* study also proves the importance of strict blood glucose control in order to reduce oxidative stress in hemodialysis patients with various complications such as arteriosclerosis[9–11].

AMPK is activated when intracellular ATP levels decrease; the enzyme phosphorylates members of the acetyl-CoA carboxylase metabolic pathway[21] and mammalian target of rapamycin[22]. Adjustment of these enzyme activities and the activity of transcription factors[23] and cofactors[24] modulates intracellular energy metabolism. Glut4 is a glucose transporter belonging to class I of the Glut family and mediates activation of AMPK and insulin. Glut4 promotes transcription and translocation to the plasma membrane, and is also involved in promoting glucose uptake. Horie *et al* treated rat cardiomyocytes with H_2O_2 and measured the time- and concentration-dependent effects. Their study showed that a long duration and high concentration of H_2O_2 increased the time required for Glut4 to travel to the cell membrane. Delayed Glut4 transport to the cell membrane in AMPK-deficient cells treated with 100 μM H_2O_2 (25 vs. 15 min for control) demonstrated the importance of AMPK in Glut4 translocation to the cell membrane after H_2O_2 treatment[25]. In our study, AMPK was also significantly higher (1.3-fold) in cells treated with 5 mM H_2O_2 for 24 h, while Glut4 was approximately 1.5-fold higher. Thus, reduction of either enzyme of glycolytic metabolism reduces intracellular ATP. Furthermore, inhibition of glycolytic metabolism is suspected to inhibit activation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) due to H_2O_2 load[20, 26]. We also demonstrated that production of lactic acid was suppressed by approximately 70%, whereas intracellular glucose increased 8-fold after H_2O_2 exposure. Decreased glycolytic metabolism due to H_2O_2 was studied by Sakai *et al*[20], who treated mouse pancreatic β cells with 50 μM H_2O_2 for 30 min and found an approximate 50% reduction in pyruvic acid, whereas the amount of lactic acid remained unchanged. In contrast, Colussi *et al*[26] treated U937 cells with 1 mM H_2O_2 for 1 h and found that intracellular glucose consumption was 97%, whereas lactic acid production was suppressed by 96%. Our study also observed these reductions, unlike the study by Sakai *et al*[20] which found no such changes. This discrepancy may be due to differences in cell type, H_2O_2 concentration, or duration of administration. Our study and that of Colussi *et al*[26] used U937 cells, but in well-differentiated cancer cells such as these, even under oxygenated conditions,

lactic acid is generated by translocation of lactate dehydrogenase from pyruvic acid produced by glycolysis, in what is known as the Warburg effect[27-29]. However, if glycolytic metabolism is strongly suppressed by H_2O_2, inhibition of lactic acid production is stronger in cancer cells, such as U937 cells with their initially high production of lactic acid, compared with other cells.

Mitochondria-derived ROS production increases under high glucose conditions, and then, resulting in inhibition of the glycolytic metabolic enzyme, GADPH. Additionally, ROS cause DNA damage, leading to activation of poly (ADP-ribose) polymerase[12]. After culturing mouse pancreatic β cells in 25 mM glucose for 2 days, Sakai and colleagues found that GADPH activity was reduced by 36% compared with cells incubated in 5 mM glucose[20]. In addition, Du *et al*[13] cultured bovine endothelial cells in 30 mM glucose for 2 days and found that GADPH activity was reduced by 66% compared with cells treated with 5 mM glucose. In these studies, GADPH activation was reduced by a high glucose load, but reduced activation of GADPH usually leads to a reduction in intracellular ATP. However, in our study, despite the increase in intracellular ROS production due to high glucose, the amounts of pyruvic acid and lactic acid were not reduced, suggesting no ATP reduction-dependent increase in AMPK or Glut4. Glycolysis downstream of pyruvic acid is maintained, as is intracellular ATP. The type of ROS produced might be different under conditions of high glucose load and hydrogen peroxide load, or it might be the unique property of cancer cells. Advanced glycation end products (AGE) can serve as an indicator for changes in GADPH activation due to high glucose load. High glucose induces various intracellular glucose pathways, including the glycolytic system by reducing GADPH activity via mitochondria-derived ROS production, activation of the hexosamine pathway, activation of protein kinase C, increased production of AGE, and activation of the polyol pathway[12]. We previously reported an increase of AGE associated with a high glucose load[30], indicating the possibility of reduced GAPDH activation.

As an *in vitro* blood cell model of DM-associated ESRD, this study has a number of limitations. Firstly, our use of malignant lymphoma-derived cancer cells as the study material, and secondly, replacing the mechanical stress of contact between blood cells and the dialysis membrane with an H_2O_2 load. A third limitation of the study is the inability to consider changes in glucose metabolism and glucose transport mechanisms due to the change in H_2O_2 concentration. Because blood cells come into contact with the hemodialysis membrane during dialysis treatment, the use of monocytic line-derived cells is a valid *in vitro* model of blood cells during hemodialysis, but there is the possibility of metabolic differences, despite the absence of the Warburg effect[27-29]. These results do not mean that mechanical stress influences ROS and glycolytic metabolism. However, despite these limitations, this model can serve as a useful *in vitro* screening tool for DM-associated ESRD.

In conclusion, this study demonstrated an increase of intracellular ROS due to H_2O_2 and high glucose load, and indicated an increase in glucose transport with reduced glycolytic metabolism due to H_2O_2. Reduced glycolytic metabolism might be induced in states of high oxidative stress in hemodialysis patients with DM.

Conflict of interest

The authors have declared no conflict of interest.

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