Insulin attenuates apoptosis induced by high glucose via the PI3-kinase/Akt pathway in rat peritoneal mesothelial cells

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Abstract

**Background.** Peritoneal mesothelial cells play an important role in peritoneal dialysis and are often exposed to dialysis fluid containing high glucose levels. Loss of peritoneal function is a major complication associated with long-term peritoneal dialysis. In this study, we hypothesized that high glucose levels induce apoptosis, and that insulin attenuates this apoptosis in peritoneal mesothelial cells. To clarify this hypothesis, we examined the effects of insulin on the phosphatidylinositol 3-kinase/Akt signaling pathway and apoptosis in rat peritoneal mesothelial cells.

**Methods.** Phosphorylated insulin receptor and Akt were detected by western blot analysis. Apoptosis was evaluated by measuring caspase 3 activity and by TUNNEL staining.

**Results.** Insulin (100 nmol/L) increased tyrosine phosphorylation of insulin receptor in peritoneal mesothelial cells. Furthermore, insulin (1-100 nmol/L) dose-dependently stimulated Akt phosphorylation. Treatment with the phosphatidylinositol 3-kinase inhibitors wortmannin (100 nmol/L) and LY294002 (10 µmol/L) attenuated insulin-induced Akt phosphorylation, indicating that insulin phosphorylates Akt via a phosphatidylinositol 3-kinase-dependent pathway. Insulin attenuated caspase 3 activity and decreased the number of TUNNEL-positive cells. The phosphatidylinositol 3-kinase inhibitors and overexpression of a dominant-negative mutant of Akt inhibited the effect of insulin on apoptosis.

**Conclusions.** The present data indicate that insulin attenuates high glucose-induced apoptosis via the phosphatidylinositol 3-kinase/Akt signaling pathway in peritoneal mesothelial cells. Therefore, the insulin signaling pathway may play a protective role in peritoneal function.

Keywords: apoptosis; glucose; insulin; peritoneal dialysis; peritoneal mesothelial cell

Introduction

Peritoneal dialysis (PD) is an efficient therapy for end-stage renal disease. However, a serious problem associated with PD is a time-dependent limitation of peritoneal function. During long-term PD, loss of peritoneal membrane integrity and loss of peritoneal ultrafiltration capacity are highly prevalent [1,2]. The peritoneal membrane surface is covered by a monolayer of peritoneal mesothelial cells (PMCs), which influence the submesothelial tissue, including the extracellular matrix, in various ways [3,4]. One of the possible mechanisms for peritoneal dysfunction is thought to be detachment of PMCs from the peritoneal membrane [5]. During PD, PMCs are continuously exposed to PD fluids (PDFs), which have high osmotic pressures due to their high glucose concentrations [6]. Numerous studies have shown that the constituents of PDFs are able to detach PMCs from the peritoneal membrane and subsequently decrease the number of intact PMCs residing on the peritoneum [7,8]. However, the molecular mechanisms of the cell detachment and signaling pathways in PMCs have not yet been established.

Apoptosis is programmed cell death and is characterized by morphological changes, such as cellular and nuclear condensation, fragmentation and shrinkage [9,10]. Many kinds of stimuli, including cytokines, hormones, infections, drugs and physical force, can induce apoptosis [11,12]. Moreover, high glucose concentrations have been reported to be a trigger of apoptosis in various cell types, including PMCs [13,14]. Although apoptotic or pre-apoptotic cells retain intact cellular membranes, they are prone to become detached from the peritoneal membrane. As PDFs contain high concentrations of glucose as an osmotic substance, it is likely that they may cause apoptosis of PMCs during PD.
Although glucose is known to have detrimental effects on the cellular function and survival of PMCs, the insulin signaling pathway and its effects in PMCs are still unclear. Generally, insulin regulates glucose metabolism and cell survival via the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in other cell types. Akt is a key molecule in this pathway and induces translocation of the glucose transporter (GLUT), regulation of cell survival and inhibition of apoptosis [15]. Insulin binds to and then activates insulin receptors and subsequently transfers signals via insulin receptor substrate (IRS), PI3K, phosphoinositide-dependent protein kinase-1 and Akt in most cell types [16–18]. Activated Akt mediates a number of metabolic actions, including glucose metabolism via translocation of GLUT and cell growth via mammalian target of rapamycin [19]. An earlier study demonstrated that activated Akt induced by insulin attenuates apoptosis by reducing caspase 3 activation in retinal neurons [20]. Furthermore, it has been established that the PI3K/Akt pathway plays key roles in regulating apoptotic changes in mesangial cells [21]. Although it has been reported that insulin plays roles in PMCs, such as induction of Na⁺/K⁺-ATPase activity [22], the insulin signaling pathway in PMCs has not yet been clarified, despite the presence of insulin receptors in these cells.

In this study, we hypothesized that high glucose concentrations induce apoptosis, and that insulin attenuates this apoptosis, via the PI3K/Akt pathway in rat PMCs (RPMCs). To clarify this hypothesis, we investigated the presence of insulin receptors in these cells.

In this study, we hypothesized that high glucose concentrations induce apoptosis, and that insulin attenuates this apoptosis, via the PI3K/Akt pathway in rat PMCs (RPMCs). To clarify this hypothesis, we investigated the presence of an insulin signaling pathway in PMCs and the effects of insulin on apoptosis induced by high glucose concentrations.

**Materials and methods**

**Materials**

Anti-phospho-Akt(Ser473), anti-Akt, anti-phospho-insulin receptor (Tyr1150/1151) and anti-insulin receptor antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). LY294002 was purchased from Calbiochem Corporation (San Diego, CA, USA). Enhanced chemiluminescence (ECL) reagents were purchased from GE Healthcare (Buckinghamshire, UK). Medium 199 containing 25 mM HEPES and 1.0 g/L glucose was purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals and reagents, including insulin, glucose, mannitol and wortmannin, were purchased from Sigma (St Louis, MO, USA).

**Cell culture**

RPMCs were isolated from the peritoneum of a male Sprague Dawley rat by enzymatic digestion as previously described [23]. Cells were grown in Medium 199 supplemented with 10% calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. RPMCs were confirmed by their morphological polygonal cobblestone appearance and their expression of the mesothelial-specific marker Hector Battifora-mesothelin-1. Cells between passages 3 and 6 were used for experiments in this study. In some experiments, RPMCs were infected with a replication-defective adenoviral vector expressing dominant-negative Akt [24], a kind gift from Dr Kenneth Walsh (Boston University, MA, USA).

**Western blotting**

RPMCs at 80–90% confluence in 100-mm dishes were made quiescent by incubation in medium 199 containing 0.1% calf serum for 24 h. Cells were stimulated with an agonist at 37°C in a serum-free medium for the specified durations. Some cells were preincubated with various inhibitors, as indicated. After the various treatments, the cells were lysed with 200 µL of ice-cold lysis buffer (50 mM HEPES pH 7.4, 5 mM EDTA, 100 mM NaCl) containing 1% Triton X-100, protease inhibitors (10 µg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin) and phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 µM microcystin). After centrifugation at 27 000 g at 4°C for 15 min, the protein concentrations in the supernatants were quantified by the Bradford assay. Proteins were separated by SDS-polyacrylamide gel electrophoresis using a 10% gel and transferred to nitrocellulose membranes. The membranes were blocked with phosphate-buffered saline (PBS) containing 5% nonfat dry milk and 0.1% Tween 20 for 1 h at room temperature, and then incubated overnight with the indicated primary antibodies at 4°C. After incubation with horseradish peroxidase-conjugated secondary antibodies, antigen–antibody complexes were detected using ECL reagents. The positive band intensities were quantified by densitometry of the immunoblots using NIH Image, version 1.61.

**Caspase 3 activity**

An APOPCYTO caspase 3 colorimetric assay kit (MBL, Nagoya, Japan) was used for the measurement of caspase activities. Briefly, RPMCs were plated in 100-mm dishes and cultured in the medium. After treatment, the cells were lysed with 150 µL of ice-cold lysis buffer. After centrifugation at 10 000 g at 4°C for 5 min, the protein concentrations in the supernatants were quantified by the Bradford assay. A 4-amino-acid sequence was labelled with p-nitroanilide (pNA) at the C-terminal side. Free pNA was released from the labelled synthetic substrate following cleavage by active caspase. The lysates were incubated with the reaction buffer, and then incubated with DEVD-pNA substrate for 2 h at 37°C. The absorbances were monitored at a wavelength of 405 nm.

**TUNNEL method**

TUNNEL staining was performed using a MEBSTAIN Apoptosis Kit Direct (MBL) according to the manufacturer’s protocol. This method detects nucleosome-sized DNA fragments by tailing their 3’-OH ends with digoxigenin nucleotides using terminal deoxynucleotidyl transferase (TdT). After treatment, the cells were fixed with 4% paraformaldehyde and incubated with the TdT buffer. The numbers of TUNNEL-positive cells were counted using fluorescence microscopy.
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Fig. 1. High glucose concentrations induce caspase 3 activation. Caspase 3 activity was measured using an APOPCYTO Caspase 3 Colorimetric Assay Kit. (A) RPMCs were incubated with high glucose (HG; 83 mM D-glucose) or normal glucose (NG; 5.6 mM D-glucose) for the indicated times. (B) RPMCs were incubated for 4 h in the presence of high glucose, normal glucose or mannitol (MAN; 83 mM D-mannitol) as an osmotic control. In all panels, the results represent means ± SE (n = 6), expressed as the relative intensity compared with untreated cells. *P < 0.05 versus normal glucose. **P < 0.05 versus high glucose.

Fig. 2. High glucose concentrations induce apoptosis. Apoptotic cells were evaluated by TUNNEL staining. RPMCs were incubated in a high-glucose medium for the indicated times. Representative photographs of TUNNEL-positive cells are shown (upper panel). In the lower panels, the data represent means ± SE (n = 4), expressed as the number of TUNNEL-positive cells. *P < 0.05 versus untreated cells. H.P.F. = high power field (×400).

[^3H]-Thymidine incorporation

Subconfluent RPMCs in six-well culture plates were made quiescent by culture in 0.1% serum M199 for 24 h, and then stimulated with several concentrations of glucose-containing medium (5.6 or 83 mM) in the presence or absence of insulin for 24 h together with 1 µCi/mL of[^3H]-thymidine. After labelling, the cells were washed twice with PBS and twice with ice-cold 5% trichloroacetic acid (TCA) to remove the unincorporated[^3H]-thymidine, solubilized in 500 µl of 0.25 N NaOH containing 0.1% SDS and neutralized. Aliquots of the samples were added to 10 ml of scintillation fluid and counted in a scintillation counter.

Statistical analysis

Results are expressed as means ± SE. The statistical significance of differences was assessed using Student’s t-test or ANOVA followed by Bonferroni’s test. A value of P < 0.05 was considered to be statistically significant.

Results

Effects of high glucose concentrations on apoptosis

First, we examined whether a high glucose concentration induces apoptosis in RPMCs. Since peritoneal dialysates containing 1.5% glucose as an osmotic substance are commonly used in clinical practice, we used 1.5% (83 mM) glucose-containing medium as the high-glucose stimulation in this study. As shown in Figure 1A, the high glucose concentration activated caspase 3, which is involved in apoptosis, compared with normal glucose concentration (5.6 mM). Next, we examined the effects of osmotic pressure on the apoptosis induced by the high-glucose medium. Mannitol, as an osmotic control for the high glucose concentration, did not affect caspase 3 activity in RPMCs (Figure 1B), indicating that the high glucose-induced apoptosis is independent of osmotic pressure. Furthermore, the high-glucose medium increased the numbers of TUNNEL-positive cells in a time-dependent manner (Figure 2A), indicating that high glucose induces apoptosis via caspase activation in RPMCs. Mannitol did not increase the number of TUNNEL-positive cells among RPMCs (data not shown), similar to the results for the caspase 3 activity.

The insulin signaling pathway in RPMCs

Although previous reports have shown that insulin plays important roles in peritoneal function [25], the insulin signaling pathway in RPMCs is still unclear. As shown in Figure 3, we confirmed the insulin receptor protein expression in RPMCs and found that insulin (100 nM) phosphorylated a tyrosine residue in the insulin receptor in a time-dependent manner. As shown in Figure 4A and B, insulin increased serine phosphorylation of Akt from 1 to 120 min in a dose-dependent manner in RPMCs. Since Akt is one of the downstream effectors of PI3K, we next examined the
effects of PI3K inhibitors on Akt phosphorylation. Pretreatment with the PI3K inhibitors wortmannin and LY294002 inhibited serine phosphorylation of Akt in dose-dependent manners (Figure 4C and D). These findings indicate that insulin induces phosphorylation of the insulin receptor and subsequently activates signal transduction via the PI3K/Akt pathway.

**Effect of insulin on apoptosis induced by high glucose**

Insulin is involved in glucose metabolism and signaling pathways via the Akt pathway. Akt is known to have an inhibitory effect on apoptosis in other cell types. To examine whether insulin attenuates the apoptosis induced by high glucose, we measured the caspase 3 activity in cells pretreated with insulin. Insulin significantly inhibited the activation of caspase 3 by high glucose (Figure 5). Insulin also decreased the number of TUNNEL-positive cells (Figure 6). Since insulin signals are known to affect the proliferation of various cell lines, we examined whether insulin could augment the cell proliferation of RPMCs. However, as shown in Figure 7, insulin did not increase [3H]-thymidine incorporation into RPMCs, indicating that it had no effect on RPMC proliferation.

Finally, to clarify the relationship between Akt and high glucose-induced apoptosis, we used cells overexpressing dominant-negative Akt, which mitigates the kinase activity

Fig. 3. Insulin induces insulin receptor phosphorylation. RPMCs were incubated with 100 nmol/L insulin for the indicated times. Western blot analysis was performed using anti-phosphorylated insulin receptor (top panel) or anti-insulin receptor (lower panel) antibodies. The results are means ± SE (n = 4), expressed as fold increases in phosphorylation over that in control cells. * P < 0.05 versus control cells.

Fig. 4. Insulin induces Akt phosphorylation via PI3K. (A) RPMCs were incubated with 100 nmol/L insulin for the indicated times. (B) RPMCs were incubated with the indicated doses of insulin for 5 min. (C) RPMCs were incubated with wortmannin at the indicated concentrations for 30 min followed by incubation with 100 nmol/L insulin for 5 min. (D) RPMCs were incubated with LY294002 at the indicated concentrations for 30 min followed by incubation in 100 nmol/L insulin for 5 min. (A–D) Western blot analyses were performed using site- and phospho-specific Akt antibodies against Ser473 (upper blot) or total Akt (lower blot). The results are means ± SE (n = 4), expressed as fold increases in phosphorylation over that in control cells. * P < 0.05 versus control cells. ** P < 0.05 versus insulin alone.
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Fig. 5. Effect of insulin on high glucose-induced caspase activation. RPMCs were incubated with normal or high glucose with or without insulin (100 nM) for 4 h (left panel). RPMCs were infected by adenoviruses to overexpress a dominant-negative Akt mutant, and then incubated with normal or high glucose with or without insulin (100 nM) for 4 h (right panel). In all panels, the results are means ± SE (n = 4), expressed as the relative intensity compared with untreated cells. *P < 0.05 versus normal glucose. **P < 0.05 versus high glucose.

Fig. 6. Effect of insulin on high glucose-induced apoptosis. RPMCs were incubated with normal or high glucose with or without insulin (100 nM) for 24 h (left panel). RPMCs were infected by adenoviruses to overexpress a dominant-negative Akt mutant, and then incubated with normal or high glucose with or without insulin (100 nM) for 24 h (right panel). Representative photographs of TUNNEL-positive cells are shown (upper panel). In the lower panels, the results are means ± SE (n = 6), expressed as the number of TUNNEL-positive cells. *P < 0.05 versus normal glucose. **P < 0.05 versus high glucose. H.P.F. = high power field (×400).

Fig. 7. Effects of insulin and high glucose on RPMC proliferation. Cell proliferation was measured by [3H]-thymidine incorporation. Cells were stimulated with a medium containing different concentrations of glucose (5.6 or 83 mM) in the presence or absence of insulin (100 nM) for 24 h together with 1 µCi/mL of [3H]-thymidine. The results are means ± SE (n = 4), expressed as the percent increase from normal glucose.

Discussion

In this study, we examined the effects of insulin on RPMC apoptosis induced by a medium containing a high glucose concentration. PMCs are exposed to non-physiological doses of glucose in PD patients. Generally, PDFs contain high concentrations of glucose as an osmotic substance, and high glucose concentrations are known to influence cell survival, growth and apoptosis, and affect cell signaling pathways in several cell types [13,26]. Although the effects of high glucose concentrations on cell survival and morphological changes in PMCs have been reported [14], the molecular mechanisms and cell signaling pathways are unclear. Previous reports revealed that high glucose concentrations can trigger human PMC apoptosis and may be involved in peritoneal damage via several mechanisms, including the hexosamine pathway, advanced glycation end-product (AGE) formation and the diacylglycerol/ceramide pathway [14]. Blockade of the interactions between AGES and their receptors partially prevented the apoptosis induced by high glucose, indicating that AGES are involved in the development of peritoneal cell damage [27]. Another possible mechanism of apoptosis is osmotic pressure induced by glucose [28]. Although osmotic pressure also affects cell signaling pathways, mannitol did not induce apoptosis in the present study, indicating that osmotic pressure had little effect on the apoptosis induced by high glucose. In the present study, we used a medium containing 1.5% (83 mM) glucose as a high-glucose stimulation because this glucose concentration is commonly used in commercially available PDFs, although more hypertonic PDFs with much higher concentrations of glucose, such as 2.5% and 4.0%, are also available. Moreover, our data showed that high glucose induced apoptosis in RPMCs via caspase 3 activation, which was independent of the osmotic pressure. We also measured caspase 9 activity, which is involved in the mitochondrial pathway of apoptosis. Caspase 9 activity was not significantly increased by high glucose (data not shown). Caspase 3 activation is the common and final
pathway of apoptosis. These data suggest that high glucose may induce apoptosis via a non-mitochondrial pathway. However, further experiments are required to elucidate the molecular mechanism of high glucose-induced apoptosis.

The number of PMCs is decreased by cell detachment from the peritoneal membrane. Apoptotic and pre-apoptotic cells are easy to detach, and necrotic cells are also separated from the membrane. Several papers have indicated that PDFs in patients contain PMCs, and it is possible to culture floating PMCs that have been collected from dialysate fluids [29,30]. Furthermore, apoptotic mesothelial cells are found in peritoneal effluents from stable dialysis patients [14]. These data strongly suggest that PMCs become detached from the peritoneal membrane through apoptosis or a pre-apoptotic state and not necessarily through necrosis. On the other hand, PDFs influence the mesothelial cell cycle and survival, including apoptosis, necrosis, cell growth and fibrosis [28,31]. A low pH (pH5.4), which is similar to that of standard PDFs, was found to induce necrosis in mesothelial cells, and high glucose had no effect on this necrosis, indicating that an acidic pH has a critical role in inducing necrosis in PMCs [14]. Since we used standard medium 199 (pH 7.2) containing a pH buffer in the present study, PMCs may not be induced to undergo necrosis after high-glucose stimulation. Cell growth induced by dialysate fluids was also reported as an inverse result to apoptosis [14]. Moreover, high glucose decreases vascular endothelial growth factor synthesis by human PMCs [32], suggesting that PDFs containing glucose may affect cell growth or fibrosis in peritoneal tissue. The present data clearly showed that high glucose induces apoptosis, and that insulin has a protective role against apoptosis in PMCs.

Insulin plays several roles in peritoneal function. Previous reports have shown that insulin stimulated Na⁺-K⁺-ATPase activity in time- and dose-dependent manners via post-transcriptional pathways [22]. These data indicate that insulin has a potential role in regulating electrolyte exchange between the abdominal cavity and the peritoneal membrane. Meanwhile, glucose uptake by human PMCs is not stimulated by insulin, whereas interleukin-1 beta increases glucose uptake, indicating that insulin has a small effect on glucose metabolism in PMCs [33]. Although the insulin signaling pathway is not well known in PMCs, the insulin receptor was found to be activated by insulin in the present study. Subsequently, activation of the insulin receptor transfers signals to PI3K and then Akt. Akt has various effects on the translocation of GLUT, cell survival and inhibition of apoptosis [15], and the present data showed that insulin inhibited apoptosis via the PI3K/Akt pathway. The previous findings together with our present data suggest that insulin has protective and beneficial effects on peritoneal function in dialysis patients. However, insulin activates biaxial signaling pathways such as the IRS/PI3K/Akt pathway and MAPKs pathway, and also induces cell growth, hypertrophy and fibrosis via MAPKs in various cell types [34]. These observations indicate that insulin is involved in peritoneal function and its complications via versatile mechanisms. In end-stage renal disease patients with diabetes mellitus, long-term PD is more often complicated by peritoneal dysfunction compared with non-diabetic patients, although the serum concentration of insulin in diabetes patients tends to be higher than that in non-diabetic patients [35,36]. In patients with diabetes mellitus, especially type II diabetes, insulin resistance is recognized as a key characteristic. Since normal insulin signaling pathways, such as the PI3K/Akt pathway, are attenuated in diabetic patients [37,38], the protective effect of insulin may be weakened, and apoptosis of PMCs may contribute to subsequent peritoneal dysfunction. Numerous previous papers clearly showed PMC apoptosis under in vitro conditions [7,14]. Clinical evidence of mesothelial cell apoptosis in patients treated with PD is rare and still unclear. Although animal experiments support the possible relevance of apoptosis to peritoneal function [7], further clinical experiments, such as peritoneal membrane biopsies or evaluation of apoptosis in dialysis fluids, are required to address these issues.

In summary, we have shown for the first time that insulin attenuates high glucose-induced apoptosis via the PI3K-Akt signaling pathway in PMCs. These results indicate that the insulin signaling pathway may play a protective role in peritoneal function in PD patients. While the potential clinical significance of these findings is unclear at present, these results should stimulate therapeutic and preventive strategies to protect against high glucose-induced apoptosis using intraperitoneal insulin administration in patients undergoing PD.

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Conflict of interest statement. None declared.

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