Original Paper

Sulfotanshinone IIA Sodium Ameliorates Glucose Peritoneal Dialysis Solution-Induced Human Peritoneal Mesothelial Cell Injury via Suppression of ASK1-P38-mediated Oxidative Stress

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Key Words

Peritoneal dialysis • Human peritoneal mesothelial cells • Apoptosis signal-regulating kinase 1 • P38 • Oxidative stress • Sulfotanshinone IIA sodium

Abstract

Background/Aims: Long-term use of high-glucose peritoneal dialysis solution (PDS) induces peritoneal mesothelial cell (PMC) injury, peritoneal dysfunction, and peritoneal dialysis (PD) failure in patients with end-stage renal disease. How to preserve PMCs in PD is a major challenge for nephrologists worldwide. In this study, we aimed to elucidate the efficacy and mechanisms of sulfotanshinone IIA sodium (Tan IIA) in ameliorating high-glucose PDS-induced human PMC injury. **Methods:** The human PMC line HMrSV5 was incubated with 4.25% PDS *in vitro* to mimic the high-glucose conditions in PD. Cellular viability was measured by Cell Counting Kit 8. Generation of superoxide and reactive oxygen species (ROS) was assessed using a Total ROS/Superoxide Detection Kit. Oxidative modification of protein was evaluated by OxyBlot Protein Oxidation Detection Kit. TUNEL (dT-mediated dUTP nick end labeling) assay and DAPI (4,6-diamidino-2-phenylindole) staining were used to evaluate apoptosis. Western blot analysis was performed to evaluate the efficacy and mechanisms of Tan IIA. **Results:** Tan IIA protected PMCs against PDS-induced injury as evidenced by alleviating changes in morphology and loss of cell viability. Consistent with their antioxidant properties, *N*-acetyl-L-cysteine (NAC) and Tan IIA suppressed superoxide and ROS production, protein oxidation, and apoptosis elicited by PDS. Apoptosis signal-regulating kinase 1 (ASK1)-p38 signaling was activated by PDS. Both Tan IIA and NAC suppressed ASK1 and p38 phosphorylation elicited by PDS. Moreover, genetic downregulation of ASK1 ameliorated cell injury and inhibited the

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phosphorylation of p38 and activation of caspase 3. **Conclusion:** Tan IIA protects PMCs against PDS-induced oxidative injury through suppression of ASK1-p38 signaling.

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Introduction

Peritoneal dialysis (PD) is one of three renal replacement therapies used in patients with end-stage renal disease (ESRD). Currently, PD is the major home-based dialysis therapy. It uses the peritoneum as the dialysis semipermeable membrane, across which ultrafiltration and diffusion occur. The function and effectiveness of PD is largely dependent on the peritoneum, which consists of peritoneal mesothelial cells (PMCs). Because of its safety, relatively low cost, and effectiveness, glucose has served as the osmotic agent in PD for nearly five decades. However, the long-term use of non-biocompatible conventional glucose-based peritoneal dialysis solution (PDS) is harmful for both the peritoneal membrane and the patients because it has a high concentration of glucose, glucose degradation products (GDPs) and lactate, high osmolality, and low pH [1, 2]. These lead to morphological and functional changes in the PMCs and limit the long-term outcome of PD in patients with ESRD. Since PMC injury is the leading cause of peritoneal dysfunction that results in PD failure [3], the development of new non-glucose-based PD solutions and novel methods for the protection and maintenance of PMCs has attracted much attention [4, 5]. Efforts aimed at improving the dialyzing capabilities of the peritoneal membrane by pharmacological intervention have not yet offered much in the way of clinical innovation.

Tanshinone IIA is one of the most abundant constituents and the main pharmacologically active natural compound of the root of *Salvia miltiorrhiza*, also known as Danshen, a widely used Chinese medicinal herb. Based on traditional Chinese medicine theory, Danshen is well known for its ability to activate blood circulation and reverse blood stasis [6]. Sulfotanshinone IIA sodium (Tan IIA) is a water-soluble derivative of tanshinone IIA. It has been used for some types of cardiovascular and cerebrovascular diseases through its pharmacological functions, including vasodilatation, anticoagulation, anti-inflammation, and anti-oxidative stress, with negligible adverse events [7-11]. In addition to its traditional uses, new findings reveal a protective role for Tan IIA in human PMCs under PD conditions [12, 13]. Anti-fibrosis, anti-senescence, anti-oxidative stress, and anti-inflammation likely underlie its pharmacological protection [14], but the detailed mechanisms need to be addressed. Oxidative stress is one of the mechanisms implicated in high-glucose-induced human PMC injury, but the specific redox signaling involved is still not well documented. In this regard, the current study aimed to evaluate the mechanisms by which Tan IIA ameliorates high-glucose PDS-induced PMC injury.

Materials and Methods

Cells

Human PMCs (HMrSV5, ATCC, BNCC340169) were purchased from BeNa Culture Collection (BNCC, Beijing, China) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) containing 100 U/mL penicillin G, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B for maintenance [15]. Cells were incubated at 37°C in 5% CO₂. All experiments were performed after cells were seeded in culture plates in PDS containing 5% FBS for 24 h.

Reagents and antibodies

Tan IIA was purchased from Shanghai Pharma No. 1 Biochemical & Pharmaceutical Co., Ltd., (Shanghai, China). Commercial PDSs were obtained from Baxter (Baxter, China). Antibodies against phospho-p38 mitogen-activated protein kinase (MAPK) (Thr180/Tyr182), phosphorylated apoptosis signal-regulating kinase 1 (phospho-ASK1) (Thr845/Ser967), β -tubulin, caspase-3, and horseradish peroxidase-conjugated anti-rabbit IgG were obtained from CST Reagents Company Limited (Shanghai, China). N-acetyl-L-cysteine

(NAC) was purchased from Sigma-Aldrich Co. LLC (Shanghai, China). Hyclone modified RPMI 1640 medium was purchased from GE Healthcare Life Science (Logan, UT) and Gibco FBS was purchased from Thermo Fisher Scientific (Scoresby, Australia). Pen-Strep Amphotericin B Solution was obtained from Biological Industries Israel Beit Haemek Ltd. (Beit Haemek, Israel).

Assessment of cell viability with Cell Counting Kit-8

HMrSV5 cells were seeded into a 96-well plate and allowed to grow for 24 h in RPMI 1640 containing 5% FBS. Cells were subsequently exposed to various stimuli in the presence or absence of the specified reagents for the indicated time. Cell Counting Kit-8 (CCK-8) reagent (Dojindo, Shanghai, China) was added to each well and incubated for additional 1 h before measurement of optical density with a spectrometer at a wave length of 450 nm. Cell viability was expressed as a percentage of the control group.

Detection of superoxide and reactive oxygen species production

The production of superoxide and reactive oxygen species (ROS) was detected by using a Total ROS/Superoxide Detection Kit (Enzo Life Sciences, New York, USA) following the manufacturer's instructions as described previously [16]. Briefly, HMrSV5 cells in 96-well plates were pre-incubated with superoxide detection reagent (orange) or oxidative stress detection reagent (green) for 1 h and exposed to various stimulants for the indicated time intervals. The immunofluorescent image was visualized and captured using an Olympus IX71 inverted fluorescence microscope (Tokyo, Japan) equipped with a standard red and green fluorescence cube.

Assessment of protein oxidation

The oxidative modification of proteins was evaluated using an OxyBlot Protein Oxidation Detection Kit (EMD Millipore, Billerica, MA) following the manufacturer's manual as described previously [16]. Briefly, the protein lysate was prepared by suspending the prewashed cells in sodium dodecyl sulfate (SDS) lysis buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol) together with freshly added proteinase inhibitor cocktail (Thermo Fisher Scientific, Shanghai, China) and 50 mM DL-dithiothreitol. Protein sample (5 µL) was transferred into Eppendorf tubes and denatured by adding 5 µL of 12% SDS for a final concentration of 6% SDS. The samples were derivatized by adding 10 µL of 2, 4-dinitrophenylhydrazine (DNPH) solution to each tube. After incubation at room temperature for 15 min, 7.5 µL of neutralization solution was added to each tube. The samples were then analyzed by western blot to detect carbonyl groups.

TdT-mediated dUTP nick end labeling (TUNEL) and 4, 6-diamidino-2-phenylindole (DAPI) staining of apoptotic cells

The apoptotic cells were detected by a TUNEL staining kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Briefly, the cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min and washed with PBS 3 times. TUNEL staining solution was added to the cells and incubated for 1 h at 37°C. After that, the media were discarded and the cells washed 3 times in PBS. Then, DAPI staining solution (KeyGEN bioTECH, Nanjing, China) was added to the cells and incubated for 5 min while avoiding light. After removing the staining solution, the cells were washed in PBS 3 times. Finally, cell fluorescence was acquired using a fluorescence photometer.

Transient transfection of cells with siRNA

HMrSV5 cells were transiently transfected with siRNA specifically targeting ASK1 (Santa Cruz Biotechnology, Dallas, TX), or a negative control siRNA (All Stars Negative Control siRNA; Qiagen, Hilden, Germany) at a final concentration of 150 nM using HiPerfect transfection reagent (Qiagen) for 24 h. Transfected cells were then either left untreated or exposed to various stimuli for the indicated times. Cellular protein was extracted and analyzed by western blot. Cell viability was evaluated by CCK-8.

Western blot analysis

Western blot analysis was performed as described previously [17]. Briefly, extracted cellular protein lysates were separated by 7.5% or 10% SDS-polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membranes using Bio-Rad (Shanghai, China) protein electrophoresis and blotting equipment. After blocking with 5% bovine serum albumin or 5% nonfat dry milk in PBS, the membranes were

incubated with primary antibody for 2 h at room temperature or overnight at 4°C. After washing, the membranes were probed with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature, and the bands were visualized using a Chemidoc Imaging System (Bio-Rad). β -tubulin or β -actin was used as an internal control to confirm the equal loading of proteins.

Statistical analysis

Values are expressed as the mean $\pm$ standard deviation. Comparison of two populations was made by Student's *t* test. For multiple comparisons, one-way analysis of variance followed by Dunnett's test was employed. Both analyses were done by using SPSS Statistics 23.0 software (IBM Corp., Armonk, NY). A *P* value of less than 0.05 was considered to indicate a statistically significant difference.

Results

Tan IIa alleviates high-glucose PDS-induced PMC injury

The PMC injury in PD is ascribed to the long-term use of nonphysiological-glucose-based PDS [18]. To mimic the *in vivo* conditions of PMCs in PD, we employed HMrSV5 cells cultured in PDS containing 5% FBS. As shown in Fig. 1A, PDS induced loss of cellular viability in a concentration-dependent manner, which mimics 1.5%, 2.5%, and 4.25% glucose PDS in PD. Incubation of HMrSV5 cells with 4.25% glucose PDS resulted in alterations in morphology such as cell shrinkage and detachment from the substrate (Fig. 1B). Notably, Tan IIa attenuated the morphological changes initiated by PDS. Loss of cellular viability triggered by PDS was also improved (Fig. 1C). These data show that pretreatment with Tan IIa ameliorates PDS-induced PMC injury.

Oxidative stress is implicated in high-glucose PDS-induced PMC injury

Since high glucose is known to be able to elicit cell injury via induction of oxidative stress [19, 20], we tested whether oxidative stress was involved in PDS-induced PMC injury. Using fluorescent probes for ROS and superoxide, we detected an increased fluorescence intensity in PDS-treated cells, reflecting an elevation in ROS and superoxide production (Fig. 2A). An increased ROS level is usually associated with oxidative modification of macromolecules such as protein, lipid, and DNA. As shown in Fig. 2B, PDS incubation indeed caused a time-dependent increase in protein carbonyl levels, an index of oxidative protein damage, as detected using the DNPH reaction followed by western blot analysis of the soluble protein fraction. Collectively, these results indicate that PDS induces oxidative stress in PMCs.

Fig. 1. (A) Effects of PDS on cell viability. HMrSV5 cells in 96-well plates were exposed to the indicated concentrations of PDS for 24 h. The cell viability was evaluated by CCK-8 assay. Data are expressed as percentage of living cells versus the untreated control (mean $\pm$ SD, *n* = 4). **P* < 0.05 versus control. (B) The role of Tan IIa in PDS-induced morphological changes. The HMrSV5 cells were pretreated with Tan IIa (20 μ g/mL) for 1 h and then challenged with PDS for 24 h. Cell morphology was analyzed using phase-contrast microscopy (magnification, $\times$ 100). (C) Effects of Tan IIa on cell viability. HMrSV5 cells in 96-well plates were pretreated with Tan IIa (20 μ g/mL) for 1 h and then challenged with the indicated concentrations of PDS for 24 h. The cell viability was evaluated by CCK-8 assay. Data are expressed as percentage of living cells versus the untreated control (mean $\pm$ SD, *n* = 4). **P* < 0.05 versus control.

Tan Ila protects PMCs through an anti-oxidative-stress mechanism

Since oxidative stress is involved in PDS-induced PMC injury and anti-oxidative stress is reported to be able to ameliorate PMC injury [21], we tested whether anti-oxidative stress was underlying the protection by Tan Ila in PMCs incubated with PDS. Similar to NAC, a classic antioxidant, Tan Ila suppressed PDS-induced ROS and superoxide generation (Fig. 3A). Protein oxidation is the result of oxidative injury. Tan Ila and NAC dramatically inhibited the oxidation of protein elicited by

Fig. 2. (A) Effects of PDS on superoxide and ROS production. HMrSV5 cells were loaded with superoxide and ROS detection reagent for 1 h and stimulated with 4.25% glucose PDS for the indicated time intervals. The cells were subsequently analyzed by fluorescence microscopy (magnification, $\times 100$). (B) Induction of oxidative modification of proteins by PDS. HMrSV5 cells were incubated for the indicated time intervals and then analyzed by OxyBlot Protein Oxidation Detection Kit and immunodetection of carbonyl groups.

Fig. 3. (A) Effects of Tan Ila and antioxidant NAC on superoxide and ROS production triggered by PDS. HMrSV5 in 96-well plates were loaded with superoxide and ROS detection reagent for 1 h, followed by pretreatment with Tan Ila (20 $\mu\text{g}/\text{mL}$) or NAC (5 mM) for 1 h and then challenged with PDS for 24 h. The cells were then analyzed by fluorescence microscopy (magnification, $\times 100$). (B) The role of Tan Ila and antioxidant NAC on oxidative modification of proteins induced by PDS. HMrSV5 cells in 12-well plates were pretreated with Tan Ila (20 $\mu\text{g}/\text{mL}$) and NAC (5 mM) for 1 h and then incubated with PDS for 4 h. Subsequently, the cellular lysates were analyzed by OxyBlot Protein Oxidation Detection Kit and immunodetection of carbonyl groups. (C) Effects of Tan Ila and NAC on cellular viability. HMrSV5 cells in 96-well plates were pretreated with Tan Ila (20 $\mu\text{g}/\text{mL}$) or NAC (5 mM) for 1 h and then challenged with PDS for another 24 h. The cell viability was evaluated by CCK-8 assay. Data are expressed as percentage of living cells versus the untreated control (mean $\pm$ SD, $n = 4$). * $P < 0.05$ versus control group. (D) Apoptosis staining of HMrSV5 cells. HMrSV5 in 48-well plates were pretreated with Tan Ila (20 $\mu\text{g}/\text{mL}$) or NAC (5 mM) for 1 h and then challenged with PDS for another 24 h. Then apoptotic cells were evaluated by TUNEL and DAPI staining (magnification, $\times 100$). (E)

The role of Tan Ila and antioxidant NAC on HMrSV5 cells apoptosis. HMrSV5 cells in 12-well plates were pretreated with Tan Ila (20 $\mu\text{g}/\text{mL}$) or NAC (5 mM) for 1 h and then incubated with PDS for another 24 h. The cellular lysates were then analyzed by western blot to detect caspase 3 and cleaved caspase 3.

Fig. 4. (A) Induction of p38 phosphorylation by PDS. HMrSV5 cells in 12-well plates were incubated with PDS for 1, 3, and 6 h. Cellular lysates were then analyzed by western blot for phosphorylated p38. (B) Effects of Tan IIA and antioxidant on p38 phosphorylation induced by PDS. HMrSV5 cells in 12-well plates were pretreated with Tan IIA (20 μ g/mL) and NAC (5 mM) for 1 h and then incubated with PDS for 4 h. Cellular lysates were then analyzed by western blot for phosphorylated p38. (C and D) Phosphorylation of ASK1 and phosphorylation of p38 induced by PDS. HMrSV5 cells in 12-well plates were incubated with PDS for 0, 0.5, 1, 4, and 12 h. Cellular lysates were then analyzed by western blot to detect phosphorylated Ser967 (C) and Thr845 (D) in ASK1 and phosphorylated p38. (E) The role of Tan IIA and NAC in phosphorylation of ASK1 on Thr845 and p38. HMrSV5 cells in 12-well plates were pretreated with Tan IIA (20 μ g/mL) and NAC (5 mM) for 1 h, and then challenged with PDS for an additional 30 min. Cellular lysates were then analyzed by western blot to detect phosphorylation on Thr845 in ASK1 and p38. β -tubulin was used as an internal control. (F) Effects of ASK1 siRNA on PDS-induced cell injury. HMrSV5 cells were transfected with either ASK1 siRNA or control siRNA for 24 h. The transfected cells were then incubated with PDS for 24 h. The cellular viability was evaluated using a CCK-8 assay. Data are expressed as percentage of living cells, compared with the siRNA control (mean $\pm$ SD, n = 4; * P < 0.05 versus siRNA control). (G and H) Effect of downregulation of ASK1 on p38 phosphorylation. HMrSV5 cells were transfected with either ASK1 siRNA or control siRNA for 24 h. The transfected cells were incubated with (H) or without (G) PDS for 4 h. Cellular lysates were analyzed by western blot for phosphorylated p38. Equal loading of protein per lane was verified by probing the blots with an anti- β -tubulin antibody.

PDS (Fig. 3B). Moreover, both Tan IIA and NAC significantly alleviated the loss of cellular viability elicited by PDS (Fig. 3C). DAPI is a dye that specifically binds DNA and can be used to detect apoptotic cells according to the strength of fluorescence and the conformation of the nucleus. PDS indeed induced HMrSV5 cell apoptosis, as shown in Fig. 3D, and both Tan IIA and NAC inhibited PDS-induced apoptosis, as evidenced by the weak TUNEL staining compared with the control. Furthermore, apoptosis induced by PDS, as evidenced by the appearance of cleaved-caspase 3, was suppressed by Tan IIA and NAC (Fig. 3E). Collectively, these results further confirm the anti-oxidative stress and anti-apoptosis role of Tan IIA.

ASK1-p38 MAPK signaling underlies the effect of Tan IIA on PMCs

Activation of oxidative-stress-sensitive kinases is the result of overproduction of ROS [22]. Indeed, PDS activated p38 in a time-dependent manner (Fig. 4A). Tan IIA and NAC attenuated the activation of p38 triggered by PDS (Fig. 4B). ASK1, a MAPK kinase kinase (MAPKKK), is activated in response to a variety of stress-related stimuli, including oxidative stress, which in turn activate c-Jun NH2-terminal kinase (JNK) and p38 [23]. It plays a crucial role in apoptosis, cell survival, differentiation, and cellular stress response. Phosphorylation on Ser967 of ASK1 suppresses cell death. Oxidative stress induces dephosphorylation of Ser967 [24] and phosphorylation of Thr845 on the activation loop of ASK1, and results in

ASK1-dependent apoptosis [25]. In the present study, PDS treatment led to dephosphorylated Ser967 and phosphorylated Thr845 on ASK1 and led to phosphorylated p38 (Fig. 4C and D). Moreover, Tan IIA and NAC suppressed the phosphorylation of ASK1 on Thr845. To further implicate ASK1 in PDS-induced PMC injury, we transfected cells with ASK1 siRNA. As shown in Fig. 4F, knockdown of ASK1 indeed ameliorated the loss of cellular viability triggered by PDS. In the presence or absence of PDS, downregulation of ASK1 suppressed the phosphorylation of p38 (Fig. 4H) and even the basal level (Fig. 4G). Furthermore, genetic downregulation of ASK1 (Fig. 5A) inhibited apoptosis, as evidenced by the suppression of caspase 3 activation (Fig. 5B) and TUNEL staining of the cells (Fig. 5C) induced by PDS. These data demonstrate that ASK1-p38 signaling is implicated in PDS-induced PMC injury and Tan IIA inhibits ASK1-p38 signal transduction.

Discussion

In this study, the two major findings are that ASK1-p38 MAPK signal transduction is involved in high-glucose-PDS-induced HMrSV5 injury and that Tan IIA protects HMrSV5 against toxicity of high-glucose PDS through suppression of ASK1-p38-mediated oxidative stress. Collectively, these data provide novel potential therapy for the maintenance and protection of PMCs in PD.

The success of PD is largely dependent on the structural and functional integrity of mesothelium, which is formed by a monolayer of PMCs [26]. However, an important problem associated with PD is a time-dependent limitation of peritoneal function including loss of peritoneal membrane integrity and peritoneal ultrafiltration capacity [27]. Accumulated studies documented that long-term use of glucose-based PDS leads to mesothelial denudation, interstitial fibrosis, neoangiogenesis, advanced glycosylated endproduct (AGE) accumulation and inflammation, increase in solute transport rate, and loss of ultrafiltration capacity. Clinically, long-term insufficient PD induces fluid and sodium retention, left ventricular hypertrophy, hyperglycemia, hyperlipidemia, hyperinsulinemia, and abdominal obesity. New PDSs have been developed over the past two decades to improve their biocompatibility and to avoid the disadvantages of glucose-based solutions. Icodextrin and amino acids have been developed and are commercially available alternatives to glucose as osmotic agents. However, the development of PMC-protective therapy is limited and most studies have focused on pharmacological interventions aimed at modulating peritoneal permeability.

Fig. 5. Effects of ASK1 siRNA on PDS-induced apoptosis. (A) HMrSV5 cells were transfected with either ASK1 siRNA or control siRNA for 24 h. The transfected or WT cells were incubated with PDS for 24 h. (B) Cellular lysates were then analyzed by western blot to detect caspase 3 and cleaved caspase 3. (C) After transfection, cells were challenged with PDS for 24 h and then analyzed by TUNEL and DAPI staining (magnification, x100).

Some antioxidants and compatible osmolytes have been developed as candidates for protection of PMCs against toxicity of high-glucose PDS.

High-glucose PDS triggers oxidative stress that is induced by both glucose itself and GDPs. The possible mechanisms can be ascribed to ROS over-generation and dysregulation of mitochondrial metabolism. Thus, some efforts have been aimed at reducing oxidative injury [4]. It has been reported that atorvastatin, a lipid-lowering agent and HMG-CoA reductase inhibitor, protected rat PMCs via suppression of oxidative-stress-mediated apoptosis under high-glucose conditions. Pyruvate, a natural hydrogen peroxide scavenger, preserved mesothelial cells incubated in medium containing hydrogen peroxide [28, 29]. Oral administration of trimetazidine, a fatty acid oxidation inhibitor, to rats has been shown to inhibit the oxidative stress triggered by high-glucose PDS. *In vitro* experiments using L-2-oxothiazolidine-4-carboxylic acid (OTZ), a glutathione precursor, increased the cytoplasmic concentration of glutathione and substantially neutralized liberation of free radicals and improved mesothelial cell growth. *In vivo* experiments on rats showed that OTZ attenuated the morphological changes induced by PDS [30].

Traditionally, oxidative stress is defined as a disturbance in the pro-oxidant-antioxidant balance. However, accumulated studies on redox signaling pathways, antioxidant intervention trials, and oxidative stress markers indicate that disruption of redox homeostatic control and signaling can trigger metabolic pathway and organ specificity in oxidative diseases rather than the direct damage to macromolecular machinery. Thus, a more useful definition of oxidative stress is “a disruption of redox signaling and control” [31]. This can be expected to enhance development of therapeutic strategies for targeted control of oxidative stress in disease prevention.

Mammalian MAPK signal transduction pathways are evolutionally conserved intracellular signaling systems that can be activated by a wide variety of stimulants and regulate cellular stress response to maintain homeostasis. ASK1 is a member of the MAPKKK family that activates both MKK4/MKK7-JNK and MKK3/MKK6-p38 MAPK signaling cascades in response to various stimuli such as endoplasmic reticulum stress, oxidative stress, infection, and calcium influx. ASK1 is activated under ROS overproduction and plays a crucial role in cellular responses to oxidative stress [32]. Phosphorylation at Ser967 causes ASK1 association with 14-3-3 protein and suppression of cell death. Oxidative stress induces dephosphorylation of Ser967 and phosphorylation of Thr845 in the activation loop of ASK1, and both are implicated in ASK1 activity and ASK1-dependent apoptosis. High glucose induces oxidative stress in PMCs and activates p38 MAPK signal transduction. Previously we reported that the ASK1-p38 pathway is involved in oxidative podocyte injury and suppression of ASK1-p38 signaling improves cell survival in response to oxidative stress [16]. In the current study, we found that ASK1-p38 signaling is also implicated in high-glucose-PDS-induced PMC injury. The ability of Tan IIA and NAC to suppress ROS and superoxide generation induced by PDS, and the fact that both Tan IIA and NAC constrained oxidative-stress-related redox signaling (ASK1-p38 signal transduction) indicated that Tan IIA protects PMCs under PD conditions via inhibition of ASK1-p38-mediated oxidative stress.

High-glucose-based PDS can induce hyperosmotic stress in PMCs as well. Hyperosmotic environments trigger compensatory molecular adaptations in cells that allow them to maintain intracellular homeostasis. Failure of these mechanisms results in inhibition of growth and increased apoptotic death. During osmotic stress, cells generate organic osmolytes to neutralize the osmotic gap and recover the physiological steady state. Betaine, one of the more relevant compatible osmolytes, is synthesized by cells in response to hyperosmotic insult. It has been reported that addition of betaine to cultured S-3T3 cells incubated in a hyperosmotic medium (0.5 osm), similar in tonicity to that of 4.25% glucose PDS (0.483 osm), prevented the 90% inhibition of cell proliferation and protected endothelial cells from apoptotic death caused by environmental hypertonicity [33]. Other studies showed that regulation of cell volume is associated with apoptosis and necrosis [34-36]. In response to the decrease in volume induced by hypertonic conditions, cells try to recover their volume by uptake of sodium and chloride ions and osmotically obliged

water, which is referred to as regulatory volume increase (RVI). However, cells undergoing apoptosis lack RVI and apoptotic cells undergo persistent cell shrinkage. This shrinkage is referred to as apoptotic volume death (AVD), which is a morphological hallmark of an apoptotic process. It is noteworthy that ASK1 is also involved in the regulation of cell volume and can be activated by hyperosmolality. ROS scavengers attenuated hypertonic condition-induced ASK1 activation. Knockdown of ASK1 by siRNA phosphorylated Akt1, restored hyperosmolality-induced RVI, and suppressed apoptosis. ROS-mediated activation of ASK1 in apoptotic situations suppresses the RVI reaction, resulting in persistent cell shrinkage under hyperosmotic conditions [37]. Notably, incubation of HMrSV5 with PDS caused cell shrinkage in our study. Further studies are needed to evaluate whether Tan IIA also regulates PMCs in response to osmotic stress.

In general, the pharmacological interventions aimed at neutralizing oxidative and osmotic injury are thus far more promising than interventions designed to improve the dialyzing capabilities of the peritoneal membrane. Our results demonstrated that Tan IIA protects human PMCs against toxicity of high-glucose PDS via inhibition of ASK1-p38 MAPK signal transduction.

Abbreviations

PD (peritoneal dialysis); ESRD (end-stage renal diseases); PMC (peritoneal mesothelial cell); GDPs (glucose degradation products); Tan IIA (sulfotanshinone IIA sodium); HMrSV5 (human peritoneal mesothelial cell line); PDS (peritoneal dialysis solution); FBS (fetal bovine serum); NAC (*N*-acetyl-L-cysteine); RPMI (Roswell Park Memorial Institute); CCK-8 (Cell Counting Kit-8); ROS (reactive oxygen species); DAPI (4,6-diamidino-2-phenylindole); ASK1 (apoptosis signal-regulating kinase 1); AGE (advanced glycosylated end product); OTZ (L-2-oxothiazolidine-4-carboxylic acid); MAPK (mitogen-activated protein kinase); MAPKKK (MAPK kinase kinase); RVI (regulatory volume increase); AVD (apoptotic volume death); JNK (c-Jun NH2-terminal kinase); ANOVA (analysis of variance); SDS (sodium dodecyl sulfate).

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Disclosure Statement

The authors declare that they have no competing interests to disclose.

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