GSK-3 β inhibition protects mesothelial cells during experimental peritoneal dialysis through upregulation of the heat shock response

K. Rusai · R. Herzog · L. Kuster · K. Kratochwill · C. Aufricht

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Abstract Non-physiological components of peritoneal dialysis fluids (PDF) lead to the injury of peritoneal mesothelial cells resulting in the failure of peritoneal dialysis (PD) potentially via inadequate induction of the protective heat shock response (HSR). Glycogen synthase kinase-3 β (GSK-3 β) is a negative regulator of cell survival partly by suppression of the HSR and is influenced by stress stimuli also present in conventional PDF. The effects of PDF on GSK-3 β activation and the impact of GSK-3 β inhibition with lithium (LiCl) were investigated on cell survival with special regard to HSR, in particular to heat shock transcription factor 1 (HSF-1) activation and Hsp72 production in an in vitro model of PD using MeT-5A and primary mesothelial cells. Incubation of cells with the PDF Dianeal® (glucose-based, low pH, high glucose degradation products (GDP)) and Extraneal® (icodextrin-based, low pH, low GDP) caused activation of GSK-3 β compared to the other tested PDF, i.e. Balance®, Physioneal® (normal pH, glucose-based, low GDP) and Nutrineal® (moderately acidic, amino acid-based). Inhibition of GSK-3 β with LiCl in Dianeal® and Extraneal®-treated cells dose-dependently decreased cell damage and death rate and was paralleled by higher HSF-1 activation and Hsp72 expression. GSK-3 β is activated by low pH GDP containing PDF with and without glucose as osmotic agent, indicating that GSK-3 β is involved in mesothelial cell signalling in response to experimental PD. Inhibition of GSK-3 β with LiCl ameliorated cell injury and improved HSR upon PDF exposure. Thus, GSK-3 β inhibitors likely have therapeutic potential as cytoprotective additive for decreasing PDF toxicity.

Keywords GSK-3 β inhibition · Heat shock response · Lithium · Mesothelial cells · PD fluids

Introduction

Peritoneal dialysis (PD) is a renal replacement therapy alternative to haemodialysis using the peritoneum as dialysis membrane offering a higher quality of life with a better cost-effectiveness (Chaudhary et al. 2011). Unfortunately, PD treatment frequently results in progressive decline of ultrafiltration capacity of the peritoneum usually associated with histological changes and loss of peritoneal integrity (Davies et al. 1998). Pathological changes in the peritoneum are mainly the result of the toxic properties of standard low pH PD fluids (PDF) containing high levels of the osmotic agent glucose and its degradation products (GDP) (Leung et al. 2005; Amore et al. 2003). Novel PDF are more biocompatible due to alternative osmotic agents (icodextrin or amino acids), buffer compositions (bicarbonate or lactate/bicarbonate) and often more sophisticated multi-chambered bags. These PDF are more expensive, and their clinical use is still limited in the global clinical setting. Searching for strategies to reduce the deleterious effects of PDF is still of high medical need and has a significant socio-economic importance.

Our group has previously shown that acute exposure of mesothelial cells to PDF not only results in injury, but also induces cellular defense mechanisms, such as the heat shock response (HSR) (Arbeiter et al. 2001, 2003; Bender et al. 2011; Bidmon et al. 2004; Boehm et al. 2010). The main effectors of the HSR are heat shock proteins (HSP), which in many cases exert chaperone functions, promoting cellular survival under stress conditions by preventing protein denaturation. Several studies of our group demonstrated that HSP-mediated cytoprotection has indeed a significant impact on mesothelial cell survival in experimental PD (Bender et al.

K. Rusai · L. Kuster · K. Kratochwill · C. Aufricht (✉)
Department of Pediatrics and Adolescent Medicine, Medical
University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria
e-mail: christoph.aufricht@meduniwien.ac.at

R. Herzog · K. Kratochwill
Zytoprotec GmbH, 1010 Vienna, Austria

2010, 2011). Cells in which HSP expression was decreased or remained unchanged after PDF exposure had a significantly higher susceptibility to PDF toxicity (Kratochwill et al. 2012). On the other hand, pharmacological (Boehm et al. 2010; Kratochwill et al. 2012) or plasmid-mediated (Bidmon et al. 2004) upregulation of the HSR or specific HSP protected cells from the toxic features of PDF.

Glycogen synthase kinase-3 β (GSK-3 β) is a serine–threonine protein kinase, involved in several signalling pathways, such as the Wnt signalling pathway, the PI3 kinase pathway or EGF receptor signalling. Its function as a central node in these pathways explains the relevance of this enzyme in important biological processes like cell differentiation, migration proliferation and cell death, inflammatory and immune responses, as well as regulation of glucose metabolism. GSK-3 β targets numerous players in the signalling cascade and directly modulates the activity of transcription factors, including heat shock transcription factor 1 (HSF-1) (Xavier et al. 2000). This key regulator of the heat shock response has been shown to be inactivated by GSK-3 β by specific phosphorylation, rendering the transcription of cytoprotective genes inactive. GSK-3 β itself is regulated through a phosphorylation step at the serine-9 residue by different upstream kinases (Juhászova et al. 2009). This regulation of GSK-3 β is influenced by cell stressors such as hypoxia, oxidative stress, heat shock, but also by factors such as changes in glucose concentration and high GDP content. Although these physicochemical properties are important non-physiological characteristics of various PDF (Pugazhenthil and Khandelwal 1995; Cuzzocrea et al. 2007), a direct association between GSK-3 β and PD has not yet been described.

The aim of our study was to investigate the effect of PDF on GSK-3 β activation in exposed mesothelial cells and the impact of GSK-3 β inhibition with lithium—a selective GSK-3 inhibitor (Ryves and Harwood 2001; Jope et al. 2007; Cohen and Goedert 2004)—on cell survival in the setting of experimental PD. As GSK-3 β is known to negatively regulate HSF-1-dependent HSR, GSK-3 β activation might suppress and GSK-3 β inhibition might enhance activation of the mesothelial HSR (He et al. 1998; Chu et al. 1998; Rowe et al. 2007).

We hypothesise that GSK-3 β —possibly activated by PDF—inhibits HSF-1-mediated transcription of cytoprotective HSP in exposed mesothelial cells. Accordingly, blocking the GSK-3 β activity with lithium might result in enhanced HSF-1-mediated HSP expression and in less cell damage, suggesting GSK-3 β inhibition as an attractive target for therapeutic intervention in PD.

Materials and methods

Standard chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. TPP tissue culture

plastics (TPP, Techno Plastics Products AG, Trasadingen, Switzerland) were used for all cell culture procedures.

For incubation with various PDF, glucose-based, low pH (pH 5.5) 3.86 % fluid (Dianeal PD4®); icodextrin-based low pH (pH 5.5) fluid (Extraneal®); amino-acid-based fluid (pH 6.7) (Nutrineal®); and glucose-based, neutral pH, lactate/bicarbonate-buffered fluid (Physioneal®) from Baxter (Vienna, Austria) were used. Glucose-based, lactate-buffered, neutral pH solution (Balance®) was obtained from Fresenius Medical Care (Bad Homburg, Germany). The control solution was M199 culture medium without FCS.

In separate experiments, pH of Dianeal® was normalised under laboratory circumstances. GDP-free PDF was laboratory-made filter-sterilised PDF of the same composition.

In vitro PDF exposure

Human mesothelial MeT-5A cells (ATCC CRL-9444 from LGC Standards (Wesel, Germany)) were cultured in M199 culture medium supplemented with 10 % FCS, 0.1 g/L L-glutamine, 2.2 g/L sodium bicarbonate, 5.96 g/L HEPES, 50 U/mL penicillin and 50 μ g/mL streptomycin and propagated at 37 °C in a humidified air containing 5 % CO₂ incubator.

Confluent cultures on 12-well plates were exposed for the indicated times (30 min or 1 h) to the PDF or kept in parallel in control medium. Recovery periods in normal culture medium containing 10 % FCS were 6 h for the HSF-1 luciferase assay, 16 h for measurements of LDH release and Western blot analysis of Hsp72 and/or p-GSK-3 β .

Human peritoneal mesothelial cells (HPMCs) were isolated from peritoneal effluent of consenting uraemic patients as described previously (Kratochwill et al. 2012). Patients were clinically stable and for more than 3 months free of peritonitis at the time of effluent collection. Briefly, peritoneal cells were concentrated by centrifugation of dialysis fluid effluent and then cultured in Earle's M199 medium supplemented to yield a final concentration of 10 % FCS, 50 U/mL penicillin and 50 μ g/mL streptomycin. Non-adherent cells were removed after 24 h by two brief washes with medium. When cells reached the confluence, they were split two times and PDF incubation experiments were performed. Only cobblestone-like epithelial cultures were used for incubation experiments, showing stable morphology during two to three cell passages.

PDF incubation of HPMCs was performed as described previously and published by our study group (Kratochwill et al. 2012). Briefly, confluent cultures on 12-well plates were exposed for 6 h to the studied PDF diluted to 1:1 with normal cell culture medium or kept in parallel in control medium, before testing for LDH release and Western blot analysis of Hsp72 and/or p-GSK-3 β were performed.

LiCl treatment

Lithium chloride (LiCl) was added for dose–response experiments in concentrations from 1 to 10 mM; for the remaining experiments, LiCl was administered at a concentration of 10 mM which is in the dose range that is applied in most in vitro studies (Lewitt et al. 2001; Mantelli and Ledda 1989; Gregory et al. 2005; Lucas et al. 2010; Li et al. 2011). LiCl treatment was started 24 h prior to incubation with PDF and was applied during the whole experiment.

Viability assays

Vital-dead staining

With the LIVE/DEAD® viability/cytotoxicity kit (Invitrogen), intracellular esterase activity (only viable cells are stained) and plasma membrane integrity (only dead cells are stained) were simultaneously stained with 4 μ M calcein AM and 2 μ M ethidium homodimer (EthD-1) in 250 μ L PBS per well of a standard 12-well plate. The plates were then analysed using an LSM-700 laser scanning microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) with the appropriate settings for excitation (Ex) and emission (Em) wavelengths of calcein AM (Ex/Em 494/517 nm) and EthD-1 (Ex/Em 528/617 nm).

LDH release

For LDH analyses, 200 μ L aliquots of supernatants were removed after the described experimental setup and kept on -80°C until analysed. Measurements were performed in duplicates using the TOX-7 LDH Kit (Sigma-Aldrich) according to the manufacturer's instructions. LDH efflux was normalised for protein content.

Neutral red uptake

To assess cell viability by neutral red uptake, cells were seeded on 96-well plates and exposed to different treatment protocols as described before. Neutral red uptake was measured using a standard reagent (Sigma-Aldrich) according to the manufacturer's protocol.

Luciferase assay

To determine the specific binding of the HSF-1, a commercially available luciferase reporter vector (LR0038, Panomics Solutions—Affymetrix, Santa Clara, CA, USA) containing the heat shock element [5' CTGGAATTTTCTAGACTGGAATTTTCTAGACTGGAATTTTCTAGA3'] as well as an empty control vector was transfected into MeT-5A cells. MeT-5A were seeded

onto 96-well cell culture plates 24 h prior to transfection and allowed to reach approximately 80–90 % confluence on the day of transfection. Before transfection per well, 0.15 μ L Eugene6 (Roche Applied Science, Mannheim, Germany) transfection reagent and 0.1 μ g HSE reporter vector or control vector were each diluted with 5 μ L of normal growth medium without FCS and incubated for 5 min. The solutions were mixed incubated for 30 min. Of this mixture, 10 μ L was added to each well incubated for 16 h at 5 % CO_2 and 37°C in a humidified atmosphere. Experimental exposures were carried out as described before, and the cells were allowed to recover for 6 h in order to facilitate protein synthesis of the luciferase enzyme. Cells were washed with PBS and lysed using a commercially available buffer (E1531, Promega, Madison, WI, USA). The cell lysates were transferred to white flat bottom microplates (Nunc, Roskilde, Denmark). The luciferase reagent (E1500, Promega) was added using an automatic pipette in 3 s intervals and the yielded signal was measured after 2 min using a luminometer plate reader (Flx800, BioTek, Winooski, VT, USA). An empty vector served as additional negative control (data not shown).

Western blot analysis

For Western blotting, protein content was determined using the 2D-Qant kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's manual and equal amounts of protein samples (2 μ g/lane) were separated by standard SDS-PAGE using a Bio-Rad Criterion cell and Criterion precast 12.5 % Tris–HCl gels (Bio-Rad, Hercules, CA, USA). In previous studies, 1 μ g of solubilised total cellular protein corresponded to $2.0 \times 10^3 \pm 0.9 \times 10^3$ (mean $\pm$ SD) cells (Bender et al. 2011). Proteins were electroblotted onto PVDF membranes (Millipore) immediately after the run by tank blotting using a Criterion blotting cell (Bio-Rad). The membranes were blocked with 5 % dry milk in TBS-Tween (10 mM Tris, 150 mM NaCl, 0.05 % Tween 20, pH 7.4). Membranes were incubated with the respective primary antibody (Hsp72: SPA810, Stress Gen Biotechnologies Corp., Victoria, BC, Canada; GSK-3 β : rabbit monoclonal antibody #9315, Cell Signaling Technologies, USA; p-GSK-3 β : (Ser9) rabbit monoclonal antibody mAb #9323, Cell Signaling Technologies) or for the loading control β -tubulin (1:2,000) (#691261, MP Biochemicals, Solon, OH, USA) for 16 h. Detection was accomplished by incubation with secondary, peroxidase-coupled antibodies (anti-mouse (Dako Cytomation, CA, USA) or anti-rabbit IgG (Santa Cruz, USA)) and enhanced chemiluminescence (ECL) using the ECL Western blotting reagent (Renaissance, NEN-Life Science Products, Boston, MA, USA) and a ChemiDoc XRS chemiluminescence detection system (Bio-Rad). Densitometric quantification of bands was accomplished using the Bio-Rad QuantityOne software.

Immunofluorescent staining

Optimal primary and secondary antibody incubation concentrations and conditions were previously determined and based on recommendations in the literature. According to the experimental setup, after incubation, cells were washed two times with PBS and fixed in ice-cold acetone/methanol. Non-specific binding sites were blocked for 45 min with 1 % bovine serum albumin in PBS (pH 7.4). p-GSK-3 β was immunodetected by incubation with monoclonal anti-p-GSK-3 β antibody (Ser9) (mAb #9323, Cell Signaling Technologies). Cells were rinsed twice in PBS, mounted and then analysed using a LSM-700 (Carl Zeiss MicroImaging GmbH).

Data analysis

All statistical analyses were performed using the Sigmaplot 11.0 software (Systat Software GmbH, Erkrath, Germany). Values from different groups were compared using *t* tests or one- or two-way ANOVA where appropriate. In case of ANOVA, Tukey's HSD was used as post hoc test. A *P* value of <0.05 was considered to be significant. The results are presented as means $\pm$ SD.

Results

Exposure to PDF Dianeal® and Extraneal® increases the level of activated GSK-3 β by a combination of low pH and GDP

The effect of PDF on GSK-3 β activation was studied in MeT-5A mesothelial cells (Fig. 1). The levels of the inactive (Ser-9 phosphorylated) and of total GSK-3 β were assessed after exposure to the following commercially available PDF: Dianeal®, Extraneal®, Physioneal®, Balance® and Nutrioneal®.

Out of the studied fluids, only Dianeal® and Extraneal® significantly decreased the level of the inactive GSK-3 β (p-GSK-3 β) analysed by Western blot. The level of total GSK-3 β remained unchanged leading therefore to a higher net level of kinase-active GSK-3 β compared to control cells or cells exposed to the alternative PDF. The cellular expression was investigated in Dianeal®- and Extraneal®-treated cultures by immunofluorescent staining confirming that there was reduced abundance of p-GSK-3 β in these cells.

The influence of pH and GDP content of the PDF on GSK-3 β activation was assessed by selective avoidance of either acidosis or heat sterilisation (Fig. 2). Both after incubation with normal pH/GDP-rich or low pH/GDP-free PDF, GSK-3 β phosphorylation was restored to the level detected in control cells. Thus, acidosis and GDPs are both needed to increase the level of activated GSK-3 β

Fig. 1 p-GSK-3 β in MeT-5A mesothelial cells exposed to commercially available PDF. Representative Western blot images of GSK-3 β , p-GSK-3 β and tubulin are shown in the *upper panel* (a). The figure was assembled from bands originating from the same blot in order to correspond to the diagram. The *middle panel* (b) shows densitometry data of p-GSK-3 β , normalised to tubulin protein expression. Control cells were treated with normal cell culture medium, PDF-treated cells were exposed for 1 h either to low pH, glucose-based (Dianeal®), to low pH, icodextrin-based (Extraneal®), to lactate-buffered, glucose-based, pH 7.4 (Balance®), to lactate/bicarbonate-buffered, glucose-based, pH 7.4 (Physioneal®) or to amino-acid based (Nutrioneal®) PDF, and allowed to recover for 16 h in control medium. **P* < 0.001 vs. control. The control was set to 100 % (*dashed line*). The *lower panel* (c) shows p-GSK-3 β immunofluorescent staining. Data are representative of three independent experiments

in mesothelial cells. Total GSK-3 β expression remained the same in all groups.

GSK-3 β inhibition by lithium leads to reduced cell damage and higher HSF-1 activation upon exposure to PDF

LDH release and HSF-1 activity were assessed in MeT-5A mesothelial cells exposed to Dianeal® or Extraneal® with or without LiCl administration to test for the effects of GSK-3 β inhibition during PDF exposure. In order to examine in which concentrations lithium effectively ameliorates mesothelial cell

Fig. 2 p-GSK-3 β in MeT-5A mesothelial cells exposed to cytotoxic physicochemical properties of PDF. Representative Western blot images of GSK-3 β , p-GSK-3 β and tubulin are shown in the upper panel. The figure was assembled from bands originating from the same blot in order to correspond to the diagram. The lower panel shows densitometry data of p-GSK-3 β , normalised to tubulin protein expression. Cells were either treated with normal cell culture medium (no acidosis, no GDP) or for 1 h with glucose-based PDF, with normal (no acidosis) or low pH (acidosis +) following filter or heat sterilisation (GDP -/+) and allowed to recover for 16 h in control medium. * $P < 0.01$ vs. control. Data are representative of three independent experiments

injury, a dose curve was analysed during incubations with either low pH glucose-based Dianeal® or icodextrin-based Extraneal®. A dose of 1, 2, 5 and 10 mM LiCl was examined (Fig. 3).

Cellular damage assessed by LDH release showed statistically significant cytoprotective effects by lithium addition to Dianeal® or Extraneal® in cells incubated with these PDF. Upon exposure to Dianeal®, addition of LiCl at doses from 1 to 5 mM did not alter the release of LDH into the supernatant, but 10 mM LiCl significantly reduced LDH release when compared to PDF exposure without the addition of lithium. Upon exposure to Extraneal®, there was a more dose-dependent LDH response curve: 1 or 2 mM LiCl did not significantly alter the release of LDH into the supernatant, whereas the addition of 5 mM LiCl decreased LDH levels and the decrease was even more marked with administration of 10 mM LiCl when compared to PDF exposure without the addition of lithium.

HSF-1 activity assessed by luciferase assay showed a clear dose-dependent upregulation of the promoter transcriptional activity by lithium addition to Dianeal® or Extraneal® in cells incubated with these PDF. Upon exposure to Dianeal®, the addition of LiCl at doses from 2 to

10 mM increasingly enhanced HSF-1 activity when compared to the severe repression following PDF exposure without the addition of lithium. Upon exposure to Extraneal®, there was also a dose-dependent increase of HSF-1 activity with increasing effects of the addition of 5 and 10 mM LiCl when compared to PDF exposure without the addition of lithium.

Lithium inhibits GSK-3 β activity and increases Hsp72 expression, leading to reduced cell injury after exposure to PDF

The effect of the addition of lithium at a cytoprotective dose of 10 mM LiCl was assessed on GSK-3 β activation and Hsp72 expression in MeT-5A mesothelial cells after exposure to Dianeal® and Extraneal® (Figs. 4 and 5). GSK-3 β activity assessed by the Ser-9 phosphorylation status of GSK-3 β was inhibited by lithium addition to Dianeal® or Extraneal® in mesothelial cells incubated with these PDF. The Ser-9 phosphorylation of GSK-3 β demonstrated control levels both in Dianeal®- and Extraneal®-treated cells after LiCl administration compared to severe decreases found in cells exposed to these PDF without lithium. There was also a significantly increased abundance of Hsp72 protein upon exposure to Dianeal® and Extraneal® with added LiCl compared to the respective PDF exposure without lithium. The cytoprotective effect of lithium addition at a dose of 10 mM LiCl was assessed by two additional independent viability tests in MeT-5A mesothelial cells after exposure to Dianeal® and Extraneal®.

Reduced cell injury following exposure to Dianeal® or Extraneal® was reflected qualitatively by improved cell morphology and a higher number of viable cells assessed by vital-dead staining and quantitatively by a significantly higher cell survival measured in independent experiments by neutral red uptake compared to cells exposed to these PDF without lithium.

Confirmation of lithium effects on inhibitory Ser-9 phosphorylation of GSK-3 β , Hsp72 levels and cell injury after exposure to PDF in PD patient derived primary mesothelial cell cultures

The effect of the addition of lithium at a dose of 10 mM LiCl on GSK-3 β activation, Hsp72 expression and cytoprotection upon exposure to Dianeal® and Extraneal® was assessed in primary mesothelial cell (HPMC) cultures grown from clinical PD effluent (Fig. 6).

GSK-3 β activity assessed by the Ser-9 phosphorylation status of GSK-3 β was inhibited by lithium addition to Dianeal® or Extraneal® in HPMC incubated with these PDF. The Ser-9 phosphorylation of GSK-3 β demonstrated increased levels both in Dianeal®- and Extraneal®-treated

Fig. 3 Dose-dependent protection and heat shock factor-1 (HSF-1) activation in MeT-5A mesothelial cells exposed to Dianeal® and Extraneal® with different concentrations of lithium chloride (LiCl). Cell damage was assessed as LDH release (a). HSF-1 promoter activity was assessed by luciferase assay (b). Cells were treated with low pH, glucose-based (Dianeal®) or with icodextrin-based PDF with low pH (Extraneal®). Data are presented as percentage of controls or relative light units. Control cells were treated with normal cell culture medium. LiCl was applied at a dose of 0, 1, 2, 5 and 10 mM as indicated (30 min exposure, 6 h recovery). Data are representative of three independent experiments. * $P < 0.01$ vs. control, # $P < 0.05$ vs. 0 mM LiCl

cells after LiCl administration compared to levels close to the control found in cells exposed to these PDF without lithium. There was also an increased abundance of Hsp72 protein upon exposure to Dianeal® and Extraneal® with added LiCl compared to the respective PDF exposure without lithium. This increase was significant for Extraneal®. Finally, LiCl-treated cells showed significantly reduced cell damage consistent with cytoprotection demonstrated by decreased LDH release compared to cells exposed to these PDF without lithium, respectively.

Discussion

This study demonstrates (a) that GSK-3 β , a central mediator of cell survival, is activated by commercially available PDF and (b) that inhibition of GSK-3 β by lithium results in

improved survival of mesothelial cells when exposed to these PDF, paralleled by enhanced HSR.

GSK-3 β is a multi-faced kinase and key component of cellular signalling involved in physiological and pathological processes (Rayasam et al. 2009). GSK-3 β is known to be a critically important regulator of cell survival, and previous studies suggest that GSK-3 β activation may be associated with cell death in stress models (Rayasam et al. 2009). Furthermore, in the last years, GSK-3 β inhibition has become a widely investigated therapeutic intervention in various experimental disease models and clinical trials.

Although several of the previously studied stressors relevant for GSK-3 β activation, such as changes in glucose concentrations and high GDP content, are also known to be major factors of PDF toxicity (Aroeira et al. 2007) (Hooper 2007), to our knowledge, there are no data yet on GSK-3 β in PD.

Fig. 4 p-GSK-3 β and Hsp72 in MeT-5A mesothelial cells exposed to Dianeal[®] and Extraneal[®] with or without lithium chloride (LiCl). Densitometry of p-GSK-3 β (**a**) and Hsp72 (**b**) protein expression normalised to tubulin protein expression. Cells were treated with low pH, glucose-based (Dianeal[®]) or icodextrin-based PDF (Extraneal[®]). LiCl was applied at a dose of 10 mM (1 h exposure, 16 h recovery). Data are presented as percentage of controls. Control cells were treated with normal cell culture medium. Data are representative of three independent experiments. * $P < 0.01$ vs. control. Pairwise comparisons with and without LiCl are indicated with their corresponding P value. The dashed line indicates the level of control cells

As a first step, we therefore investigated the activation and expression of GSK-3 β in mesothelial cells in an in vitro PD model, established recently by our group in order to study the cellular stress responses upon exposure to PDF (Kratochwill et al. 2012). Following exposure to different PDF, cells demonstrated selective GSK-3 β activation upon Dianeal[®] (high glucose, high GDPs, low pH) and upon Extraneal[®] (icodextrin-based, low GDPs, low pH) exposure. In contrast, Physioneal[®], Balance[®] (both high glucose, low GDPs, normal pH) and Nutrineal[®] (amino acid-based, no GDPs, moderate acidic pH) did not influence the activation of GSK-3 β .

These results are in accordance with previous observations in other systems, reporting upregulated GSK-3 β activity by glucose-related toxicity (Lin et al. 2008). In our study, the glucose-based PDF Dianeal[®] with a high level of

Fig. 5 Cell survival in MeT-5A mesothelial cells exposed to Dianeal[®] and Extraneal[®] with or without lithium chloride (LiCl). Light microscopy (**a**) shows representative images of control cells treated with medium with or without LiCl. Cell survival of cells incubated with low pH, glucose-based PDF (Dianeal[®]) with or without LiCl, icodextrin-based PDF with low pH (Extraneal[®]) with or without LiCl was independently assessed by two additional methods. Representative fluorescence images of vital/dead staining experiments are shown (**b**). The lower panel (**c**) shows results of neutral red uptake (NRU) experiments. LiCl was applied at a dose of 10 mM (1 h exposure, 16 h recovery). Data are presented as percentage of control. Control cells were treated with normal cell culture medium without LiCl. Data are representative of three independent experiments. * $P < 0.01$ vs. control

GDPs did, whereas glucose-based PDF with low GDPs (Physioneal[®], Balance[®]) did not activate GSK-3 β , indicating that high glucose in the PDF alone does not suffice to

Fig. 6 p-GSK-3 β , Hsp72 expression and cell damage in human peritoneal mesothelial cells (HPMC) exposed to Dianeal® and Extraneal® with or without lithium chloride (LiCl). p-GSK-3 β (a) and Hsp72 (b) were assessed by Western blot. Cell damage was assessed as LDH release (c). Cells were treated with low pH, glucose-based (Dianeal®) or with icodextrin-based PDF with low pH (Extraneal®). LiCl was applied at a dose of 10 mM (6 h exposure, PDF diluted to 1:1 with medium). Protein expression was normalised to tubulin protein expression. Data are presented as percentage of controls. Control cells were treated with normal cell culture medium. Data are representative of five cultures. The dashed line indicates the level of control cells

activate GSK-3 β (Leng et al. 2010). Interestingly, GSK-3 β was also activated by the icodextrin-based PDF (Extraneal®), which contains lower levels of GDPs as Dianeal®, but is formulated at a similar acidic pH. Therefore, it might be the interplay of these physicochemical properties of these PDF that have led to enhanced GSK-3 β activation. Although the situation in experimental models of PD is quite exceptional because the insult of PDF exposure to the peritoneal monolayer can be closely simulated in vitro, the composition of PDF represents a major challenge for stress research. The chemical components of commercially available PDF including the pH of the fluid represent at least four different stressors with interdependent cytotoxic potential. In this study, we tried to compare stress responses under the influence of acidosis, GDP or combinations thereof. Indeed, our experiments with pH normalisation and GDP elimination also demonstrated that acidosis as well as some GDP content was needed to activate GSK-3 β in this in vitro setting of PD. Data on the association of acidosis and GSK-3 β are scarce. However, Beauloye et al. also reported that insulin (a classical inhibitor of GSK-3 β) (Grimes and Jope 2001) does not decrease GSK-3 β activity in an acidotic intracellular environment, suggesting that low pH might indeed result in a higher propensity for GSK-3 β activation (Beauloye et al. 2001).

The role of GSK-3 β as a potential drug target has become more and more prominent in the last years (Phukan et al. 2010). Several studies reported that inhibition of GSK-3 β by selective small molecules is protective in clinically relevant disease models: GSK-3 β inhibition was shown to improve insulin resistance in type II diabetes (Gum et al. 2003; Ring et al. 2003), to have beneficial effects in neurological disorders like Alzheimer's disease (Hsiung et al. 2003) and to reduce cardiac hypertrophy and ischaemia (Morisco et al. 2001). Despite increasing evidence for the therapeutic potential of GSK-3 β inhibition to counteract various cytotoxic stressors, to our knowledge, there are no studies investigating GSK-3 β inhibition in the model of experimental PD.

In the next step, we therefore investigated the impact of adding a GSK-3 β inhibitor to those PDF that had shown to induce GSK-3 β activity. Lithium has been reported to be a selective inhibitor of GSK-3 β catalytic activity by competition for magnesium (Ryves et al. 2002) and to indirectly increase inhibitory phosphorylation of GSK-3 β (De Sarno et al. 2002). We decided to focus on LiCl as this compound is broadly investigated as standard GSK-3 β inhibitor both in clinical and experimental research (Carmichael et al. 2002; Chuang et al. 2011; Li et al. 2011). In our study, LiCl was found to be most effective at concentrations of 10 mM, which is the dose used in the majority of in vitro studies (Lewitt et al. 2001; Mantelli and Ledda 1989; Gregory et al. 2005; Lucas et al. 2010; Li et al. 2011).

Indeed, addition of lithium to the cytotoxic PDF Dianeal® and Extraneal® prior to experimental PD resulted in significant cytoprotection of the exposed mesothelial cells, as reflected by several assessments of cellular viability, such as dose-dependent decrease of LDH release, reduced cell death rate in the live/dead assay and improved cell survival measured as neutral red uptake. These results also demonstrate that—at least in the *in vitro* setting of experimental PD—GSK-3 β activation is to a major extent responsible for the cytotoxic effects observed in mesothelial cells after treatment with Dianeal® and Extraneal®, two commercially available PDF used widely in the clinical setting.

The next set of experiments focused on the cellular mechanisms mediating the observed cytoprotective effects of GSK-3 β inhibition in our model. One of the major pathways influenced by GSK-3 β is the cytoprotective HSR (Kline and Morimoto 1997; He et al. 1998). The HSPs, which are the effector proteins of the HSR, are molecular chaperones playing an important role in the establishment of proper protein conformation and prevention of unwanted protein aggregation. Furthermore, they are able to directly prevent cells from cell death and play therefore a central role in cell fate (Hightower 1991).

Therefore, we examined if lithium-mediated cellular protection was associated with an enhanced HSR. In well accordance with its effect on cytoprotection, addition of LiCl to the PDF indeed resulted in dose-dependent upregulation of HSF-1 transcriptional activity demonstrated by increased luciferase activity in this experimental PD model. The addition of lithium normalised the levels of phospho-GSK-3 β both in Dianeal®- and Extraneal®-treated cells and significantly increased Hsp72 levels in cells recovering from both cytotoxic PDF exposure. Thus, the addition of the GSK-3 β inhibitor LiCl to Dianeal® and also to Extraneal® directly improved the HSR in mesothelial cells by increasing HSF-1 activation following exposure to PDF.

Our results agree with previous reports that lithium-associated protection by GSK-3 β inhibition was mediated to a significant extent by the upregulation of the HSR. The study of Bijur was the first to examine the influence of GSK-3 β manipulation on the HSR (Bijur et al. 2000). In a neuronal model system, they found that overexpression of GSK-3 β severely impaired activation of HSF-1 and subsequent expression of Hsp72. Similar to our study, the addition of lithium also enhanced the activation of neuronal HSF-1 and increased HSP expression by releasing the inhibitory influence of GSK-3 β on the HSR (Bijur et al. 2000). These findings were later confirmed demonstrating that overexpression of GSK-3 β impaired activation of HSF-1, whereas HSF-1 activation was facilitated by lithium (Bijur et al. 2000). Recently, protective lithium effects were also shown to be associated with improved HSP levels in ischaemic brain injury (Ren et al.

2003; Xu et al. 2006, 2007; Rowe and Chuang 2004; Ryves and Harwood 2001; Carmichael et al. 2002; Chuang et al. 2011).

In the final part of the present study, we set out to confirm the biological role of GSK-3 β activation by the PDF Dianeal® and Extraneal® in the human *ex vivo* PD model, based on effluent-derived primary peritoneal mesothelial cells from uraemic patients. Albeit such *ex vivo* exposure to diluted PDF still represents an artificial system, this model likely reflects intraperitoneal conditions more closely to those prevalent during an intraperitoneal PD dwell and has been previously shown to be relevant to assess the balance of mesothelial cell injury and repair processes (Mantelli and Ledda 1989; Li et al. 2011; Ring et al. 2003; Kratochwill et al. 2012). Confirming the data of acute PDF exposure in the MeT-5A mesothelial cell line, the addition of lithium to PDF resulted in increased levels of phospho-GSK-3 β —reflecting inhibition of GSK-3 β activation—and in decreased cellular damage in peritoneal effluent-derived primary mesothelial cells. Interestingly, exposure to Dianeal® and Extraneal® resulted in inadequately low HSP expression in the primary mesothelial cells despite marked cellular injury, confirming our recent findings of an inadequate HSR in this human *ex vivo* model of PD (Kratochwill et al. 2012). Validating the cellular mechanism responsible for the cytoprotective effects observed in immortalised mesothelial cells, the addition of the GSK-3 β inhibitor lithium resulted in enhanced expression of Hsp72—reflecting an improved adequacy of the HSR in human peritoneal mesothelial cells derived from the dialysed peritoneal cavity.

Taken together, these data therefore identify GSK-3 β as a promising therapeutic target for the preservation of peritoneal membrane integrity in PD. In the clinical setting, lithium has been used for the treatment of bipolar mood disorders since the 1950s, demonstrating an overall favourable pharmaceutical profile (Quiroz et al. 2004). In addition to mood disorders, lithium is now in phase II clinical trial for the treatment of amyotrophic lateral sclerosis and for the treatment of intestinal graft versus host disease. There are several alternative GSK-3 β inhibitors available on the market, some of them already under clinical investigation and/or in clinical use for various indications without relevant toxicity during the phase II–III trials. Therefore, GSK-3 β inhibitors might emerge as promising pharmacological agents to introduce HSP-mediated cytoprotection in the clinical setting of PD.

In conclusion, this study demonstrates that GSK-3 β is significantly activated by commercially available PDF. Inhibition of GSK-3 β by lithium enhanced the HSR, demonstrated by increased HSF-1 activation and upregulated Hsp72, and reduced cell injury in human mesothelial cells upon exposure to Dianeal® and Extraneal®. GSK-3 β

inhibitors might be attractive cytoprotective additives to these PDF.

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Conflict of interest Christoph Aufricht is cofounder of Zytotec GmbH, a spin-off of the Medical University Vienna that holds the patent “Peritoneal dialysis fluid” (Application number: EP11195786).

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