Insulin Inhibits Caspase-3 Activity in Human Renal Tubular Epithelial Cells via the PI3-Kinase/Akt Pathway

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Key Words

Apoptosis • Renal tubular cells • Acute kidney injury •
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Abstract

Apoptotic mechanisms in proximal renal tubular epithelial cells (PTEC) are crucial in the pathogenesis of acute kidney injury. We investigated whether insulin alters anti-apoptotic signalling in human PTEC. Cells were deprived of insulin for 0, 24 or 48 h and then stimulated with insulin for 0 or 5 min. Apoptosis was induced by camptothecin incubation. Insulin receptor kinase (IR-kinase) activity, phosphorylation of insulin receptor substrate-1 (IRS-1), IRS-1-associated PI3-kinase (p85), Ser²⁷³-phosphorylation of Akt and caspase-3 activity (C3-activity) were determined. Insulin stimulation increased the activity of IR-kinase, IRS-1 phosphorylation, p85 association with IRS-1 and Ser²⁷³-phosphorylation of Akt by at least 250%, respectively and decreased the C3-activity by 45% ($p < 0.01$, respectively). Deprivation of insulin for 24 and 48 h reduced basal and insulin-stimulated IR-kinase activity, IRS-1 phosphorylation, p85 association with IRS-1 and Ser²⁷³-phosphorylation of Akt by 30-40% and increased C3-activity by 15-20%

($p < 0.01$, respectively). Incubation with camptothecin increased C3-activity by 250-300% ($p < 0.001$). Subsequent insulin stimulation reversed the camptothecin induced increase of C3-activity. Our data indicate that apoptosis in PTEC is regulated by the insulin dependent PI3-kinase/Akt pathway. The enhancement of tubular-specific cell survival signals might represent a potential therapeutic tool for the protection of renal function in acute kidney injury.

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Introduction

Despite significant scientific and medical effort, acute kidney injury and acute renal failure still causes considerable patient morbidity and mortality. In most studies mortality rates for acute renal failure in medical and surgical patients continues to exceed 30% [1]. The underlying mechanism for the development of acute renal failure is not completely understood but appears to involve disturbances of renal tubular epithelial cell function [2]. Particularly apoptotic mechanisms seem to represent a crucial regulator of cell survival in this context [3-5]. Recent studies suggest that insulin signalling plays an

important role in the apoptotic regulation of renal tubular and mesangial cells [5, 6].

Van den Berghe et al. showed that intensive insulin therapy designed to maintain blood glucose at or below 110 mg/dl reduces morbidity and mortality among critically ill patients in a surgical intensive care unit. The development of acute renal failure was reduced by approximately 40% by using this therapeutic concept [7]. A subsequent multivariate logistic regression analysis revealed that the prevention of acute renal failure was related to the insulin dose and not the metabolic control, as reflected by normoglycemia [8]. This clinical finding of a beneficial insulin dose-dependent effect on the adaptation and / or recovery of renal function fits to recent findings of experimental studies in mesangial cells concerning protective insulin effects on apoptosis [6].

Survival of cells after injury depends on the up-regulation of intracellular anti-apoptotic pathways and inhibition of intracellular apoptotic enzymes. Such enzymes predominantly include caspases, a family of at least 14 cell death proteases that are crucial in the execution of apoptosis [9]. They cleave and inactivate various structural and functional intracellular proteins that are necessary for cell survival and proliferation [10]. In the renal tubular epithelium the caspase-3 represents the main apoptotic enzyme that gets activated during ischemia or the early period of reperfusion [10-12]. There is increasing evidence that insulin signalling might play a role in the regulation of caspases and, by this, in apoptosis [13].

In general, insulin signalling is initiated by binding to its specific cell surface receptor (IR), followed by rapid phosphorylation of tyrosine residues on the receptor beta-subunit. This results in phosphorylation of several intracellular substrates including insulin-receptor substrates 1-4. Binding of tyrosine-phosphorylated insulin receptor substrates to the 85kD regulatory subunit of the lipid kinase phosphatidylinositol-3 (PI3)-kinase then results in an activation of this enzyme. PI3-kinase is necessary for the insulin-mediated stimulation of the serine/threonine kinase Akt which is activated by phosphorylation at the Thr³⁰⁸ and Ser⁴⁷³ residues [14]. Upon activation, Akt phosphorylates enzymes leading to the initiation of the metabolic insulin effects as glycogen synthesis and glucose transport. Hiromura et al. found that the insulin signalling pathway might also serve as a "survival pathway" that is activated after withdrawal of important growth factors and able to impair apoptosis in mice mesangial cells [6]. Moreover, Shimamura et al. demonstrated in rat mesangial cells that the key enzymes

in insulin signaling, the phosphatidylinositol-3 (PI3)-kinase and protein kinase B (Akt), are involved in the regulation of anti-apoptotic signals by modifying caspase activity [15].

In order to investigate, whether insulin signalling is related to anti-apoptotic mechanisms in proximal human epithelial tubular cells (PTEC), we studied the effects of long term insulin deprivation and / or short term insulin stimulation on insulin signaling and caspase-3 activity in PTEC.

Materials and Methods

Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM) and HAM's F-12 medium were purchased from Biochrom (Berlin, Germany). Porcine monocomponent insulin was from Novo Biolabs (Bagsvaerd, Denmark), [¹²⁵I-Tyr-A¹⁴]-monoiodoinsulin from Amersham (Braunschweig, Germany), ³²P-ATP and ¹⁴C-glycogen UDP-glucose were from NEN Life Science Products (Boston, NE, USA). Goat anti-mouse IgG came from Dianova (Hamburg, Germany) and peroxidase-labelled antibodies from Dako (Glostrup, Denmark). Rabbit polyclonal antibodies against IRS-1, PI3-Kinase 85.000 M_r-regulatory subunit and recombinant IRS-1 were from Upstate Biotechnology (Lake Placid, NY, USA). Antibody against Ser²⁷³-phosphorylation of Akt and Caspase-3 (Asp175) Sandwich ELISA Kit (7190) were obtained from Cell Signaling (Beverly, MA, USA). Other chemicals were from Sigma (Deisenhofen, Germany) or Merck (Mannheim, Germany).

Culture of human renal proximal tubular epithelial cells

Human renal proximal tubular epithelial cells (PTEC) were cultured as described previously [16]. In brief, normal renal tissue was obtained from kidneys removed for renal cell carcinoma. Cells were grown from 1 mm³ pieces of renal cortex not involved by disease on a matrix of fetal calf serum (FCS). PTEC for maintenance and experiments were cultured in serum-free Dulbecco's modified Eagle's medium (DMEM)/HAM's F-12 medium in a 1:1 ratio. The medium was supplemented with insulin (1 µg/mL ≈ 15 nmol/L final concentration), transferrin (5 µg/mL), selenium (5 ng/mL), hydrocortisone (36 ng/mL), triiodothyronine (4 pg/mL), and epidermal growth factor (10 ng/mL). Passages 2 to 6 were used for our studies. Previous studies showed that these cells feature the characteristic properties of proximal renal tubular cells by employment of enzyme histochemistry, immunohistochemistry and ultrastructural examination. [16, 17].

Cell viability and apoptosis

At the end of the incubations, cells were counted in a Neubauer-chamber and viability was tested with trypan blue staining. Apoptosis in one dish was defined as reduction in cell number of more than 20% and / or detection of more than 20% trypan blue positive cells associated with an increase of caspase-3 activity of at least 50% compared to viable cells.

Preparation of camptothecin stock solution and apoptosis induction

To prepare the camptothecin stock solution 50 mg camptothecin (Sigma, Deisenhofen, Germany) was dissolved in 2 ml DMSO/methanol. Stock solution for controls was similarly prepared except that the DMSO/methanol did not contain camptothecin. For apoptosis induction PTEC were grown to near confluency. Then medium was replaced by 9.99 ml DMEM/HAM's F-12 medium containing the supplements mentioned above and 10 μ l of camptothecin stock solution (final concentration 2mmol/L) were added. Cells were then incubated for the desired time intervals. Subsequently, the medium was removed and cells were incubated in a medium containing 9.99 mL *incubation buffer* (20 mmol/L HEPES, 130 mmol/L NaCl, 4.8 mmol/L KCl, 1.3 mmol/L KH_2PO_4 , 1 g/L D-glucose, 1.3 mmol/L MgSO_4 , 1.2 mmol/L CaCl_2 , 2% BSA, pH 7.4) and 10 μ L camptothecin stock solution. Medium for controls contained 10 μ L of DMSO/methanol without camptothecin. After an incubation period of 15 min at 37°C cells were stimulated with insulin as described below.

Insulin deprivation

For insulin deprivation experiments culture medium was removed and cells were incubated in DMEM/HAM's F-12 medium that contained the same supplements as described above with or without 15 nmol/L insulin (final concentration). After the different deprivation periods the medium was replaced by 20 mL *incubation buffer* and cells were incubated for 15 min at 37°C and then stimulated with insulin as described below.

Wortmannin stock solution and PI-3 kinase inhibition

For 1 mmol/L stock solution with a wortmannin concentration of 0.1 mmol/L 1 mg wortmannin was dissolved in 2.32 ml DMSO and then 1:10 diluted with *incubation buffer*. The medium was replaced by 9.99 ml DMEM/HAM's F-12 medium containing the supplements mentioned above and 10 μ L of the 0.1 mmol/L wortmannin stock solution was added (final concentration 100 nmol/L). Cells were then incubated for 12 h at 37°C. Insulin stimulation was performed as described below.

Insulin stimulation and solubilization of cells

For insulin stimulation 0 or 100 nmol/L insulin was added and cells were incubated for 5 min at 37°C. Subsequently, the buffer was removed and the dishes with the cells rapidly frozen in liquid nitrogen. The thin ice layer that contained the cells was scraped off from the dishes at 20°C and homogenised with a motor-driven Potter homogenizer in a solution that contained 2% Triton X-100, 5 mmol/L phenylmethylsulfonylfluoride, 800 U/mL trypsin inhibitor aprotinin, 8 mmol/L EDTA, 30 mmol/L benzamidine, 2.5 μ g/mL pepstatin, 2.5 μ g/mL leupeptin, 160 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 0.2 mmol/L sodium vanadate, 2 mmol/L dichloroacetic acid, and 20 mmol/L HEPES, pH 7.4 (final concentrations) [18]. Samples were then centrifuged at 10^5 g (4°C) to remove insoluble material. Protein concentration was measured using the BioRad protein-dye binding assay.

Western blots

Solubilized cells (300 μ g protein) were incubated overnight with 1 μ g anti-IRS-1-antibody and then added to 30 μ L packed agarose beads crosslinked with protein G. Bound proteins were detached with Laemmli-buffer, boiled, and subjected to SDS-PAGE. For determination of Ser²⁷³-phosphorylated Akt, cell lysates (100 μ g protein) were directly subjected to SDS-PAGE (7.5%). Proteins were then transferred to nitrocellulose membranes. Membranes were blocked in BSA (3% in TBS-T) and incubated with the specific antibodies for 2 h. Bound antibodies were detected by peroxidase-labeled antibodies and chemiluminescence.

Insulin receptor kinase and binding capacities

These were measured as previously described [19]. Briefly, 30 μ g protein of cell sample lysates were added to microwells coated with anti-insulin receptor antibody for 16 h at 4°C. The wells were washed and receptor-mediated ³²P-incorporation into recombinant IRS-1 (17 nmol/L) measured at 120 nmol/L ³²P-ATP. [¹²⁵I-Tyr-A¹⁴]-monoiodoinsulin binding to immobilized insulin receptors was also measured in the wells [19]. Insulin binding activity (BA) was defined as the amount of specifically bound insulin at a concentration of 8.7 nmol/L and analyzed as described earlier [20]. Finally, insulin-receptor kinase activity was expressed as attomoles phosphate incorporated into IRS-1 per minute per femtomole of insulin binding.

Caspase-3 activity

Cells were incubated in *apoptosis induction medium* or insulin deprived as described above. Then cells were stimulated with insulin and solubilized with lysis buffer (1 mM β -glycerophosphate, 1 mM EDTA disodium salt, 1 mM EGTA, 1 μ g/ml leupeptin, 150 mM sodium chloride, 2.5 mM sodium phosphate, sodium orthovanadate, 20 mM Tris-Cl, Triton X-100). Subsequently, 100 μ L of the cell lysate was added to 100 μ L dilution buffer (0.1% Tween-20, 0.1% sodium azide in 20x PBS) and pipetted into microwells coated with anti-caspase-3 antibody and incubated for 2 h at 37°C. Wells were washed and HRP-linked streptavidine was added to the wells. Then 100 μ L of detection reagent (0.05% 3,3',5,5' tetramethylbenzidine peroxide, 0.1% hydrogen peroxide) was added and wells were incubated for 10 min at 37°C. Finally, 100 μ L of stop solution (0.05% α and β unsaturated carbonyl compound) were added and color reaction was determined spectrophotometrically at 450 nm.

Data analysis

In every Western blot all bands were compared (2-3 time points +/- insulin) by scanning densitometry. Relative units represent the ratio between the intensity of the single bands and the mean intensity of all bands in the same blot. To compare the different experiments of the caspase-3 activity, absolute values (optical density) of one ELISA experiment (2-3 time points +/- insulin) were compared. Densitometric units represent the ratio between the optical density of a single sample in a microtiter well compared to the mean intensity of all samples in the same

Fig. 1. Effects of insulin (IS) stimulation and deprivation on insulin receptor kinase (IR-kinase) activity. Renal proximal epithelia tubular cells (PTEC) were deprived of insulin for the indicated times. Then cells were stimulated without (□) or with (■) 100nmol/L insulin for 5 minutes. Insulin binding activity (BA) was analyzed. IR kinase activity was expressed as attomoles phosphate incorporated into IRS-1 per minute per femtomole of insulin binding. Shown are means $\pm$ SEM (n=5); * p<0.05, insulin stimulated cells compared to non insulin-stimulated cells (baseline values); # p<0.05, baseline values of insulin deprived cells compared to non insulin-stimulated and non insulin-deprived cells.

ELISA experiment. Differences were tested by *t*-statistics for unpaired data, Mann-Whitney rank sum test or ANOVA on ranks (SigmaStat 3.0, SPSS Inc. Chicago, USA). We considered a p-value of 0.05 or less to be significant.

Results

Cell number, viability and apoptosis

Insulin deprivation for less than 48 h had no effect on cell number ($1.22 \times 10^6 \pm 0.19$; and $1.25 \times 10^6 \pm 0.13$ cells/dish deprived of insulin for 0 or 48 h, respectively) or cell viability (8.5 ± 0.16 and 9.1 ± 0.12 % trypan blue positive cells/dish, respectively). Insulin deprivation for more than 48 h as well as camptothecin incubation for more than 8 h resulted in a significant reduction of cell number (>20%, p<0.001) and viability (>20% trypan blue positive cells/dish, p<0.001) associated with morphological changes (e.g. loss of cell to cell contacts).

Insulin deprivation and insulin stimulation

In cells deprived of insulin for 0, 24 or 48 h insulin stimulation lead to a significant increase of IR phosphorylation (270%, 450%, 526%, figure 1), IRS-1 phosphorylation (95%, 289% and 412%, respectively),

Fig. 2. Effects of insulin (IS) stimulation on IRS-1 phosphorylation (A), association of p85 with IRS-1 (B) and Ser²⁷³-phosphorylation of Akt (C). Cells were deprived of insulin for the indicated times. Then PTECs were stimulated without (□) or with (■) 100 nmol/L insulin for 5 minutes. IRS-1 phosphorylation, p85 and Ser²⁷³-phosphorylated Akt in cell lysates were measured by immunoblotting with specific antibodies. Relative units were calculated as described. Shown are means $\pm$ SEM (n=5); * p<0.05, insulin stimulated cells compared to non insulin-stimulated cells (baseline values); # p<0.05, baseline values of insulin-deprived cells compared to non insulin-stimulated and non insulin-deprived cells. Insets show representative blots at the respective time points.

association of PI 3-kinase (p85) with IRS-1 (23%, 35% and 45%, respectively) and Ser²⁷³-posphorylation of Akt (18%, 24% and 45%, respectively)(figure 2A-C). Deprivation of insulin for 24 or 48 h resulted in a significant reduction of the baseline phosphorylation of IR-

Fig. 3. Effects of insulin (IS) stimulation on Caspase-3 activation. Cells were deprived of insulin for the indicated times. Then PTECs were stimulated without (□) or with (■) 100 nmol/L insulin for 5 minutes. Control experiments were performed by additional incubation of cells with 2mmol/L camptothecin for 12h (white box, black-striped). Cells were then solubilized and 100μl of lysate was added to microwells coated with anti-caspase-3 antibody. Reaction was stopped after 2h and wells were washed. Enzyme activity was determined spectrophotometrically at 450nm. Shown are means ± SEM, n=6 *: p<0.05.

phosphorylation by 15-25% (figure 1), IRS-1 by 19-55%, association of p85 with IRS-1 by 24-64% and Ser²⁷³-phosphorylation of Akt by 15-46% compared to cells not deprived of insulin (figure 2A-C). Deprivation of insulin as well as insulin stimulation did not alter the amount of the signalling proteins IRS-1 and Akt (data not shown). The stimulation time of 5 min with 100 nmol/L insulin was chosen according to preceding experiments that revealed that stimulation with 10 μmol/L or 500 μmol/L insulin for 0, 2, 5, 10 or 60 min did not lead to further increase of the activation of the signalling parameters IR, IRS-1 and Ser²⁷³-phosphorylation of Akt (data not shown).

Moreover, insulin stimulation significantly decreased caspase-3 activity in PTECs deprived or not deprived of insulin by approximately 30-60% (figure 3). On the other hand insulin deprivation for 24 or 48 h led to a significant increase of the caspase-3 enzyme activity by 15% and 58%, respectively. Camptothecin incubation for 48 h resulted in maximal caspase-3 activity approximately twofold above the maximal enzyme activity observed after 48 h of insulin deprivation (figure 3).

Fig. 4. Effects of insulin, camptothecin and wortmannin on IRS-1 phosphorylation (A), and Ser²⁷³-phosphorylation of Akt (B). Cells were incubated without (□) or with (■) 100 nmol/L insulin for 12 h and additionally without (white box, black-striped) or with (black box, white-striped) 2 mmol/L camptothecin for 12 h or without (white box, black chequered) or with (black box, white chequered) 100 nmol/L PI3-kinase inhibitor wortmannin for 12 h, respectively. IRS-1 phosphorylation, p85 and Ser²⁷³-phosphorylated Akt in cell lysates were measured by immunoblotting with specific antibodies. Relative units were calculated as described. Shown are means ± SEM (n=4); * p<0.05.

Apoptosis induction with camptothecin

In cells incubated with camptothecin similar insulin-induced activation characteristics of IRS-1 phosphorylation, association of PI3-kinase with IRS-1 (data not shown) and Ser²⁷³-phosphorylation of Akt were noticed and no statistical differences were detected between cells treated with or without the apoptosis inductor (figure 4). Camptothecin incubation resulted in an approximately threefold increase of the caspase-3 activity in the cells (figure 5) associated with a significant decrease of cell number and a significant increase of

Fig. 5. Effects of insulin, camptothecin and wortmannin on Caspase-3 activation. PTECs were incubated without (□) or with (■) 100 nmol/L insulin for 12 h and additionally without (white box, black-striped) or with (black box, white-striped) 2 mmol/L camptothecin for 12 h or without (white box, black chequered) or with (black box, white chequered) 100 nmol/L PI3-kinase inhibitor wortmannin for 12 h, respectively. Cells were then solubilized and 100 µl of lysate was added to microwells coated with anti-caspase-3 antibody. Reaction was stopped after 2 h and wells were washed. Enzyme activity was determined spectrophotometrically at 450nm. Shown are means ± SEM, $n = 5$; $p < 0.05$.

trypan blue positive cells per dish ($23 \pm 5\%$ and $21 \pm 7\%$ compared to cells not incubated with camptothecin, $p < 0.001$ respectively). The amount of IRS-1 or Akt was not significantly altered by incubation with the apoptosis inducer (data not shown).

Simultaneous insulin incubation for 12 h partially reversed the apoptotic effect of camptothecin reflected by a decrease of caspase-3 activity of approximately 38% (figure 5) associated with an increase of cell number ($17 \pm 4\%$, $p < 0.01$) and an decrease of trypan blue positive cells per dish ($14 \pm 3\%$, $p < 0.01$).

PI-3 kinase inhibition with wortmannin

Incubation with wortmannin did not alter the insulin-induced increase of IRS-1 phosphorylation (figure 4 A) but resulted in a significant decrease of both baseline and insulin stimulated Akt-phosphorylation by 65% and 45% ($p < 0.01$, respectively; figure 4 B). Wortmannin had no effect on the amount of IRS-1 or Akt (data not shown). Furthermore, PI3-kinase inhibition by wortmannin incubation increased baseline caspase-3 activity approximately 2.5 fold ($p < 0.001$) compared to cells not incubated with the inhibitor and was associated with a decrease of cell number ($19 \pm 4\%$ $p < 0.01$) and an increase of trypan blue positive cells/dish ($21 \pm 5\%$, $p < 0.01$). Insulin stimulation of these cells did not reverse the wortmannin effect reflected by a non significant decrease of caspase-3 enzyme activity (figure 5).

Discussion

In the present study we found that insulin stimulation of human proximal epithelial tubular cells (PTEC)

activates the insulin signaling PI3-kinase/Akt pathway and impairs apoptosis by reducing caspase-3 activity.

The effect of insulin stimulation in PTEC on the activation of essential insulin signalling proteins and on apoptosis has not been investigated so far. Other previous studies tested the pro-survival properties of insulin [6] and / or insulin-like growth factor-1 (IGF-1) [6, 21] in rodent cell types. These studies demonstrated a role of the PI3-kinase/Akt pathway in the inhibition of cellular apoptosis. Kulik et al. found that incubation of rat fibroblasts with IGF-1 resulted in the activation of the signaling proteins PI3-kinase/Akt and a consecutive inhibition of ultraviolet-light induced apoptosis [21]. In a study by Hiromura et al. the incubation of mice mesangial cells with insulin or IGF-1 reduced the ultraviolet-light and serum-free media induced apoptosis in association with an activation of the PI3-kinase /Akt pathway, respectively [6]. Other experimental studies in rat mesangial cells [15] or rabbit renal proximal tubular cells [5] further support a potential role of the PI3-kinase/Akt signalling pathway in the prevention / induction of apoptosis. Shimamura et al. found that the inhibition of PI3-kinase as well as a dominant-negative mutant of Akt increased the apoptotic signals in rat mesangial cells while a dominant-active mutant of Akt prevented apoptosis that was induced by using serum-free medium [15]. Zhuang et al. found in rabbit renal proximal tubular cells that inhibition of the PI3-kinase by wortmannin consequently decreases Akt activity resulting in an increase of the H_2O_2 -induced apoptosis [5].

Overall, the data of these studies strongly support the result of our study in PTEC that the activation of the PI3-kinase/Akt signalling pathway is important for the regulation of apoptosis. It is, however, not clear, whether

the effects on apoptosis via an activation of PI3-kinase/Akt are predominantly mediated by the insulin or the IGF-1 receptor. Both, insulin and IGF-1 are capable of binding to each other receptors with subsequent activation of IRS-1 and PI3-kinase/Akt [22]. It has been shown that the insulin receptor gets autophosphorylated at almost physiologic concentrations of IGF-1 [23], whereas insulin has a 10 to 100-fold lower IGF-1 receptor affinity compared to IGF-1 [22]. Since we used a medium for cell culture that did not contain IGF-1, we can exclude a general IGF-1-mediated effect of apoptosis in our study with PTEC. Nevertheless, it might be possible, that the insulin mediated autophosphorylation of the IGF-1 receptor that is highly expressed in renal tubular epithelial cells [24] predominantly activates the PI3-kinase/Akt pathway.

Beyond the potential important role of the PI3-kinase/Akt pathway for the regulation of cellular apoptosis, the mechanism downstream of Akt that leads to the inhibition of apoptosis is not clear. The data of the present study strongly suggest that Akt exerts its anti-apoptotic effects by inhibition of the caspase-3 activity in PTEC. A possible mechanism represents a direct downstream Akt-mediated phosphorylation of Bad, a member of the Bcl-2 family [25]. Phosphorylation of Bad at Ser-136 by activated Akt results in its dissociation from Bcl-X_L and association with the adapter protein 14-3-3 [25, 26]. The free Bcl-X_L released from sequestration by Bad promotes cell survival predominantly by inhibition of caspase activation [27, 28].

Furthermore, Kaushal et al. showed in porcine renal tubular cells that cisplatin-induced Akt-phosphorylation is associated with a phosphorylation of Bad and a suppression of caspase-9 and caspase-3 [11, 12]. Finally, Kiley et al. demonstrated in rat proximal tubular cells that incubation of the cells with IGF-1 after a mechanical strain induced cell injury resulted in a restored Bad-phosphorylation [29]. Another possible mechanism for anti-apoptotic effects of Akt includes direct effects of Akt on anti-apoptotic transcription factors. Brunet et al. showed in 293 epithelial kidney cells that the activation of Akt by IGF-1 resulted in a phosphorylation of FKHRL1, a member of the forkhead transcription factor family [30]. The authors hypothesized that the phosphorylation of FKHRL1 may inhibit the transcription of death genes (e.g. Fas ligand) and, by this, inhibit apoptosis. A further study by Khawaja et al. suggests that Akt may activate nuclear transcription factor kappa-b (NFkappaB) that also

regulates the expression of anti-apoptotic genes [31]. Such effects on gene regulation are presumably highly cell specific and poorly understood in renal tubular cells. In any case, a major role of gene expression appears not important for the results of our study since we investigated cellular effects already after short-time insulin stimulation (5 min).

Another potential mechanism for the observed anti-apoptotic insulin effects in PTEC involves the activation of the extracellular signal regulating kinase (ERK). Hiromura et al. showed that ERK is activated during apoptosis in mice mesangial cells and that insulin stimulation partially rescued these cells from apoptosis by suppressing ERK activation [6]. In our study the inhibition of the PI3-kinase with wortmannin induced apoptosis and was associated with a 50% decrease of insulin-stimulated Akt phosphorylation and a 65% increase of caspase-3 activity. Thus, our data strongly support a central role of the PI-3 kinase/Akt pathway in PTEC as a major signalling cascade that is responsible for the anti-apoptotic insulin effects.

According to the results of our study that apoptosis of human proximal tubular cells is regulated by the insulin dependent PI3-kinase/Akt pathway enhancement of tubular-specific cell survival signals via activation of this cascade might represent a potential therapeutic option for the preservation or recovery of renal function in acute kidney injury. Even if transfer from our *in vitro* results to *in vivo* conditions is difficult our data might help to explain the clinical finding that insulin itself is the beneficial factor related to the prevention of acute renal failure in critically ill patients [8].

Disclosure

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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