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Radiocontrast media cause dephosphorylation of Akt and downstream signaling targets in human renal proximal tubular cells

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ABSTRACT

Radiocontrast medium induced nephrotoxicity is a major clinical problem. There is considerable interest in reducing the incidence of acute renal failure due to the use of radiocontrast media (RCM). Reduction of renal blood flow and direct toxic effect on renal tubular epithelial cells have been postulated as major causes of RCM nephropathy. Understanding the molecular mechanisms by which RCM cause cell damage may allow the development of pharmacological therapy to prevent their nephrotoxicity. In this work we have investigated the signaling pathways that may be affected by RCM.

The incubation of human renal tubular proximal cells with sodium diatrizoate, iopromide and iomeprol caused a marked dephosphorylation of the kinase Akt on Ser473 within 5 min of incubation. RCM also caused a decrease in cell viability, which was substantially alleviated by transfecting the cells with a constitutively active form of Akt. Further downstream targets of Akt, including the Forkhead family of transcription factors FKHR and FKHRL1, were also dephosphorylated by RCM at Thr24 and Thr32, respectively. The P70S6 kinase was also dephosphorylated at Thr389 and Ser371 by RCM. However there was a more dramatic decrease in phosphorylation of the phosphorylated form of mammalian target of rapamycin (mTOR) and of the extracellular-signal regulated kinases (ERK) 1/2 caused by sodium diatrizoate than by iopromide.

These results demonstrate the effect of RCM on some intracellular signaling pathways that may allow understanding of the mechanism of their toxicity and may allow the development of strategies to overcome their adverse effects.

1. Introduction

Radiographic contrast media (RCM) are widely used in clinical practice. In recent years their utilization in radiographic examinations has even increased. However, renal impairment can frequently follow the use of RCM, especially in patients whose renal function is already compromised, particularly in diabetic patients [1–5]. Given the high incidence of acute renal

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failure (ARF) associated with the use of contrast media [6–13], which nowadays accounts for 12% of in-hospital cases of ARF [14], there is considerable interest in its prevention that has led to the empiric suggestion to avoid dehydration or even perform fluid infusion before contrast injection.

The pathophysiology of ARF secondary to contrast media has not been elucidated as yet. Reduction in renal blood flow and/or a direct toxic effect on renal tubular epithelial cells have been postulated as major causes of contrast media nephropathy [15,16] with radiographic contrast agents reported to induce apoptosis both in glomerular cells and in renal tubular epithelial cells [17–20].

Since kinase-dependent intracellular signaling pathways can modulate cell growth, proliferation and death [21–23] it is important to characterize at least some of these pathways in cells undergoing a particular stress. The knowledge of which pathways are involved in determining cell injury/survival may help in finding a way to reduce the deleterious side effects of RCM.

Akt is known to be a critical regulator of cell survival [21]. The transfection of a variety of cell types with constitutively active Akt alleles (and in some cases with wild-type Akt) blocks apoptosis induced by a large number of apoptotic stimuli, including growth factor withdrawal, ultraviolet irradiation, matrix detachment, cell-cycle discordance, DNA damage and treatment of cells with anti-Fas antibody or transforming growth factor β (TGF-β) [24–31]. The identification of the Akt consensus phosphorylation sequences in proteins involved in the apoptotic process has raised the hypothesis that Akt regulates cell survival by directly phosphorylating components of the cell death apparatus [32,33]. The extracellular-signal regulated kinase (ERK), members of the mitogen-activated protein kinase (MAPK) family, have also been reported to play a role in cell proliferation and cell-cycle progression [34].

In the last few years new contrast media (monomeric non-ionic), like iomeprol and iopromide, have been used, often replacing the old ones (e.g. Hypaque®, whose main compound is sodium diatrizoate) with a reported reduction in their toxic effects on renal tubular cells.

In the present study we have evaluated the effects of some commonly used RCM on the viability of renal tubular cells and investigated their action on the kinases that are implicated in cell survival, namely ERK and Akt. With respect to Akt, we have also tried to identify which downstream targets are affected by RCM.

2. Materials and methods

2.1. Materials

The radiocontrast media used in our study were iomeprol (Iomeron 400™, Bracco S.p.A, Milan, Italy), iopromide (Ultravist 370™, Schering, Milan, Italy) and sodium diatrizoate (Sigma Chemical Co.; Milan, Italy). The dose of the radiocontrast media in our study (75 mg I/ml) was chosen on the basis of the dosage used in clinical practice. Usually the RCM is administered at doses of 1.5–2.5 mg I/kg b.w., leading to plasma concentrations of 15–20 mg I/ml [35]. Since in the kidney 70–80% of the glomerular ultrafiltrate is reabsorbed by the proximal convoluted tubule, the RCM concentration in this region will range between 75 and 100 mg I/ml.

The constitutively active Akt plasmid (CA-Akt) and pcDNA3 were kind gifts from Dr. J. Haendeler, University of Frankfurt, Germany.

2.2. Cell culture

In our experiments we have used HK-2 cells (a human renal proximal tubular epithelial cell line) and primary human renal tubule proximal (pHRTP) epithelial cells. HK-2 cells were obtained from the American Type Culture Collection and grown in 100 mm culture dishes (Corning; New York, USA) as described by others [36]. In brief, they were cultured in DMEM containing Glutamax (Gibco, Invitrogen; Milan, Italy) supplemented with 10% fetal calf serum and 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma; Milan, Italy) in an atmosphere of 5% CO_2 in air at 37 °C, up to a confluence of approximately 90%. pHRTP cells were isolated from fragments of normal tissue (1–2 mm) obtained from kidneys excised from patients with renal cancer but having normal renal function and not affected by hepatitis viruses; the cells were grown as previously described [37].

2.3. Cell transfection

HK-2 cells were grown in 6-well plates (Corning) to approximately 70% confluency and were transfected with 1 μg DNA using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The transfected plasmid DNA encoded the constitutively active form of Akt in which the Ser473 and Thr308 sites have been modified to an aspartic acid residue [38]. Transfection was allowed to proceed for approximately 18 h; then the transfection medium was removed and replaced by minimal medium for a further 18 h prior to experimentation. A parallel set of cultured cells, used as a control, were transfected with the backbone plasmid vector pcDNA3 lacking the Akt gene.

2.4. Cell viability

Cell viability was measured by the ability of viable cells to reduce MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) (Sigma) [39]. Cells were grown in 6-well plates; after treatments with the radiocontrast media, the cells were washed once with sterile PBS and incubated with 1 mg/ml MTT (in sterile PBS) for 3 h at 37 °C; they were then dissolved in dimethyl sulfoxide (DMSO). Measurements of the coloured product as a result of MTT reduction were made at 540 nm using a Beckman DU 800 (Beckman-Coulter; Milan, Italy) spectrophotometer.

2.5. Western blot analysis

HK-2 and pHRPT cells were washed with cold PBS and then lysed in buffer containing: 20 mM HEPES (pH 7.4), 2 mM EGTA, 1 mM DTT, 1 mM NaVO4, 1% (v/v) Triton X-100, 2 μM leupeptin, 2 μM microcystin, 1.5 μM aprotinin and 400 μM PMSF. The samples were then centrifuged at 10,000 × *g* for 10 min and the supernatant was retained (lysate). Some of the

supernatant was used to determine the protein content and the rest utilized for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined by using a modified Bradford protein assay protocol [40] in order to obtain an equal loading (approximately 30 μg of each sample were loaded).

Protein extracts were resolved again by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond C® extra, Amersham Biosciences, GE Healthcare; Cologno Monzese, Italy) followed by Western blotting as previously described [41]. Briefly, the membrane was incubated for 1 h at room temperature with 5% (w/v) non-fat powdered milk in a "TBS-Tween buffer" {"TBST": 20 mM Tris and 137 mM NaCl, pH 7.6, containing 0.1% (v/v) Tween 20}. The primary antibody, diluted in TBST with 5% (w/v) non-fat powdered milk, was then added to the membrane and incubated overnight at 4 °C. The membrane was then washed three times, 5 min each, at room temperature with TBST and incubated for 1 h with a secondary antibody conjugated with horseradish peroxidase (Dako; Glostrup, Denmark), diluted 1:5000 in TBST with 1% (w/v) non-fat powdered milk at room temperature, followed by washing three times as above. The secondary antibodies, conjugated with horseradish peroxidase, were detected by the enhanced chemiluminescence system (Amersham biosciences) according to the manufacturer's instructions. The primary antibodies included the following: anti-phospho-ERK1/2 (p44/p42 MAP kinase, Cell Signaling, Beverly, MA, USA); anti-phospho-Akt (Ser473 and Ser308, Cell Signaling); anti-total Akt (Cell Signaling); anti-phospho-FKHRL1 (Thr32)/anti-phospho-FKHR (Thr24) (Cell Signaling); anti-total FKHRL1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-caspase-3 (Cell Signaling); anti-phospho-$p70^{S6}$ kinase (Thr389) and (Ser371) (Cell Signaling); anti-phospho-mTOR(Ser2448) (Cell Signaling). All the experiments were performed at least three times under the same conditions (the data shown in the figures are representative of at least three separate experiments).

2.6. Statistical analysis

All results were expressed as mean ± S.E. Statistical analysis was performed using *t*-test. Statistical significance was defined as p less than 0.05.

3. Results

3.1. Cell viability after RCM exposure

Incubation of HK-2 cells with two different radiocontrast agents, sodium diatrizoate (NaD) and iopromide (IOP), resulted in a loss in cell viability as determined by the MTT assay (Fig. 1). The loss in cell viability was significantly ($p < 0.05$) greater with sodium diatrizoate (30%) than with iopromide (22%).

3.2. Dephosphorylation of Akt after RCM exposure

A dramatic decrease in the phosphorylation of Akt was observed within 5 min of incubation of HK-2 cells with sodium diatrizoate (NaD), iomeprol (IOM) and iopromide (IOP) and remained suppressed throughout the incubation period of 2 h

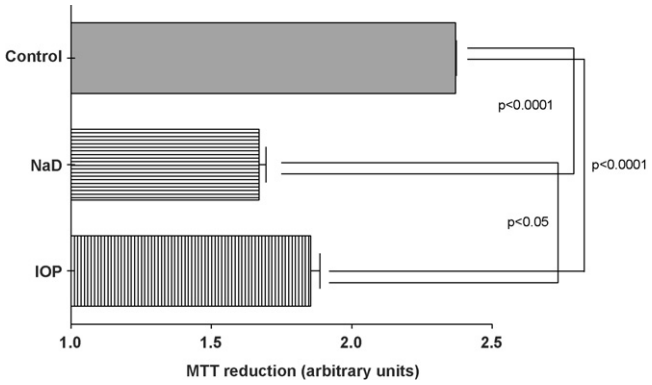

Fig. 1 – Effects of sodium diatrizoate and Iopromide on viability of HK-2 cells. The columns indicate cell viability (assessed by MTT assay) after overnight starvation and subsequent incubation of HK-2 cells with sodium diatrizoate ("NaD") (bar with horizontal lines) or Iopromide ("IOP") (bar with vertical lines), both at a concentration of 75 mg I/ml for 2 h. The values for the chemical reduction of MTT on the *x*-axis are expressed in arbitrary units. Both "NaD" ($p < 0.0001$ vs. control) and "IOP" ($p < 0.0001$) significantly decreased cell viability vs. "control". The decrease in cell viability was significantly greater with "NaD" than with "IOP" ($p < 0.05$).

(Fig. 2). Both phosphorylation sites of Akt (Ser473 and Thr308) were affected similarly by both types of RCM, sodium diatrizoate and monomeric non-ionic RCM iopromide and iomeprol. The total protein levels of Akt were unchanged throughout the timecourse. Two bands were observed when the blots were probed with the phospho-Akt antibody. However, when total Akt antibody was used, only one band was observed and corresponded with the lower band seeing in the phospho-

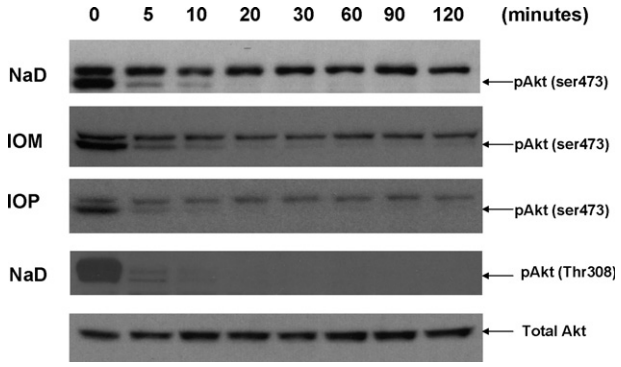

Fig. 2 – Dephosphorylation of phospho-Akt (pAkt) of HK-2 cells by RCM. Cells were cultured in 100 mm dishes and incubated with the respective RCM (75 mg I/ml) for the times shown. They were then lysed and lysates were processed; SDS-PAGE was carried out on 10% (w/v polyacrylamide) resolving gels followed by Western blotting. All RCM ("NaD": sodium diatrizoate; "IOM": iomeprol; "IOP": iopromide) markedly reduced pAkt levels. The panel showing total Akt refers to the experiment using iopromide ("IOP"). (The total Akt levels were also similar for the other RCM experiments.)

Akt immunoblot (data not shown). The presence of a doublet in Western blots of samples obtained from HK-2 cells and then subsequently probed with a phospho-Akt antibody has been previously reported by Sharples et al. [42] who also suggested that the lower of the two bands was that of Akt.

3.3. *Effects of transfection of HK-2 cells with constitutively active Akt (CA-Akt) plasmid on cell viability after RCM exposure*

To investigate whether the reduction in cell viability, as determined by the MTT assay, was due to a reduction in phosphorylation of Akt and consequently to a decrease in activity of this kinase, HK-2 cells were transfected with a plasmid encoding the constitutively active form of Akt and exposed to RCM (iopromide or sodium diatrizoate); cell viability was then assessed by the MTT assay. With both sodium diatrizoate and iopromide, transfection of the cells with the constitutively active Akt plasmid resulted in greater reduction of MTT compared with the cells transfected with the backbone vector (Figs. 3 and 4). Interestingly, it was observed that this recovery in MTT reduction in CA-Akt transfected cells was greater in cells incubated with iopromide than with sodium diatrizoate. Hence, transfection with CA-Akt could significantly recover the loss in cell viability (as measured by the reduction of MTT) upon exposure of the cells to the RCM.

3.4. *Dephosphorylation of Forkhead proteins FKHR and FKHRL1 after RCM exposure*

The Forkhead family of transcription factors have been reported to be substrates downstream of Akt [21], and may play a role in cell survival and death. A reduction in phosphorylation of FKHR (at Thr24) and FKHRL1 (at Thr32) was observed under the effect of RCM, the levels of phosphoproteins gradually decreasing during the 2 h period of incubation with either sodium diatrizoate or iopromide (Fig. 5).

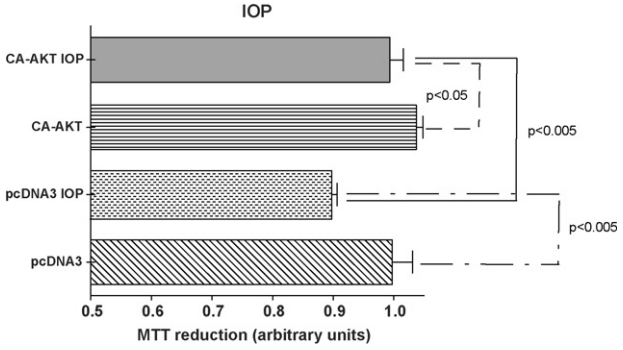

Fig. 3 – Protective effect of Akt on HK-2 cells incubated with Iopromide ("IOP"). Cells were transfected with CA-Akt or pcDNA3 (backbone) and then incubated with iopromide ("IOP"; 75 mg I/ml) for 1 h, followed by assaying for viability using the MTT assay. The values of chemical reduction of MTT on the *x*-axis are expressed in arbitrary units. CA-Akt significantly increased cell viability after incubation with "IOP" compared with cells transfected with pcDNA3 ("CA-AKT IOP" vs. "pcDNA3 IOP"; $p < 0.005$). However, CA-Akt could not fully prevent the reduction in cell viability induced by iopromide ("CA-AKT IOP" vs. "CA-AKT"; $p < 0.05$). The extent of loss in cell viability was higher for pcDNA3 transfected cells than for CA-Akt transfected cells ("CA-Akt IOP" vs. "CA-Akt", $p < 0.05$; "pcDNA3 IOP" vs. "pcDNA3", $p < 0.005$).

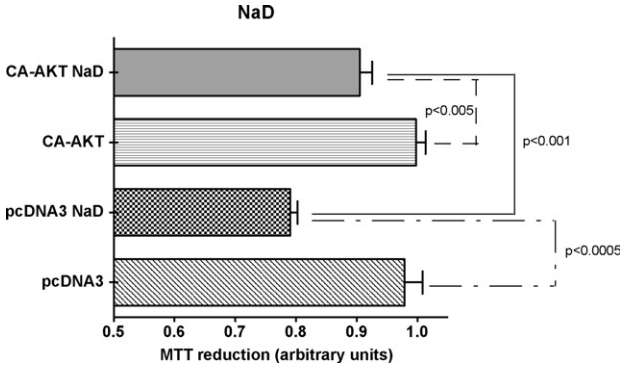

Fig. 4 – Protective effect of Akt on HK-2 cells incubated with sodium diatrizoate ("NaD"). Cells were transfected with CA-Akt or pcDNA3 (backbone) and then incubated with sodium diatrizoate ("NaD"; 75 mg I/ml) for 1 h, followed by assaying for viability using the MTT assay. The values of chemical reduction of MTT on the *x*-axis are expressed in arbitrary units. CA-Akt significantly increased cell viability after incubation with sodium diatrizoate compared with cells transfected with pcDNA3 ("CA-AKT NaD" vs. "pcDNA3 NaD"; $p < 0.005$). However, CA-Akt could not fully prevent the reduction in cell viability induced by sodium diatrizoate ("CA-AKT NaD" vs. "CA-AKT"; $p < 0.005$). The extent of loss in cell viability was higher for pcDNA3 transfected cells than for CA-Akt transfected cells ("CA-Akt NaD" vs. "CA-Akt", $p < 0.005$; "pcDNA3 NaD" vs. "pcDNA3", $p < 0.0005$).

3.5. *Dephosphorylation of p70 S6 kinase ($p70^{S6K}$) and mTOR after RCM exposure*

The serine/threonine kinases 70 kDa ribosomal protein S6 kinase ($p70^{S6K}$) and mammalian target of rapamycin (mTOR) both contain putative Akt phosphorylation sites and are believed to be activated by Akt upon phosphorylation. In the case of $p70^{S6K}$ the phosphorylation site is Thr389 [43], whilst for mTOR it is Ser3448 [44]. Another site on $p70^{S6K}$ at Ser371 is believed to be a phosphorylation site for mTOR [45]. As shown in Fig. 6 both $p70^{S6K}$ sites that we looked at are dephosphorylated upon incubation with the RCM (sodium diatrizoate or iopromide). The dephosphorylation of $p70^{S6K}$ at Thr389 is more rapid than dephosphorylation of at Ser371 and follows a similar pattern to that of Akt dephosphorylation. Both iopromide and sodium diatrizoate exhibit similar patterns of dephosphorylation of these kinases. However, incubation of the cells with sodium diatrizoate led to a steady decrease in mTOR phosphorylation at Ser2448 during the 2-h incubation period, whereas incubation with IOP shows an initial decrease in phosphorylation at Ser2448 followed by recovery (Fig. 7).

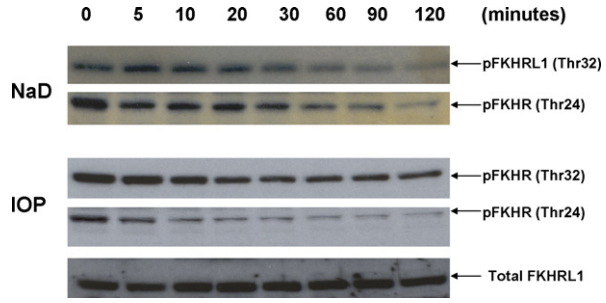

Fig. 5 – Dephosphorylation of Forkhead proteins of HK-2 cells by RCM. Samples were prepared and analysed as described in Fig. 2. RCM ("NaD": sodium diatrizoate; "IOP": iopromide) reduced phospho-FKHRL1 (Thr32) and phospho-FKHR (Thr24) levels. The panel showing total FKHRL1 is for the experiment using IOP. (The total FKHRL1 levels were also similar for the other RCM experiments.) (pFKHR = phospho-FKHR and pFKHRL1 = phospho-FKHRL1).

3.6. *Effect of RCM on ERKs phosphorylation*

Iopromide and sodium diatrizoate have different effects on ERKs phosphorylation (Fig. 8). Iopromide caused an initial decrease in the phosphorylation of ERK1/2 that was followed by a recovery within 60 min. Sodium diatrizoate, on the contrary, induced a prolonged decrease in phosphorylation; it remained, in fact, suppressed throughout the incubation time.

3.7. *Effect of adenosine RCM-mediated dephosphorylation of Akt and FKHR/FKHRL1 via phosphatidyl-inositol 3-kinase(PI 3-K)*

The addition of adenosine to the cell culture caused increased phosphorylation levels of both Akt (at Ser473) and Forkhead proteins (at Thr24 for FKHR and Thr32 for FKHRL1) following RCM exposure (Figs. 9 and 10). However the increase of Akt, FKHR/FLHRL1 phosphorylation by adenosine was prevented by the specific PI 3-K inhibitor LY294002. DMSO, in which

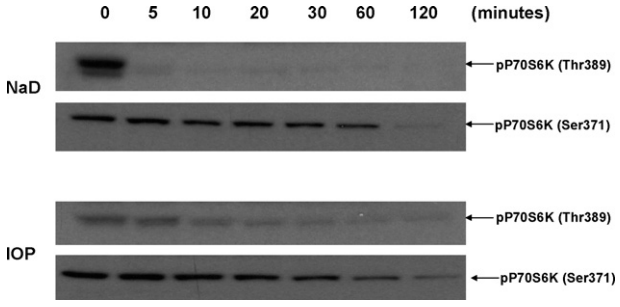

Fig. 6 – Dephosphorylation of $p70^{S6K}$ of HK-2 cells by RCM. Samples were prepared and analysed as described in Fig. 2. The dephosphorylation of $p70^{S6K}$ at Thr389, caused by both sodium diatrizoate (NaD) and iopromide (IOP), is more rapid than dephosphorylation of at Ser371 and follows a similar pattern of Akt dephosphorylation (pP70S6K = phospho-$p70^{S6K}$).

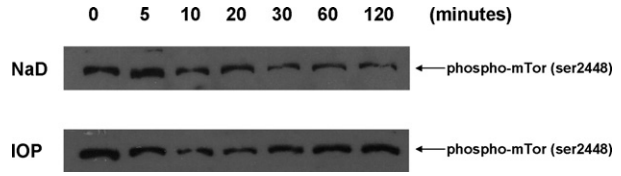

Fig. 7 – Dephosphorylation of mTOR of HK-2 cells by RCM. Samples were prepared and analysed as described in Fig. 2, except that the resolving gels used were 8% (w/v) total polyacrylamide. Sodium diatrizoate ("NaD") led to a steady decrease in mTOR phosphorylation (at Ser2448) during the 2-h incubation period, whereas incubation with iopromide ("IOP") shows an initial decrease in phosphorylation at Ser2448 followed by recovery of the signal.

LY294002 had been dissolved, did not change the phosphorylation levels of all the three proteins (Akt, FKHR and FKHRL1). Interestingly, whilst the addition of adenosine led to a full recovery of the phospho-Akt (Ser473) levels in cells incubated with iopromide, this was not the case in cells incubated with sodium diatrizoate (Figs. 9 and 10).

3.8. *Effect of RCM on phosphorylation levels of Akt, FKHR/FKHRL1 and $p70^{S6K}$ in primary human renal tubular proximal (pHRTP) cells*

Phosphorylation levels of Akt, FKHR/FKHRL1, mTOR and $p70^{S6K}$ decreased after incubation of pHRTP cells with either sodium diatrizoate or Iopromide (Fig. 11).

3.9. *Effect of RCM on cleavage of caspase-3 in HK-2 cells*

RCM did not change levels of 35 kDa caspase-3 protein and no evidence of the cleavage products (17 and 19 kDa) was observed (Fig. 12).

4. Discussion

The aim of our study was to identify some of the intracellular signaling pathways that may mediate a direct proximal renal tubular damage after exposure to some RCM. The first important result was the observation that exposure of HK-2 cells to RCM caused a decrease in cell viability (Fig. 1). This confirms the results obtained by others [20,35] using a proximal renal tubular cell line of porcine origin exposed to high RCM concentrations. This toxic effect of RCM on renal cells was not due to different levels of tonicity, as other investigators have already demonstrated [35,46].

When evaluating the RCM effect on intracellular signaling pathways, we have first focused our attention on the Akt kinase, an important regulator of cell survival. In our experiments the phosphorylation (and activation) of Akt was decreased dramatically within a few minutes of incubation of both HK-2 and pHRTP cells with sodium diatrizoate and iopromide (Figs. 2 and 11).

The transfection of HK-2 cells with CA-Akt appeared to partially prevent the damaging effect of RCM (Figs. 3 and 4).

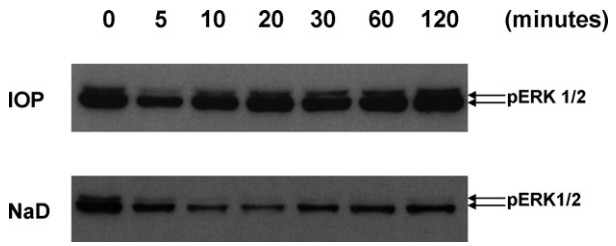

Fig. 8 – Effect of RCM on phosphorylation of ERKs of HK-2 cells. Samples were prepared and analysed as described in Fig. 2. Iopromide ("IOP") caused an initial decrease in the phosphorylation of ERK1/2 that was followed by a recovery in 60 min. Sodium diatrizoate ("NaD"), on the contrary, induced a permanent decrease in phosphorylation which actually remained suppressed throughout the incubation time (pERK1/2 = phospho-ERK1/2).

Our results are consistent with those of Yano et al. who have shown that phosphorylation of Akt with cyclic AMP alleviates the cytotoxic effects of RCM [20]. We have to consider, however, that the use of agents that prolong activation of Akt may have undesirable side effects, since Akt has been implicated in oncogenesis [21] and in cardiac hypertrophy [47]. Thus it appeared important to find downstream components of intracellular signaling pathways that may play a role in the pathophysiology of renal damage by RCM. Furthermore, since CA-Akt did not completely prevent the effects of RCM on cell viability we also attempted to identify other possible pathways that might be involved in RCM nephrotoxicity.

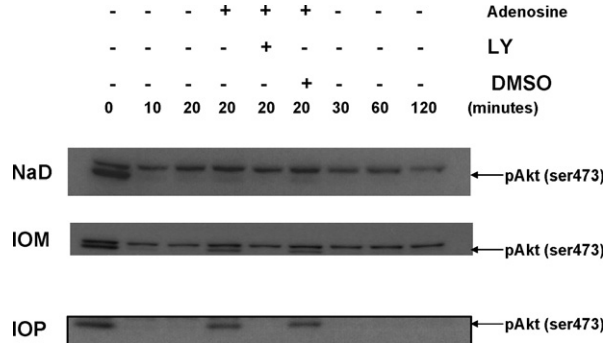

Fig. 9 – Adenosine attenuates the RCM-induced dephosphorylation of Akt of HK-2 cells. Adenosine [final concentration 100 μM] was added to the cell culture at the same time as the RCM (sodium diatrizoate: "NaD"; iopromide: "IOP"). LY294002 inhibitor (LY) [final concentration: 10 μM] and dimethyl sulfoxide (DMSO) were preincubated with the cells for 30 min prior to incubation with the RCM. The addition of adenosine to the cell culture caused an increased phosphorylation level of Akt (at Ser473) following RCM exposure. The adenosine-induced increase in Akt phosphorylation at Ser473 was prevented by LY294002 incubation. DMSO, in which LY294002 had been dissolved, was used as control: it did not change the phosphorylation levels of Akt (pAkt = phospho-Akt).

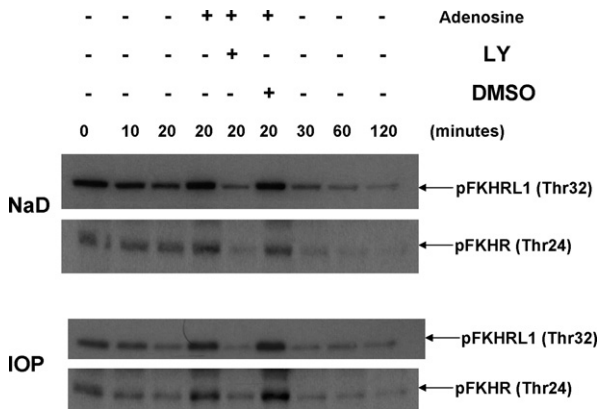

Fig. 10 – Adenosine attenuates the RCM-induced dephosphorylation of FKHR/FKHRL1 of HK-2 cells. Adenosine (final concentration 100 μM) was added to cell culture at the same time of RCM (sodium diatrizoate: "NaD"; iopromide: "IOP"). LY294002 inhibitor (LY) [final concentration: 10 μM] and dimethyl sulfoxide (DMSO) were preincubated with the cells for 30 min prior to incubation with the RCM. The addition of adenosine to cell culture caused increased phosphorylation levels of Forkhead proteins following RCM exposure. The increase in FKHR/FKHRL1 phosphorylation at Thr24/Thr32 was prevented by LY294002 incubation. DMSO, in which LY294002 had been dissolved, was used as control: it did not change the phosphorylation levels of Forkhead proteins (pFKHR = phospho-FKHR and pFKHRL1 = phospho-FKHRL1).

Thus we have investigated the effects of RCM on Forkhead family members and found dephosphorylation of Forkhead proteins by RCM (Figs. 5 and 11). When FKHR and FKHRL1 are phosphorylated on Ser24 and Ser32, respectively, they are inhibited [48]; but dephosphorylation of these sites may activate them to regulate expression of genes that are involved in apoptotic cell death [21]. However, we did not observe caspase-3 activation as evidenced by caspase-3 cleavage (Fig. 12), although we cannot rule out apoptotic cell death via a caspase-independent pathway [49].

The addition of adenosine to cell cultures together with RCM alleviated the decrease in phospho-Akt (Ser473) (Fig. 9) and Pfkhr (Thr24)/pFKHRL1 (Thr32) (Fig. 10). In addition, incubation with the PI 3-K inhibitor resulted in decreased levels of the phosphoproteins. An explanation for the latter observation may be that adenosine causes an enhancement in the activity of the PI 3-K pathway that results in phosphorylation of Akt and subsequently of FKHR/FKHRL1. The protective effect of adenosine on renal cells after an acute insult has been already reported. Using HK-2 cells, that express all the subtypes of adenosine receptors, Lee and Emala have, in fact, demonstrated that adenosine is able to protect the cells from oxidative injury [50].

Other downstream targets of Akt have been implicated in cell division and proliferation, namely the mammalian target of rapamycin (mTOR) and the 70 kDa ribosomal protein S6 kinase ($p70^{S6K}$) [44]. Upon phosphorylation of the putative Akt phosphorylation site at Ser2448, mTOR is activated. It was

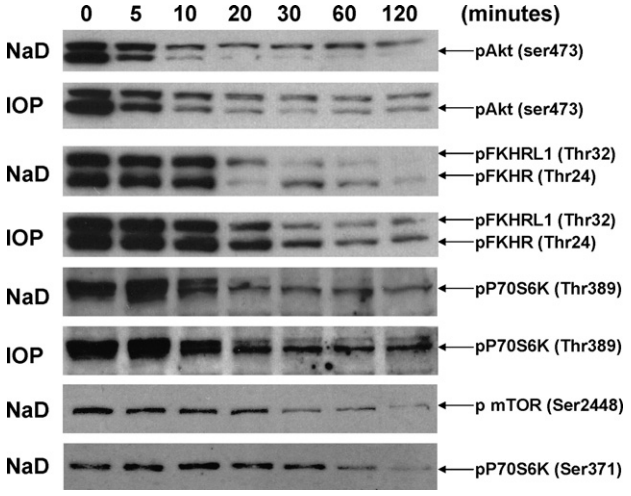

Fig. 11 – RCM-induced dephosphorylation of Akt, FKHR/FKHRL1 and p70^{S6K} in primary human renal tubular proximal (pHRTP) cells. pHRTP cell cultures were prepared and analysed as described in Fig. 2. Phosphorylation levels of Akt, FKHR/FKHRL1, mTOR and p70^{S6K} decreased after incubation of pHRTP cells with either sodium diatrizoate ("NaD") or iopromide ("IOP") (pAkt = phospho-Akt; pFKHR = phospho-FKHR; pFKHRL1 = phospho-FKHRL1; pP70S6K = phospho-p70^{S6K}; p mTOR = phospho-mTOR).

observed that some dephosphorylation of this site occurs upon incubation with RCM, albeit this takes place only transiently with iopromide/iomeprol. Akt may also regulate mTOR activity by phosphorylating and relieving the inhibitory effect of tuberin on mTOR [44]. Active mTOR phosphorylates amongst its substrates the translational repressors and, in so doing, it overcomes inhibition of protein synthesis. The consequent ability to increase the protein synthesis capacity of the cell is responsible, at least in part, for the ability of TOR proteins to drive cell growth and proliferation [51]. This inactivation of mTOR may result in decrease in cell proliferation and hence lead to the loss in the observed cell viability. As mentioned above, p70^{S6K} is also a target of mTOR as it is of Akt. Here we focused on two phosphorylation sites in p70^{S6K}, at Thr389 and Ser371. The site at Thr389 has also been reported to be an Akt phosphorylation site as well as a rapamycin-sensitive and hence a target of mTOR [43].

We have found a considerable decrease in phosphorylation of p70^{S6K} and mTOR upon incubation with RCM (Figs. 6 and 7). In the case of the Thr389 site, the dephosphorylation was more dramatic than that at Ser371 and followed more closely that of Akt (Fig. 6). Phosphorylation at Thr389 is critical for p70^{S6K} activity [43] and dephosphorylation at this site would therefore result in inactivation of p70^{S6K} and consequently in the inhibition of protein synthesis and cell growth [44]. The fact that there was a gradual decrease in phosphorylation of Ser371 (Fig. 6) but the levels of phospho-mTOR were back to normal in the case of iopromide incubation (Fig. 7), suggests that, despite the phosphorylation status of mTOR, its activity may be controlled by a mechanism other than phosphorylation as already mentioned.

It is important to note that in pHRTP cells (Fig. 11) the dephosphorylation of Akt, Forkhead proteins and p70^{S6K} followed the same pattern of HK-2 cells, thereby confirming previous reports demonstrating that HK-2 cells retain the phenotypic expression and functional characteristics of human proximal tubular cells [52,53].

The observation that CA-Akt did not prevent the loss in cell viability following the incubation with RCM suggested that other pathways, independent of Akt, may be involved in causing loss in viability. We have therefore decided to investigate the effect of RCM on the phosphorylation status of ERK1/2. Our experiments have shown that whilst iopromide causes a transient decrease in phosphorylation of ERK1/2, sodium diatrizoate induces a significant decrease in the phosphorylation status of these kinases for a long period of time (Fig. 8). Since these kinases have about 160 different identified substrates [34] the impact on the cell may be considerable. It is interesting to note that the ERKs have also been implicated in translational control, possibly cooperating with the mTOR pathway [44]; thus, their inactivation will impact on cell growth and proliferation.

In summary we have identified several key signaling components that are dephosphorylated as a result of incubation with RCM. These components are key signaling molecules that regulate cellular growth and proliferation. Their inactivation by dephosphorylation may contribute to the nephrotoxicity of RCM. The dephosphorylation of intracellular signaling components by sodium diatrizoate was greater than that of iopromide. Akt may play an important role in the toxic cellular effect of RCM, since transfection of CA-Akt was able to partially improve renal cell viability after incubation with RCM. Our results represent an important step in the direction of prevention of radiocontrast nephrotoxicity. By knowing the effects on such molecules we can develop ways to overcome the detrimental effects of contrast media.

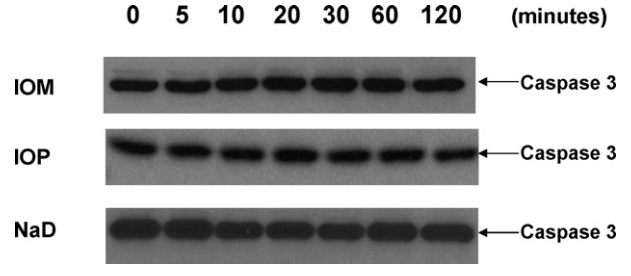

Fig. 12 – Caspase-3 levels of HK-2 cells are unaffected by RCM. Samples were prepared and analysed as described in Fig. 2. RCM did not change levels of 35 kDa caspase-3 protein throughout the timecourse. The band seen here is the full length uncleaved 35 kDa caspase-3 protein.

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