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Differential Activation of Signaling Pathways by Low-Osmolar and Iso-Osmolar Radiocontrast Agents in Human Renal Tubular Cells

Michele Andreucci,[1]* Teresa Faga,[1] Domenico Russo,[2] Bernardo Bertucci,[3] Oscar Tamburrini,[3] Antonio Pisani,[2] Massimo Sabbatini,[2] Giorgio Fuiano,[1] and Ashour Michael[1]

[1]*Chair of Nephrology, Department of Health Sciences, "Magna Graecia" University, Catanzaro I-88100, Italy*

[2]*Chair of Nephrology, Department of Public Health, "Federico II" University, Naples I-80131, Italy*

[3]*Chair of Radiology, Department of Experimental and Clinical Medicine, "Magna Graecia" University, Catanzaro, I-88100, Italy*

ABSTRACT

Radiocontrast media (RCM)-induced nephrotoxicity (CIN) is a major clinical problem accounting for 12% of all hospital-acquired cases of acute kidney injury (AKI). The pathophysiology of AKI due to RCM is not well understood, but direct toxic effects on renal cells have been postulated as contributing to CIN. It is believed that iso-osmolar RCM (IOCM) are less nephrotoxic than low-osmolar RCM (LOCM) but clinical data have been controversial. We have investigated the intracellular signaling pathways that may be affected by the LOCM iomeprol (IOM) and the IOCM iodixanol (IOD). Both IOM and IOD caused a dramatic decrease in phosphorylation of the kinase Akt at Ser473 and Thr308 in human renal tubular (HK-2) cells, with IOM having a greater effect; IOM also caused a greater decrease in cell viability. IOM also had a greater effect on phosphorylation of p38 MAP kinases, JNKs, and NF-kB (Ser276), and caused a marked decrease in the phosphorylation of forkhead box O3a (FOXO3a) and signal transducer and activator of transcription 3 (STAT3). However, IOD caused a greater decrease in the phosphorylation of mTOR (Ser2448) and phospho-ERK 1/2 while both RCM caused a similar decrease in the phosphorylation of phospho-p70S6 kinase (Ser371). In vivo studies showed that both IOM and IOD caused a significant decrease in both pAkt (Ser473) and pERK 1/2 in rat kidneys. Our study gives an insight into the possible mechanism of toxicity of RCM via their action on intracellular signaling pathways and may help in developing pharmacological interventions for their side-effects. J. Cell. Biochem. 115: 281–289, 2014. © 2013 Wiley Periodicals, Inc.

KEY WORDS: SIGNALING; RADIOCONTRAST MEDIA; RENAL CELL; IODIXANOL; KINASE

Radiographic contrast media (RCM) are widely used in clinical practice. Their use, however, may lead to contrast-induced nephropathy (CIN) especially in patients with renal function already reduced and in patients with diabetes [Rich and Crecelius, 1990; Rudnick et al., 1995; Lin and Bonventre, 2005]. The continued growth in radiographic examinations means that increasing number of patients are exposed to RCM, which has resulted in a high incidence of acute kidney injury (AKI) associated with RCM use [Katzberg, 2005; Persson et al., 2005; Solomon, 2005], being the third leading cause of hospital-acquired AKI accounting for 12% of all cases [Nash et al., 2002]. Hence, there is considerable and evermore growing interest in the prevention of CIN, but unfortunately few pharmacologic interventions have been found to be beneficial, and only extracellular volume expansion via intravenous fluid administration has shown some success [Weisbord and Palevsky, 2008]. While all types of RCM may contribute to the incidence of CIN, it is acknowledged that high-osmolar RCM (HOCM) are more nephrotoxic than the low-osmolar RCM (LOCM) and iso-osmolar RCM (IOCM) [Morcos, 2009]. It has been suggested that IOCM may be less nephrotoxic than LOCM, but to date clinical studies have been

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*Correspondence to: Prof. Michele Andreucci, MD, PhD, Chair of Nephrology, "Magna Graecia" University, Viale Europa, loc. Germaneto, Campus "Salvatore Venuta", I-88100 Catanzaro, Italy. E-mail: andreucci@unicz.it

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controversial [Jo et al., 2006; Mehran et al., 2009; Reed et al., 2009; Shin et al., 2011a; Bolognese et al., 2012]. While the pathophysiology of AKI due to RCM administration is not well understood, the reduction of renal blood flow and/or direct toxic effects on renal tubular epithelial cells have been postulated as major causes of CIN [Heyman et al., 1991; Tervahartiala et al., 1997], with RCM reported to induce apoptosis both in glomerular cells and in renal tubular epithelial cells [Zhang et al., 1999; Hizoh and Haller, 2002; Yano et al., 2003].

Since kinase mediated intracellular signaling pathways can modulate cell growth, proliferation, death, and inflammation [Datta et al., 1999; Cross et al., 2000; Woodgett, 2000; Kyriakis and Avruch, 2001], characterization of some of these intracellular pathways in cells exposed to RCM may shed some light on the extent and mechanism of their toxicity and possibly help in future development of pharmacological therapies in order to reduce the risk of CIN. We have previously found that in human renal proximal tubular (HRPT) cells both HOCM and LOCM caused the dephosphorylation (inactivation) of Akt, a kinase that is known to play a central role in cell survival, together with a decrease in phosphorylation of its downstream targets [Andreucci et al., 2006]. Further work showed that HOCM also caused a greater activation of the c-Jun N-terminal and p38 mitogen activated protein kinases (JNKs and p38 MAPKs, respectively) together with a corresponding increase in the pro-inflammatory cytokine IL-8 compared with LOCM [Andreucci et al., 2011]. In the present study we have used a similar approach to compare the effects of two of the most recently developed classes of RCM on intracellular signaling pathways known to play a role in cell death, survival/proliferation, and inflammation: an LOCM, iomeprol (IOM), and an IOCM, iodixanol (IOD).

MATERIALS AND METHODS

MATERIALS

The RCM used in our study were IOM (Iomeron 400™, Bracco S.p.A, Milan, Italy), IOD (Visipaque 320™) and sodium diatrizoate (NaD) (Sigma–Aldrich Co., St.Louis, MO). The chosen concentrations of each used in this study are physiologically relevant and were calculated based on the dosage commonly used in clinical practice as mentioned in previous studies [Andreucci et al., 2011].

ANIMAL STUDIES

Preliminary studies on normal rats, on the basis of data from current literature, were performed to ascertain the doses, timing, and route of administration of radiographic contrast media. This study was carried out in 18 male Sprague-Dawley rats (3 months-old, Charles River) fed a standard diet (19% protein content as casein) and tap water ad libitum. The experimental protocol and surgical procedures using animals were carried out according to Italian law and were approved by the Italian Ministry of Health.

After light i.p. anesthesia with Zoletil (25 mg/kg) and Xylazine (25 mg/kg), the right kidney was nephrectomized under sterile conditions. Seven days later, the rats were randomly assigned to one of the following groups (n = 6 each group): control (C), IOCM and LOCM. Seven days later, after a 4-day period of water deprivation, the rats were anaesthetized with sodium pentobarbital (4 mg per 100 g body weight injected intraperitoneally) and 10 mg/kg indomethacin was administered via the femoral vein. After a period of 15 min, 0.9% normal saline (for control rats), 6 ml per kg of IOD (320 mgI/ml, Visipaque, GE Healthcare, Milano, Italy) [IOCM] or IOM (400 mgI/ml, Iomeron Santa Farma, Italy) [LOCM] were injected via the femoral artery cannulation.

After 24 h, the rats were anaesthetized again (Inactin, 80 mg/kg intraperitoneally) and the left kidneys were removed and immediately snap frozen in liquid nitrogen and stored at −80°C.

CELL CULTURE

In our experiments we have used HK-2 cells (a HRPT epithelial cell line), obtained from the American Type Culture Collection (ATCC®) and grown in 100 mm culture dishes (Corning) as described before [Andreucci et al., 2006]. In brief, they were cultured in DMEM containing Glutamax™ (Gibco) supplemented with 10% Fetal Calf Serum and 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma) in an atmosphere of 5% CO_2 in air at 37°C, up to a confluence of approximately 90%.

CELL VIABILITY

Cell viability was measured by the ability of viable cells to reduce MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma) [Andreucci et al., 2009]. Cells were grown in six-well plates; after treatments with the RCM, the cells were washed once with sterile PBS and incubated with 1 mg/ml MTT (in sterile PBS) for 1 h at 37°C; they were then dissolved in dimethyl sulphoxide (DMSO). Measurements of the coloured product as a result of MTT reduction were made at 540 nm using a Beckman DU 800 spectrophotometer.

WESTERN BLOT ANALYSIS

HK-2 cells, at each time point, 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, 400 μM PMSF. The samples were then centrifuged at 10,000*g* for 10 min and the supernatant was retained (lysate). Part of the supernatant was used to determine the protein content and part utilized for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein concentrations were determined by using a modified Bradford protein assay protocol [Bradford, 1976] in order to obtain an equal loading (approximately 30 μg of each sample were loaded). Tissue extracts were prepared from whole rat kidneys as previously described [Andreucci et al., 2010].

Protein extracts were resolved by SDS–PAGE and transferred to a nitrocellulose membrane (Hybond C® extra, Amersham Biosciences), as previously described [Andreucci et al., 2003]. 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), diluted

1:5,000 TBST with 1% (w/v) non-fat powdered milk at room temperature. It was then washed as above (i.e., three times). 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 Signalling, Beverly, MA); anti-phospho-Akt (Ser473 and Thr308, Cell Signalling); anti-phospho-p38 (Cell Signalling); anti-phospho-FOXO3a (Thr32)/anti-phospho-FOXO1 (Thr24) (Cell Signalling); anti-caspase-3 (Cell Signalling); anti-phospho-p70S6 kinase (Ser371) (Cell Signalling); anti-phospho-NF-κB [p65 subunit] (Ser276); anti-phospho STAT3 (Tyr705) (Cell Signalling); anti-Pim-1 (Cell Signalling); anti-phospho-mTOR (Ser2448) (Cell Signalling); anti-phospho JNK1/2 (anti-ACTIVE, Promega); anti-β-actin (Sigma).

STATISTICS

All results were expressed as mean ± SE. Statistical analysis was performed using ANOVA for unpaired data (GraphPad 4.0 software). Statistical significance was defined as $P < 0.05$.

RESULTS

VIABILITY OF HK-2 CELLS AFTER EXPOSURE TO IOM AND IOD

HK-2 cells were exposed to IOM and IOD for 3 h at concentrations of 75 and 100 mgI/ml for each RCM, after which the media containing the RCM was removed and cell viability determined using the MTT assay. The results in Figure 1A show that compared to control (non-treated) cells, both RCM caused a significant decrease in cell viability at both concentrations used ($P < 0.005$). Furthermore, cells treated with 75 mgI/ml of IOM showed a more significant decrease in cell viability than those treated with the corresponding concentration of IOD ($P < 0.005$) and, similarly, cells treated with 100 mgI/ml of IOM showed a more significant decrease in cell viability compared with cells treated with the same concentration of IOD ($P < 0.005$). There was no statistically significant difference in cell viability between the cell populations treated with different concentrations of IOD. Although cells treated with 100 mgI/ml IOM appeared to show a greater decrease in cell death compared with those treated with 75 mgI/ml it was not statistically significant (Fig. 1A).

In another experiment HK-2 cells were exposed to IOM and IOD at a concentration of 100 mgI/ml for 3 h after which the RCM stimulus was removed and the cells incubated for a further 21 h (i.e., 24 h after the initial incubation with the RCM) with fresh medium and cell viability measured. The results in Figure 1B show that the viability of both IOM- and IOD-stimulated cells were the same as in the control non-treated cells.

IOM AND IOD CAUSE DEPHOSPHORYLATION OF Akt

Incubation of HK-2 cells with both IOM and IOD at concentrations of 75 and 100 mgI/ml caused a drastic dephosphorylation of Akt at Ser473 (Fig. 2A), starting at 5 min after incubation with each RCM. In the case of IOM, the decrease in phospho-Akt (Ser473) ("pAkt") was greatest at 30 min followed by a slight recovery up to 3 h after incubation. This slight recovery, however, was not present at the higher concentration of IOM. Similarly for IOD, the pAkt signal was weakest after 30 min of incubation with the RCM after which there was a slight recovery of the signal; again, the recovery was less in cells treated with the higher concentration of IOD. For both RCM concentrations, the dephosphorylation of pAkt was greater in IOM-treated than IOD-treated cells (Fig. 2A). Dephosphorylation of Akt at Thr308 was also observed in RCM-treated HK-2 cells (Fig. 2B). The pAkt (Thr308) signal was completely reduced within 5 min post-incubation with the RCM, and in cells treated with IOM there was no observable signal for the whole 3 h period of incubation with the RCM at both concentrations used, whereas in IOD-treated cells there was a slight recovery in the signal with less recovery noted at the higher concentration used. It should be noted that while the protein band representing pAkt (Thr308) is obvious in the farthest left lane of Figure 2B, it is not apparent in the rest of the lanes in the IOM panel and is only evident as the lower band of the doublet seen in the IOD panel. HK-2 cells treated with NaD show a dramatic decrease in both pAkt (Ser473) and pAkt (Thr308) comparable to IOM-treated cells.

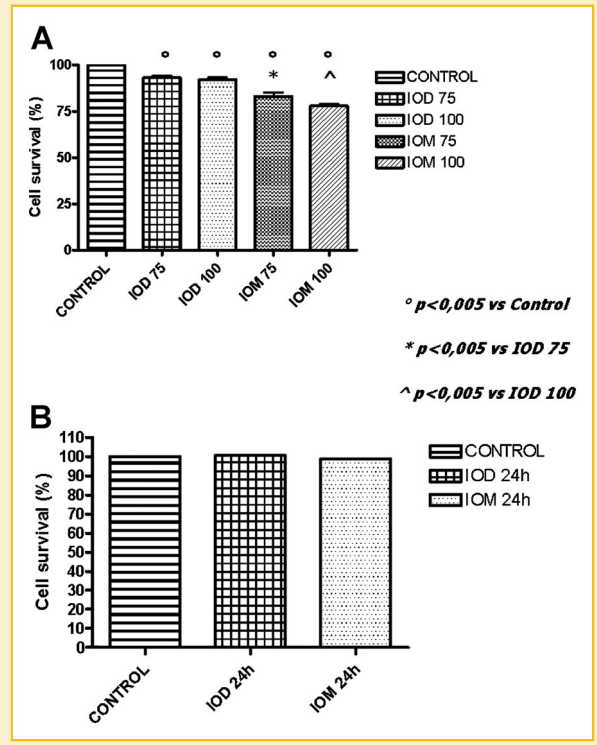

Fig. 1. Effect of iomeprol and iodixanol on cell viability in HK-2 cells. The columns indicate cell viability after overnight culture in serum-free medium and subsequent incubation of HK-2 cells with iomeprol (IOM) or iodixanol (IOD) at two concentrations (corresponding to 75 and 100 mgI/ml) for 3 h. Cell viability was assessed by the MTT assay at the end of the 3-h incubation period (A) or 21 h after removal of the RCM (B). The *chemical reduction* of MTT is expressed as a percentage of the control. In A both RCM at both concentrations used, reduced cell viability compared to untreated (control) HK-2 cells ($P < 0.005$ shown as °). Cell viability decreased more in IOM-treated cells than in IOD-treated cells at both the respective concentrations used ($P < 0.005$ shown as * for IOM 75 mgI/ml vs. IOD 75 mgI/ml $P < 0.005$ shown as ^ for IOM 100 mgI/ml vs. IOD 100 mgI/ml).

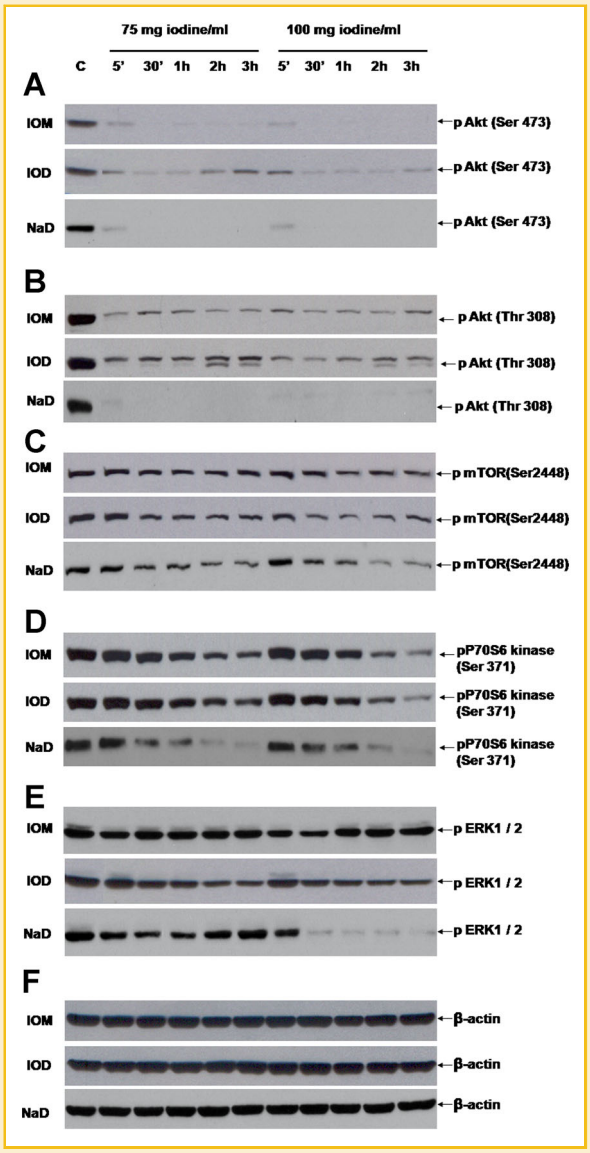

Fig. 2. Effect upon the phosphorylation status of kinases involved in cell survival and proliferation in HK-2 cells exposed to iomeprol and iodixanol. HK-2 cells were incubated with each of iomeprol (IOM), iodixanol (IOD), and sodium diatrizoate (NaD) at two concentrations (75 and 100 mgI/ml) for each RCM, for the shown times. NaD-treated cells were included as a reference to see changes induced by a very toxic RCM. Then cell lysates were prepared and subjected to SDS–PAGE and Western blotting as explained in the Materials and Methods Section. Panel A: blots probed with anti-phospho-Akt (Ser473) antibody; Panel B: blots probed with anti-phospho-Akt (Thr308) antibody; Panel C: blots probed with anti-phospho-mTOR (Ser2448) antibody; Panel D: blots probed with anti-phospho-p70S6 kinase (Ser371) antibody; Panel E: blots probed with anti-phospho-ERK1/2 antibody; Panel F: blots probed with anti-β-actin antibody. The data shown are representative of blots of three experiments.

IOM AND IOD CAUSE DEPHOSPHORYLATION OF mTOR

A dephosphorylation of the serine/threonine kinase mammalian target of Rapamycin (mTOR) was observed in HK-2 cells incubated with IOM and IOD (Fig. 2C). A decrease in the phospho-mTOR (Ser2448) signal was evident after 30 min of incubation with the RCM. Interestingly, IOD-treated cells showed a greater decrease in phospho-mTOR than IOM-treated cells. In both cases the decrease was greater at the higher concentration of RCM. NaD-treated cells exhibited a greater decrease in phospho-mTOR (Ser2448) that was both time and concentration dependent.

IOM AND IOD CAUSE DEPHOSPHORYLATION OF p70S6 Kinase

The serine/threonine kinase p70S6 kinase was dephosphorylated at Ser371 in cells treated with IOM and IOD (Fig. 2D). Both RCM seemed to have a similar effect with levels of phospho-p70S6 kinase (Ser371) decreasing gradually with time of incubation with the higher concentration of RCM having a greater effect especially at the longer incubation times. Again, cells incubated with NaD showed a more dramatic decrease in phospho-p70S6 kinase (Ser371) that was time and concentration dependent.

EFFECT OF RCM ON PHOSPHORYLATION OF ERKs

The levels of phospho-ERK 1 and 2 ("pERK 1/2") in cells incubated with 75 mgI/ml IOM decreased markedly after 5 min before increasing again after 30 min while those incubated with 100 mgI/ml decreased for at least 30 min before increasing again after 1 h (Fig. 2E). Interestingly, IOD appeared to cause a decrease in pERK 1/2 that was time and concentration-dependent. Cells incubated with 75 mgI/ml NaD caused a marked decrease in pERK 1/2 but appeared to recover after 3 h; however, a dramatic decrease was observed in cells incubated at the higher concentration of NaD after 30 min and did not change for the remainder of the 3 h incubation period.

EFFECT OF RCM ON PHOSPHORYLATION OF NF-kB, p38, AND JNK MAP KINASES

Incubation of HK-2 cells with IOM and IOD also caused differential changes in the phosphorylation status of the JNK (Fig. 3A) and p38 MAP kinases (Fig. 3B) and the transcription factor NF-kB (Fig. 3C), molecules that have been implicated in cell death and inflammation. While an increase in the phosphorylation of all three molecules was observed in cells treated with IOM, IOD-treated cells only showed an obvious increase in the phosphorylation of JNKs albeit much less than in IOM-treated cells. Only small increases in phospho-NF-kB ("pNF-kB") and phospho-p38 kinase (at 5 and 30 min of incubation) were observed in IOD-treated cells. It should be noted that levels of phospho-JNKs and phospho-p38 returned to basal levels at the end of the 3 h period of incubation. Significantly greater increases in the phosphorylation status of all three molecules were observed in NaD-treated cells.

EFFECT OF RCM ON FOXO3a, STAT3, AND CASPASE-3

No changes were noted in the levels of caspase-3 in cells treated with RCM at both concentrations (Fig. 4A), with no cleavage products detected. However, dramatic decreases in the phosphorylated levels of the transcription factors forkhead box protein O3a (FOXO3a) (Fig. 4B) and STAT3 (Fig. 4C) were observed with time in cells treated with IOM, and the decrease was greater at the higher concentration used. In IOD-treated cells a slight decrease in pFOXO3a levels was observed at both concentrations used, while a more noticeable reduction in phospho-STAT3 ("pSTAT3") levels was observed. In NaD-treated cells the decrease in pFOXO3a levels was more dramatic than that observed in

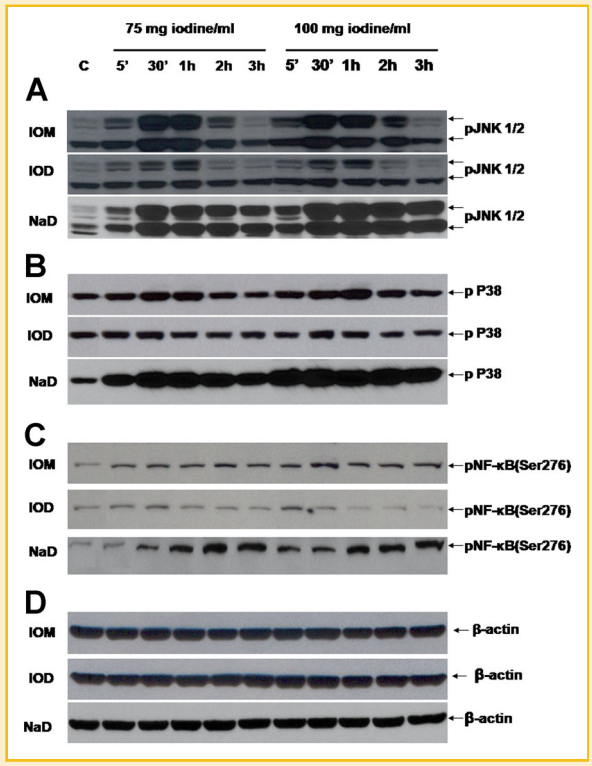

Fig. 3. Phosphorylation of JNKs, p38, and NF-kB in HK-2 cells exposed to iomeprol and iodixanol. Legend as for Figure 2. Panel A: blots probed with anti-phospho-JNK1/2 antibody; Panel B: blots probed with anti-phospho-p38 antibody; Panel C: blots probed with anti-phospho-NF-κB (Ser276) antibody; Panel D: blots probed with anti-β-actin antibody. The data shown are representative of blots of three experiments.

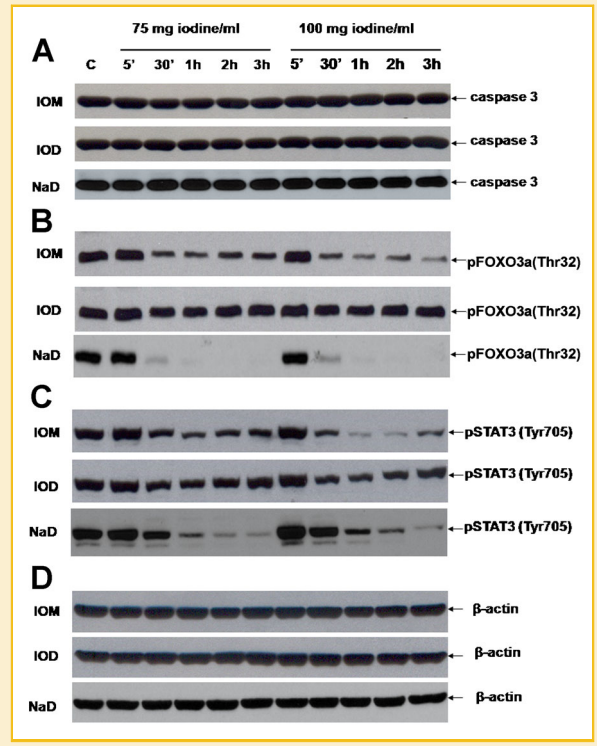

Fig. 4. Effect of iomeprol and iodixanol on caspase-3, and phosphorylation of the transcription factors FOXO3a and STAT3 in HK-2 cells. Legend as for Figure 2. Panel A: blots probed with anti-caspase-3 antibody; Panel B: blots probed with anti-phospho-FOXO3a (Thr32) antibody; Panel C: blots probed with anti-phospho-STAT3 (Tyr705) antibody; Panel D: blots probed with anti-β-actin antibody. The shown data are representative of blots of three experiments.

IOM-treated cells, while the decrease in pSTAT3 was also more marked than IOM-treated cells at 75 mgI/ml.

LONG-TERM EFFECT ON SIGNALING MOLECULES AFTER AN INITIAL EXPOSURE TO IOM AND IOD

HK-2 cells were incubated with 100 mgI/ml of either IOM or IOD for 3 h, after which the medium containing the RCM was removed and replaced with fresh serum-free medium, and the phosphorylation/activation of various signaling molecules were assessed by Western blotting at various time intervals afterwards. A slight increase in pAkt (Ser473) was observed 1 h after removal of the RCM, after which the signal decreased gradually, falling to below basal levels at 5 h before recovering and showing greater than basal levels at 21 h after removal of the RCM (Fig. 5, panel A).

The expression of the serine/threonine kinase Pim-1 was also observed to be upregulated in IOM-treated but not in IOD-treated cells; its expression was observed to be strongest at 1 h after removal of the RCM and then gradually decreasing thereafter. Pim-1 expression was also not observed in NaD-treated cells (Fig. 5, panel B).

The levels of pERK1 and pERK2 also increased 1 h after removal of the RCM but then reverted to basal levels (Fig. 5, panel C). Interestingly, pSTAT3 (Tyr705) levels decreased for the first 3 h in IOM-treated cells before gradually increasing back to the basal levels found in non-treated cells (Fig. 5, panel D). In stark contrast, pERK 1 and 2 were upregulated in NaD-treated cells up to 5 h after removal of the RCM while levels of pSTAT3 (Tyr705) were significantly reduced; the levels of both molecules were absent in samples 21 h after removal of the RCM. Phosphorylated levels of mTOR (at Ser2448) increased 1 h after removal of the RCM, but in IOD-treated cells, those levels decreased to below those of the control levels before recovering again at 21 h after removal of the RCM (Fig. 5, panel E), whereas in NaD-treated cells the levels were elevated above those of the control for up to 5 h after removal of the RCM, with none detected in the 21 h sample.

In IOD-treated cells levels of pFOXO3a increased up 2 h after removal of the RCM but then reverted to basal levels; both IOD- and IOM-treated cells showed increased levels of phospho-FOXO3a ("pFOXO3a") 21 h after removal of the RCM (Fig. 5, panel F). However, in NaD-treated cells, pFOXO3a levels were less than in control cells 1 h after removal of RCM, decreasing with time and were not detected in the 21 h sample.

There were no significant changes in phospho-p38 in IOD- and IOM-treated cells but the increase observed in NaD-treated cells was remarkable (Fig. 5, panel G). Similarly, while phospho-JNKs were not

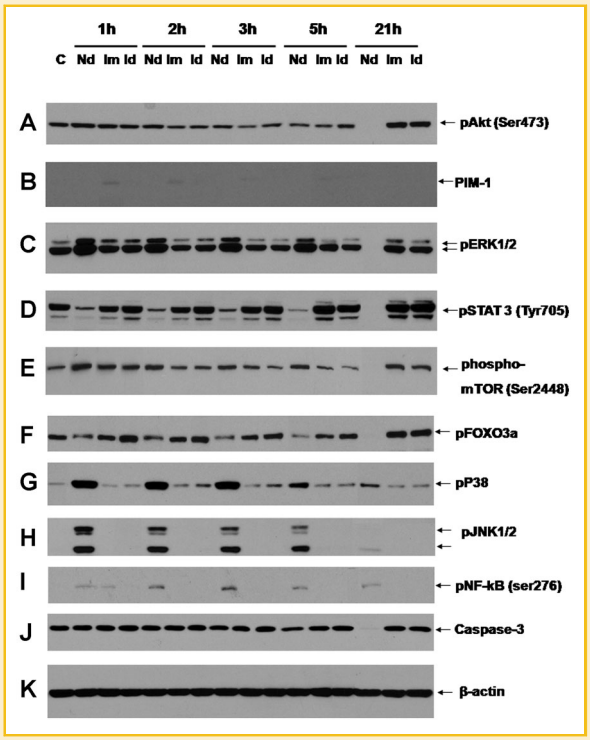

Fig. 5. Modification of signaling molecules in HK-2 cells after an initial exposure to iomeprol, iodixanol, and sodium diatrizoate. HK-2 cells were incubated with iomeprol (Im), iodixanol (Id), and sodium diatrizoate (Nd) at a concentration of 100 mgI/ml for 3 h. Nd-treated cells were included as a reference to see changes induced by a very toxic RCM. The medium containing the RCM was then removed and replaced with fresh serum-free medium; cell lysates were then prepared at the time points indicated (1, 2, 3, 5, and 21 h after removal of each of the RCM) and subjected to SDS–PAGE followed by Western blotting. Cells treated with NaD at 21 h were found to be detached and "floating" in the medium; cell lysates were prepared from these floating cells and subjected to SDS–PAGE/Western blotting. The data shown are representative of blots of three experiments. Panel A: blots probed with anti-phospho-Akt (Ser473) antibody; Panel B: blots probed with anti-PIM-1 antibody; Panel C: blots probed with anti-phospho-ERK1/2 antibody; Panel D: blots probed with anti-phospho-STAT3 (Tyr705) antibody; Panel E: blots probed with anti-phospho-mTOR (Ser2448) antibody; Panel F: blots probed with anti-phospho-FOXO3a (Thr32) antibody; Panel G: blots probed with anti-phospho-p38 antibody; Panel H: blots probed with anti-phospho-JNK1/2 antibody; Panel I: blots probed with anti-phospho-NF-κB (Ser276) antibody; Panel J: blots probed with anti-caspase-3 antibody; Panel K: blots probed with anti-β-actin antibody.

detected in IOD- and IOM-treated cells after removal of the RCM, significant levels were observed in NaD-treated cells 1 h after removal of the RCM which then decreased gradually with time (Fig. 5, panel H). A slight signal in pNF-κB (Ser276) was detected in IOM-treated cells at 1 h after removal of the RCM (Fig. 5, panel I) whereas pNF-κB (Ser276) levels were present in all the samples obtained from NaD-treated cells at all the time points after RCM removal.

Levels of caspase-3 remained unchanged (Fig. 5, panel J) in IOM- and IOD-treated cells but a decrease was observed 5 h after removal of the RCM in NaD-treated cells with only a slight trace at the 21 h time point. No degradation product was observed (not shown in Fig. 5, panel J).

EFFECT OF IOM AND IOD ON PHOSPHORYLATION STATUS OF SIGNALING MOLECULES IN RAT KIDNEYS

Whole tissue lysates, prepared from rat kidneys excised from animals exposed to IOD and IOM, and subjected to Western blotting are shown in Figure 6. Both IOM and IOD caused a dramatic decrease in the phosphorylation status of the pro-survival kinases Akt and ERK 1 and 2. However, no significant changes were observed for the phospho-p38 MAP kinase, while no signal was detected for pJNKs, pNF-kB (Ser276), or pFOXO3a (data not shown).

DISCUSSION

Our cell viability studies indicate that IOD is toxic to HK-2 cells but less so than IOM. Other in vitro studies have suggested that IOD may be slightly more or equally toxic to IOM [Hardiek et al., 2001; Heinrich et al., 2005], although the cells and experimental conditions used were different to ours. In previous work from our laboratory, we have shown the effects of both HOCM and LOCM on intracellular signaling pathways when incubated with HK-2 cells [Andreucci et al., 2006, 2011]. In particular, we observed the dramatic dephosphorylation at Threonine 308 and Serine 473 (and hence inactivation) of Akt, a kinase known to play a role in cell survival/proliferation [Datta et al., 1999]. In our present work we observed that the LOCM, IOM, caused a greater decrease in pAkt than the IOCM, IOD, and this was also dependent on the concentration of the RCM used. One could correlate the effect on Akt with the greater decrease in cell viability, particularly considering our previous observations that transfection of HK-2 cells with constitutively activated Akt can improve cell viability in RCM-treated cells [Andreucci et al., 2006]. When the RCM was removed, Akt levels eventually returned to basal levels 21 h later, coinciding with a recovery in cell viability.

Investigation of other signaling pathways that are crucial to cell survival/death also showed differences in their phosphorylation (activation) status in HK-2 cells incubated with the different RCM. Phosphorylated forms of ERK1 and ERK2 were more dramatically decreased in IOD-treated cells. The ERK MAP kinases have a wide range of substrates and play an important role in cell survival,

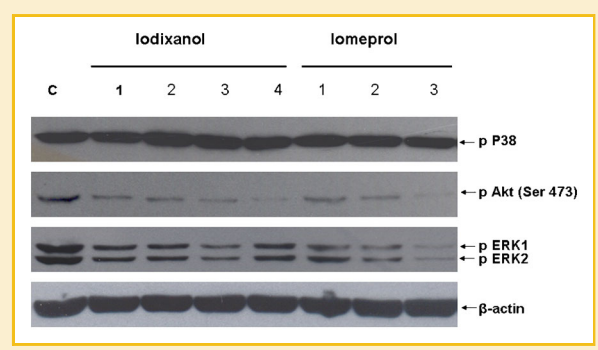

Fig. 6. Effect of iomeprol and iodixanol on the phosphorylation of Akt, ERK 1/2, and p38 in rat kidneys. Rat kidney lysates were prepared as in the Materials and Methods Section, from kidneys excised from animals treated with iomeprol and iodixanol. The lysates were then subjected to SDS–PAGE followed by Western blotting.

proliferation, and differentiation [Roskoski, 2012], and their dephosphorylation (inactivation) would again have consequences upon cell fate, given that it has been estimated that they may regulate the function of more than a thousand proteins in the cell [Avruch, 2007]. However, after removal of the RCM, the levels of both kinase isoforms returned to normal basal levels within 24 h.

Another kinase that is at the centre of an intracellular signaling pathway that regulates cell growth, proliferation and survival is mTOR, which forms the catalytic core of two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [Foster and Fingar, 2010]. These complexes contain some shared and other different partner proteins reflecting the various cellular processes which they control in response to diverse physiological stimuli. mTORC1 is mainly phosphorylated at Ser2448 while mTORC2 is phosphorylated at Ser2481 [Copp et al., 2009]. Although Akt was believed to mediate Ser2448 phosphorylation of mTORC1 it is now thought that p70S6 kinase 1 is the mTOR Ser2448 kinase [Foster and Fingar, 2010]. Our observations that the phospho-mTOR (Ser2448) signal does not decrease in line with the decrease of pAkt agrees with this. However, it has been suggested that Akt may regulate mTORC1 by phosphorylating and thereby inactivating TSC2, a negative regulator of mTORC1 [Hay and Sonenberg, 2004] although it is believed that the data supporting this hypothesis are weak, while at the same time it is believed that TSC2 may promote mTORC2 signaling [Foster and Fingar, 2010]. mTORC1 promotes protein synthesis by phosphorylating the eukaryotic initiation factor 4E (eIF4E)-binding protein 1, 4EBP1, causing it to dissociate from eIF4E and allow cap-dependant translation [Foster and Fingar, 2010]. p70S6-kinase was believed to be directly phosphorylated on Ser371 by mTORC1 [Saitoh et al., 2002] but recent work has suggested that glycogen synthase kinase (GSK)-3β is responsible for its phosphorylation, with mTORC1 playing a role in inhibiting the PP2A-like phosphatases that de-phosphorylate the Ser371 site [Shin et al., 2011b]. Thus if one were to use levels of phospho-p70S6-kinase (Ser371) as a guide to mTORC1 activity, levels of phospho-p70S6-kinase (Ser371) in HK-2 cells incubated with IOD and IOM suggest that the effect on mTORC1 activity is similar, even though the phosphorylation at Ser2448 is not. However, the effects on phospho-GSK-3β were not investigated, so any inferences would be tentative. The Ser/Thr kinase PIM-1 was also upregulated in the first few hours after removal of IOM from HK-2 cells and given its role in cell survival and proliferation [Amaravadi and Thompson, 2005] it may also contribute to cell recovery. However, while recovery in the cell population was also observed in IOD-treated cells, Pim-1 expression was not observed after removal of the RCM. This suggests that Pim-1 expression may be necessary to suppress or induce a factor(s) involved in cell death or survival respectively in IOM-treated cells.

Both IOM and IOD treatment of HK-2 cells decreased the levels of phospho-FOXO3a and phospho-STAT3 (Tyr705), two transcription factors that play a role in cell death/survival and proliferation. When FOXO3a is phosphorylated, it is sequestered in the cytoplasm but when de-phosphorylated it is translocated to the nucleus where it may control the expression of genes involved in apoptosis, such as the Fas ligand gene [Brunet et al., 1999]. Hence, one would expect IOM-treated cells to be more vulnerable to cell death as a result of dephosphorylation of this protein. Since the serum- and glucocorticoid kinases (SGK) also phosphorylate the Thr32 site of FOXO3a, it may be possible that in IOD-treated cells the higher levels of phospho-FOXO3a (Thr32) may be due to the action of SGK which by inference would mean is also deactivated in IOM-treated cells.

STAT3 has been implicated in cell survival and proliferation and is activated by phosphorylation on Tyr705, which leads to its dimerization, nuclear translocation, and binding to its target genes [Levy and Lee, 2002]. Again, one would expect a greater negative effect on cell viability in IOM-treated cells.

No cleavage of caspase-3 levels was detected in RCM-treated HK-2 cells, indicating that the decrease in cell viability occurs via other mechanisms.

The p38 and JNK subfamilies of the MAP kinases and the transcription factor NF-kB have been implicated in cell death and inflammation [Kyriakis and Avruch, 2001]. The greater activation (phosphorylation) of all these molecules may contribute to the greater loss in cell viability observed in IOM-treated cells. Moreover, activated p38 and JNK MAP kinases, and NF-kB (phosphorylated at Ser276) have been implicated in the upregulation of IL-8 [Li and Nord, 2002; Nowak et al., 2008]; hence IOM-treated cells may be more likely to be prone to inflammation. The changes shown in Figure 5 suggest that HK-2 cells may eventually recover normal levels of signaling pathways after an initial exposure to IOD (IOCM) and IOM (LOCM), whereas the dramatic changes seen in the same pathways upon exposure to NaD (HOCM), as a reference of a very toxic RCM, leads to cell death as seen by the detachment of cells from culture dishes (also noted in the legend to Fig. 5). However, in cells that may already be suffering an existing stress, exposure to an additional stress such as an IOCM or LOCM may cause perturbations in the signaling pathways that may lead to a different cellular outcome.

The animal studies carried out yielded both confirmatory and contradictory results to those expected based on the in vitro human cell culture experiments. While phospho-p38 levels remained relatively unchanged in the animal studies, the lowered levels of pAkt (Ser473) confirmed our observations in HK-2 cells when incubated with the RCM for up to 3 h. Also the lowered levels of pERK 1/2 confirmed the effect in IOD-treated HK-2 cells, but were contrary to what was expected from IOM-treated cells. Furthermore, our long-term incubations after an initial (3 h) exposure to RCM suggested a recovery in both the pAkt and pERK1/2 signals. Notwithstanding the limitations of any in vitro study, the discrepancy may be due to the doses administered in the in vivo studies being higher than commonly used in clinical settings. Nonetheless, the effect on both pAkt and pERK 1/2 is remarkable, especially since these effects are noted 24 h after RCM administration and in whole kidneys that are composed of different types of tissues and cells suggesting that RCM may have a similar effect in mesangial, podocyte, vascular smooth muscle, and endothelial cells. Furthermore, in some clinical situations the amount of RCM used may be higher such as in coronary computed tomography angiography, and 5 ml/kg of RCM volume may be used [Davidson et al., 2006]. In any case, some in vivo studies using clinical doses of RCM have reported a urinary concentration of iodine as high as 120–130 mgI/ml (cited in Beaufils et al. [1995], which is much higher than the maximum concentration used in our study. Furthermore, consideration ought to be given also to the fact that

in patients with renal failure the elimination time of RCM may be considerably longer than normal healthy individuals [Donnelly et al., 1993] and so the time of contact of RCM with cells and tissues may also be significantly prolonged. The decrease in phosphorylation of Akt and ERKs 1 and 2 is also intriguing, given that they have both been implicated in iNOS expression [Oh et al., 2008; Korkmaz et al., 2011] and their inactivation (dephosphorylation) would therefore be correlated with a decrease of this enzyme leading to lower vasodilation of blood vessels and hence reduced blood flow to the kidneys. This would be in addition to the deleterious effect on cell survival by their inactivation, as key members of pro-survival signaling pathways. The observations made in this study should also be taken into consideration when assessing how RCM may affect kidneys that may already be compromised. For example, in an in vivo study using gentamicin-induced kidney failure in rats, it was found that administration of RCM (ioversol) caused necrosis of proximal tubules that was not present in gentamicin-only treated groups [Jensen et al., 2013]. Since gentamicin may induce apoptotic pathways in renal tubules [Quiros et al., 2011], it is feasible that such apoptotic processes will be augmented by RCM via their effects on signaling pathways that may decide the fate of the cell.

Our findings show that both LOCM and IOCM are both toxic and cause changes in major intracellular signaling pathways involved in cell survival, death and inflammation that may explain their toxic actions. Delineating their molecular mechanism of action may help in development of ways to reduce their harmful and common side-effects. However, despite improvements in the development of "safer" RCM, such as IOD, caution still needs to be exercised with respect to their use.

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