Chemico-Biological Interactions 229 (2015) 17–25

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Reversal of radiocontrast medium toxicity in human renal proximal tubular cells by white grape juice extract

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ARTICLE INFO

Article history:
Received 2 October 2014
Received in revised form 16 December 2014
Accepted 22 December 2014
Available online 17 January 2015

Keywords:
Signaling
Renal cell
Vitis spp.
Kinase
Cell injury

ABSTRACT

Radiocontrast media (RCM)-induced nephrotoxicity (CIN) is a major clinical problem accounting for 12% of all hospital-acquired cases of acute kidney injury. The pathophysiology of CIN is not well understood, but direct toxic effects on renal cells have been postulated as contributing to CIN. We have investigated the effect of a white grape (*Vitis vinifera*) juice extract (WGJe) on human renal proximal tubular (HK-2) cells treated with the radiocontrast medium (RCM) sodium diatrizoate.

WGJe caused an increase in phosphorylation of the prosurvival kinases Akt and ERK1/2 in HK-2 cells. Treatment of HK-2 cells with 75 mg I/ml sodium diatrizoate for 2.5 h and then further incubation (for 27.5 h) after removal of the RCM caused a drastic decrease in cell viability. However, pre-treatment with WGJe, prior to incubation with diatrizoate, dramatically improved cell viability. Analysis of key signaling molecules by Western blotting showed that diatrizoate caused a drastic decrease in phosphorylation of Akt (Ser473), FOXO1 (Thr24) and FOXO3a (Thr32) during the initial 2.5 h incubation period, and WGJe pre-treatment caused a reversal of these effects. Further analysis by Western blotting of samples from HK-2 cells cultured for longer periods of time (for up to 27.5 h after an initial 2.5 h exposure to diatrizoate with or without WGJe pre-treatment) showed that WGJe pre-treatment caused a negative effect on phosphorylation of p38, NF-κB (Ser276) and pERK1/2 whilst having a positive effect on the phosphorylation of Akt, FOXO1/FOXO3a and maintained levels of Pim-1 kinase.

WGJe may alleviate RCM toxicity through modulation of signaling molecules that are known to be involved in cell death and cell survival and its possible beneficial effects should be further investigated.

1. Introduction

Radiocontrast media (RCM) are commonly used in medical practice, but their use may lead to contrast-induced nephropathy (CIN) [1]. The continued growth in radiographic procedures has resulted in an increase in incidences of CIN, accounting for 12% of all cases of hospital-acquired kidney failure [2], and the only non-controversial therapy for prevention of CIN is hydration [3–10]. Whilst the toxicity of RCM is not fully understood, it is believed to be due to several factors with the two possible principal mechanisms being their effects on renal haemodynamics and direct toxic effects on renal cells [6,11–14].

Work carried out by us and others has shed some light on the possible molecular mechanisms of RCM toxicity [10], in particular highlighting signaling pathways that may be affected by RCM [13,15–19]. It has been observed that all classes of RCM (high-, low- and iso-osmolar) cause a dramatic decrease in the phosphorylation (activation) of the Akt kinase [15,17], and given the important role of this kinase in cell survival and proliferation [20,21], this may explain in part the cytotoxic effects of RCM. Indeed, the decrease in cell viability due to RCM exposure of human renal tubular cells transfected with a plasmid encoding

Abbreviations: WGJe, white grape juice extract; RCM, radiocontrast medium; ERK1/2, extracellular signal-regulated kinases 1 and 2; FOXO1/3a, forkhead box O1/O3a; NF-κB, nuclear factor kappa-light-chain enhancer of B cells; Pim-1, proviral insertion site in Moloney murine leukemia virus-1; MTT, (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NaD, sodium diatrizoate; JNK1/2, c-Jun N-terminal kinase 1 and 2.

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http://dx.doi.org/10.1016/j.cbi.2014.12.035
0009-2797/

constitutively active Akt was lessened [15]. Furthermore, Yano et al. [22], suggested that Akt may play a role in reversing the up-regulation of pro-apoptotic molecules by RCM in porcine renal tubular cells. It has also been proposed that reactive oxygen species (ROS) play a role in RCM toxicity [23–26] and *in vivo* studies using rats fed with the antioxidants alpha-tocopherol [27] and grape seed proanthocyanidin extract [28] were seen to be protective against renal tissue damage due to RCM. Given that grape juice is a rich source of antioxidants and has been shown to increase serum antioxidant capacity in adults [29] and given that it has also been shown that Concord grape juice may activate Akt kinase by increasing its phosphorylation at Ser 473 [30], it seemed feasible that grape juice may afford protection against the toxic effects of RCM. On this basis, we set out to test the action of a white grape juice extract (WGJe) on culture human renal proximal tubule cells (HK-2) treated with the high-osmolar contrast medium sodium diatrizoate and in particular to focus on any changes in the signaling pathways in these cells.

2. Materials and methods

2.1. Materials

Sodium diatrizoate (also denoted as NaD or diatrizoate in this manuscript; Sigma–Aldrich Co., St. Louis, MO – USA) was dissolved in RPMI cell culture medium and used at a final concentration of 75 mg iodine/ml when incubated with the cells. This concentration chosen for this study is physiologically relevant and calculated based on the dosage commonly used in clinical practice as mentioned in previous studies [15,16]. All other materials and reagents used were of the highest purity available from their respective manufacturers. The white grape (*Vitis vinifera*) juice extract (hereafter referred to as WGJe) was provided by the company "Bono & Ditta" (Campobello di Mazzara, Trapani, Italy). The extract in liquid form has been produced by passing the must-mute columns equipped with adsorbent resins which retain polyphenolic compounds. Then, molecules were eluted with 4% NaOH and immediately passed through cationic resins which allows to change the biomolecules in acid form. The product were collected, filtered and stored at +4 °C for a short time. Finally, liquid extract was transformed into a dry powder by the method of spray drying, and small aliquots were stored at −20 °C until its use. At the beginning of each experiment an aliquot of WGJe was defrosted and dissolved in RPMI cell culture medium at the desired concentrations.

2.2. Chemical characterization of WGJe

The chemical composition of WGJe was examined using a UPLC/QqQ-MS/MS, as previously described [31]. Separation of the phenolic compounds was executed by a Waters Acquity HSS T3 column 1.8 μm, 150 mm × 2.1 mm (Milford, MA, USA), held at 40 °C. Water containing 0.1% formic acid was the mobile phase A, while the mobile phase B was acetonitrile containing 0.1% formic acid. During the analysis, samples were maintained at 4 °C, and the flow at 0.4 mL/min. Mass spectrometry detection was performed on a Waters Xevo TQMS Triple quadrupole mass spectrometer equipped with an electrospray (ESI) source.

2.3. Cell culture

In our experiments we have used HK-2 cells (a human renal proximal tubular epithelial cell line), obtained from the American Type Culture Collection (ATCC®) and grown in 100 mm culture dishes (Corning, New York, USA) as described previously [15]. In brief, they were cultured in DMEM containing Glutamax™ (Gibco, Life Technologies, Monza, Italy) supplemented with 10% Fetal Calf Serum and 100 units/ml Penicillin and 100 μg/ml Streptomycin (Sigma–Aldrich) in an atmosphere of 5% CO_2 in air at 37 °C, up to a confluence of approximately 90%.

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-2H-tetrazolium bromide; Sigma–Aldrich). Cells were grown in 6-well plates; after the appropriate treatment with WGJe (250 μg/ml for 15 min), sodium diatrizoate was added, where appropriate, at a final concentration of 75 mg iodine/ml. After 2.5 h, the media was removed and the cells were washed once with sterile PBS and incubated with 1 mg/ml MTT (in sterile PBS) for 1 h at 37 °C. Then, the supernatants were removed and crystals of formazan (MTT metabolic product) were dissolved in dimethyl sulphoxide (DMSO). Measurements of the absorbance (of the colored product as a result of MTT reduction) were made at 540 nm using a Beckman DU 800 spectrophotometer.

2.5. Western blot analysis

HK-2 cells, at each time point, were washed with cold PBS and then lysed in buffer containing: 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM EGTA, 1 mM DTT, 1 mM $NaVO_4$, 1% (v/v) Triton X-100, 2 μM microcystin, 400 μM PMSF with one protease inhibitor cocktail tablet (cOmplete Mini™, Roche GmbH, Germany) added for every 10 ml of lysis buffer as per manufacturers' instructions. The samples were then centrifuged at 10000×*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 [32] protocol in order to obtain an equal loading (approximately 30 μg of each sample were loaded).

Protein extracts were resolved by SDS–PAGE and transferred to a nitrocellulose membrane (Hybond C® extra, Amersham Biosciences), as previously described [33]. 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, Denmark), diluted 1:5000 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 incubation of the membrane with an enhanced chemiluminescence reagent [100 mM Tris–HCl pH 8.8, 2.5 mM luminol, 0.4 mM ρ-coumaric acid, 2.9 mM hydrogen peroxide (freshly added before use)] for 1 min. The primary antibodies included the following: anti-phospho-ERK1/2 (p44/p42 MAP kinase, Cell Signaling Technology, Beverly, MA – USA); anti-Total ERK2 (Santa Cruz Biotechnology, USA); anti-phospho-Akt (Ser 473 Cell Signaling Technology); anti-Total-Akt (Cell Signaling Technology) anti-phospho-p38 (Cell Signaling Technology); anti-phospho-FoxO3a (Thr 32)/anti-phospho-FoxO1 (Thr 24) (Cell Signaling); (Cell Signaling Technology); anti-phospho-NF-κB [p65 subunit] (Ser276) (Cell Signaling); anti-Pim-1 (Cell Signaling Technology); anti-phospho JNK1/2 (anti-ACTIVE, Promega); anti-β-actin (Sigma–Aldrich).