Uranyl acetate modulates gene expression and protein levels of the type 2, but not type 1 inositol 1,4,5-trisphosphate receptors in mouse kidney

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Abstract. Nephrotoxic effect of uranium is already well documented. Nevertheless, little is known about the effect of uranium on calcium homeostasis and calcium transport systems. Calcium released from endoplasmic reticulum through special calcium release channels – inositol 1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) – serves as a main source of cytosolic calcium signaling in the majority of cell types. To contribute to understanding mechanism of toxicity of the uranyl acetate (UA), we focused on modulation of the gene expression, protein levels and activity of IP_3 receptor's intracellular calcium channels by UA in mouse kidney. We have found that UA did not affect mRNA and protein levels of the type 1 IP_3Rs, but increased mRNA and also protein levels of the type 2 IP_3 receptors in kidney. Nevertheless, IP_3-induced calcium release was decreased by addition of UA. We assume that decreased activity of IP_3 receptors due to the acute exposure to UA results in feedback, which triggers activation of IP_3R2 expression. Thus, inhibition of calcium release and increased levels of the type 2 IP_3 receptors might participate, at least partially, in UA-induced nephrotoxicity.

Key words: Kidney — Uranyl acetate — Inositol 1,4,5-trisphosphate receptors

Introduction

Uranium is a dense silvery metal used in both civil and military industries. The best-known use of uranium is as a fuel in nuclear power-reactors and nuclear weapons. Uranium is also commonly used in analytical and clinical chemistry. Depleted uranium has also been used during wartime in heavy tank armor, armor-piercing bullets, and missiles, producing uranium dust from fired munitions, by which several land areas are contaminated (Craft et al. 2004; Mitchel and Sunder 2004). Although it is well established that uranium exposure can result in both chemical and radiological toxicity, in general, chemical toxic effects from uranium compounds occur at lower exposure levels than radiological toxicity (Hartmann et al. 2000). Thus, the major negative effect of uranium is chemical kidney toxicity rather than radiation hazard (Leggett 1989). Nephrotoxic damage occurs mainly in the renal proximal tubular epithelium. Many questions about the fine mechanisms of nephrotoxicity or mutagenicity remain unanswered and these mechanisms are not easily accessible *in vivo.*

Perturbation in calcium homeostasis might contribute to uranium toxicity (Taulan et al. 2006). An increase in intracellular calcium is a well known cell reaction to many toxic agents. Tissue calcium levels increase when lethal cell injury develops in a tissue, following massive calcium release from mitochondria and endoplasmic reticulum (Prat et al. 2005). Several calcium transport systems that are localized either in plasma membrane or in the membranes of intracellular stores, are able to selectively increase or decrease intracellular calcium concentrations. Release of calcium ions from intracellular stores (e.g. sarcoplasmic/endoplasmic reticulum) is the key step in many

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signal transduction processes and thus plays a role in etiology of certain pathological states (Berridge 1993).

Endoplasmic reticulum serves as a major intracellular calcium store. The efflux of calcium from the endoplasmic reticulum into the cytosol is regulated by two sets of ligand-gated intracellular channels, residing in endomembrane – inositol 1,4,5-trisphosphate (IP_3) receptor calcium releasing channel (IP_3Rs) and ryanodine receptor calcium releasing channel (RyRs) (for review see Verkhratsky 2006). Both types of these channels are sensitive to cytosolic free calcium – RyRs are activated by an increase in cytosolic calcium, whereas fluctuations in cytosolic calcium modulate the IP_3 receptors sensitivity to its main activator, IP_3. So far, three types of IP_3Rs (type 1, 2 and 3) were determined. Individual types of these calcium channels differ in the localization and probably modulate different metabolic pathways.

In various tissues, IP_3 receptors are known to be modulated by different stimuli, such as stress (Zacikova et al. 2000; Lencesova et al. 2002; Krizanova et al. 2004), hypoxia (Jurkovicova et al. 2007), etc., on the level of gene expression, protein and activity. To contribute to understanding mechanism of toxicity of uranyl acetate (UA), we focused on modulation of the gene expression, protein levels and activity of IP_3 receptor's intracellular calcium channels by UA in mouse kidney.

Materials and Methods

Animals

Male adult mice C57B1/129SV, 10 weeks old (weighing 25–30 g) were used for experiments. Prior to experiments, animals were housed for 1 week in a controlled environment (22 ± 2°C, 12 h light/dark cycle, light on at 6.00 a.m.). Food and water were available *ad libitum*. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Animals were divided into five groups; each group was composed of 5 animals. First two groups served as a control, to the third group UA (2 mg per mouse/day) and to the fourth group UA (4 mg per mouse/day) was added in the drinking water for 24 h and fifth group was drinking UA (2 mg per mouse/day) for five days. No measurable differences were observed in the amount of water drunk by control and treated animals. After the end of exposure, animals were decapitated, kidney was withdrawn, separated into the medulla and cortex and stored frozen at –70°C until use.

RNA isolation and relative quantification of mRNA levels by reverse transcription and subsequent polymerase chain reaction (RT-PCR)

Population of total RNAs was isolated by TRI Reagent (MRC Ltd.). Briefly, tissue samples were homogenized by tissue homogenizer (Biospec Products Inc.) in TRI Reagent and after 5 min the homogenate was extracted by chloroform. RNAs in the aqueous phase were precipitated by isopropanol. RNA pellet was washed with 75% ethanol and stored in 96% ethanol at –70°C. Purity, integrity and quantity of isolated RNAs was checked on GeneQuant Pro spectrophotometer (Amersham Biosciences). Reverse transcription was performed using 1.5 μg of total RNAs and Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences) with $pd(N)_6$ primer. PCR specific for the type 1, 2 and 3 of IP_3 receptor (IP_3R1, IP_3R2 and IP_3R3) was carried out afterwards using primers (Table 1) described by Genazzani et al. (1999), Hudecova et al. (2004). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as a housekeeper gene control for semi-quantitative evaluation of PCR. All used primer sequences, together with

Table 1. Sequences of primers, their position and product size

Primer	Primer sequences	Position	Product
IP_3R1A	5'- GTG GAG GTT TCA TCT GCA AGC -3'	70–90	
IP_3R1B	5'- GCT TTC GTG GAA TAC TCG GTC -3'	56–476	535/410 bp
IP_3R2A	5'- GCT CTT GTC CCT GAC ATT G -3'	3284–3306	
IP_3R2B	5'- CCC ATG TCT CCA TTC TCA TAG C -3'	3624–3645	361 bp
IP_3R3A	5'- CTG CCC AAG AGG AG AGG AAG -3'	6445–6466	
IP_3R3B	5'- GAA CAG CGC GGC AAT GGA GGA G -3'	6897–6919	452 bp
SERCA2A	5'- ATT GTT CGA AGT CTG CCT TCT GTG G -3'	1489–1522	
SERCA2B	5'- CAT AGG TTG ATC CAG TTA TGG TAA A -3'	1648–1672	174 bp
GAPDH1	5'- AGA TCC ACA ACG GAT ACA TT -3'	795–814	
GAPDH2	5'- TCC CTC AAG ATT GTC AGC AA -3'	506–525	309 bp

IP_3R1, (GI 1055286) type 1 IP_3 receptor; IP_3R2, (GI 13752805) type 2 IP_3 receptor; IP_3R3, (GI 6981109) type 3 IP_3 receptor; SERCA2, (GI 8392934) sarco(endo)plasmic reticulum Ca^{2+} ATPase; GAPDH, (GI 56187) glyceraldehyde 3-phosphate dehydrogenase.

corresponding position and fragment size for each amplified gene, are listed in Table 1. Each PCR program started with initial denaturation at 94°C for 5 min, followed by 30 (for GAPDH), 25/30 (for IP_3R1/IP_3R2) and 38 (for IP_3R3) cycles of denaturation at 94°C for 1 min, annealing at 56–60°C for 1 min, and polymerization at 72°C for 1 min. PCRs were terminated by final polymerization at 72°C for 7 min. All PCR products were analyzed on 2% agarose gels.

Western blot analysis

The IP_3R1 and IP_3R2 proteins were determined in crude membrane fraction from the mouse kidney and cerebellum by Western blot analysis. Protein concentration was determined according to Lowry et al. (1951). Twenty five micrograms of protein extract from kidney was used for Western blot analysis. Using a polyclonal antiserum specific for IP_3R1 receptor (dilution 1 : 1000) we detected immunoreactive protein of approximately 240 kDa. Specific anti-IP_3-receptor polyclonal antibody (Abcam) was raised against a synthetic peptide corresponding to the C-terminus of human IP_3 receptor (amino acids 1829-1848). This sequence is 100% conserved in human, mouse, and rat type 1 IP_3 receptor protein. Antibody cross-reacts with dog and rat and is expected to cross-react with human and mouse due to sequence homology (100%). Specific anti-IP_3-receptor 2 polyclonal antibody (dilution 1 : 1000; Chemicon International, Inc.) was used for determination of the type 2 IP_3 receptor. The antibody detected protein of 250 kDa. After the incubation with secondary anti-rabbit antibody conjugated to the horseradish peroxidase (1 : 5000) immunoreactive proteins were visualized by ECL detection system (Amersham Biosciences).

Preparation of endoplasmic reticulum vesicles and determination of IP_3-induced and ryanodine- induced calcium release

Membranes from kidney were prepared by ultracentrifugation as described previously (Monkawa et al. 1998; Krizanova et al. 1990; Thurzova et al. 1995). Microsomes containing 250 μg of proteins were loaded 30 min with $^{45}Ca^{2+}$ in the presence of ATP. IP_3-induced calcium release and ryanodine-induced calcium release was measured 2 min after addition of either 1 μmol/l IP_3 and/or 1 μmol/l ryanodine.

Statistical analysis

Each value represents the average of 5 animals. Results are presented as means ± S.E.M. Statistical differences among groups were determined by one-way analysis of variance (ANOVA). Statistical significance $p < 0.05$ was considered to be significant. For multiple comparisons, an adjusted *t*-test with *p* values corrected by the Bonferroni method was used (Instat, GraphPad Software, USA).

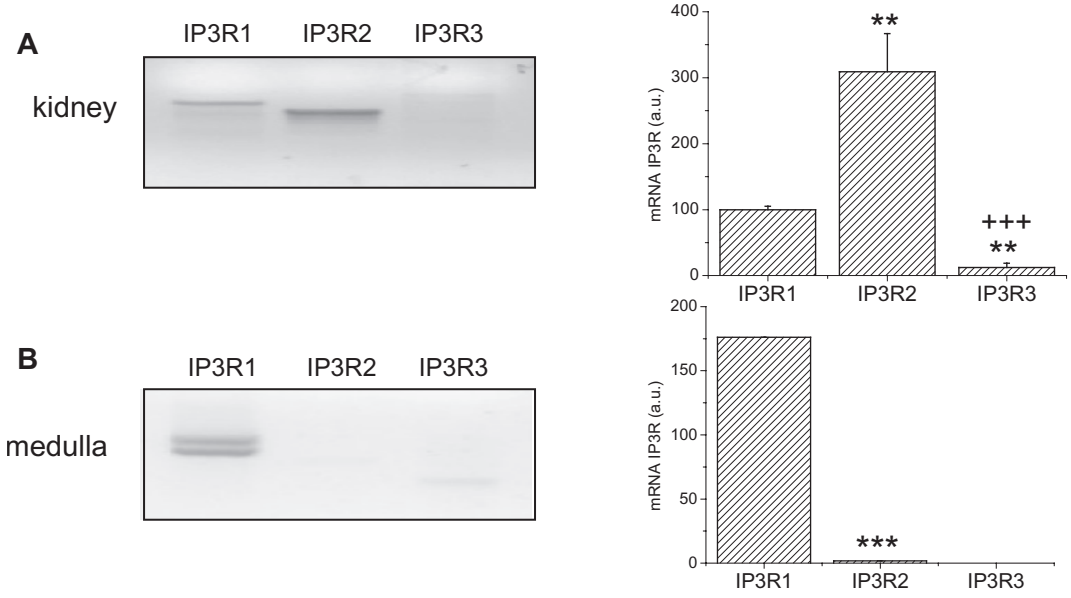

Figure 1. Relative distribution of the individual types of IP_3 receptors in murine kidney (A) and medulla (B). To each graph the typical result of the gel is placed. In kidney, mRNA of type 2 IP_3 receptors (IP_3R2) was the most abundant, but also nonneuronal form of the type 1 IP_3 receptor (IP_3R1) (410 bp) was amplified. In renal medulla, mRNA of the type 1 IP_3 receptor is highly predominant. Type 3 IP_3 receptor's (IP_3R3) mRNA is rare in the whole kidney and medulla as well. Columns are displayed as mean ± S.E.M. Statistical significance ** $p < 001$, *** $p < 0.0001$ compared to IP_3R1 and $^{+++}$ $p < 0.0001$ compared to IP_3R2.

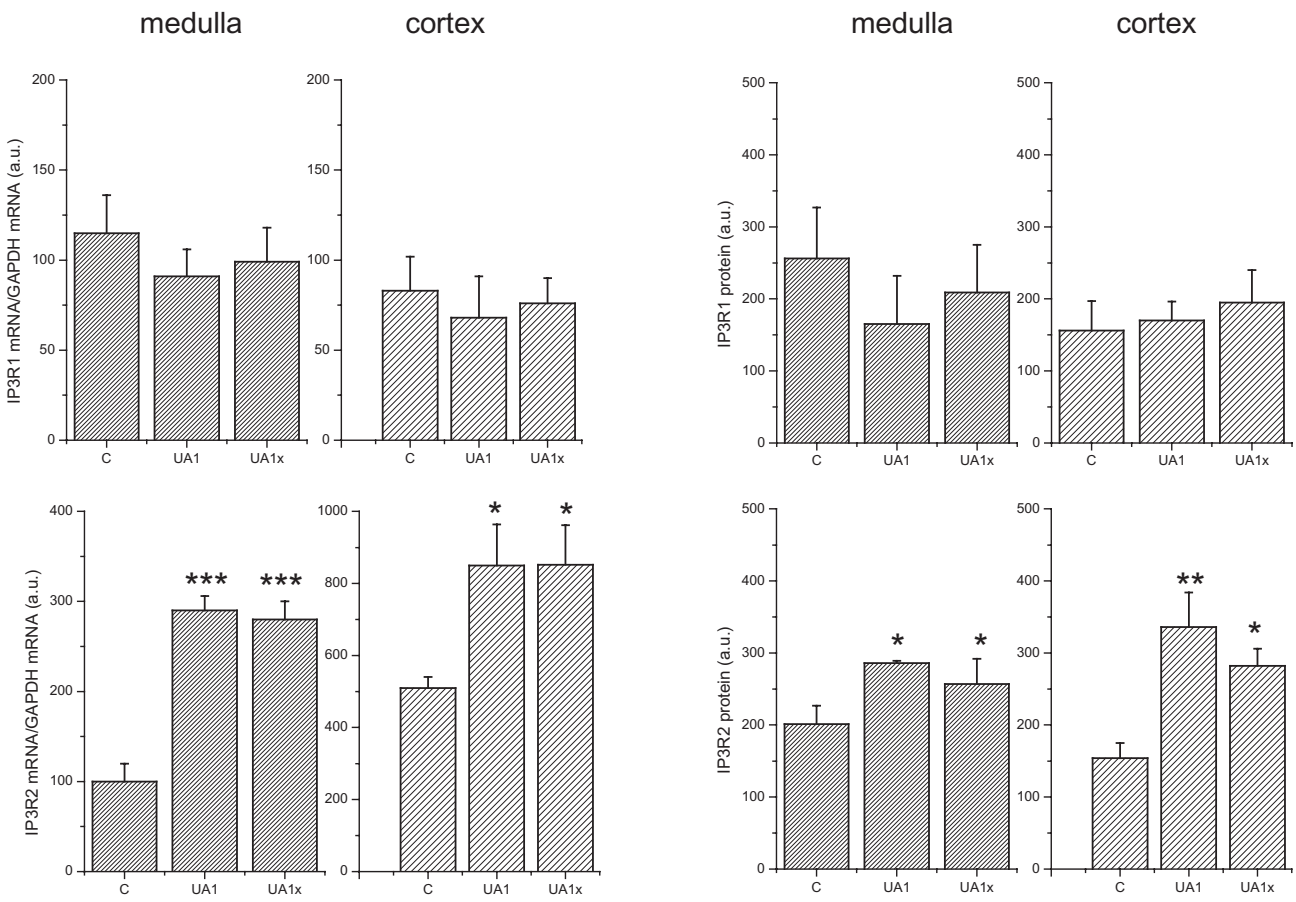

Figure 2. IP_3R1 and IP_3R2 levels of mRNA (left) and protein (right) after uranyl acetate (UA) treatment in kidney medulla and cortex. Mice were treated 2 (UA1) and 4 (UA1x) mg/kg body weight of UA for 24 h. IP_3R1 mRNA and protein levels were not changed, but both, IP_3R2 mRNA and protein were increased significantly after UA treatment. Columns are displayed as mean ± S.E.M. Statistical significance * $p < 0.05$, ** $p < 001$ and *** $p < 0.0001$ compared to untreated controls.

Results

In the whole kidney, mRNA of type 2 IP_3 receptors predominated compared to IP_3R1 mRNA (Figure 1A). The mRNA of type 3 IP_3 receptors was almost not detectable. On contrary, type 1 IP_3 receptors were highly abundant in renal medulla (Figure 1B).

UA added to drinking water for 24 h (UA1) did not affect gene expression and protein levels of IP_3R1 in renal medulla and cortex (Figure 2, upper part). Nevertheless, it significantly increased mRNA and protein levels of the type 2 IP_3 receptors in renal medulla and cortex (Figure 2, lower part). When the dose of UA was doubled (UA1x), no additional increase was observed. However, in the whole kidney, additional increase in the protein levels of the type 2 IP_3 receptor was observed after 5 days of UA treatment (from 111 ± 2 a.u. in control group to 376 ± 19 a.u. in UA exposed group), compared to 1 day (205 ± 2 a.u., Figure 3).

In order to assess the activity of IP_3 receptors and RyRs, we measured IP_3-induced calcium release and RyR-induced calcium release from microsomes loaded with $^{45}Ca^{2+}$ (Figure 4). We have found that IP_3-induced calcium release was markedly decreased in kidney after addition of UA (from 16,824 ± 458 cpm/2 min in control microsomes to 2621 ± 977 cpm/2 min in microsomes with UA). RyR-induced calcium release was unchanged in kidney microsomes after addition of UA (Figure 4).

We measured also mRNA of other intracellular calcium transporters, the phospholamban-dependent calcium ATPase 2 (SERCA2) and RyRs in control and uranium acetate-treated mice. While mRNA of the type 2 RyRs was unchanged, mRNA of the SERCA2 was significantly increased after UA treatment (Table 2).

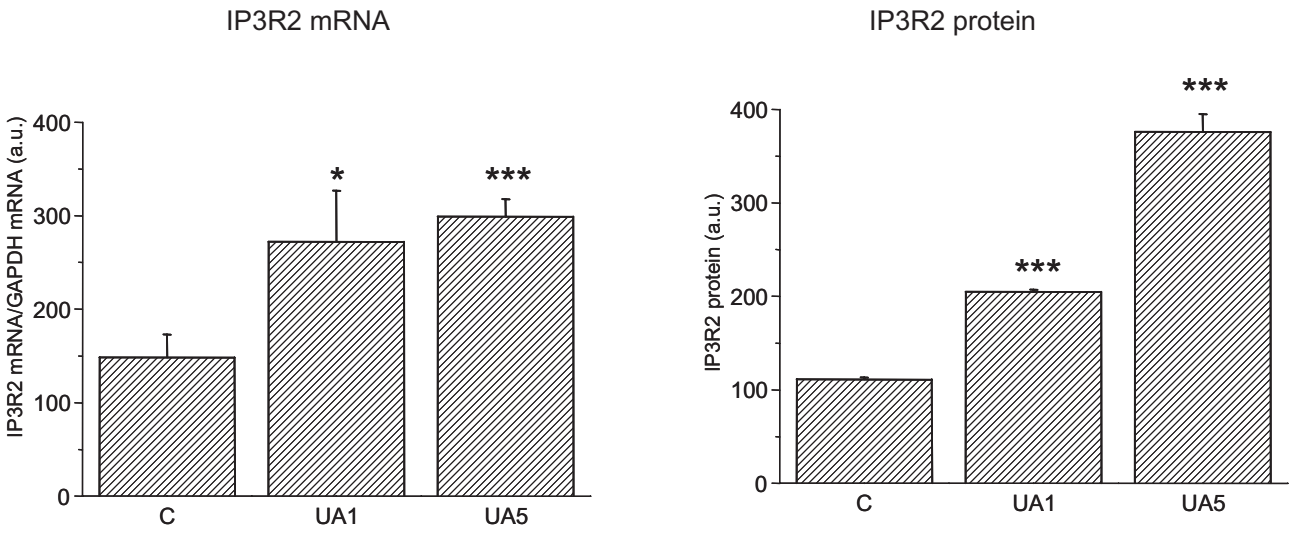

Figure 3. Effect of single (UA1) and repeated (UA5) treatment with uranyl acetate (UA) on mRNA levels of the type 2 IP_3 receptors (IP_3R2) in the whole kidney. Significant changes in mRNA (left) and protein (right) were observed after both, single and repeated treatment with UA. Columns are displayed as mean ± S.E.M. Each value represents an average of 5 animals. Statistical significance * $p < 0.05$ and *** $p < 0.0001$ compared to untreated controls.

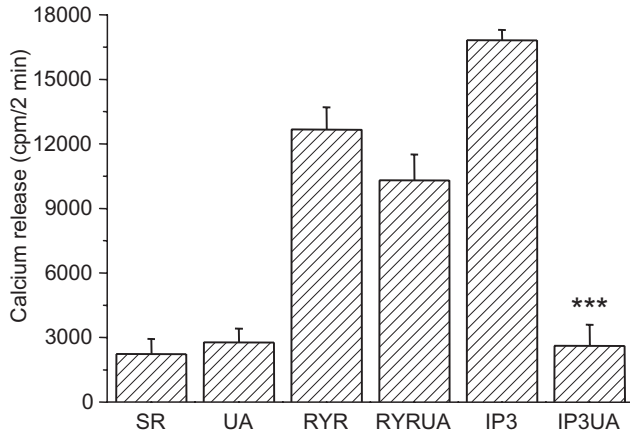

Figure 4. IP_3-induced and ryanodine induced calcium release from kidney microsomes. SR, spontaneous release; UA, spontaneous calcium release after uranyl acetate treatment; RyR, ryanodine-induced calcium release; RyRUA, ryanodine-induced calcium release after UA treatment; IP3, IP_3-induced calcium release; IP3UA, IP_3-induced calcium release after UA treatment. Columns are displayed as mean ± S.E.M.

Table 2. The mRNA levels of RyR2 and SERCA2 in mouse kidney after the exposure to uranyl acetate (UA)

	Control (a.u.)	UA treatment (a.u.)	*p*
SERCA2	3.88 ± 0.34	6.91 ± 1.01	<0.05
RyR2	3.42 ± 0.5	4.2 ± 0.4	ns

ns, non significant.

Discussion

We have found that gene expression and protein levels of the type 1 IP_3 receptors were not changed significantly by UA treatment, while significant increase was observed in mRNA and protein levels of the type 2 IP_3 receptors. IP_3-induced calcium release in membrane vesicles loaded with $^{45}Ca^{2+}$ was impaired in samples from mice treated with UA. Calcium transport through the ryanodine receptors was unaffected, pointing to lower vulnerability of these receptors to harmful stimuli. Based on these results we propose that increased expression of the type 2 IP_3 receptors might be due to initial impaired activity due to UA treatment.

The industrial use of uranium has pin-pointed the need to review its chemical impact on human health. The long half-lives of uranium radioisotopes means that natural uranium is not considered being a major radiological hazard (Prat et al. 2005). However, its chemical toxicity has been documented since the 1940s and its nephrotoxicity is well established at organ level. Nephrotoxic damage occurs mainly in the renal proximal tubular epithelium (L´Azou et al. 2002). Kurttio and coworkers (2002) studied the renal effects of uranium in drinking water. They have found an association between increased uranium exposure through drinking water and excretion of several solutes in urine. The effect was consistent with reduced reabsorption in kidney tubules. Effect of uranium is highly dependent not only on the time of exposure, but also on dose. Both functional and histologic damage to the proximal tubulus has been demonstrated (Haley et al. 1982; Diamond et al. 1989; Gilman et al. 1998) with higher doses of UA. A more precise

knowledge of uranium interaction mechanisms at the cellular level can help to find compounds that can be able to compete with uranium at its biological targets.

An increase in intracellular calcium is a well known cell reaction to many toxic agents. Tissue calcium levels increase when lethal cell injury develops in a tissue, following massive calcium release from mitochondria and endoplasmic reticulum. Since using DNA microarrays and HEK 293 cells Prat and collegues (2005) showed that IP_3 cascade was highly disturbed, we focused on IP_3 receptors in renal cells. Nevertheless, phospholamban-dependent SERCA2 that is localized on endoplasmic reticulum was studied as well.

In kidney, IP_3 receptors are localized in different cells and thus might play a different role. Immunostaining of IP_3R1 was seen in glomerular mesangial cells and in vascular smooth muscle cells of arteries and arteriols (Monkawa et al. 1998). These cells are believed to have a contractile function. The IP_3R1 is thought to be a major isoform regulating the contractility of smooth muscle (Monkawa et al. 1998). Furuichi and coworkers (1990) examined the localization of mRNA for IP_3R1 by *in situ* hybridization. They reported a strong signal observed by vessels, while no signal was detected in glomerular mesangial cells. Cells positive for IP_3R2 antibody were localized in the intercalated collecting ducts. The intercalated cells regulate excretion and reabsorption of acid and base (Schuster 1993). Thus, IP_3R2 might be involved in this process. The immunostaining of IP_3R3 was observed in cortical collecting ducts, vascular smooth muscle cells and glomerular mesangial cells (Monkawa et al. 1998). We detected majority of type 1 IP_3 receptors in renal medulla, while in the whole kidney the type 2 IP_3 receptor was more abundant.

Preliminary results showed that there were no changes in the type 3 IP_3 receptors in kidney after exposure to UA (data not shown). However, since abundance of these receptors was low, we were not able to quantify precisely the amount of IP_3 receptors of type 3 and therefore we did not include these results. Nevertheless, from our results we can conclude that even a one day exposure to UA affects the gene expression and protein levels of the type 1 and 2 IP_3 receptors in different manner. Since both these receptors are localized on different cell types and thus probably play a different role in kidney function, UA can cause dysregulation on different pathways. It is widely accepted that calcium release from the endoplasmic reticulum serves as a main source for cytosolic calcium signaling in the majority of cell types. Disruption of the endoplasmic reticulum calcium homeostasis initiates endoplasmic reticulum stress, which may, if not compensated, be detrimental to the cell (Verkhratsky and Petersen 2002; Paschen and Mengesdorf 2005). IP_3 receptors and their dysregulation might be involved in this process.

In summary, acute UA dose results in decrease of IP_3-induced calcium release, while RyR-induced calcium release was unaffected. Prolonged treatment of animals with UA results in increased type 2 IP_3 receptors on both, mRNA and protein levels, while type 1 IP_3 receptors was unaffected. We propose that decreased activity of IP_3 receptors due to the acute exposure to UA results in feedback, which triggers activation of IP_3R2 expression and also SERCA2. We propose that both effects of UA on the type 2 IP_3 receptors on activity and expression level might participate in the kidney cell's damage by UA.

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