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Identification of type 1 IP_3 receptors in the rat kidney and their modulation by immobilization stress

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Abstract

Inositol 1,4,5-trisphosphate receptor (IP_3-receptor) is a calcium channel, transporting calcium from intracellular stores to the cytoplasm. In kidney, IP_3-receptors are involved in the signal transduction of various hormones. In our work we studied the effect of immobilization stress on the IP_3-receptor's protein content in renal cortex and the medulla of normotensive and hypertensive rats. We detected both mRNA and type 1 IP_3-receptor protein in medulla, but not in renal cortex. We found that this receptor was approximately twice as abundant in normotensive as in genetically hypertensive rat kidney. Immobilization stress decreased the amount of type 1 IP_3-receptor in the renal medulla of normotensive rats approximately five times, while no effect due to single and/or repeated stress was observed in the renal medulla of spontaneously hypertensive rats. The results indicate that expression of type 1 IP_3-receptor in renal medulla is modulated by hypertension and immobilization stress.

Keywords: Immobilization; Stress; Calcium; Inositol trisphosphate

1. Introduction

An increase in the cytosolic free calcium occurs as an integral component in the alteration of cellular responses produced by a variety of hormones. Part of this increase originates from an intracellular compartment. Inositol 1,4,5-trisphosphate (IP_3) is a second messenger, which mobilizes calcium from intracellular stores [1–3]. IP_3 binds to its receptors (IP_3-receptors), which induces the release of calcium from the endoplasmic reticulum [4]. To date, three genes for mammalian IP_3-receptor were found, giving three IP_3-receptor subtypes, type 1, 2 and 3 [5,6]. These types of IP_3-receptor are approximately 70% identical at the amino acid level and were observed in many different tissues and cells, with various subtypes and distribution [7,8]. In kidney, all three types of IP_3-receptor were found [9]. Type 1 was detected in glomerular mesangial cells and vascular smooth muscle cells. Type 2 was expressed exclusively in intercalated cells of collecting ducts from the cortex to the inner medulla. Type 3 was expressed in vascular smooth muscle cells, glomerular mesangial cells and some cells of cortical collecting ducts, probably principal cells [10]. Yang et al. [11] proposed that different types of IP_3 receptors might have different functions in the rat kidney.

IP_3-receptors serve as intracellular calcium release

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channels involved in signal transduction of various hormones in the kidney. Alterations in IP_3-receptor amount or in its distribution in the kidney may contribute to its dysfunction. For example, it was already shown that there is down-regulated type 1 IP_3-receptor in glomerular and vascular cells in diabetic kidney, which may contribute to the renal vasoregulation and renal hypertrophy during diabetes [12].

Stress is a stimulus, which could alter the protein expression and/or the function of the IP_3-receptor. Stress is known to be deeply involved in the development of serious health complications, such as hypertension or heart failure. Immobilization is proved to be one of the most potent stress models [13], since it activates both components of the sympathoadrenal system, i.e. adreno-medullar and sympathoneural. Moreover, immobilization was found to affect enzymes, receptors and membrane proteins not only on the activity level (catecholamines [14], plasma renin activity [15]), but also in the protein amount (tyrosine hydroxylase [16], Na^+/Ca^{2+} exchanger, Ca^{2+}-channel [17]) and/or the gene expression (α_1 subunit of Ca^{2+}-channels, catecholamine biosynthetic enzymes, renin, etc. [18]).

The aim of the current study was to investigate a possible correlation between the amount of mRNA and type 1 IP_3-receptor in kidney under control and pathophysiological conditions. We focused on the comparison of the IP_3-receptor in renal cortex and/or medulla in normotensive and spontaneously hypertensive rat strains. Since the immobilization stress is a strong stimulus affecting the sympathoadrenal system, we also compared the mRNA and protein levels of IP_3-receptor of type 1 in the kidney of immobilized animals in comparison to unstressed animals.

2. Materials and methods

2.1. Animals

The Animal Care Committee of the Slovak Academy of Sciences, Bratislava, Slovak Republic approved the protocol used. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. Normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) three-month-old male rats were used for all experiments. Before initiation of the experimental procedures, the animals were housed 3–4 per cage for at least 7 days. The room temperature was maintained at 23 ± 2°C and periodic 12-h alternation of light and dark was performed during the whole experiment. Food and water were available ad libitum. During the experiment, animals were weighed on a laboratory balance. Blood pressure was measured by the tail-cuff procedure.

2.2. Immobilization stress

In our experiments we used the immobilization protocol described by Kvetňanský and Mikulaj [13]. Rats were immobilized one or seven times for 30 min or 2 h, except for a control group. Animals were killed either immediately after the last immobilization or 3 and 24 h later. The renal cortex and medulla were dissected, dried from blood, washed in ice cold physiological solution and frozen in liquid nitrogen.

As a positive control for IP_3 receptor type 1, the rat brain cerebellum and hypothalamus of unstressed controls were used. The hypothalamus was removed as a complete area. One third of the right hemisphere of the cerebellum was separated by scissors. Tissues were rapidly frozen in liquid nitrogen.

2.3. Western blot analysis

Protein assays were done by the Lowry method [19]. A sample of each protein extract (20 μg of total protein) was separated by electrophoresis on a 6.5% sodium dodecyl sulfate–polyacrylamide gel [20] and then transferred to a nitrocellulose membrane, using a semi-dry blotting. The blot was blocked in 5% non-fat dry milk diluted in TBST and then incubated with the polyclonal antibody against IP_3 type 1 receptor (a gift of Professor Walaas and Dr. L.S. Haug, University of Oslo, Norway). This antibody was raised against an 18 amino acid synthetic peptide corresponding to the carboxy-terminal end of the type 1 IP_3 receptor from rat. Since the carboxy-termini of the IP_3 receptor's subtype are different, this antibody recognizes very specifically only type 1 IP_3

receptor [21,22]. We used a secondary anti-rabbit antibody labeled by peroxidase (Calbiochem). The protein bands (240 kDa) were detected with a sensitive chemiluminescent detection system-ECL (Amersham, UK). A calibration curve with different amounts of protein extract (2, 5, 10, 15, 20 and 30 mg of total protein) was made to check the accuracy of protein loaded. The optical density of the individual bands was quantified using a Kodak camera and IMAGE software.

2.4. RNA preparation

RNA was isolated from the renal cortex and medulla according to the procedure of Chomczynski and Sacchi [23], using guanidine isothiocyanate (Fisher Scientific, USA) and phenol–chloroform extraction. The concentration and purity of RNA was determined in triplicate spectrophotometrically on Shimadzu UV 3000.

2.5. Relative quantification of mRNA levels by reverse transcription and polymerase chain reaction (RT-PCR)

Reverse transcription was performed from 5 μg of total RNA using Ready-To-Go™ You-Prime First Strand Beads (Amersham Pharmacia Biotech) and pd(N)$_6$ primer. Specific PCR for the type 1 IP$_3$-receptor was done afterwards using primers as described in Genazzani et al. [24] (forward: 5′-GTG GAG GTT TCA TCT GCA AGC-3′; reverse: 5′-GCT TTC GTG GAA TAC TCG GTC-3′). After initial denaturation at 94°C for 5 min we used 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and polymerization at 72°C for 1 min. PCR was completed by the final polymerization at 72°C for 7 min. These primers are known to amplify two splice variants of type 1 IP$_3$ receptor, a neuronal 535 bp fragment and nonneuronal 410 bp fragment [24].

Since it is known that the fragment amplification by PCR is linear only at a limited number of cycles, prior to the experiment we amplified the fragment of type 1 IP$_3$-receptor using 10, 15, 17, 20, 23, 25, 27, 30, 32, 35, 37, 40 and 45 cycles. The amplification curve was linear between 27–45 cycles. Therefore, 35 cycles used were appropriate for our experiments.

2.6. Statistical analyses

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

3. Results

The blood pressure and body weight are important parameters for animal characterization. Therefore,

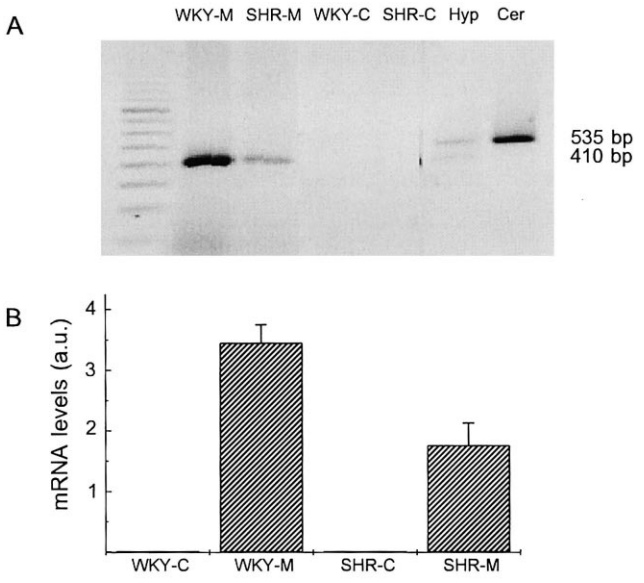

Fig. 1. mRNA levels of type 1 IP$_3$-receptor in renal cortex and medulla. In the upper part (A) there is a typical result of PCR of IP$_3$ receptor fragment from the cortex (WKY-C, SHR-C) and medulla (WKY-M, SHR-M) of WKY and SHR rats. As a positive control, cerebellum (cer) and hypothalamus (hyp) were amplified. In cerebellum, a longer splice variant (535 bp) was observed, while in hypothalamus, both variants (535 and 410 bp) were detected. In renal medulla, only the 410 bp splice variant of type 1 IP$_3$-receptor was noticed. The graph in part (B) represents a statistical evaluation of PCR results in renal cortex and medulla. Each column is displayed as mean ± S.E.M. and each value represents an average of six animals. In WKY renal medulla, the amount of mRNA of type 1 IP$_3$-receptor was almost twice as high as in SHR ($P < 0.001$). In renal medulla, no signal for mRNA of IP$_3$-receptor of type 1 was observed.

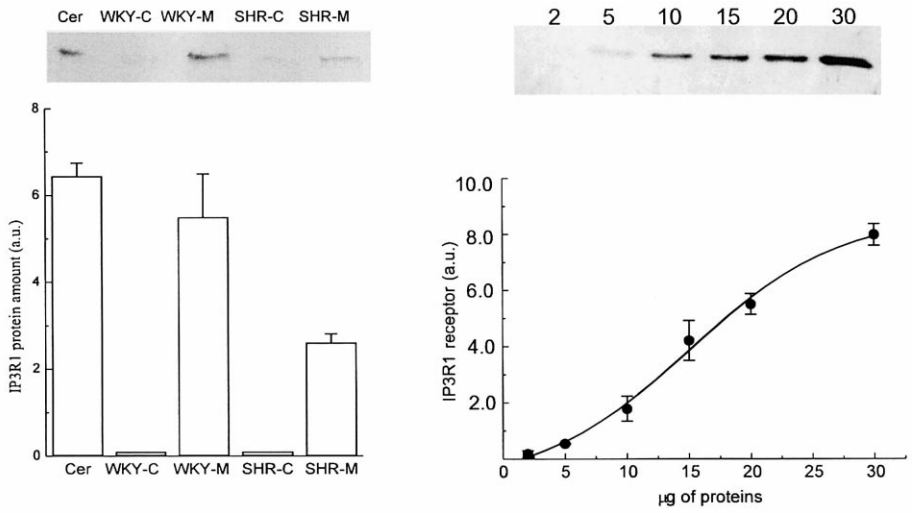

Fig. 2. IP_3 receptor type 1 protein in kidney cortex (-C) and medulla (-M) of normotensive WKY and hypertensive SHR rats. Cerebellum (Cer) serves as a positive control. Typical result of Western blot and subsequent hybridization with polyclonal antibody against this receptor is in the left part of the figure. Concentration curve with different amounts of proteins loaded on gel is shown in the right part of the figure. Graph represents an average from six animals and results are displayed as mean ± S.E.M.

we checked the body weight and blood pressure of both strains of rats, in order to confirm the developed hypertension in SHR. The body weight of SHR animals was significantly lower compared to normotensive WKY rats (273.0 ± 3.3 g in SHR versus 320.3 ± 6.9 g in WKY). Nonetheless, the blood pressure was significantly elevated in SHR, compared to WKY (from 105.1 ± 4.4 to 167.3 ± 2.3 mm Hg). Neither body weight, nor blood pressure was significantly changed by the immobilization.

We determined type 1 IP_3-receptor on both mRNA and protein levels. In the renal medulla of both WKY and SHR rats, we have found mRNA of a shorter splice variant of type 1 IP_3-receptor (Fig. 1). No signal was obtained from the renal cortex. As a positive control, we used mRNA from cerebellum and hypothalamus. In these parts of the brain, a longer splice variant was detected. Moreover, in the hypothalamus we detected also a shorter splice variant.

Kidney cortex and medulla membrane proteins were subjected also to the Western blot analysis to determine whether antibody against the IP_3-receptor of type 1 will recognize corresponding protein in these fractions. Since the IP_3-receptor of type 1 is highly expressed in cerebellum, we used the cerebellum microsomes as a positive control. Polyclonal antibody against the IP_3-receptor of type 1 labeled the protein with Mw 240 kDa in the cerebellum and in the kidney medulla, but not in the kidney cortex (Fig. 2, left). The concentration dependence of type 1 IP_3-receptor in the renal medulla of WKY rats is shown in Fig. 2, right. Immobilization stress down-regulated the protein of the IP_3-receptor of type 1 rat

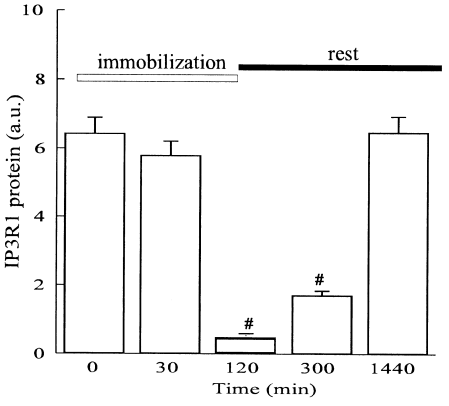

Fig. 3. The effect of immobilization stress on the IP_3-receptor of type 1 in renal medulla of normotensive rats. In renal medulla of normotensive rats, immobilization decreased the amount of IP_3-receptor of type 1 after 120 min of immobilization. This amount decreased approximately five times after single (IMO1) immobilization ($^{\#}P < 0.001$) compared to control values at time 0 min. After 3 h of rest, a significant decrease in the amount of this receptor was still observed. After 24 h of rest, the protein amount of the IP_3-receptor of type 1 returned to the control levels.

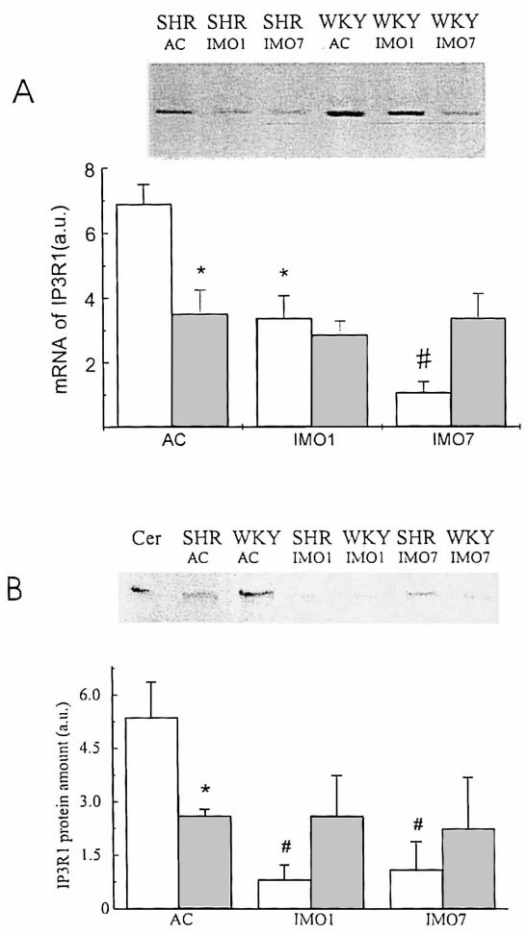

Fig. 4. mRNA (A) and protein (B) levels of type 1 IP_3-receptors in renal medulla of WKY (empty columns) and SHR (filled columns) rats during immobilization. AC, absolute controls; IMO1, animals were immobilized once for 2 h and decapitated immediately after the end of stimulus; IMO7, animals were immobilized seven times for 2 h with a subsequent 22 h rest and decapitated immediately after the end of the last immobilization. Gels represent a typical result and graphs represent an average of six animals. Results are displayed as mean ± S.E.M.

medulla in normotensive WKY rats after 2 h of immobilization (Fig. 3). No IP_3-receptor of type 1 was observed in the renal cortex of WKY and SHR after immobilization (not shown). When animals were immobilized only for 30 min, no significant decrease of type 1 IP_3-receptor was observed. After a 24 h rest, the amount of IP_3-receptor of type 1 returned to the original level (Fig. 3). We also compared the mRNA and protein levels of type 1 IP_3-receptor between WKY and SHR rats in renal medulla under the control conditions and after the immobilization stress. Both the mRNA and protein levels of IP_3R1 were significantly higher ($P < 0.01$) in the control group (AC) of WKY rats compared to SHR rats (Fig. 4). Although the effect of immobilization stress on the mRNA (Fig. 4A) and protein (Fig. 4B) level of the IP_3-receptor of type 1 in the renal medulla was observed in WKY rats, immobilization had no effect on the IP_3-receptor of type 1 in SHR rats. On the mRNA level, single immobilization of WKY rats causes approximately a two-fold down-regulation of type 1 IP_3 receptor, while after repeated immobilization for seven times, a profound decrease was observed (Fig. 4A). The single immobilization (IMO1) for 2 h and repeated immobilization (IMO7) significantly decreased the protein level of the IP_3R1 compared to the control groups (from 5.37 ± 0.99 to 1.1 ± 0.78 arbitrary units, Fig. 4B).

4. Discussion

The present results indicate for the first time that (i) the IP_3-receptor of type 1 was down- regulated in renal medulla of SHR compared to WKY animals on both mRNA and protein levels and (ii) in WKY renal medulla, a rapid down-regulation of the IP_3-receptor of type 1 occurred after the single and repeated immobilization for 2 h.

Immobilization stress is known to regulate several Ca^{2+}-transport systems in different tissues [17,25,26]. However, these Ca^{2+}-transport systems were found to be up-regulated by immobilization. The possible mechanism of this effect might involve catecholamine or glucocorticoid regulation, since both catecholamines and glucocorticoids are rapidly increased by this stress stimulus [27]. Down-regulation of type 1 IP_3 receptor in renal medulla might point to a different regulatory mechanism of this Ca^{2+}-transport system and to its specific role during immobilization stress in renal medulla. The physiological relevance of this observation remains to be proven.

Down-regulation of the IP_3-receptor of type 1 in kidney may decrease the local intracellular calcium concentration and might affect the signal transduction of renal hormones. Sharma et al. [12] demonstrated that the IP_3-receptor protein of type 1 is reduced in glomerular cells and vascular smooth muscle cells of the diabetic rat in association with

development of renal hypertrophy and creatinine clearance. Regulation of IP_3-receptor expression is likely to have functional consequences. Reduction of the type 1 IP_3-receptor protein expression has previously been shown to result in decreased IP_3 sensitivity and $[Ca^{2+}]_i$ release in response to IP_3 mobilizing agonists in neuroblastoma cells and rat liver epithelial cells [28].

In renal medulla of SHR rats, the type 1 IP_3-receptor was down-regulated on both mRNA and protein levels. The physiological relevance of this observation is not clear yet. However, it was found that the renal alpha 1-adrenergic receptor coupling to phospholipase C is less efficient in SHR than in WKY [29]. This would result in lower IP_3 production and thus in a decreased requirement of IP_3 receptors. Nevertheless, this hypothesis remains to be tested.

The signal for the down-regulation of the IP_3-receptors is probably caused by IP_3 binding [30]. Since the sensitivity of individual IP_3-receptor subtypes to IP_3 is different [31], it might be suggested that the sensitivity of these IP_3-receptor subtypes to down-regulation is different. It is tempting to speculate that immobilization stress might thus elevate the phosphatidylinositol 4,5-bisphosphate turnover, possibly by activation of phosphoinositidase C. In renomedullary interstitial cells, angiotensin II was also shown to increase the intracellular IP_3 concentration as well as the intracellular calcium concentration [32]. In SHR renal medulla, increased angiotensin II levels might further elevate the intracellular IP_3 concentration, which would result in the down-regulation of IP_3-receptors of type 1.

In summary, we have found both mRNA and protein of type 1 IP_3-receptor in renal medulla, but not in renal cortex of WKY and SHR rats. We have shown that in renal medulla, this receptor is modulated by immobilization stress in normotensive, but not in hypertensive animals. The physiological relevance of this observation remains to be elucidated.

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