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Cytokine 33 (2006) 138–144

Tumor necrosis factor α down-regulates the Na^+-K^+ ATPase and the Na^+-K^+2Cl^- cotransporter in the kidney cortex and medulla

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Received 23 March 2005; received in revised form 21 December 2005; accepted 24 December 2005

Abstract

The effect of TNF-α on the renal Na^+-K^+ pump and the Na^+-K^+2Cl^- cotransporter was investigated in the rat. Animals were injected with the cytokine, and 4 h later, a homogenate from the cortical and medullary tissues was prepared and used to assay the activity of the Na^+-K^+ ATPase and the protein expression of the pump and symporter. TNF-α reduced the activity and expression of the pump in both cortex and medulla, and its effect disappeared when animals were pre-treated with indomethacin, suggesting that TNF-α acts via PGE_2. Higher levels of PGE_2 were detected by enzyme immunoassay, in kidney tissues isolated from rats treated with PGE_2, thus confirming this hypothesis. The cytokine also down-regulated the Na^+-K^+2Cl^- cotransporter but this effect was not abrogated by indomethacin. PGE_2, injected into animals, exerted a dose-dependent effect. Low doses did not have any effect on the two transporters in the cortex while high doses inhibited and down-regulated the pump and up-regulated the cotransporter. In the medulla low doses increased the activity and expression of the pump but down-regulated the cotransporter while high doses exerted an exactly opposite effect on the two transporters. It was concluded that the effect of TNF-α on the pump is mediated via PGE_2 which is released at relatively high doses. The effect of the cytokine on the cotransporter is, however, independent of PGE_2.

Keywords: TNF-α; PGE_2; Na^+-K^+2Cl^-; Na^+-K^+ATPase; Kidney

1. Introduction

Tumor necrosis factor α (TNF-α) is a pro-inflammatory cytokine with a wide range of biological effects. It plays a central role in shock due to sepsis [1,2] and has been implicated, directly or indirectly, in the pathogenesis of renal failure [3,4].

The cytokine is released by the thick ascending limb of the loop of Henle in rats challenged with LPS, and was shown to exert cytotoxic effects and interfere with ion transport mechanisms across epithelia [5]. Dogs infused with TNF-α had marked nartriuresis and polyuria [6].

Since one of the major functions of the kidney is the regulation of body fluid osmolality and electrolyte balance, TNF-α may be altering renal functions by targeting electrolyte transport processes along the nephron and interfering with the activity of the transporters involved. The Na^+-K^+ ATPase also known as the Na^+-K^+ pump is a key transporter that regulates the activity of many others. It is a highly conserved integral membrane protein, which couples ATP hydrolysis to Na^+ and K^+ transfer across the plasma membrane against their electrochemical gradient [7]. By establishing Na^+ and K^+ gradients across the cell membrane, it provides the driving force for the activity of other transporters like the Na^+/H^+ and the Na^+/Ca^{2+} exchangers and the Na^+-K^+2Cl^- cotransporter that regulates, respectively, cytoplasmic pH, Ca^{2+} levels, and cellular volume. The Na^+-K^+ pump also drives a variety of secondary active transport processes in the nephron like those involving amino acids and glucose reabsorption. The ATPase is a heterodimer expressed in all the different segments of

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doi:10.1016/j.cyto.2005.12.007

the kidney tubules and is composed of two functional subunits: a catalytic α subunit (~110–113 kDa) containing the binding sites for ATP, sodium, potassium, phosphate and cardiac glycosides [8] and a β subunit (~35–55 kDa glycoprotein) needed for the activity of the enzymatic complex [9]. The β subunit also facilitates plasma membrane localization and is responsible for the early structural and functional maturations of the α subunit in the endoplasmic reticulum [8]. Several isoforms of both α and β subunits have been identified and all of them are capable of forming functionally active enzymes [10]. They are expressed in different cell types contributing in each to multiple specialized properties [11]. The α1 and β1 subunits perform housekeeping functions, and are highly expressed in the kidney. In the rat, their abundance is highest in cortical and medullary thick ascending limbs of the loop of Henle and the proximal convoluted tubule [12].

Another important transporter in the kidney is the Na^+-K^+2Cl^- (NKCC) cotransporter which couples the movement of $1Na^+$ with $1K^+$ and $2\ Cl^-$. The symporter uses the potential energy released by the downhill movement of Na^+ and Cl^- to drive the uphill movement of K^+ into the cell. Two isoforms have been identified: NKCC1, present in the basolateral membrane of a wide variety of secretory epithelia; and NKCC2, present only in the medullary region of the kidney [13,14], and localized to the apical membrane of the epithelial cells [15]. In epithelia, the cotransporter is responsible for the net transcellular Cl^- movement from one side of the cell to the other. By raising intracellular Cl^- above electrochemical equilibrium, it provides the driving force needed for Cl^- exit on the other side of the cell.

It is apparent that both the Na^+–K^+ pump and the Na^+-K^+2Cl^- symporter play a crucial role in the regulation of electrolyte movements across kidney tubules, and in the indirect maintenance of body fluid osmolality. The importance of these two transporters incited us to investigate whether they constitute target sites for TNF-α action. The aim of this work was to study the effect of TNF-α on the Na^+–K^+ pump and the Na^+-K^+2Cl^- (NKCC1 in particular) transporter in kidney cells isolated from rats treated in vivo with the cytokine.

2. Materials and methods

2.1. Animal treatment

Male Sprague–Dawley rats (*Rattus norvegicus*) were used and were handled all through in accordance with the Guide for Laboratory Animal Facilities and Care, US Department of Health, Education and Welfare.

Rats weighing around 250 g were injected i.p. with TNF-α (170 ng/100 g body weight) 4 h prior to the beginning of the experiment, or with different doses of PGE_2 (21 and 0.37 μg/100 g BW) 15 min before the beginning of the experiment. These time points were chosen based on a similar work studying the effect of TNF-α in the rat distal colon (unpublished data). When indomethacin was used, it was injected i.p. (1 mg/100 g BW) 10 min before TNF-α. TNF-α was dissolved in physiological saline. Ethanol and dimethyl sulfoxide (DMSO) were used as respective vehicles for PGE_2 and indomethacin. A similar volume of the vehicles was injected into control rats.

To ensure that any observed effect is due to TNF-α and not due to a contaminant like the lipopolysaccharide, TNF-α was heated for 20 min at 70 °C and its effect on the pump was studied.

2.2. Preparation of kidney cortical and medullary homogenates

Rats were anesthetized by i.p. injection of pentobarbital (5 mg/100 g body weight) and their abdomen were opened through a lateral incision. The intestine and stomach were pushed aside to expose the left kidney for its perfusion with Tris buffer (buffer A: 200 mM NaCl; 5 mM $MgCl_2 \cdot 6H_2O$; 2 mM EGTA; 5 mM KCl; 200 mM Tris–HCl, pH 7.4). A heparinized catheter was introduced into the aorta just above the level of the left renal artery to allow the infusion of the buffer. The mesenteric artery and the right renal artery were both ligated. The left renal vein was cut for drainage. The left kidney was perfused for 10 min with Tris buffer at a rate of 5 ml/min until cleared from blood. The bleached kidney was excised, its capsule removed, cut longitudinally and small pieces from the cortex and medullary tissues were then removed and homogenized in Tris buffer for around 5 min with a polytron (20 000–22 000 rpm) at 4 °C. Protease inhibitors were added to the samples which were kept at −20 °C for later use.

2.3. PGE_2 assay

Kidneys were isolated from animals treated with TNF-α. Cortical and medullary tissues were immediately excised, cut into small pieces, and homogenized in presence of indomethacin at 4 °C to inhibit any additional PGE_2 synthesis. Total PGE_2 (intracellular and extracellular) extraction and assay were conducted using an enzyme immunoassay kit from Amersham Biosciences (PGE_2 Biotrak EIA system) following exactly the protocol described by the manufacturer.

2.4. Na^+–K^+ ATPase assay

A crude membrane homogenate was prepared by spinning homogenized cells at 3300 × *g* for 10 min at 4 °C. Membrane homogenates were then diluted in Tris buffer (buffer A) to a final concentration of 2 mg/ml protein and incubated for 30 min with saponin (0.02% final concentration) at room temperature. After a pre-incubation at 37 °C for 10 min in the presence or absence of ouabain (4 mM final concentration), the reaction was initiated by addition of ATP to a final concentration of 1.25 mM, and terminated after a 1-h incubation at 37 °C by addition of trichloroacetic acid (200 μl, 11%). The amount of inorganic phosphate liberated was measured colorimetrically according to the method of Taussky and Shorr [16], and the activity of the enzyme was determined by measuring the ouabain-inhibitable inorganic phosphate liberated.

The percent inhibition of the enzyme activity was calculated as follows:

$$1 - \frac{\text{Pi(treatment)} - \text{Pi(treatment + ouabain)}}{\text{Pi(control)} - \text{Pi(control + ouabain)}} \times 100.$$

2.5. Western blot analysis

Membrane proteins were quantified using the Bio-Rad reagent and were equally loaded and resolved on 8% polyacrylamide gels, then transferred onto a PVDF membrane (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547, USA) . Protein expression of β actin was used to check the equal loading. The PVDF membrane was then washed, blocked and incubated overnight at 4 °C with a rabbit anti-Na^+-K^+ ATPase α-1 IgG (Upstate Biotechnology, Lake Placid, NY 12946, USA), or with an anti-rat sodium potassium chloride cotransporter antibody (Alpha Diagnostic International, TX, USA). Detection of the signal was by enhanced chemiluminescence using luminol reagent (Santa Cruz Biotechnology Inc., CA, USA).

Autoradiographs were analyzed by densitometry using a Gel-Pro(2) Analyzer software. The intensities of the signals are reported as a percentage of the control value.

2.6. Statistical analysis

Results are reported as mean ± SEM. Statistical significance was tested by a one-way analysis of variance followed by a Tukey–Kramer multiple comparison.

3. Results

3.1. Effect of TNF-α and indomethacin on the activity and protein expression of the Na^+-K^+ ATPase

TNF-α decreased the protein expression of the Na^+-K^+ ATPase in both kidney cortex and medulla (Fig. 1). This effect did not appear when heated TNF-α was used. Indomethacin, an inhibitor of COX enzymes, did not have any effect on the expression of the pump and in its presence, the down-regulatory effect of the cytokine did not appear.

The effect of TNF-α and indomethacin on the pump activity followed a similar trend. The cytokine reduced the ATPase activity down to around 40% of its control value (Fig. 2). This inhibitory effect disappeared, however, in the presence of indomethacin.

3.2. Effect of heated TNF-α, TNF-α, and indomethacin on the protein expression of the Na^+-K^+2Cl^- cotransporter

The protein expression of the Na^+-K^+2Cl^- cotransporter was not affected by indomethacin in both cortex and medulla (Fig. 3), but was significantly reduced by TNF-α even in the presence of indomethacin.

3.3. Effect of TNF-α on PGE_2 levels in the kidney

TNF-α caused a significant increase in the level of PGE_2 in both kidney cortex and medulla (Fig. 4).

3.4. Dose-dependent effect of PGE_2 on the activity and protein expression of the Na^+-K^+ ATPase

Low doses of PGE_2 (0.37 μg/100 g BW) injected into animals up-regulated the pump in the kidney medullary cells (Fig. 5) and increased its activity (Fig. 6), but were without any effect on the ATPase in cortical cells (Figs. 5 and 6). High doses (21 μg/100 g BW), however, reduced the activity and protein expression of the Na^+-K^+ ATPase in both cortex and medulla (Figs. 5 and 6).

3.5. Dose-dependent effect of PGE_2 on the protein expression of the Na^+-K^+2Cl^- cotransporter

In the cortex (Fig. 7), low doses of PGE_2 did not have any effect on the expression of the cotransporter while high doses caused an up-regulation.

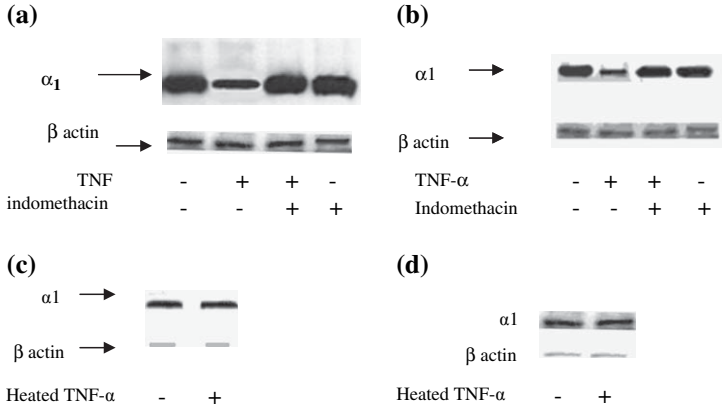

Fig. 1. Effect of heated TNF-α, TNF-α, and indomethacin on the expression of the Na^+-K^+ ATPase in the renal cortex (a) and (c) and medulla (b) and (d). Results are representative of an experiment repeated three times.

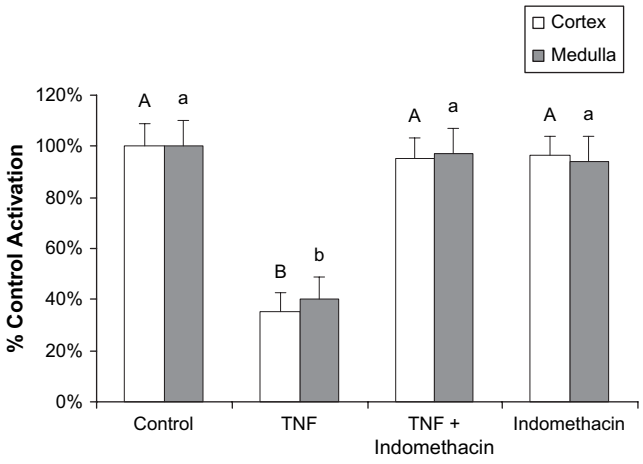

Fig. 2. Effect of TNF-α (i.p. 170 ng/100 g body weight) in presence or absence of indomethacin (i.p. 1 mg/100 g BW) on the activity of the Na^+-K^+ ATPase in kidney cortex and medulla ($n = 3$). Letters above the bars indicate significant differences. Bars sharing a common letter are not significantly different; $P < 0.05$.

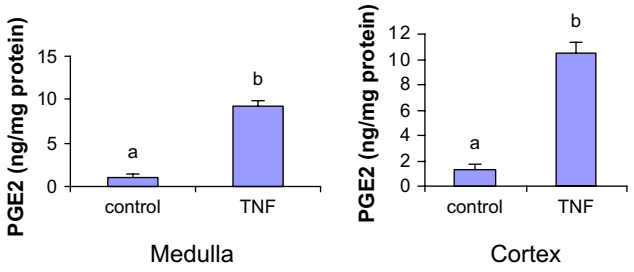

Fig. 4. Effect of TNF-α on PGE_2 levels in kidney cortex and medulla ($n = 3$). Letters above the bars indicate significant differences. Bars sharing a common letter are not significantly different; $P < 0.01$.

In the medulla (Fig. 7), PGE_2 affected the protein expression of the cotransporter in a dose-dependent manner. The protein expression was reduced at low doses and increased at high doses.

4. Discussion

This work demonstrated a TNF-α-induced decrease in the activity and protein expression of the Na^+-K^+ pump in both renal cortex and medulla. The decrease in activity was ascribed to the reduction in the abundance of enzyme molecules.

This effect did not appear, however, when the rat was pre-injected with indomethacin, an inhibitor of COX enzymes, suggesting that the effect of the cytokine is mediated through PGE_2. This hypothesis was further confirmed by the observed increase in renal PGE_2 levels induced by TNF-α, and supported by many other works reporting a PGE_2-mediated effect of TNF-α. The cytokine for example was reported in the literature to induce cyclo-oxygenase 2 (COX-2) expression in primary cultures of the medullary thick ascending limb [17] and increase PGE_2 synthesis in mesangial cells [18] and bovine chondrocytes [19].

Another effect of TNF-α demonstrated in this work was the decrease in protein expression of the Na^+-K^+2Cl^- (NKCC) cotransporter in the cortex and medulla (Fig. 3), an effect that was not abrogated by indomethacin and which is not consequently dependent on PGE_2.

Two isoforms of the Na^+-K^+2Cl^- cotransporter (NKCC) are known to be present in mammalian tissues: NKCC1 and NKCC2 [20–24].

The isoform studied in this work is the NKCC1. A decrease in the abundance of this cotransporter is expected to reduce the transepithelial NaCl transport from the serosal to the mucosal side into the tubular lumen, and may come as a consequence of the TNF-α-induced decrease in the Na^+-K^+ ATPase activity which provides the driving force for this symporter. It is also in line with the reported diuretic and natriuretic effects of TNF-α [6].

TNF-α signaling is a very complex process. TNF-α can activate mitogen-activated protein kinases (MAPK), nuclear factor kappa B (NF-κB), Jun N-terminal kinase (JNK), proteases, ceramides, and reactive oxygen species [25]. On the other hand the Na^+-K^+2Cl^- is regulated by phosphorylation/dephosphorylation or changes in its gene expression [26]. The abundance of the Na^+-K^+2Cl^- cotransporter in the thick ascending limb of the kidney was found to be regulated by vasopressin via a cAMP-mediated increase in the transcription of its gene, which contains a cAMP regulatory element [27]. Recently, TNF-α was shown to reduce cAMP levels by targeting the enzyme adenylate cyclase through a mechanism involving NF-κB [28]. Thus in this study, TNF-α may have down-regulated the cotransporter by reducing the basal level of cAMP, thus decreasing the transcription

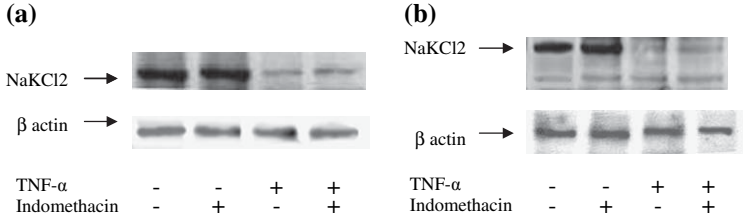

Fig. 3. Effect of TNF-α and indomethacin on the protein expression of the Na^+-K^+2Cl^- in renal cortex (a) and medulla (b). (Protein load: 40 μg.) Results are representative of an experiment repeated three times.

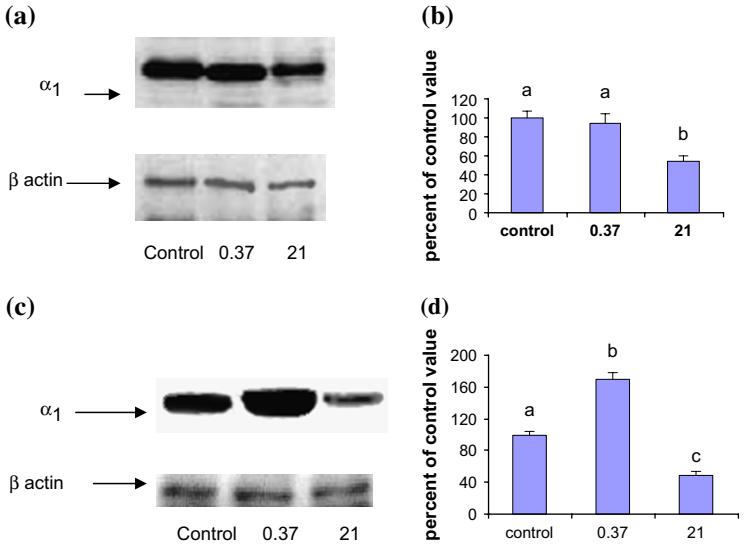

Fig. 5. (a) and (c): Effect of different doses of PGE_2 (0.37 and 21 μg/100 g body weight) on the protein expression of the Na^+-K^+ ATPase in the renal cortex (a) and medulla (c). Results are representative of an experiment repeated three times. (b) and (d): Quantification of the results by densitometry. Results are reported as a percentage of the control value. Letters above the bars indicate significant differences. Bars sharing a common letter are not significantly different; $P < 0.01$.

of its gene which has a cAMP regulatory element. This pathway, however, is still speculative and would need to be confirmed by future experimental work.

It can be concluded that TNF-α affects the pump and the symporter via, respectively, PGE_2-dependent and PGE_2-independent pathways.

Injection of the animals with a low dose (0.37 μg/100 g BW) of the prostaglandin did not affect the activity nor the protein expression of the Na^+-K^+ pump in the kidney cortex, but increased both significantly in the medulla. A higher dose (21 μg/100 g BW), however, reduced the ATPase activity and protein expression in both cortex and medulla, and thus exerted a similar effect to that observed with TNF-α. The results suggest that the cytokine induces the release of PGE_2 at levels high enough to induce inhibition and down-regulation of the pump. The decrease in pump activity correlated here again very well with the decrease in its expression and was ascribed to a reduction in the number of the ATPase molecules.

This dose-dependent response to the prostaglandin may be attributed to the activation of different types of PGE_2 receptors (EP receptors) having different signal transduction pathways and different affinities for the prostaglandin. EP1 receptors are known to signal via an increase in intracellular calcium [29–31] and are present in the collecting duct, increasing in abundance from the cortex to the papillae [32]. They were also detected in the nuclear envelope where they can regulate

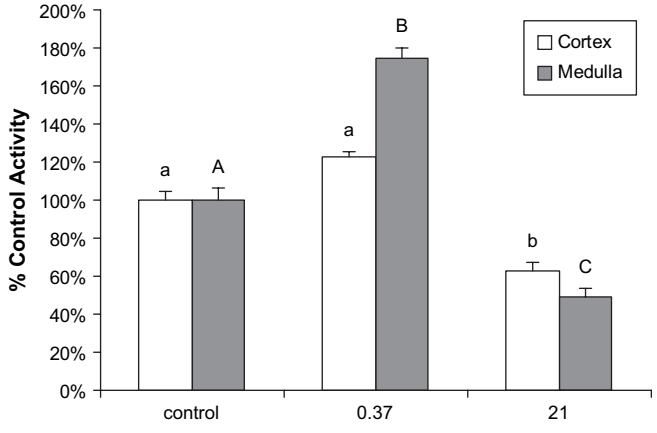

Fig. 6. Dose-dependent effect of PGE_2 (0.37 and 21 μg/100 g BW) on the activity of the Na^+-K^+ ATPase in kidney cortex and medulla ($n = 3$). Letters above the bars indicate significant differences. Bars sharing a common letter are not significantly different. $P < 0.05$: (a, b); (A, C), $P < 0.01$: (A, B), $P < 0.001$: (B, C).

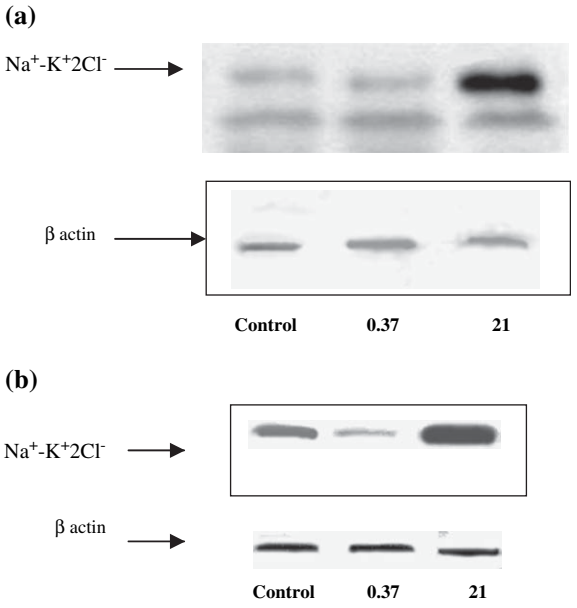

Fig. 7. Dose-dependent effect of PGE_2 (0.37 and 21 μg/100 g BW) on the expression of the Na^+-K^+2Cl^- cotransporter in kidney cortex (a) and medulla (b). (Protein load: 12 μg.) Results are representative of an experiment repeated three times.

gene expression [33]. Activation of these receptors was shown to inhibit renal Na^+ and water reabsorptions and contributes to natriuresis and diuresis. EP2 and EP4 are coupled to a Gs protein and cause an increase in cAMP levels [34,35]. EP2 receptors exhibit a low level of expression in the kidney and their precise localization is uncertain [36] while EP4 was detected in the collecting duct [32] and in the glomerulus [37].

EP3 receptors are expressed in the medullary thick ascending limb and collecting duct. When activated they inhibit cAMP generation via Gi [33] and are consequently expected to exert an opposite effect to that of EP2 and EP3.

Many hormones and mediators are known to exert a long- or a short-term regulatory effect on the Na^+-K^+ ATPase. Short-term regulation usually involves changes in the kinetics of the enzyme or its translocation between the plasma membrane and intracellular stores. Protein kinases, mainly PKC and PKA, were shown to play a major role in this regulation.

In the kidney, inhibition of the sodium pump in proximal segments of the nephron was reported to be mediated through G protein-linked PKC-dependent pathways [38,39], whereas in distal segments, PKA-associated pathways are involved [40,41]. Activation of the pump by PKA in the renal proximal tubule was demonstrated to be secondary to an increase in its translocation to the plasma membrane [42]. Similarly inhibition of the pump was shown to come as a consequence of its phosphorylation by PKC at Ser-23 [43–45].

In some cases PKA was shown to regulate the pump indirectly by altering the activity of other Na^+ transporters and consequently intracellular Na^+ which in turn leads to a change in the ATPase activity [46].

It has also been suggested [47] that phosphorylation of the rat Na^+-K^+ ATPase at Ser-23 by PKC requires prior phosphorylation by PKA at Ser-943.

In this work EP1 and EP4 are suspected to mediate the effect of high doses of PGE_2 because they have a lower affinity for the prostaglandin than EP3 [35] and activate, respectively, PKC and cAMP signaling which have been shown to inhibit both Na^+ and water reabsorptions in the thick ascending limb of the loop of Henle and the collecting duct [48]. EP3 may be involved in the up-regulatory effect PGE_2 observed at low doses in kidney medulla due to its higher affinity for PGE_2 and its mechanism of action which involves a decrease in cAMP production resulting in an increase in Na^+ transport [36].

Different doses of PGE_2 also exerted a dual trend on the expression of the Na^+-K^+2Cl^- symporter. Low doses had no effect in the cortex while high doses caused an up-regulation of the cotransporter. In the medulla down-regulation was observed at low doses while an opposite increase in the symporter expression was observed at high doses. Thus, at all doses studied, the effect of PGE_2 on the cotransporter was opposite to its effect on the pump. When the pump was down-regulated and inhibited, the cotransporter was up-regulated and vice versa. The two transporters are thus working in concert to regulate NaCl uptake from the tubular lumen and consequently water. The opposite effect observed at high doses may come as a form of negative feedback mechanism, or a way by which the same mediator can be used, depending on its level to exert opposite effects on salt reabsorption depending on body needs.

Acknowledgments

This work was supported by a grant from the University Research Board.

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