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The signal transduction pathway that mediates the effect of interleukin-1 beta on the Na⁺-K⁺-ATPase in LLC-PK₁ cells

Received: 30 December 2003 / Accepted: 9 January 2004 / Published online: 17 February 2004
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Abstract IL-1 β reduces the activity and protein expression of Na⁺-K⁺-ATPase in rat kidney cells. The aim of the present study was to elucidate the signalling pathway involved, using the LLC-PK₁ cell line. In these cells IL-1 β caused a time and concentration-dependent decrease in the protein expression of the Na⁺-K⁺-ATPase. Inhibition of extracellular signal-regulated kinase (ERK), nuclear factor- κ B (NF- κ B) and cyclooxygenase (COX), but not p38 mitogen-activated kinase (MAPK), abolished the effect of the cytokine on the pump. The activation of NF- κ B by IL-1 β was maximal at 20 min and declined thereafter. Inhibition of the transcription factor by pyrrolidinediethyl-dithiocarbamate (PDTC) down-regulated the ATPase. The effects of IL-1 β on the pump and NF- κ B were prevented by the COX inhibitor indomethacin. Exogenous PGE₂ reduced protein expression of the ATPase within 15 min, even in presence of an ERK inhibitor. It is concluded that IL-1 β stimulates the mitogen and extracellular signal regulated protein kinase kinase/extracellular signal regulated protein kinase (MEK/ERK) pathway. This activates NF- κ B, thus leading to increased COX-2 expression and PGE₂ release. PGE₂ in turn inhibits NF- κ B and reduces the protein expression of Na⁺-K⁺-ATPase.

Keywords IL-1 β · MEK · NF- κ B · PGE₂ · Na⁺-K⁺-ATPase · LLC-PK₁

Introduction

Renal epithelia play a key role in the excretion of ions and molecules and in the maintenance of salt and water balance. The principal regulator of transepithelial ion transport is the Na⁺-K⁺-ATPase, a ubiquitously expressed pump responsible for the generation of the Na⁺ gradient

that drives many cellular activities such as active transport processes and the production of action potentials.

The Na⁺-K⁺-ATPase has two subunits, α and β . The catalytic α -subunit (~113 kDa) contains the binding sites for Na⁺, K⁺, ATP and ouabain [19]. The β -subunit is needed for the activity of the complex and for the insertion of the pump in the plasma membrane. Many α - and β -subunit isoforms have been identified [8] and can assemble in different combinations. The α 1 β 1 association is found nearly in every tissue and is considered to be the housekeeping isozyme. It is also the principle isozyme in the kidney [25].

Interleukin-1 beta (IL-1 β) is a proinflammatory cytokine produced by macrophages. It alters renal electrolyte reabsorption and induces natriuresis [3, 4, 12, 15, 22, 30] and diuresis [16]. Recently, we have unravelled the mechanism underlying these effects of the cytokine by demonstrating a decrease in the activity and protein expression of Na⁺-K⁺-ATPase in both medullary and cortical kidney cells isolated from rats treated with IL-1 β [16]. Inhibition of the pump is expected to decrease the Na⁺ gradient and Na⁺ reabsorption, thus leading to natriuresis, and to decrease the osmotic gradient, thus leading to diuresis. Although the effect of the cytokine on the pump has been well established, the signalling pathway involved is still ill-defined.

IL-1 β is known to exert a diversity of biological effects through an activation of the mitogen-activated protein (MAP) kinase (MAPK) cascades, including the extracellular signal-regulated kinase (ERK), c Jun amino-terminal kinase (JNK) and the p-38 MAPK [23]. Once activated, MAPKs in turn phosphorylate a variety of intracellular substrates including certain transcription factors like nuclear factor- κ B (NF- κ B) [14, 15, 23, 24].

NF- κ B is a nuclear transcription factor that resides in its inactive state in the cytoplasm, complexed to an inhibitory protein I κ B. Upon activation of the complex, I κ B undergoes sequential phosphorylation on Ser-32 and Ser-36 by I κ B kinase, resulting in its subsequent ubiquitination and degradation, thus releasing NF κ B for transloca-

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tion to the nucleus where it activates transcription of various genes [2].

Prostaglandins (PG) are also thought to mediate part of the response to IL-1 β . By activating JNK or p38 MAPK, IL-1 β induces cyclooxygenase-2 (COX-2) expression and PGE₂ production [10, 14]. Although there are two COX isoforms (COX-1 and -2), only COX-2 is inducible by cytokines, growth factors and bacterial endotoxins [27, 29]. COX-1 is constitutively active and unaffected by IL-1 β or tumour necrosis factor- α (TNF- α) [9]. Activation of NF- κ B is necessary for the induction of COX-2 expression in many, but not all cells. Although TNF- α activates NF- κ B and induces COX-2 gene expression in rat vascular smooth muscle cells [6], the two effects are unrelated to each other, suggesting that the transcriptional regulation of COX-2 by NF- κ B is dependent on the type of cell [6].

It is apparent that although many of the mediators of IL-1 β signalling have been identified, they are not ubiquitous and their positions relative to each other in the signalling pathway are still ill-defined. The aim of this work was thus to determine the signal transduction pathway activated by IL-1 β through which the cytokine exerts its effect on renal Na⁺-K⁺-ATPase. For this the porcine renal proximal tubule cell line (LLC-PK₁) was used and the involvement of already established intermediates of IL-1 β action like NF- κ B, MAPK and PGE₂ was studied. Knowing the mode of action of the cytokine would help in modulating the different undesirable effects and symptoms that accompany some inflammatory reactions and may help also in circumventing the natriuresis and diuresis that always accompany high levels of IL-1 β .

Materials and methods

Culture media, fetal bovine serum (FBS), penicillin/streptomycin, trypsin-EDTA and Hanks balanced salt solution (HBSS) were purchased from Gibco (Paisley, UK). The MAPK inhibitors PD98059 and SB 202190 were from Calbiochem (San Diego, Calif., USA). Pyrrolidinediethyldithiocarbamate (PDTTC), indomethacin, cycloheximide, actinomycin D and prostaglandin E₂ (PGE₂) were obtained from Sigma (St Louis, Mo., USA). Recombinant human interleukin-1 β was from Endogen (Woburn, Mass., USA). Rabbit anti-Na⁺-K⁺-ATPase α 1 polyclonal antibody and anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody were from Upstate Biotechnology (Lake Placid, N.Y., USA, Cat. No. 06-520 and 12-348 respectively). Anti-I κ B α monoclonal antibody was from Biosource International (Camarillo, Calif., USA), while goat anti-mouse IgG HRP-conjugated antibody and the enhanced chemiluminescence (ECL) kit were from Santa Cruz Biotechnology, (Santa Cruz, Calif., USA). Polyvinylidene difluoride (PVDF) membranes, protein assay reagent and rainbow marker were from Biorad (Hercules, Calif., USA). The LLC-PK₁ cell line (porcine renal proximal tubule cells) was a generous gift from Dr. Rula Barhoumi-Mouneimbeh from Texas A&M University, USA.

Cell culture and treatments

Cells were cultured in DMEM-F12 medium supplemented with 10% heat-inactivated FBS, penicillin (100 μ g/ml) and streptomycin (100 μ g/ml) in a humidified incubator (95% air, 5% CO₂) at 37°C

in 100-mm culture dishes. The medium was replenished twice a week and at 80% confluence the cells were treated with IL-1 β . Prior to treatment, cells were washed with HBSS and then incubated for different periods (30, 60, 90, 120, 150 or 180 min) in growth medium to which IL-1 β was added at different concentrations (5, 7.5, 10, 15 or 20 ng/ml), to determine the optimal concentration and incubation period. Once determined, they were employed in all experimental treatments. The specificity of the response was investigated by studying the effect of heat-inactivated IL-1 β (heated for 3 min to 120°C) on the pump expression. For this LLC-PK₁ cells were incubated with the heated cytokine at the optimal concentration and time (15 ng/ml for 2 h).

When the cells were to be treated with PGE₂, the latter was added to the incubation medium at 1 nM for 15, 30 or 45 min. Inhibitors or their vehicle were added, when their effect was to be studied, 15 min prior to the addition of IL-1 β or PGE₂. The MAPK inhibitors, PD98059 and SB202190 were added at a final concentration of 50 μ M; indomethacin (dissolved in DMSO) and PDTTC were added at 100 μ M. Actinomycin D and cycloheximide were added at 3 and 5 μ g/ml respectively. When activation of NF κ B was to be studied, cells were incubated for 20 min with IL-1 β or the inhibitors.

At the end of the incubation period, the cells were harvested, lysed, homogenized in a Polytron homogenizer at 4°C for 4 min and assayed for protein using the Bio-Rad assay and samples were used for Western blot analysis.

Na⁺-K⁺-ATPase assay

Cell homogenates were diluted in TRIS buffer (in mM): 200 NaCl; 5 MgCl₂; 2 EGTA; 5 KCL; 200 TRIS-HCl, pH 7.4, to a final protein concentration of 5.2 mg/ml and incubated with saponin (0.02%) for 30 min at room temperature. Aliquots (50 μ l) were then incubated 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 μ l, 11%). The samples were centrifuged at 3,000 g for 5 min and the amount of inorganic phosphate (P_i) liberated in the supernatant measured colourimetrically according to [28]. The activity of the enzyme was determined as the ouabain-inhibitable P_i liberated and the percentage recovery was calculated as follows:

$$\frac{P_i(\text{IL} - 1\beta) - P_i(\text{IL} - 1\beta + \text{ouabain})}{P_i(\text{control}) - P_i(\text{control} + \text{ouabain})} \times 100$$

Western blot analysis

Protein expression of Na⁺-K⁺-ATPase was determined by Western blot analysis. Membrane proteins (30 μ g) were loaded equally and resolved on 8% polyacrylamide gels using the Mini-Protean 3 system (Bio-Rad). Samples were warmed to 37°C for 30–45 min before loading and then transferred to a PVDF membrane using a Bio-Rad Mini-Trans-blot cell. The PVDF membrane was washed, blocked and incubated with a rabbit polyclonal primary anti-Na⁺/K⁺-ATPase α -1 IgG and with a goat anti-rabbit HRP conjugated secondary antibody. Signals were detected using enhanced chemiluminescence with luminol reagent.

The state of activation of NF- κ B was determined by monitoring changes in the protein expression of the inhibitory I κ B protein by Western blot analysis, and was conducted as above except that the membrane was incubated with an I κ B- α antibody added at 1 μ g/ml and the secondary antibody used was an HRP-conjugated goat anti-rabbit IgG.

The expression of β actin was used to check for equal loading. Autoradiographs were quantified densitometrically using appropriate software (Gel-Pro Analyzer v. 2, Media Cybernetics, Silver Spring, Md., USA). Signal intensities were normalized with respect to that of β -actin and results reported as percentages of the control value.

Statistical analysis

The significance of differences between means was established by a one-way ANOVA followed by a Tukey-Kramer multiple comparisons test.

Results

IL-1 β caused a concentration and time-dependent decrease in the activity and protein expression of Na⁺-K⁺-ATPase in LLC-PK₁ cells (Figs. 1, 2). The maximal effect was observed at 2 h and 10 ng/ml. Heat-inactivated IL-1 β did not have any effect on the pump when added at the optimal concentration and for the optimal period.

Involvement of MAPK

To study the involvement of the MAPK cascades in the cytokine effect on the pump, cells were incubated in the simultaneous presence of IL-1 β and PD98059 or SB202190, inhibitors of mitogen and extracellular signal regulated protein kinase kinase/extracellular signal regulated protein kinase (MEK/ERK) and p38 MAPK respectively (Fig. 3). The effect of the cytokine was still apparent in the presence of the p38 MAPK inhibitor, but not when MEK was inhibited.

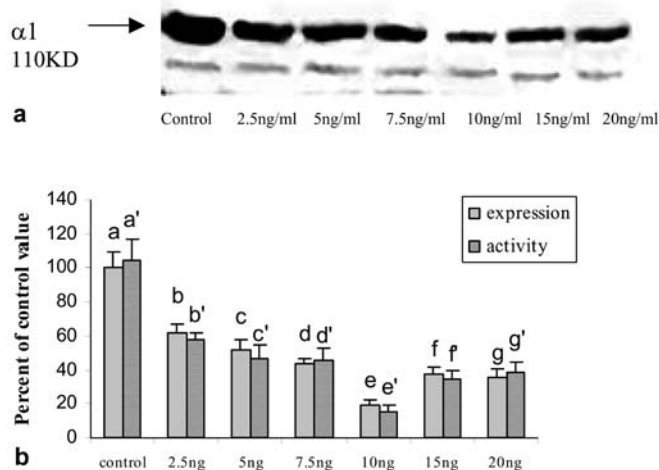


Fig. 1 **a** Effect of different concentrations of interleukin-1 β (IL-1 β) on protein expression of the $\alpha 1$ -subunit of the Na⁺-K⁺-ATPase in LLC-PK₁ cells. Cells were incubated for 2 h. Results are representative of an experiment repeated 3 times. **b** Effect of different concentrations of IL-1 β on the activity and protein expression of the Na⁺-K⁺-ATPase. Means \pm SEM. Protein expression was quantified using Gel-Pro Analyzer (v. 2) software and is shown as a percentage of the signal obtained with the control. The letters identify the columns for purposes of comparison: $P < 0.05$: c vs. e; $P < 0.01$: a vs. b, b vs. e; $P < 0.001$: a vs. c, a vs. d, a vs. e, a vs. f, a vs. g. Activity was assayed by measuring the ouabain-inhibitable release of inorganic phosphate: $P < 0.05$: b' vs. e'; $P < 0.01$: a' vs. b'; $P < 0.001$: a' vs. c', a' vs. d', a' vs. e', a' vs. f', a' vs. g'

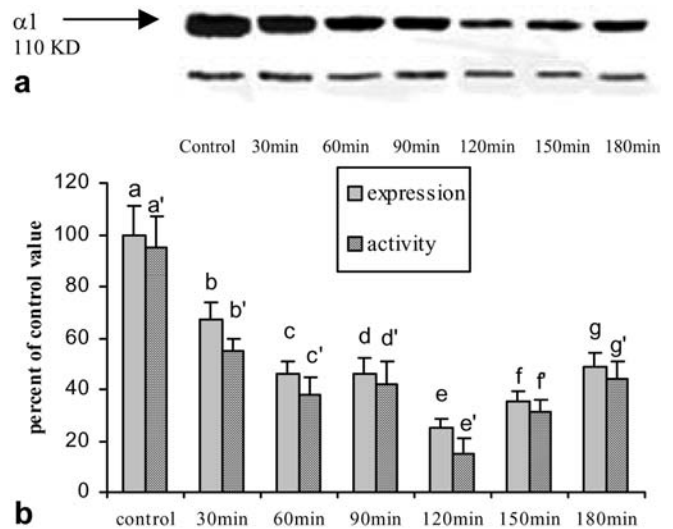


Fig. 2 **a** Time course effect of the effect of IL-1 β on the protein expression of the $\alpha 1$ -subunit of Na⁺-K⁺-ATPase. Cells were incubated for different periods with IL-1 β (10 ng/ml). Results are representative of an experiment repeated 3 times. **b** Effect of duration of incubation with IL-1 β on the activity and protein expression of Na⁺-K⁺-ATPase. Means \pm SEM; quantification details as in Fig. 1. $P < 0.05$: b vs. c, b vs. d, b vs. g, c vs. e, d vs. e; $P < 0.01$: e vs. g; $P < 0.001$: a vs. b, a vs. c, a vs. d, a vs. e, a vs. f, a vs. g. Activity is reported as the percentage recovery of the enzyme activity. $P < 0.05$: a' vs. b', b' vs. e'; $P < 0.01$: a' vs. c', a' vs. d', a' vs. g'; $P < 0.001$: a' vs. e', a' vs. f'

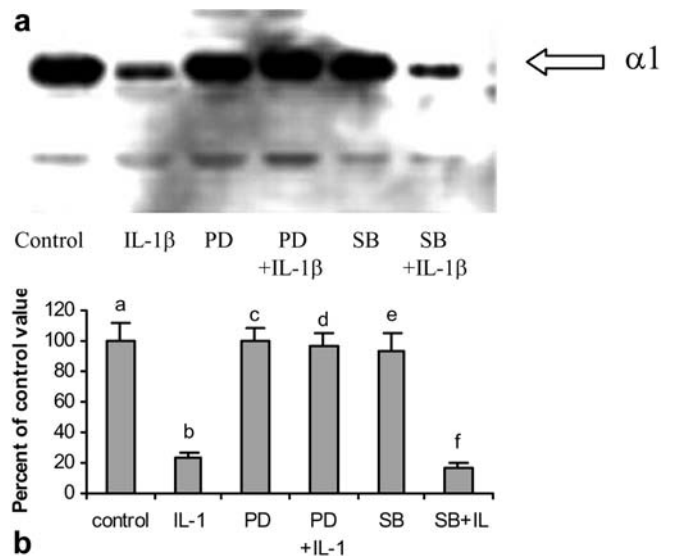


Fig. 3 **a** Effect of PD 98058 (50 μ M) and SB 202190 (50 μ M) on protein expression of the $\alpha 1$ -subunit of the Na⁺-K⁺-ATPase. The inhibitors were added 15 min before the addition of IL-1 β after which the cells were incubated for 120 min. Results are representative of an experiment repeated 3 times. **b** Quantification of the results, quantification details as in Fig. 1. $P < 0.001$: a vs. b, a vs. f, b vs. c, b vs. d, b vs. e, c vs. f, d vs. f, e vs. f

Involvement of NF- κ B

In this study, NF κ B activation was studied by monitoring changes in the protein expression of I κ B α , which is degraded upon activation of the transcription factor. IL-1 β

(10 ng/ml) activated NF- κ B maximally by 20 min and the activation decreased thereafter (Fig. 4) until it returned to basal levels. Incubation of LLC-PK₁ cells for 2 h with PDTC, an inhibitor of NF- κ B, down-regulated the pump (Fig. 5) and in the simultaneous presence of PDTC and IL-1 β , the protein expression of the ATPase was not different from that seen in the presence of PDTC or IL-1 β alone.

Position of MEK/ERK relative to NF- κ B in the signalling pathway

IL-1 β added in presence of PD98059, an inhibitor of the MEK/ERK pathway, did not cause any activation of NF- κ B at 20 min (Fig. 6).

Effect of indomethacin

The IL-1 β -induced down-regulation of the Na⁺-K⁺ pump and NF- κ B activation were prevented by indomethacin, the classic, non-selective inhibitor of COX (COX-1 and -2) (Fig. 7).

Effect of PGE₂

Exogenous PGE₂ added to the cells reduced protein expression of the pump within 15 min (Fig. 8) and inactivated NF- κ B (Fig. 9c, d). These responses were not influenced by PD98050, a MEK/ERK inhibitor (Fig. 9). PDTC, an inhibitor of NF- κ B, decreased the protein expression of Na⁺-K⁺-ATPase, and a further decrease was elicited by the simultaneous presence of PGE₂ (Fig. 10).

Effect of PGE₂ in presence of inhibitors of translation or transcription

To determine whether PGE₂ affects transcription or translation of the pump, cells were incubated with PGE₂ in presence or absence of actinomycin D or cyclohexim-

Fig. 4 **a** Time course effect of IL-1 β (10 ng/ml) on the protein expression of the inhibitor of nuclear factor- κ B (*I κ B*) unit. Results are representative of an experiment repeated 3 times. **b** Changes in protein expression of I κ B compared with control. Means \pm SEM, details as in Fig. 1. *P*<0.05: *a* vs. *i*; *P*<0.01: *a* vs. *b*, *c* vs. *d*, *d* vs. *e*, *c* vs. *f*, *c* vs. *g*, *c* vs. *h*; *P*<0.001: *a* vs. *c*

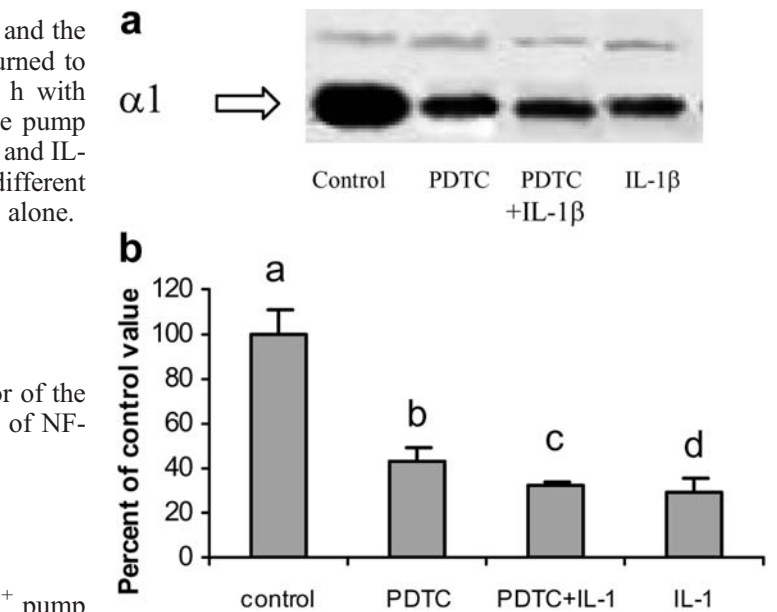
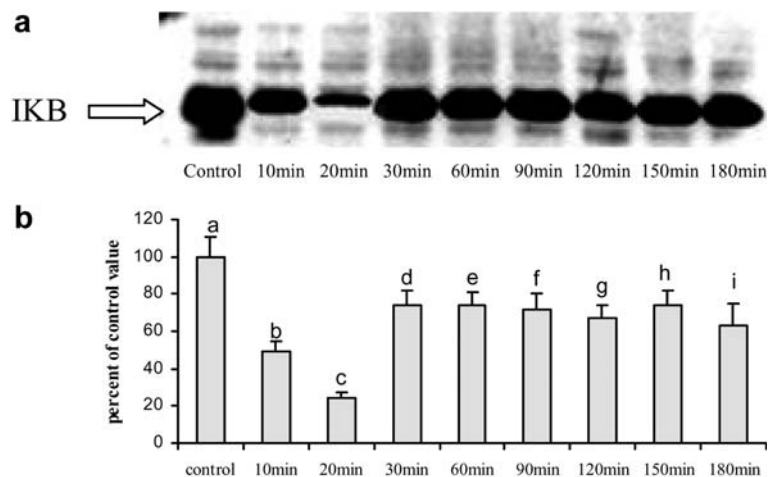


Fig. 5 **a** Effect of pyrrolidinediethyldithiocarbamate (PDTC) (50 μ M) on protein expression of the α 1-subunit of the Na⁺-K⁺-ATPase in the presence or absence of IL-1 β (10 ng/ml). Results are representative of an experiment repeated 3 times. **b** Quantification of the results. Means \pm SEM, details as in Fig. 1. *P*<0.01: *a* vs. *b*; *P*<0.001: *a* vs. *c*, *a* vs. *d*

ide, inhibitors of transcription and translation respectively. Either inhibitor, added alone, decreased the expression of the pump and further inhibition was observed in the simultaneous presence of PGE₂, which was similar to that obtained with PGE₂ alone (Fig. 11).

Discussion

IL-1 β caused in LLC-PK₁ cells a concentration and time-dependent decrease in the activity and protein expression of Na⁺-K⁺-ATPase (Figs. 1, 2). This finding is consistent with similar findings in kidney cortical cells isolated from IL-1 β -treated rats [16] and with the inhibitory effect on ATPase activity and ouabain binding in MDCK cells [7].



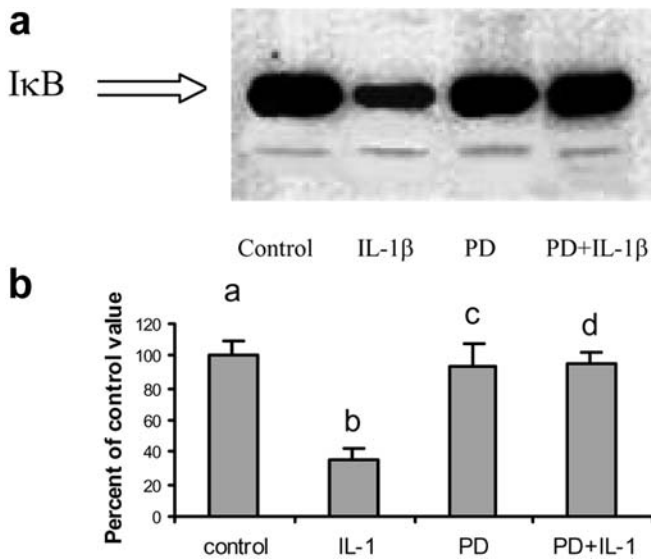


Fig. 6 a Effect of mitogen-activated protein kinase (MAPK) inhibitor PD98059 (*PD*) on protein expression of the IκB subunit. *PD* (50 μM) was added to cells 15 min before the addition of IL-1β and cells were incubated thereafter for 20 min. Results are representative of an experiment repeated 3 times. **b** Quantification of the results; means±SEM; details as in Fig. 1. *P*<0.01: *a* vs. *b*, *b* vs. *c*, *b* vs. *d*

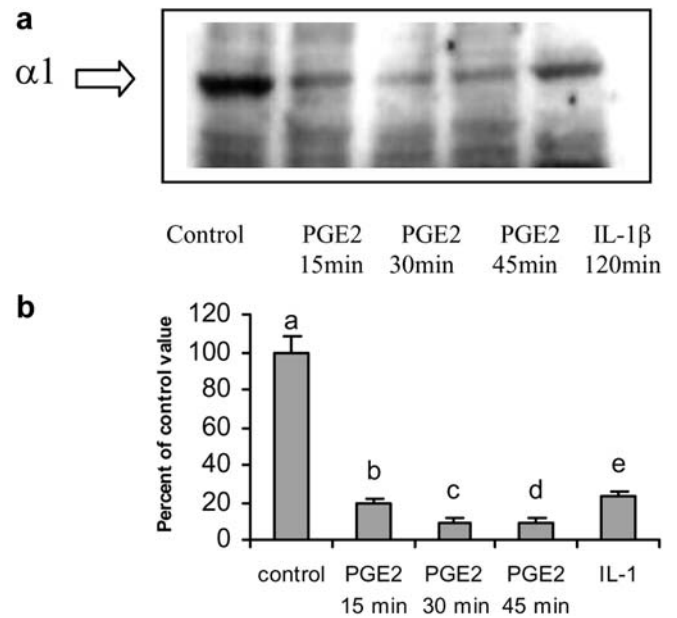


Fig. 8 a Time course of the effect of 1 nM prostaglandin E₂ (*PGE*₂) on the protein expression of the α1-subunit of the Na⁺-K⁺-ATPase. Results are representative of an experiment repeated 3 times. **b** Quantification of results; means±SEM; details as in Fig. 1. *P*<0.001: *a* vs. *b*, *a* vs. *c*, *a* vs. *d*, *a* vs. *e*

The inhibition in activity correlated closely with the decrease in the pump expression and can thus be ascribed to a reduction in the number of enzyme molecules.

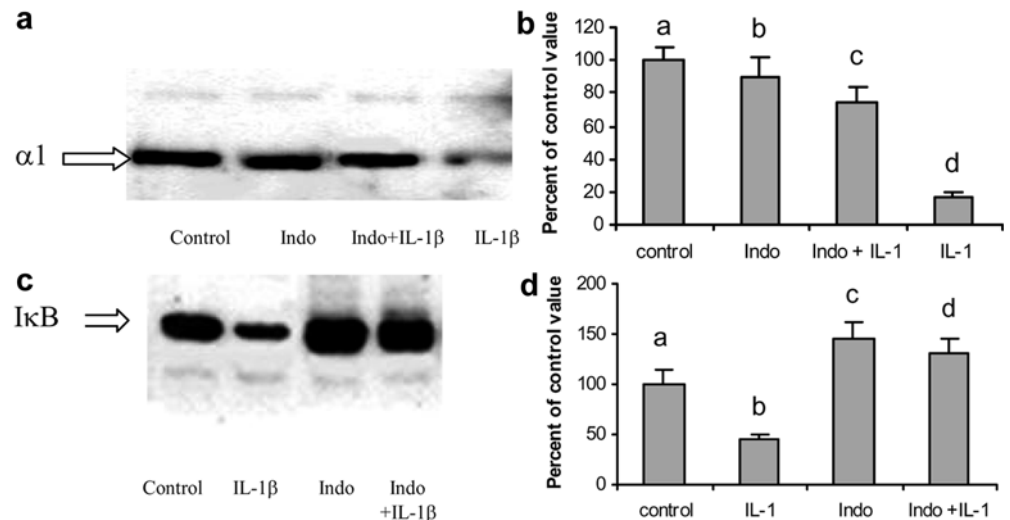
IL-1β is known to signal through activation of the MAPK cascades [17, 23] that have as their terminal effectors the ERKs, JNKs and p38 MAPKs. IL-1β modulates the three types of MAPK in mammalian cells [23]. The kinases are activated by distinct upstream MAPK kinases (MEK, SEK and MKK3) that recognize and phosphorylate threonine and tyrosine residues within a tripeptide motif (Thr-X-Tyr) on MAPK, a process that is required for their activation.

To determine whether the effect of IL-1β on the Na⁺-K⁺-ATPase is mediated through the MAPK cascades, cells

were incubated in the simultaneous presence of IL-1β and PD98059 or SB202190, inhibitors of MEK/ERK and p38 MAPK respectively (Fig. 3). Inhibition of MEK, but not p38 MAPK, abrogated the effect of IL-1β on the pump, suggesting that ERK, but not p38 MAPK, is involved in regulating pump expression. In contrast, p38 MAPK rather than ERK mediates the effect of IL-1β on the pump in Caco-2 cells [1]. It can be concluded that different MAPKs are involved in the signalling pathways of the cytokine in different cells.

Activated MAPKs are known to phosphorylate a variety of intracellular substrates including certain transcription factors like NF-κB [11]. NF-κB resides in its inactive form in the cytosol, complexed with inhibitory proteins known

Fig. 7 a Effect of indomethacin (100 μM) on the protein expression of the α1-subunit of the Na⁺-K⁺-ATPase in the presence and absence of IL-1β (10 ng/ml). Results are representative of an experiment repeated 3 times. **b** Quantification of results; means±SEM; details as in Fig. 1. *P*<0.001: *a* vs. *d*; *P*<0.01: *b* vs. *d*, *c* vs. *d*. **c** Effect of IL-1β (10 ng/ml) on the expression of IκB subunit in presence or absence of indomethacin (100 μM). Results are representative of an experiment repeated 3 times. **d** Quantification of results; means±SEM; details as in Fig. 1. *P*<0.01: *a* vs. *b*, *b* vs. *d*; *P*<0.001: *b* vs. *c*



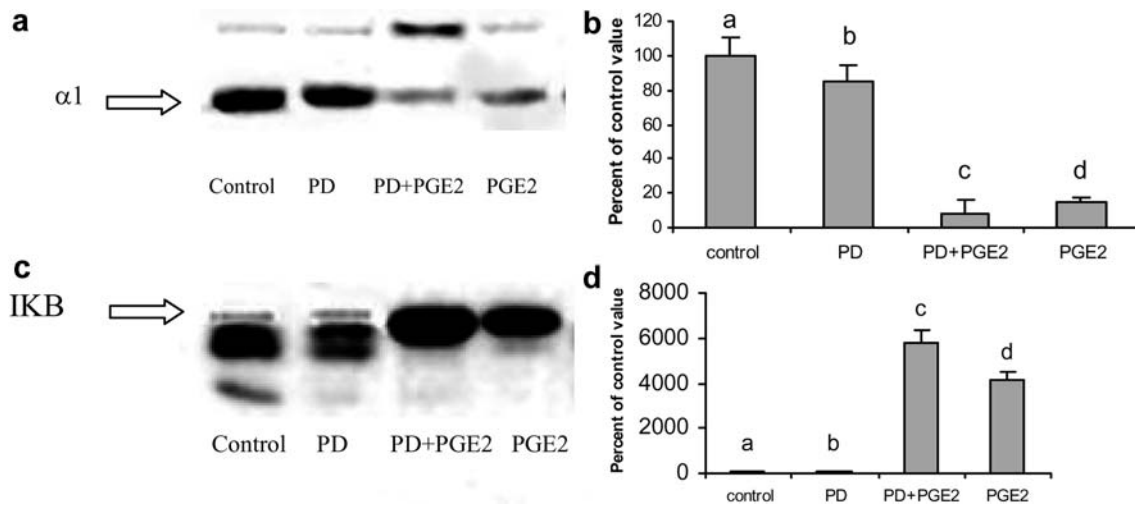


Fig. 9 **a** Effect of 1 nM PGE₂ on the protein expression of the $\alpha 1$ -subunit of the Na⁺-K⁺-ATPase in presence of the MEK/ERK inhibitor PD98059 (50 μ M). Results are representative of an experiment repeated 3 times. **b** Quantification of results; means \pm SEM; details as in Fig. 1. $P < 0.001$: *a* vs. *c*, *a* vs. *d*, *b* vs. *c*; $P < 0.01$:

b vs. *d*. **c** Effect of 1 nM PGE₂ on the activation of NF- κ B in the presence of PD98059 (50 μ M). Results are representative of an experiment repeated 3 times. **d** Quantification of results; means \pm SEM; details as in Fig. 1. $P < 0.001$: *a* vs. *c*, *a* vs. *d*, *b* vs. *c*, *b* vs. *d*

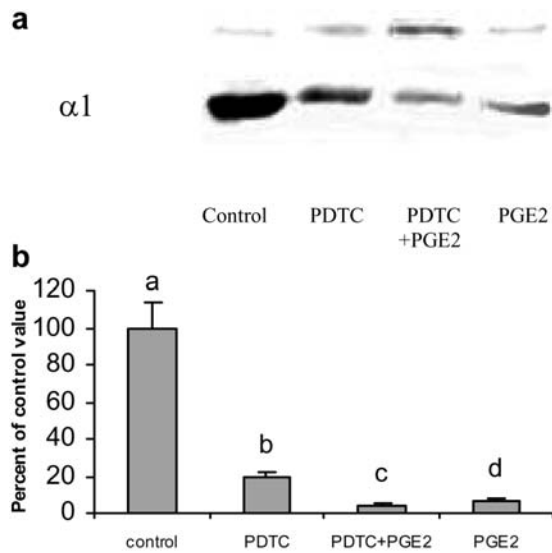


Fig. 10 **a** Effect of PGE₂ (1 nM) in presence and absence of PDTC (100 μ M) on protein expression of the $\alpha 1$ -subunit of Na⁺-K⁺-ATPase. **b** Quantification of results; means \pm SEM; details as in Fig. 1. $P < 0.001$: *a* vs. *b*, *a* vs. *c*, *a* vs. *d*

as I κ Bs [21]. Phosphorylation of I κ B by I κ B kinase leads to its dissociation from the complex and its subsequent degradation by proteasomes. The liberated NF- κ B then translocates into the nucleus, where it activates the transcription of various genes. Leung et al. [18] have reported that IL-1 β induces, within 5 min, I κ B phosphorylation, degradation and nuclear translocation in numerous cell types [26]. Our results showed also that activation of NF- κ B by IL-1 β was apparent as early as 10 min, reached its peak at 20 min and decreased in magnitude thereafter (Fig. 4).

Activated NF- κ B is known to increase in turn the expression of I κ B, and newly synthesized I κ B enhances the dissociation of NF- κ B from the DNA, because the

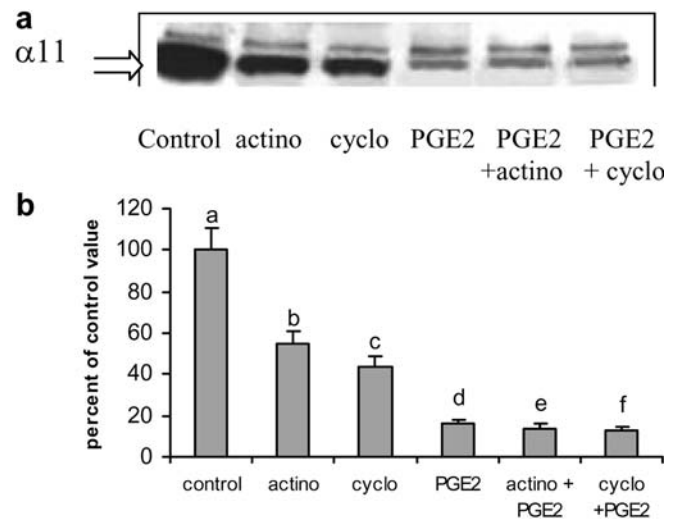


Fig. 11 **a** Western blot showing the effect of PGE₂ in presence or absence of actinomycin or cycloheximide on the protein expression of the $\alpha 1$ subunit of the Na⁺-K⁺-ATPase. **b** Quantification of results; means \pm SEM; details as in Fig. 1. $P < 0.01$: *a* vs. *b*, *a* vs. *c*, *a* vs. *d*, *a* vs. *e*, *a* vs. *f*, *b* vs. *d*, *b* vs. *e*, *b* vs. *f*; $P < 0.05$: *c* vs. *d*, *c* vs. *e*, *c* vs. *f*

affinity of NF- κ B for I κ B is higher than its affinity for the κ B sites on DNA, initiating thus a negative feedback loop [21]. This may be one reason for the transient activation of NF- κ B observed at 20 min.

To determine whether such activation is necessary for basal expression of the pump or for the effect of the cytokine, LLC-PK₁ cells were incubated for 2 h with PDTC, an inhibitor of NF- κ B, in presence or absence of IL-1 β (Fig. 5). The inhibitor alone reduced the protein expression of the ATPase indicating that the transcription factor NF- κ B is involved in its basal expression. When IL-1 β was added simultaneously with PDTC, no further decrease in the pump expression was observed beyond that seen with PDTC or IL-1 β alone. This suggests that if IL-

IL-1 β acts through NF- κ B, its effect should be inhibitory, and because the transcription factor was already inhibited no further effect was observed. Since the activation of the transcription factor preceded the down-regulatory effect of the cytokine on the pump, it can be postulated that transient NF- κ B activation is needed for the cytokine effect on the ATPase.

IL-1 β added in presence of PD98059, an inhibitor of the MEK/ERK pathway, did not activate NF- κ B at 20 min (Fig. 6) implying that MAPKs are involved in the activation process and are situated upstream from the transcription factor in the signalling pathway. This finding is in line with the well-established activation of NF- κ B by the MAPK cascades [24]. On the other hand, IL-1 β induces, through activation of some MAPK, COX-2 expression and PGE₂ production [12, 13], which in turn inhibits NF- κ B translocation to the nucleus [20]. In the present study, the IL-1 β -induced down-regulation of the Na⁺-K⁺ pump was prevented by indomethacin, a COX inhibitor (Fig. 7). Since IL-1 β enhances PGE₂ production by inducing gene expression of COX-2 with little effect on COX-1 [10], PGE₂ may be a potential mediator of the cytokine action on the ATPase. In support of this hypothesis, exogenous PGE₂ added to the cells also down-regulated the pump within 15 min (Fig. 8) and inactivated NF- κ B (Fig. 9c, d). Both responses were unaffected by PD98050, an inhibitor of MEK/ERK, inferring that the MAPK is upstream from PGE₂ (Fig. 9).

Although PGE₂ inhibited NF- κ B, the transcription factor may still not necessarily be involved in the pathway leading to down-regulation of Na⁺-K⁺-ATPase. To clarify this point, the effect of PGE₂ on the pump was studied in presence of PDTC, an inhibitor of NF- κ B. PDTC alone reduced the expression of the pump, and a further reduction was observed in the simultaneous presence of PGE₂ (Fig. 10), which could be due to an additional inhibition of NF- κ B by the prostaglandin over and above that due to PDTC, or to the activation of another pathway not involving NF- κ B.

It could be concluded that NF- κ B is involved in the basal expression of the Na⁺-K⁺-ATPase and that IL-1 β exerts its effect on the pump by activating the MEK/ERK cascade, which in turn activates NF- κ B leading to an increase in the gene expression of COX-2 and consequently PGE₂ release. The increase in PGE₂ in turn inhibits NF- κ B, leading to a decrease in the synthesis of new pump molecules. The basal level of PGE₂ is relatively low and does not inhibit the basal activity of NF- κ B. The time frame for the whole pathway is consistent with the proposed sequence of events. IL-1 β needed 120 min to exert its effect on the pump, whereas, PGE₂ needed only 15 min to exert the same effect.

Transitory activation of NF- κ B is thus needed to increase the level of PGE₂, which is the key and end player in the cytokine action on the Na⁺-K⁺-ATPase. Since its effect appeared as early as 15 min, and because the turnover rate of the Na⁺-K⁺-ATPase molecules could not be as short as 15 min, there may be a further mechanism by which PGE₂ decreases protein expression of the pump, in addition to the inhibitory effect on basal expression mediated through inhibition of NF- κ B. This mechanism may include recruitment of pump molecules to cytosolic

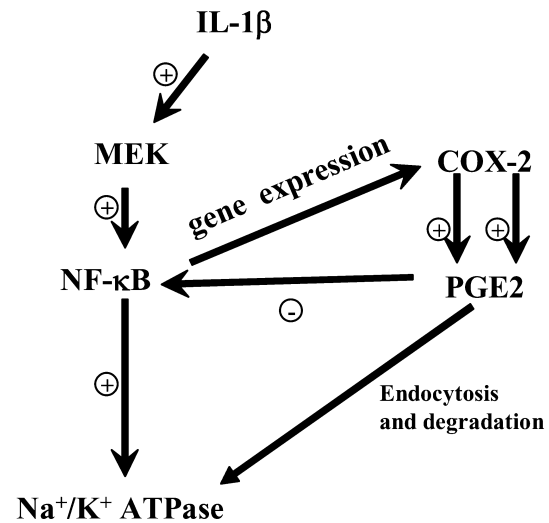


Fig. 12 Signal transduction pathway mediating the effect of IL-1 β on Na⁺-K⁺-ATPase in LLC-PK₁ cells

compartments and their degradation, in an endocytotic process that could be similar to that described by Chibalin et al. [5] to explain the dopamine-induced decrease in Na⁺-K⁺-ATPase activity in proximal tubule cells. Although this hypothesis is speculative and remains to be tested, it is supported by the data obtained with inhibitors of transcription and translation. Since the simultaneous addition of PGE₂ and the inhibitors produced a further decrease in the pump protein expression over that obtained with the inhibitors alone (Fig. 11), it can be inferred that PGE₂ has a mechanism of action that does not involve transcription or translation. On the other hand because the addition of actinomycin D or cycloheximide to PGE₂ did not exert any further down-regulatory effect beyond that exerted by PGE₂ alone, it could be speculated that translation and transcription were already inhibited by PGE₂.

In conclusion, IL-1 β activates NF- κ B transiently, leading to PGE₂ production. PGE₂, at high levels, acts along two lines: on one hand it inhibits NF- κ B and contributes to the termination of its activation, initiated by IL-1 β . On the other hand it promotes endocytosis and degradation of Na⁺-K⁺-ATPase molecules through an as yet unknown mechanism. It should be noted however that the signal transduction pathway activated by the cytokine is not the same in all types of cells. In Caco-2 cells [1], IL-1 β increases PGE₂ production and exerts a similar effect on the pump, but via different mediators. In these cells, AP-1 contributes to the basal expression of the pump in addition to other still unidentified transcription factors, while NF- κ B is not involved. PGE₂ acts independently of NF- κ B and AP-1 and promotes endocytosis and degradation. Whether the unidentified transcription factors are inhibited in parallel is unknown.

The signal transduction pathway of IL-1 β on the Na⁺-K⁺-ATPase in LLC-PK₁ is shown in Fig. 12.

Acknowledgements This work was supported from a grant from the University Research Board and the National Lebanese Council for Scientific Research.

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