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β-Agonists regulate Na,K-ATPase via novel MAPK/ERK and rapamycin-sensitive pathways

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Abstract We studied whether the β-adrenergic agonist, isoproterenol (ISO), regulates Na,K-ATPase in alveolar epithelial cells (AEC) via a mitogen-activated protein kinase (MAPK)/extracellular signaling related kinase (ERK) dependent pathway. ISO increased ERK activity in AEC by 10 min via a β-adrenergic receptor, protein kinase A (PKA)-dependent mechanism. Activation of the MAPK pathway by ISO, resulted in increased Na,K-ATPase β1 and α1 subunit protein abundance in whole cell lysates, which resulted in functional Na,K-ATPases at the basolateral membranes. ISO did not change the α1 or β1 mRNA steady state levels, but rapamycin, the inhibitor of the mammalian target of rapamycin, also blocked the ISO-mediated increase in Na,K-ATPase total protein abundance, suggesting a posttranscriptional regulation. We conclude that ISO, regulates the Na,K-ATPase in AEC via PKA, ERK and rapamycin-sensitive mechanisms.

Key words: Isoproterenol; cAMP; Protein kinase A; Mammalian target of rapamycin; Lung

1. Introduction

Pulmonary edema resolution depends on the ability of the lung to clear fluid from the alveolar space. This process occurs by active Na^+ transport across the alveolar epithelium [1–4]. Vectorial Na^+ movement in alveolar epithelial cells (AEC) is regulated in part by Na,K-ATPases located at the cell basolateral membranes (BLMs). [1,4–8]. The Na-K-ATPase is a membrane bound protein comprised of a catalytic α subunit, with a molecular mass of ∼112 kDa and a regulatory β subunit, which has a molecular weight of ∼40 and 60 kDa [9–12].

β-Adrenergic agonists have been shown to increase active Na^+ transport in isolated rat lungs by increasing Na,K-ATPase function [13–15]. Isoproterenol (ISO) increased Na,K-ATPase activity by recruiting Na,K-ATPase proteins from intracellular pools to the AEC BLMs within 15 min [16]. However, the long-term mechanisms of the Na,K-ATPase regulation by β-adrenergic agonists have not been elucidated.

The mitogen-activated protein kinase/extracellular signaling regulated kinase (MAPK/ERK) cascade is activated by a variety of signals, including catecholamines [17–21]. This cascade regulates the expression of specific genes via transcriptional and/or post-transcriptional mechanisms [18]. β-Adrenergic agonists have been found to activate the MAPK/ERK pathway [22–24], however, there are no studies relating this pathway to Na,K-ATPase regulation. Thus, we set out to study whether β-adrenergic agonists regulate Na,K-ATPase via MAPK/ERKs by transcriptional or post-transcriptional mechanisms. Our data suggest that β-adrenergic agonists regulate Na,K-ATPase α1 and β1 subunits via a cAMP, protein kinase A (PKA), ERK-dependent pathway, by post-transcriptional, rapamycin-sensitive mechanisms.

2. Materials and methods

2.1. Materials

Propanolol, H-89 and rapamycin, were purchased from Calbiochem (La Jolla, CA, USA) and forskolin, ISO and ouabain were purchased from Sigma (St. Louis, MO, USA). U0126, anti-P-ERK antibody and anti-ERK antibody were purchased from Promega (Madison, WI, USA) and [γ-^{32}P]ATP and Percoll was from Amersham (Arlington Heights, IL, USA). PD98059, Elk-1 fusion protein and antibodies against P-(Thr202/Tyr204)-p44/42 and P-Elk-1, were purchased from New England Biolabs (Beverly, MA, USA). Antibodies against Na,K-ATPase β1-and α1-subunits were a generous gift from Dr. Martin-Vasallo (University of La Laguna, Spain) and Dr. M. Caplan (Yale University, CT, USA) respectively.

2.2. Cell isolation and culture

AEC were isolated from pathogen-free male Sprague–Dawley rats (200–225 g), as previously described [6,16], according to the method of Dobbs et al. [25]. Briefly, the lungs were digested with elastase (3 U/ml; Worthington Biochemical, Freehold, NJ, USA), AEC were purified by differential adherence to IgG-pretreated dishes and cell viability was assessed by trypan blue exclusion (>95%). Cells were resuspended in Dulbecco's modified Eagle's medium (Cellgro, VA, AK, USA) containing 10% fetal bovine serum with 2 mM glutamine, 200 U/ml penicillin, 200 μg/ml streptomycin and 0.5 μg/ml amphotericin B. Cells were seeded in 6 cm plates at 8 million per plate or 10 cm plates at 18 million per plate (for Na,K-ATPase activities and BLM isolation experiments) and incubated in a humidified atmosphere of 5% CO_2, 95% air at 37°C. The day of isolation is designated day 0. All experimental conditions were tested in day 2 serum-starved cells. Media was changed every 2 days.

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Abbreviations: ISO, isoproterenol; AEC, alveolar epithelial cell; MAPK, mitogen-activated protein kinases; ERK, extracellular signaling related kinases; mTOR, mammalian target of rapamycin; PKA, protein kinase A; BLM, basolateral membrane

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2.3. Cell lysate and BLM isolation

AEC were treated for the desired times, medium was aspirated, cells were washed twice with cold phosphate-buffered saline (PBS) and total protein was isolated using 0.5 ml of lysis buffer (20 mM Tris, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 2.5 mM sodium pyrophosphate; 1 mM β-glycerolphosphate; 1 mM Na_3VO_4; 1 μg/ml leupeptin, 1 mM PMSF, New England Biolabs) per 60 mm plate and centrifuged at 14 000×*g*. BLMs were isolated using a Percoll gradient as have been previously described [13,16,26]. Briefly, cells were scraped in PBS, centrifuged and resuspended in homogenization buffer (300 mM mannitol in 12 mM HEPES, pH 7.6), homogenized and centrifuged twice to discard the nuclear and mitochondrial pellet. The membrane pellet was resuspended in 1 ml of HB by gentle pipetting. To form a Percoll gradient, 0.19 g of undiluted Percoll (Pharmacia Biotech, Piscataway, NJ, USA) was added to a 1 ml suspension. The suspension was gently mixed and centrifuged at 48 000×*g* for 30 min, and the ring of BLMs was collected.

2.4. Western blot analysis

Total cell lysates or BLM proteins were measured by Bradford assay (Bio-Rad, Hercules, CA, USA), resolved in 10% SDS–PAGE and transferred to nitrocellulose membranes (Optitran, Scleicher and Schuell, Keene, NH, USA). Incubation with specific antibodies was performed overnight at 4°C. Blots were developed with an ECL-plus detection kit (Amersham, Arlington Heights, IL, USA) used as recommended by the manufacturer and bands were analyzed by densitometric scan (Stratagene EagleSight, La Jolla, CA, USA).

2.5. ERK assay

AEC seeded in 6 cm plates, were serum-starved for 18–24 h and treated with the different drugs for the desired time. Cell lysates were obtained as described and the ERK activity was determined as described in the p44/p42 MAPK assay kit manual by New England Biolabs, which is based on the immunoprecipitation of the active ERK with a phosphospecific p44/p42 MAP kinase (Thr202/Tyr204) monoclonal antibody in order to do an in vitro kinase assay using Elk-1 as substrate. Samples (25 μl) were fractionated in a 12.5% SDS–PAGE and analyzed by immunoblotting using a phosphospecific Elk-1 antibody (New England Biolabs, Beverly, MA, USA) as a probe.

2.6. Determination of Na,K-ATPase activity in AEC cells

Na,K-ATPase activity was determined as described before [16]. Briefly, after cells were incubated with the desired conditions for 7 days, they were placed on ice and washed twice with cold PBS, and aliquots (~10 μg of protein) were transferred to the Na,K-ATPase assay medium, containing (in mM) 50 NaCl, 5 KCl, 10 $MgCl_2$, 1 EGTA, 50 Tris–HCl, and 7 Na_2ATP and [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) in tracer amounts (3.3 nCi/μl). Cells were transiently exposed to a thermic shock to render the membrane permeable to ATP. The samples were incubated at 37°C for 15 min and the reaction was terminated by the addition of 700 μl of a trichloroacetic acid:charcoal (5:10 w/v) suspension and rapid cooling to 4°C. After the charcoal phase containing the unhydrolyzed nucleotide was separated (12 000×*g* for 5 min), the liberated ^{32}P was counted in an aliquot. The Na,K-ATPase activity was calculated as the difference between the samples assayed in the same medium but devoid of Na^+ and K^+ and in the presence of 2.5 mM ouabain (ouabain-insensitive ATPase activity).

2.7. RNA isolation and analysis by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

AEC total RNA was isolated using Rneasy total RNA (Qiagen, Valencia, CA, USA) as described by the manufacturer. RNA was quantified by measurement of absorbance at 260 nm. The RT reaction was performed using the Superscript Preamplification System (Gibco-BRL, Grand Island, NY, USA) following the manufacturer's instructions. The resultant cDNA were amplified by PCR using specific primers (β1 subunit: oligonucleotides that correspond to the positions 418–437 and 786–805, respectively, of the cDNA from the rat gene (considering number 1 the 'A' of the first ATG of the gene); α1 subunit: oligonucleotides that correspond to the positions 37–54 and 1483–1500, respectively, of the cDNA from the rat gene) and analyzed by agarose gel electrophoresis. The amplified bands were quantified by densitometric scan (Stratagene EagleSight, La Jolla, CA, USA) normalizing against the internal control gene G3PDH (glyceraldehyde-3-phosphate dehydrogenase). The conditions of the PCR were 94°C, 1 min; 53°C 1 min 30 s; 72°C, 2 min; 25 cycles. For the amplification of the control gene G3PDH, we used the rat G3PDH Control Amplimer Set from Clontech (Palo Alto, CA, USA).

2.8. Statistical analysis

Data is reported as mean ± S.E.M. Statistical analysis was done by one-way ANOVA and Tukey correction. Results were considered significant when $P < 0.05$.

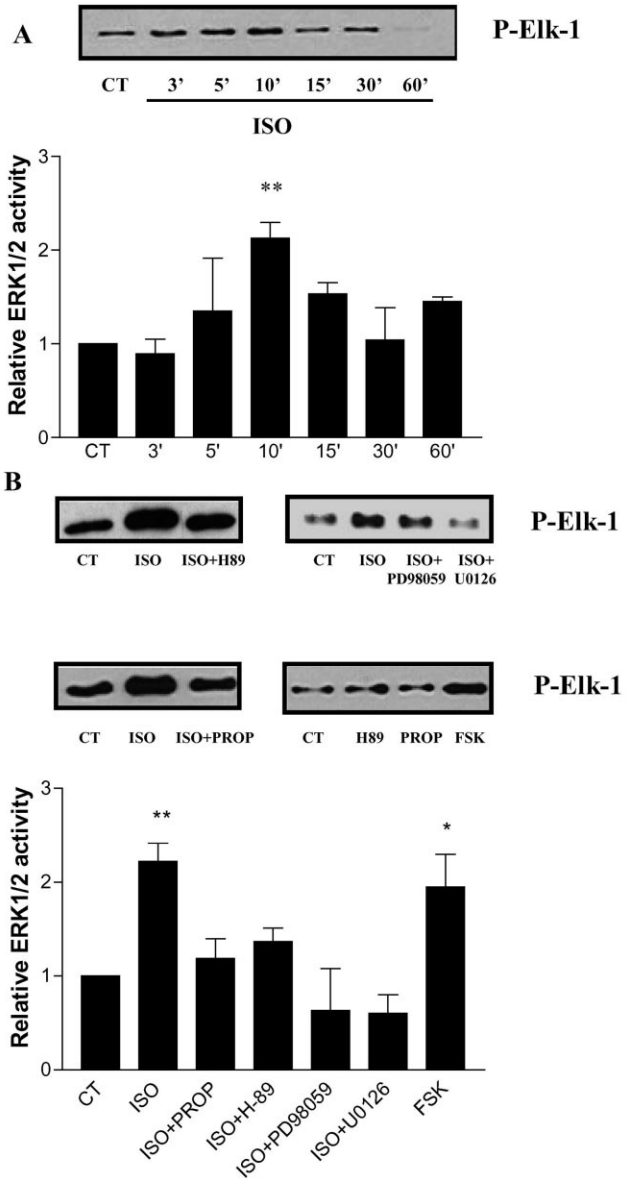

Fig. 1. ISO activated ERK1/2 in AEC via a β-adrenergic receptor, PKA-dependent pathway. A: Serum-starved AEC were incubated with ISO at the indicated times and the ERK activity was determined as phosphorylation of the transcription factor Elk-1. B: Serum-starved AEC were incubated with the adenyl cyclase activator forskolin (FSK), 50 μM, for 15 min or with 1 μM ISO for 10 min in the presence or absence or either the β-adrenergic receptor antagonist propanolol (PROP) (1 μM), the PKA inhibitor, H-89 (0.5 μM), or the MEK inhibitors PD98059 (PD) (50 μM) or U0126 (U0) (10 μM). Antagonists were added 15 min prior to ISO, except for the MEK inhibitors, which were added 1 or 2 h prior to ISO. Representative Western blots against phospho-Elk-1 and composite graph that depicts the data as mean ± S.E.M. ($n = 4$). Control values were assigned as 1. $^{**}P < 0.01$ versus CT, $^{*}P < 0.05$ versus CT.

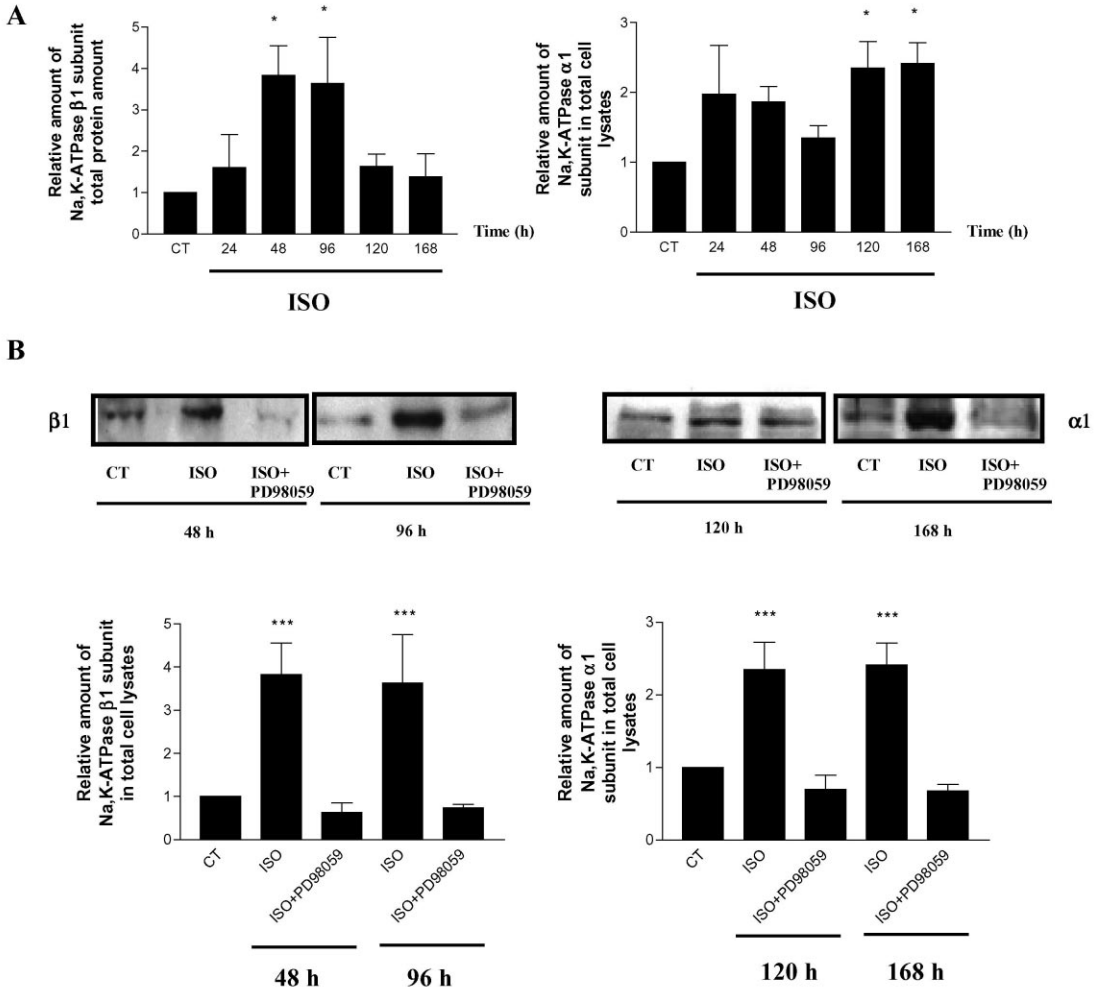

Fig. 2. ISO increased whole cell Na,K-ATPase α1 and β1 subunits protein abundance via a MAPK/ERK-dependent mechanism. Serum-starved AEC were incubated with 1 μM ISO in the presence or absence of 50 μM PD for the indicated times. Medium and treatment were changed every 2 days. Total protein was analyzed by Western blot against both subunits of the pump. A: Composite graphs showing the time course for increase in total protein obtained for the different subunits expressed as mean ± S.E.M., with control values assigned as 1. $*P < 0.05$. B: Composite graphs and representative blots showing that the increase in total Na,K-ATPase protein was ERK-dependent. $***P < 0.0001$ as compared to CT ($n = 3$ for 12 and 24 h, $n = 4$ for 48, 96, 120 and 168 h).

3. Results and discussion

β-Adrenergic agonists have been shown to regulate alveolar fluid reabsorption in mammalian lungs [13–15,27]. The short-term regulation of the Na,K-ATPase by ISO occurs by translocating intracellular Na,K-ATPase proteins to the AEC BLMs within 15 min of ISO stimulation [16]. Recently, it has been reported that terbutaline increases the Na,K-ATPase α1 subunit in AEC after 5 days of incubation [28] however, the mechanisms involved in the Na,K-ATPase long-term regulation have not been elucidated. We sought to determine the role of the MAPK/ERK pathway in the long-term regulation of the Na,K-ATPase by β-adrenergic agonists.

As shown in Fig. 1A, ISO stimulated ERKs activity in AEC by 10 min of incubation. This effect was blocked by the MEK1/2 inhibitors, PD98059 (50 μM) and U0126 (10 μM), the β-adrenergic antagonist propanolol (1 μM) and by the PKA inhibitor H-89 (1 μM) (Fig. 1B). Forskolin (50 μM), an adenyl cyclase activator, also activated ERK1/2 in AEC (Fig. 1B), confirming the involvement of PKA in ERK activation in AEC. These results suggest that ISO activates the MAPK/ERK pathway via a cAMP, PKA-dependent pathway in AEC, which is consistent with previous reports that show PKA-dependent activation of MAPK/ERKs by β-adrenergic agonists, either by the classical or by an alternative pathway [22,29].

As shown in Fig. 2A, ISO increased Na,K-ATPase β1 subunit protein abundance after 48 h of incubation and α1 subunit after 120 h of incubation in whole cell homogenates, these effects were completely blocked by PD98059 (Fig. 2B) and U0126 (Fig. 4), while cells incubated only with the MEK inhibitor U0126 (Fig. 4) showed a non-significant decrease in the Na,K-ATPase total protein amount. These results suggest that ISO increases the Na,K-ATPase subunits total protein abundance via a MAPK/ERK-dependent mechanism.

Because our cells needed to be incubated for 96 or 168 h in some of our experiments, we studied the effect of ISO and the MEK inhibitors on the MAPK pathway at this time points. For these experiments we used Western blots against the active (phosphorylated) MAPK and against the total MAPK, finding that neither ISO nor the MEK inhibitor U0126 had any effect on the total MAPK protein or the active MAPK

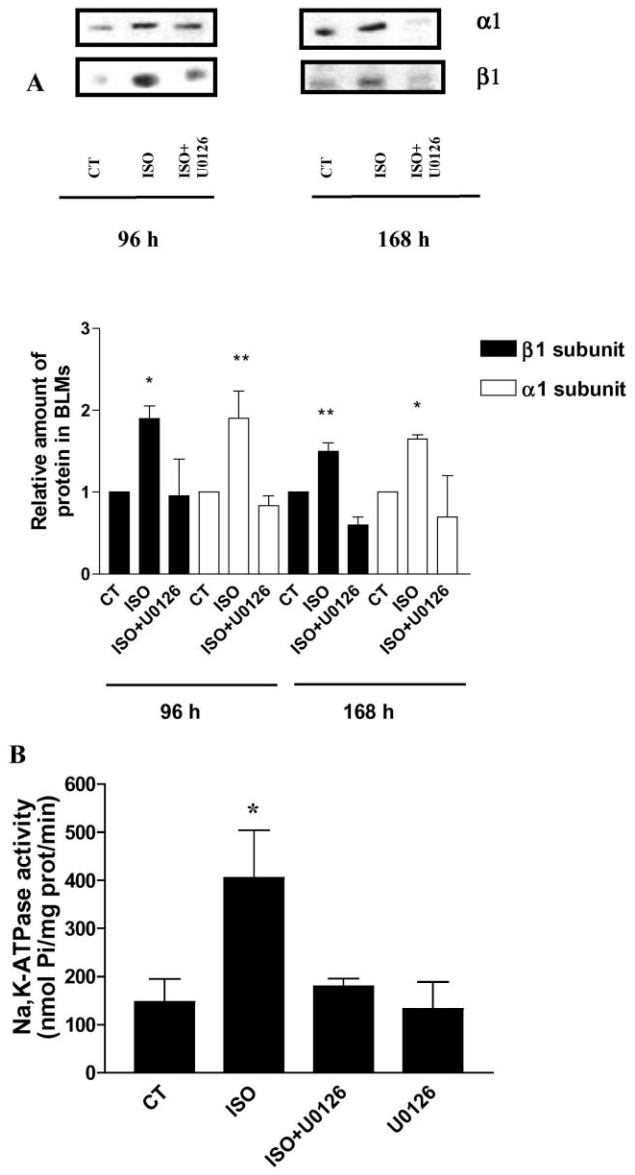

Fig. 3. ISO increased Na,K-ATPase protein amount at the BLMs and Na,K-ATPase activity via MAPK. A: Serum-starved AEC were incubated with ISO in the presence or absence of U0126 (U0) (10 μM) for either 96 or 168 h and BLMs were analyzed by Western blot against both subunits of the Na,K-ATPase. Results are expressed as mean ± S.D. $*P < 0.05$ as compared to CT ($n = 3$). Representative blots are also shown. B: Serum-starved AEC were incubated with ISO in the presence or absence of U0 for 7 days and the Na,K-ATPase activity was assessed by [^{32}P]ATP hydrolysis as described. Results are expressed as mean ± S.E.M. $*P < 0.05$ ($n = 3$).

(data not shown). These results suggest that the ISO-mediated effect on MAPK is transient.

Although the maximal increase in total protein was observed at different time points for α1 and β1 Na,K-ATPase subunits, both subunits were increased at the BLMs when cells were incubated with ISO for either 96 or 168 h (Fig. 3A) resulting in a ISO-mediated, MAPK/ERK-dependent increase in Na,K-ATPase activity (Fig. 3B), while the MEK inhibitor by itself, did not affect the basal activity of the Na,K-ATPase. These findings further support that ISO increases the total amount of functional Na,K-ATPases via a MAPK/ERK pathway. There are previous studies reporting that the Na^+ pump subunits can be regulated differentially in time [11,30] or that overexpressing one subunit can lead to an increase of both subunits at the cell BLMs [8,31]. We postulate that when the intracellular stores for one subunit are depleted, the cell, triggered by ISO, synthesizes new protein, replenishing these intracellular stores. Alternatively, the increased β1 subunit could stabilize the α1 subunit, increasing its half life, supporting a previously proposed role for the Na,K-ATPase β1 subunit [9,32].

The increase in total protein abundance can occur by transcriptional or post-transcriptional mechanisms. We did not

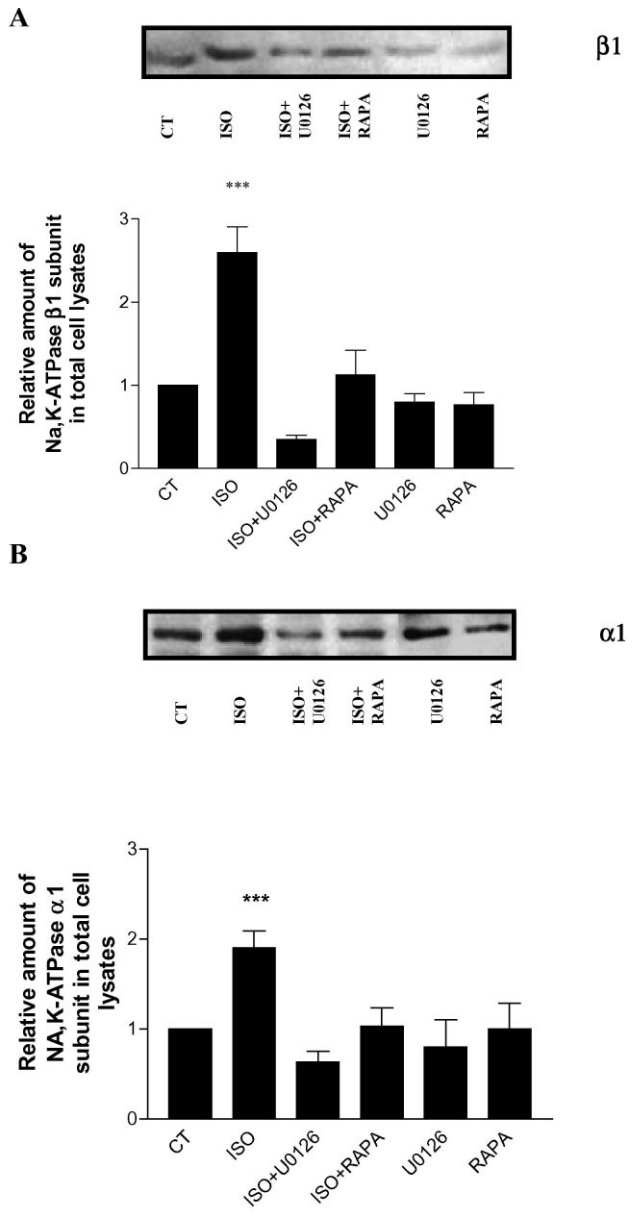

Fig. 4. Rapamycin blocked ISO-mediated regulation of the Na,K-ATPase. Serum-starved AEC were incubated with ISO in the presence of rapamycin (RAPA) (50 ng/ml) or the MEK inhibitor U0126 (U0) (10 μM) for either 96 h, for the analysis of the β1 subunit (A) or 168 h, for the analysis of the α1 subunit (B). Total protein was analyzed by Western blotting. Results are expressed as mean ± S.D. $***P < 0.0001$ as compared to CT ($n = 4$) and representative blots are shown.

find significant changes in mRNA steady state levels after incubation with ISO at the times studied (data not shown), suggesting that the increase in Na,K-ATPase protein abundance was post-transcriptionally regulated. This contrasts with the recent report of Minakata et al. [28] where terbutaline, after 4 days of incubation, increased the α1 subunit mRNA 1.5-fold as compared to controls. It is possible that we failed to detect the increase in the mRNA levels, however, more likely, the differences can be explained by the different experimental conditions used in the two studies. In our experiments, cells were serum-starved before being incubated with ISO because serum has different growth factors that have been shown to stimulate MAPK, and serum by itself has also been shown to upregulate Na,K-ATPase gene expression [33].

Although MAPK/ERKs are best known for activating transcription factors [34], this cascade has also been reported to regulate post-transcriptional mechanisms related to the translational machinery, specifically, via the regulation of the eukaryotic initiation factor 4E (eIF4E), the eIF4E-binding protein (eIF4E-BP) and the $p70^{s6K}$ [35–38]. The immunosuppressant rapamycin inhibits the mammalian target of rapamycin (mTOR) [39] and two important steps in translational regulation of protein synthesis are known to be downstream of TOR, the eIF4E-BP and the $p70^{s6K}$ [35,36,40,41]. Thus, to examine whether the post-transcriptional regulation could be mediated by rapamycin-sensitive mechanisms, serum-starved AEC were incubated with ISO for 96 h (for the β1 subunit analysis) or 168 h (for the α1 subunit analysis), in the presence or absence of rapamycin (50 ng/ml). As shown in Fig. 4, rapamycin inhibited the ISO-mediated increase in Na,K-ATPase total cell protein abundance without affecting the basal Na,K-ATPase protein amount. These results suggest that, the β-AR–PKA–MAPK pathway could be regulating either the $p70^{s6K}$, the eIF4E or both components of the translational machinery to increase the Na,K-ATPase protein abundance. ISO, rapamycin or U0126 did not change actin protein abundance in any of the groups (data not shown), suggesting that the ISO-mediated effect on the Na,K-ATPase was specific.

In summary, ISO post-transcriptionally regulates the Na,K-ATPase via MAPK/ERK and rapamycin-sensitive pathways in AEC. Further studies are warranted to elucidate the specific pathways and the post-transcriptional mechanisms that are involved in this regulation.

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