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Insulin up-regulates epithelial sodium channel in LPS-induced acute lung injury model in rats by SGK1 activation

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ABSTRACT

Activity of the epithelial sodium channels (ENaCs) in the lung tissue plays a critical role on sodium/fluid homeostasis and the lung fluid clearance. The serum- and glucocorticoid-inducible kinase-1 (SGK1), one of the critical regulation proteins of ENaC, is activated by insulin and growth factors possibly through 3-phosphoinositide-dependent kinase PDK1 or/and phosphatidylinositol 3-kinase (PI3K). However, it is uncertain whether insulin shows its stimulatory action on ENaC by activation of SGK1 in lipopolysaccharide (LPS)-induced acute lung injury (ALI) condition. In our study, Wistar rats were injected with LPS to induce ALI. Evans blue dye albumin (EBA) concentration was used to measure pulmonary oedema. For detecting the ratio of phospho-SGK1/SGK1 and α-ENaC protein, Western blot was performed. Real-time polymerase chain reaction (RT-PCR) was used to assess α-ENaC messenger RNA (mRNA). Immunohistochemistry was used to locate and quantitate α-ENaC expression. The EBA concentration was markedly increased by LPS alone but significantly reduced in rats that also received insulin injection. The ratio of phospho-SGK1/SGK1 was raised significantly in the insulin group and insulin + LPS group, compared with the control group and the LPS group, respectively. Furthermore, α-ENaC was up-regulated by insulin treatment. Simultaneously, injection with LPS significantly reduced α-ENaC expression. These findings demonstrated that insulin up-regulates ENaC in vivo possibly resulting from activation of SGK1.

Introduction

Acute lung injury (ALI), a serious lung condition, is defined as a clinical syndrome of lung injury with hypoxaemic respiratory failure induced by intense pulmonary inflammation that develops after a severe physiologic insult.[1,2] Recent studies suggested a lethality of around 40% for ALI or acute respiratory distress syndrome (ARDS).[3] According to the American-European Consensus Conference (AECC), ALI is classified into two different types, the direct type or pulmonary type that directly affects lung parenchyma, and the indirect type or extrapulmonary type that results from an acute system inflammatory response.[4]

Pulmonary oedema, with normal cardiac filling pressure, is one of the major pathological features of ALI.[1,2] The epithelial sodium channel (ENaC) plays a critical role in pulmonary oedema by participating in the clearance of fluid from the alveolar space.[5,6] ENaC was found on alveolar epithelial type I and II cells both *in vivo* and in vitro.[7,8] The native ENaC has been shown to be a multimeric protein made up of at least three homologous subunits (α, β and γ) that together build the functional channel.[9,10] Whilst the α subunit is critical to form functional ENaC, β and γ subunits significantly promote channel activity and substantively change the gating characteristics of the channel.[11]

The serum- and glucocorticoid-inducible kinase-1 (SGK1), one of the critical regulation proteins of ENaC, is widely expressed and under genomic control by stress condition and hormones (including insulin, endothelin-1 and gluco- and mineralocorticoids).[12,13] SGK1 is activated by insulin and growth factors through 3-phosphoinositide-dependent kinase (PDK1) and phosphatidylinositol 3-kinase (PI3K).[12–15] SGK1 activates ENaC through phosphorylation of several key substrates. First, phosphorylation of the channel proteins itself increases its opening probability. The second substrate is Nedd4-2. Activation of SGK1 causes inhibition of Nedd4-2 through recruitment of inhibitory 14-3-3 chaperone proteins and subsequent decrease of channel internalisation and degradation. The next is through AF9. Phosphorylated SGK1 inhibits the Dot1a–AF9 complex and diminishes H3 hypermethylation in the vicinity of the ENaCα promoter.[15]

In a previous study, we found that insulin up-regulated expression of α-ENaC in A549 cells (an epithelial cell line from human lung adenocarcinoma) via activation of SGK1.[16] Further, another report also demonstrated that insulin was a powerful regulator of ENaC in the A6 cell line, which was derived from *Xenopus laevis* kidney.[17] Meanwhile, other research found that insulin and insulin-like growth factor-1 (IGF-1) stimulate activity

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of SGK1 by phosphoinositide-dependent kinases (PDK-1 and -2) in kidney principal cell.[18,19] Furthermore, SGK1 regulates the amiloride-sensitive sodium channel (epithelial sodium channel or ENaC) that was also identified in kidney principal cells.[18,19] Accordingly, we hypothesised that lipopolysaccharide (LPS)-induced changes in rat lung capillary leak would be attenuated by insulin treatment as calculated by the EBA extravasation from the vascular space into surrounding lung tissue via activation of SGK1.

Materials and methods

Animal

Specific pathogen-free (SPF) Wistar rats (males) aged about 6 weeks (180–220 g) were obtained and maintained in a pathogen-free environment in the facilities of the animal centre in our university. The rats were maintained on a standard diet and water ad libitum with a temperature-controlled room and 12 h dark/light cycles. The study protocol was approved by our university Animal Care and Use Committee, and the care and handling of the animals were in accordance with the US guidelines (NIH publication #85-23, revised in 1985). Animals underwent an acclimatisation period of at least 7 days before use in these experiments.

LPS-induced ALI/ARDS model in rats

We randomly divided 80 rats into four groups, control group, insulin group, LPS group and insulin + LPS group, with 20 rats (10 for Evans blue analysis, 10 for other analysis methods) in each. ALI was induced by LPS according to previous reports.[20,21] In brief, after anaesthesia with an intra-peritoneal injection of 2% sodium pentobarbital in saline (40 mg kg^{-1}), insulin + LPS group and LPS group animals were injected with LPS (*E. coli* LPS serotype 0111: B4, Sigma) 5 mg kg^{-1} via the jugular vein. Control animals received saline injections instead of LPS. At the time of LPS exposure, the insulin group and insulin + LPS group injected with insulin maintaining blood glucose between 1.5 and 4.5 mmol l^{-1}. Moreover, the LPS group and control group received saline instead of insulin in the same way. The blood glucose levels were recorded at 5 time points (0, 0.5, 1, 3 and 6 h). Six hours later, rats were sacrificed. The lung vasculature was perfused through the right cardiac ventricle with 10 ml of cold 0.9% NaCl; later the lungs were removed.

EBA concentration

To evaluate pulmonary oedema, Evans blue dye albumin (EBA) concentration was used.[22–24] According to the established protocol, at the time of the first LPS injection, and before insulin treatment, Evans blue (Sigma–Aldrich, St. Louis, MO, USA) (20 mg kg^{-1}) was injected via a tail vein.[20] To eliminate residual blood and EBA from the pulmonary bed, the saline (37 °C) was used to perfuse the left lung through the pulmonary artery for 4 min at 0.04 ml g^{-1} body weight per minute by an infusion pump. At last, the left lung was excised, with the outer portion rinsed with saline and blotted dry. The lung was weighed, placed in an oven and heated at 90 °C for 16 h. The dried tissue was incubated in 2 ml of formamide at 37 °C for 24 h. The dye concentration of the eluent was evaluated by spectrophotometry at 620 nm. The concentration of EBA extracted from the lungs was calculated against a standard curve and was expressed as mg of EBA g^{-1} of lung.

Western blot

To detect α-ENaC, SGK1 and phospho-SGK1 expression, Western blot was performed. After completion of treatment, lung tissues were removed from sacrificed rats and then were quick-frozen with liquid nitrogen. The homogenates were centrifuged for 15 min at 15,000 revolutions min^{-1} (r m^{-1}) and 4 °C. After collection of the supernatants, protein concentration was measured with a bicinchoninic acid assay kit using BSA as standard (Pierce Biotechnology, Rockford, IL, USA). An equal amount of protein (125 μg) was resolved in Tris–glycine sodium dodecylsulphate (SDS) polyacrylamide gel (10%). Protein bands were blotted to nitrocellulose membranes. After 1.5 h of incubation in blocking solution (5% dry milk in Tris–buffered saline with Tween 20), the membranes were performed using the following primary antibodies (abs): anti-α-ENaC (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-SGK1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-phospho-SGK1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane was then incubated with secondary Ab (goat anti-rabbit Ab at 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. Peroxidase labelling was detected by the enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and analysed by a densitometry system. Detection of a housekeeping gene was also performed on the same membrane by using anti-β–actin Ab (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) to confirm that the same amount of each protein was loaded. The ratio of the α-ENaC/β-actin was calculated for the relative expression of α-ENaC protein. In addition, the ratio of phospho-SGK1/total SGK1 was calculated.

Real-time reverse-transcribed polymerase chain reaction (RT-PCR)

For detection of α-ENaC mRNA, RT-PCR was used. Total RNA was extracted from lungs with Trizol reagent (Invitrogen, CA, USA). The first strand complementary DNA (cDNA) was synthesised using a reverse transcription kit (KeyGEN, Biotech, Nanjing, China). A PCR (35 cycles) was performed in a 50-μl reaction volume (5 μl 10× Taq Buffer, 4 μl 2.5 mM deoxynucleotide triphosphate (dNTP) and 4 μl 25 mM $MgCl_2$). Using forward and backward primers (α-ENaC or β-actin) 2 μl each, cDNA template 2 μl, Taq polymerase (KeyGEN, Biotech, Nanjing, China) 0.5 μl, the reaction was performed with a DNA thermal cycler. The rat β-actin housekeeping gene was used as

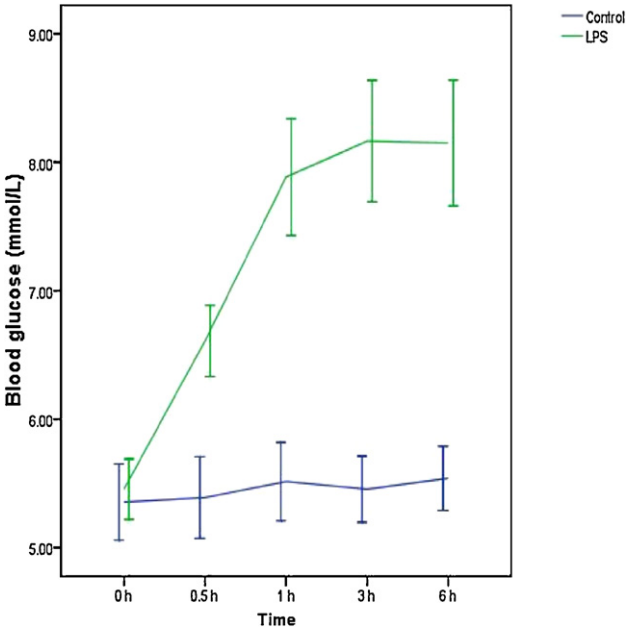

Fig. 1. LPS up-regulates the blood glucose level in vivo. LPS injection caused a noticeably increase of blood glucose after 0.5 h. Blood glucose reached maximum at 3 h after LPS injection. Data are presented as the mean ± SEM ($n = 20$). *$P < 0.01$ compared with control at the corresponding time points.