Evolutionary Conserved Role of c-Jun-N-terminal Kinase in CO_2-induced Epithelial Dysfunction

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Supporting Information

Materials and Methods

Reagents

Cell culture reagents were from Mediatech Inc (Herndon, VA). $^{22}Na^{+}$ was from GE Healthcare (Piscataway, NJ) and ^{3}H-mannitol was from Perkin Elmer (Life Sciences, Inc, Boston, MA). The myristoylated PKC-ζ peptide inhibitor was from Biomol International (Plymouth Meeting, PA). Lipofectamine 2000 and Opti-MEM I reduced serum medium were from Invitrogen, (Carlsbad, CA). A monoclonal antibody directed against the Na,K-ATPase α_1 subunit (clone 464.6) was from Upstate Biotechnology (Lake Placid, NY; catalog number: 05-369). The a5 antibody against the *Drosophila* Na,K-ATPase was from the Developmental Studies Hybridoma Bank, University of Iowa). The antibody against Toll was from Santa Cruz Biotechnology (Santa Cruz, CA; catalog number: sc 33-741). Anti-p-JNK, -JNK and -p-c-Jun were from Cell Signaling (Danvers, MA; catalog number: #9251, #9252 and #9261, respectively). The antibody against JNK that was used in A549 cells was from BD Biosciences (San Jose, CA; catalog number: BD 554285) Secondary goat anti-mouse-horseradish peroxidase (HRP) was from Bio-Rad (Hercules, CA) and goat anti-rabbit-HRP was from Cell Signaling (Danvers, MA). All other chemicals were purchased from Calbiochem (San Diego, CA).

Animals

Pathogen-free adult male Sprague-Dawley rats were from Harlan (Indianapolis, IN). Animals were provided with food and water *ad libitum*, were maintained on a 12:12-h light-dark cycle.

Isolation and culture of alveolar epithelial cells

Alveolar epithelial type II cells were isolated from the lungs of Sprague-Dawley rats weighing 200-225 g, as previously described [1]. The day of isolation and plating was designated culture *day 0*. All experiments were conducted on *day 3*. A549 cells (ATCC CCL 185) were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin. A549 cells expressing an empty vector or DN PKC-ζ were grown in the presence of G418 [2]. Experiments were performed on confluent monolayers of A549 cells. All cells were incubated in a humidified atmosphere of 5% CO_2-95% air at 37 °C.

CO_2 media

The composition of media for various CO_2 levels has been previously described [2,3]. Briefly, initial solutions were prepared with DMEM-Ham's F-12 medium-Tris base (3:1:0.5) containing 10% fetal bovine serum with 100 U/ml penicillin, and 100 μg/ml streptomycin. The buffering capacity of the media was modified by changing its initial pH with a Tris base in order to obtain a pH of 7.4 with the various CO_2 levels (pCO_2 of 40, 60, 80 and 120 mmHg). In some experiments, modeling extracellular acidosis, an initial pH of 6.8 was used, resulting in a final pH of 7.2 and a pCO_2 of 40 mmHg. Medium was placed overnight in a humidified chamber (C-174 Chamber, Biospherix, Ltd., Redfield, NY) to achieve the desired CO_2 levels and pH prior to starting the experiments.

Biotinylation of cell surface proteins

Cells were labeled for 20 min using 1 mg/ml EZ-link NHS-SS-biotin (Pierce Chemical Co., Rockford, IL) and surface proteins were pulled-down with streptavidin-Sepharose beads (Pierce Chemical Co., Rockford, IL) as described previously [4,5,6]. Proteins were analyzed by SDS-PAGE and Western blot analysis.

Western blot analysis

Protein concentration was quantified by Bradford assay (Bio-Rad, Hercules, CA) and proteins were resolved in 10%-12.5% polyacrylamide gels. Thereafter, proteins were transferred to nitrocellulose membranes (Optitran, Schleider & Schuell, Keene, NH) using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA). Incubation with specific antibodies was performed overnight at 4 °C. Blots were developed with a chemiluminescence detection kit (PerkinElmer Life Sciences, Boston, MA) used as recommended by the manufacturer. The bands were quantified by densitometric scanning (Image J 1.29X, National Institutes of Health).

RNA knockdown in A549 cells

A specific siRNA against JNK_1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A549 cells (American Type Culture Collection; Manassas, VA) were plated in 60-mm cell culture dishes and transfected with 100 pmol siRNA or scrambled (scr) RNA using lipofectamine RNAiMax (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Experiments were performed 24 h later.

***In vitro* JNK kinase assay**

The in vitro kinase assay was performed as described previously [3,7]. To assess whether JNK_1 directly phosphorylated the Na,K-ATPase purified Na,K-ATPase α_1-subunit (Sigma-

Aldrich, St. Louis, MO) or an ATII whole cell lysate were incubated with activated JNK_1 (Cell Signaling, Danvers, MA) in the presence of 10 μCi [γ-^{32}P]–ATP and100 μM ATP for 30 min at 30°C in 35 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM EGTA, 1 mM $CaCl_2$, and 10 mM β-glycerolphosphate. The reactions were stopped by adding SDS sample buffer and boiling the samples. The proteins were separated by SDS-PAGE, and radio-labeled areas were detected by autoradiography.

Isolated-perfused rat lung model

The isolated lung preparation has been described in detail previously [2,3,8]. Briefly, the lungs and heart of anesthetized rats were removed en bloc. The pulmonary artery and left atrium were catheterized and perfused continuously with a solution of 3% bovine serum albumin (BSA) in buffered physiological salt solution (135.5 mM Na^+, 119.1 mM Cl^-, 25 mM HCO_3^-, 4.1mM K^+, 2.8 mM Mg^+, 2.5 mM Ca^{2+}, 0.8 mM SO_4^{2-}, 8.3 mM glucose). Trace amounts of FITC-albumin was also added to the perfusate. The recirculating volume of the constant pressure perfusion system was 90 ml; arterial and venous pressures were set at 12 and 0 cm H_2O respectively. The vascular pressures were recorded every 10 sec with a multichannel recorder (Cyber Sense Inc. Nicholasville, KY). The lungs were immersed in a "pleural" bath (100 ml) filled with the same BSA solution. The entire system was maintained at 37 °C in a water bath. Perfusate pH was maintained at 7.40 by bubbling with a gas mixture of 95%O2/5%CO2. The lungs were then instilled via the tracheal cannula in two sequential phases with a total of 5 ml volume of the BSA solution containing 0.1mg/ml EBD-albumin, 0.02 μCi/ml of $^{22}Na^+$ and 0.12 μCi/ml of ^{3}Hmannitol. Samples were taken from the instillate, perfusate, and bath solutions after an equilibration time of 10 minutes from the instillation and again 60 min later. To ensure a homogenous sampling of the instillate, a volume of 2 ml was aspirated and reintroduced into the airspaces three times before removing each sample. All samples were centrifuged at 3000 g for 10 minutes. Absorbance analysis of the supernatant or

EBD albumin was performed at 620 nm in a Hitachi model U2000 spectrometer (Hitachi, San Jose, CA). Analysis of FITC-albumin (excitation 487 nm and emission 520 nm) was performed in a Perkin-Elmer fluorometer (model LS-3B, Perkin-Elmer, Oakbrook, IL). Scintillation counts for $^{22}Na^{+}$ and ^{3}H-mannitol were measured in a Beckman beta counter (model LS 6500,Beckman Instruments Inc., Fullerton, CA).

Maintenance of *Drosophila* S2 cells and CO_2 exposure

Drosophila S2 cells were grown at room temperature in Schneider's insect medium containing 10% FBS (Valley Biomedical) and 0.2% Penicillin-Streptomycin (GIBCO). For cell attachment, plates were treated with 1N HCl for 1h, washed 3 times with sterile water, 0.5mg/mL Concanavalin A (Calbiochem) for 1h, washed once with sterile water, and S2 cells allowed to attach for 1hr. CO_2 treatments were performed at 15% CO_2 using pre-equilibrated media buffered to pH equal to air condition, with S2 cells attached to 6-well plates. Positive control DJNK (Basket) activation was achieved using soluble *E. coli* peptidoglycan (InvivoGen) at 25mg/ml final concentration for 15 min.

Maintenance of *C. elegans* strains and CO_2 exposure

C. elegans strains were handled as described previously [9].N2, *jnk-1(gk7)* and *kgb-2(gk361)* null mutations were obtained from the *C. elegans* Genetic Center. DYNAMENT CO_2 controller with a mini infrared sensor (0–20% CO_2) was connected to a sealed Perspex incubator. CO_2 was flowed to the incubator via the controller until reaching the desired level and was balanced using air.

References

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