Inhibition of G protein-coupled proton sensor GPR68 ameliorates acid and LPS-induced lung endothelial dysfunction and acute lung injury

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INTRODUCTION

Tissue acidification resulting from dysregulated cellular bioenergetics accompanies various inflammatory states. GPR68, along with other members of proton-sensing G protein-coupled receptors, responds to extracellular acidification and has been implicated in chronic inflammation-related diseases such as ischemia, cancer, and colitis. The present study examined the role of extracellular acidification on human pulmonary endothelial cell (EC) permeability and inflammatory status *per se* and investigated potential synergistic effects of acidosis on endothelial dysfunction caused by bacterial lipopolysaccharide (LPS, *Klebsiella pneumoniae*). Results showed that medium acidification to pH 6.5 caused delayed increase in EC permeability illustrated by a decrease in transendothelial electrical resistance and loss of continuous VE-cadherin immunostaining at cell junctions. Likewise, acidic pH induced endothelial inflammation reflected by increased mRNA and protein expression of EC adhesion molecules VCAM-1 and ICAM-1, upregulated mRNA transcripts of tumor necrosis factor-α, IL-6, IL-8, IL-1β, and CXCL5, and increased secretion of ICAM-1, IL-6, and IL-8 in culture medium monitored by ELISA. Among the GPCRs tested, acidic pH selectively increased mRNA and protein expression of GPR68 and only GPR68-specific small molecule inhibitor OGM-8345 rescued acidosis-induced endothelial permeability and inflammation. Furthermore, acidic pH exacerbated LPS-induced endothelial permeability and inflammatory response in cultured lung macrovascular as well as microvascular endothelial cells. These effects were suppressed by OGM-8345 in both EC types. Moreover, OGM-8345 mitigated acute lung injury induced by LPS and mechanical ventilation in mouse. Altogether, these results suggest that GPR68 is a critical mediator of acidic pH-induced dysfunction of human pulmonary vascular endothelial cells and mediates the augmenting effect of tissue acidification on LPS-induced endothelial cell injury and acute lung injury induced by endotoxin.

METHODS

Cell Culture and Acidosis Stimulation Primary cultures of human pulmonary artery endothelial cells (HPAECs), human lung microvascular endothelial cells (HLMVEC), and EGM-2 growth media kit were obtained from Lonza (Allendale, NJ, USA). Cells were used at passages 5–8, and experimental stimulations were performed in EGM-2 containing 2% fetal bovine serum. For acidosis stimulation, the pH of 20 mM HEPES-buffered culture medium was adjusted to desired values with HCl or NaOH using an electronic pH meter. Medium was equilibrated in a cell culture incubator (37 ◦C, 5% CO2) for 2–3 h prior to use.

Endothelial Permeability Measurements Electric cell–substrate impedance sensing system (ECIS, Applied Biophysics, Troy, NY, USA) was employed to measure transendothelial electrical resistance (TER) across EC monolayers as described previously [19]. On the day of the TEER experiment, the culture medium in the EC monolayer grown on ECIS plates was replaced with EGM-2 containing 2% serum, and TEER equilibration was reached within 30–45 min. At this point, TEER recording restarted, and after 10–15 min, desired stimulations were performed. TEER measurements were continued for an additional 16–25 h. Similarly, EC permeability to macromolecules was determined by express permeability testing (XPerT) assay developed by our group [20]. Briefly, FITC-avidin solution was added directly to the culture medium for 3 min before termination of the experiment, and unbound FITC-avidin was washed out with PBS (pH 7.4, 37 ◦C). Cells were fixed with 3.7% formaldehyde in PBS (10 min, room temperature), and images of FITC-avidin bound to the biotinylated gelatin matrix were captured using a Nikon Eclipse TE 300 microscope (Nikon, Tokyo, Japan).

Immunocytochemistry Immunostaining with VE-cadherin antibody was performed to visualize EC junctions. In brief, cells were fixed in 3.7% formaldehyde solution for 10 min. at 4 ◦C, followed by permeabilization with 0.1% Triton X-100 for 30 min at room temperature. Blocking was performed with 2% BSA in PBS for 30 min followed by incubation with VE-cadherin antibody for 1 h at room temperature. Secondary antibody staining was performed with appropriate Alexa 488-conjugated antibody, and Texas Red-conjugated phalloidin was also added to visualize actin filaments. Slides were analyzed using a Nikon Eclipse TE300 inverted microscope connected to a SPOT RT monochrome digital camera and image processor (Diagnostic Instruments, Sterling Heights, MI, USA). Images were processed using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA). 2.5. GPR68 Activation Assay The PRESTO-Tango assay was run to determine GPR68 activation as described earlier [21]. Briefly, cells were seeded on 6-well dishes, and transfection was carried out with a combination of GPR68 Tango, β-arrestin, and luciferase plasmids using LTX Lipofectamine reagent (Thermo Fisher Scientific) for 24 h. At the end of desired periods of agonist stimulation, a luciferase assay was performed with Bright-Glo reagent from Promega by measuring luminescence on a VICTOR X5 multiplate reader (PerkinElmer, Waltham, MA, USA). 2.6.

Western Blotting Cells were washed with PBS and lysed directly in Laemmli sample buffer. Cell lysates were run on 8% SDS-PAGE and transferred onto polyvinylidene fluoride membranes using a semi-dry transfer system (17 V, 1 h, Bio-Rad, Hercules, CA, USA). Blocking of membranes was performed in 3% BSA for 1 h at room temperature and incubated with desired primary antibodies at 4 ◦C overnight. Horseradish peroxidase-conjugated secondary antibodies were added at room temperature for 1 h. Immunoreactive protein bands were detected using an enhanced chemiluminescent system (Thermo Fisher Scientific). Image J software (Version 1.54, NIH, Bethesda, MD, USA) was utilized to calculate image intensities.

Quantitative Real-Time PCR RNeasy Plus Kit (Qiagen, Germantown, MD, USA) was used to isolate total RNA, andone microgram of cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad,Hercules, CA, USA). Then, quantitative real-time PCR was performed on a Bio-Rad CFX96 real-time PCR system with SYBR green (Quantabio, Beverly, MA, USA). Ct values werenormalized to GAPDH, and fold changes in gene expression were calculated using the ΔΔCt method.

ELISA was performed to determine the secretory levels of sICAM, IL-6, and IL-8 in conditioned media with commercially available kits (R&D Systems, Minneapolis, MN). Briefly, supernatants obtained after brief centrifugation (800 g, 3 min.) were run for ELISA following manufacturer's instructions. Absorbance was read at 450 nm within 30 min. and the concentration of cytokines was calculated by generating a standard curve.

Statistical analysis Results are presented as means ± S.D. of three to six independent experiments. Control and stimulated groups were compared using unpaired Student's *t* test. For multiple groups comparison, one-way ANOVA followed by the post hoc Fisher's test was used with P<0.05 considered as statistically significant.

RESULTS

Endothelial dysfunction is induced by acidosis

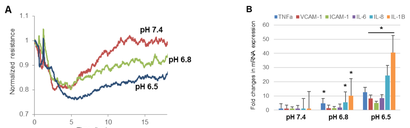

Figure 1. Acidosis induces endothelial dysfunction. (A) TER measurements in HPAEC monolayers incubated at normal (pH 7.4) or lowered (pH-6.8, pH-6.5) pH levels. Shown are normalized resistance values over time. (B) qRT-PCR analysis of mRNA expression of inflammatory marker genes: TNF-α, VCAM-1, ICAM-1, IL-6, IL-8, and IL-1β in HPAEC cultured in media with normal and acidic pH for 3 h. *p<0.05, vs. pH 7.4, n=3. (C) Western blot analysis of ICAM-1 and VCAM-1 protein expression by HPAEC incubated for 6 h in control (pH 7.4) or acidic (pH 6.5; pH 6.5) conditions for 6 h; probing for α-tubulin was used as loading control. Shown are representative data of 5 independent experiments.

Acidosis activates GPR68 in endothelial cells

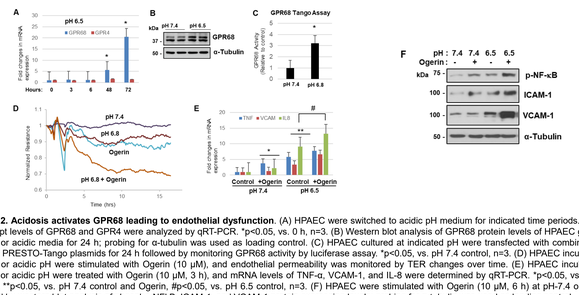

Figure 2. Acidosis activates GPR68 leading to endothelial dysfunction. (A) HPAEC were switched to acidic pH medium for indicated time periods. mRNA transcript levels of GPR68 and GPR4 were analyzed by qRT-PCR. *p<0.05, vs. 0 h, n=3. (B) Western blot analysis of GPR68 protein levels of HPAEC grown in normal or acidic media for 24 h; probing for α-tubulin was used as loading control. (C) HPAEC cultured at indicated pH were transfected with combination of GPR68 PRESTO-Tango plasmids for 24 h followed by monitoring GPR68 activity by luciferase assay. *p<0.05, vs. pH 7.4 control, n=3. (D) HPAEC incubated at normal or acidic pH were stimulated with Ogerin (10 μM), and endothelial permeability was monitored by TER changes over time. (E) HPAEC incubate at normal or acidic pH were treated with Ogerin (10 μM, 3 h), and mRNA levels of TNF-α, VCAM-1, and IL-8 were determined by qRT-PCR. *p<0.05, vs. pH 7.4 control, **p<0.05, vs. pH 7.4 control and Ogerin, #p<0.05, vs. pH 6.5 control, n=3. (F) HPAEC were stimulated with Ogerin (10 μM, 6 h) at pH-7.4 or pH-6.5 followed by western blot analysis of phospho-NFkB, ICAM-1, and VCAM-1 protein expression levels; probing for α-tubulin was used as loading control.

Specific inhibition of GPR68 rescues acidosis-induced endothelial barrier disruption

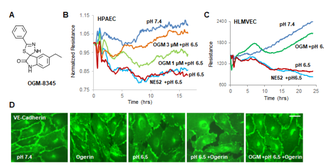

Figure 3. Inhibition of GPR68 but not GPR4 rescues acidosis-induced endothelial barrier disruption. (A) Chemical structure of GPR68 inhibitor OGM-8345. (B) HPAEC were switched to acidic pH media and treated with OGM-8345 (1μM and 3μM) or NE52-QQ57 (1 μM). TER was monitored over time and normalized resistance is presented. (C) Human lung microvascular endothelial cells (HLMVEC) were exposed to acidic pH medium and treated with OGM-8345 (3 μM) or NE52-QQ57 (1 μM). Endothelial permeability was determined by measuring TER over time. (D) HPAEC were exposed to acidic pH or Ogerin alone or to their combination, with or without OGM-8345 (3μM, 6h). Immunostaining for VE-cadherin was performed to visualize endothelial adherens junctions. Bar: 10 μm.

OGM-8345 but not NE52 attenuates acidosis-induced endothelial inflammatory responses

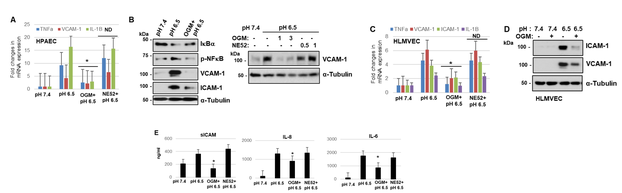

Figure 4. OGM-8345 but not NE52 attenuates acidosis-induced endothelial inflammatory responses. (A) HPAECs were switched to acidic pH medium alone or supplemented with indicated GPCRs inhibitors. After 3 h of incubation, mRNA expression analysis of TNF-α, VCAM-1, and IL-1β was performed by qRT-PCR. *p<0.05, vs. pH 6.5, ND- statistically not significant compared to pH 6.5, n=3. (B) HPAEC were incubated in acidic pH media with or without OGM-8345 (3μM, 6h); and protein expression levels of IkBa, phospho-NFkB, VCAM-1, and ICAM-1 were determined by western blotting; α-tubulin was used as a loading control (left panel). VCAM-1 protein levels were determined in cells incubated at acidic pH media with indicated multiple doses of inhibitors (right panel). (C, D) HLMVEC were incubated in acidic pH medium alone or with OGM-8345 or NE52-QQ57 where indicated. (C) After 3 h, mRNA levels of TNF-α, VCAM-1, ICAM-1, and IL-1β were determined by qRT-PCR *p<0.05, vs. pH 6.5, ND- statistically not significant compared to pH 6.5, n=3. (D) Western blot analysis of ICAM-1 and VCAM-1 protein expression; reprobing with α-tubulin served as loading control. (E) HPAEC exposed to acidic pH media with or without indicated inhibitors for 6 h were subjected to ELISA analysis to determine the secretory levels of sICAM, IL-8 and IL-6; *p<0.05, vs. pH 6.5, n=3.

LPS exacerbates acidosis-induced endothelial dysfunction

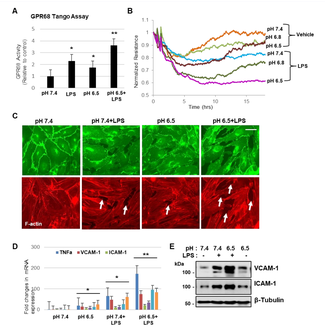

Figure 5. LPS exacerbates acidosis-induced endothelial dysfunction. (A) HPAEC incubated at normal or acidic pH were stimulated with LPS (100 ng/ml, 6h) followed by measurement of GPR68 activity by Tango assay. *p<0.05, vs. pH 7.4 control, **p<0.05, vs. pH 7.4+LPS, n=4. (B) Cells incubated in the indicated pH media were stimulated with vehicle or LPS, and TER was monitored over time. (C) HPAEC were stimulated with LPS (100 ng/ml, 6h) in normal (pH 7.4) or acidic (pH 6.5) medium. Immunofluorescence staining with VE-cadherin antibody was performed to visualize adherens junctions, staining for F-actin was used to monitor actin cytoskeleton remodeling and paracellular gap formation (shown by arrows). Bar: 10 μm. (D, E) HPAEC exposed to normal or acidic pH were treated with LPS (50 ng/ml) followed by qRT-PCR analysis of mRNA expression of indicated pro-inflammatory marker gens (D); and by western blotting analysis of VCAM-1 and ICAM-1protein levels with β-tubulin being used as loading control (E). *p<0.05, vs. pH 7.4 control, **p<0.05, vs. pH 7.4+LPS, n=3.

OGM-8345 attenuates LPS-induced endothelial barrier disruption at normal and acidic pH

Figure 6. OGM-8345 attenuates LPS-induced endothelial barrier disruption at normal and acidic pH. (A) HPAEC grown in normal or acidic pH media were pre-treated with OGM-8345 followed by addition of LPS for 6 h. XPerT assay was performed to determine endothelial macromolecular permeability. FITC fluorescence micrographs are presented; DAPI-counterstaining depicts cell nuclei. (B) HLMVEC were stimulated with LPS (100 ng/ml, 6h) in normal or acidic media in the presence or absence of OGM-8345. F-actin staining was performed to monitor cytoskeletal remodeling and paracellular gap formation (shown by arrows). Shown are representative results of 4 independent experiments; Bar: 10 μm.

OGM-8345 but not NE52-QQ57 attenuates acidosis- and LPS-induced endothelial inflammation

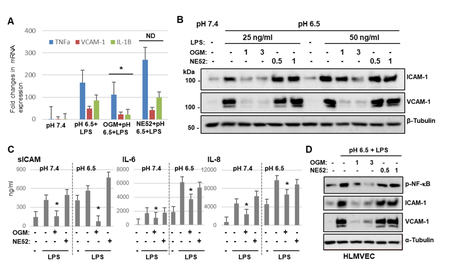

Figure 7. OGM-8345 but not NE52-QQ57 attenuates acidosis- and LPS-induced endothelial inflammation. (A) HPAEC pre-treated with OGM-8345 or NE52 (30 min) were switched to media with pH 6.5 followed by stimulation with LPS (50 ng/ml, 3 h). qRT-PCR analysis was carried out to determine mRNA transcript levels of TNF-α, VCAM-1, and IL-1β. *p<0.05, vs. pH 6.5+LPS, n=3, ND- statistically not significant. (B) HPAEC were pre-incubated with OGM-8345 (1 or 3 μM) or with NE52-QQ57 (0.5 or 1 μM) at acidic pH followed by addition of LPS (50 ng/ml, 6 h). Protein expression of ICAM-1 and VCAM-1 was determined by western blot; β-tubulin was used as loading control. (C) HPAEC pre-treated with OGM-8345 or NE52 for 30 min were incubated at normal or acidic pH followed by addition of LPS (50 ng/ml, 6 h). Secretory protein levels of sICAM, IL-6, and IL-8 in conditioned media were evaluated by ELISA assay. *p<0.05, vs. corresponding LPS groups, n=3. (D) HLMVEC were pre-incubated with indicated doses of inhibitors at pH 6.5 media for 30 min followed by LPS stimulation (100 ng/ml, 6 h). Cell lysates were subjected to western blot analysis to determine protein levels of phospho-NFkB, ICAM-1, and VCAM-1; reprobing for α-tubulin was used as loading control. Shown are representative results of 4 independent experiments.

Inhibition of GPR68 ameliorate acute lung injury induced by LPS and mechanical ventilation

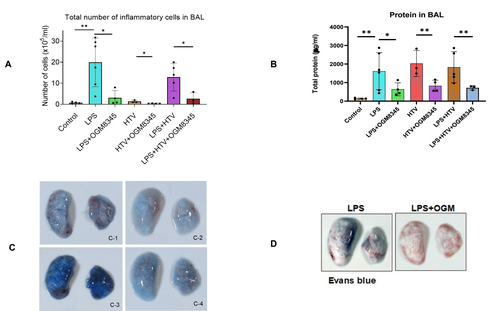

Figure 8. OGMs inhibit ALI induced by LPS and mechanical ventilation. Mice with or without LPS treatment were exposed to high tidal ventilation for 4 hrs ± OGMs. OGM was administered into mice 15 min before ventilation started. Count of cells (A), protein concentration (B) in BAL, and Evans blue retention in lungs were determined (C). C1. Mice were subject to HTV treatment; C2. Mice were subject to HTV treatment after OGM administration; C3. Mice were subject to LPS and HTV treatment; C4. Mice were subject to LPS and HTV treatment with OGM administration. D. Evans blue staining of lungs treated with LPS ± OGM.

SUMMARY

1. A shift towards an acidic pH is a strong trigger for lung endothelial dysfunction that may have important clinical relevance since acid accumulation is a feature of various disease states such as ischemia.

2. Acidosis also seems to intensify existing inflammatory responses and GPR68 turns out to be an essential mediator of acidosis-driven lung endothelial injuries.

3. Acute lung injury induced by LPS and mechanical ventilation that trigger acidosis are ameliorated by inhibition of GPR68.

4. Most importantly, GPR68 targeting small molecule inhibitor OGM-8345 exerts robust protection against acidosis-induced endothelial dysfunction suggesting that it could be considered as a potential therapeutic drug candidate to restore endothelial function and treat associated lung injuries.

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