GPR68 Inhibition with a Novel Group of Ogremorphin Inhibitors Upregulate Endothelial Barrier Function and Protect Against Bacterial Pathogens or Acidosis-induced Inflammation in Lung Endothelium

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INTRODUCTION

- Endothelial dysfunction characterized by an increase in endothelial permeability and hyperinflammatory responses is a pathological hallmark of several lung disorders including acute lung injury, acute respiratory distress syndrome, sepsis, and more importantly the current global pandemic COVID-19.
- Precise control of endothelial barrier and inflammatory status is especially important to avoid devastating complications of infections, traumatic tissue injury, and other pathologic conditions, and prevent development of pulmonary or brain edema.
- In this regard, drugs targeting the prevention and restoration of endothelial function is of great clinical interest to treat endothelial dysfunction-derived cardiopulmonary diseases.
- Recent findings have suggested a role of G protein-coupled receptors (GPCRs), especially a sub-family of proton-sensing GPCRs including GPR4 and GPR68, in modulation of endothelial function.
- GPR68 was originally described as a proton-sensing/acid-sensing GPCR that is inactive at pH 7.4 and fully activated at pH 6.4. Interestingly, GPR68 was recently shown to be a mechanosensor of shear stress in human and mouse vascular endothelial cells.
- In this study, we examined barrier protective and anti-inflammatory effects of two recently developed novel class of GPR68 inhibitors: ogremorphins OGM-8345 and OGM-1 using cultured human lung endothelial cells (HPAECs) and mouse models of LPS or bacterial pathogen-induced acute lung injury.

MATERIALS AND METHODS

Cell culture: Human pulmonary artery endothelial cells (HPAECs) were cultured using the complete growth media obtained from Lonza. Cells were used at passages 5-7 and all the agonists stimulation were carried out at the media containing 2% FBS.

Analysis of endothelial cell monolayer permeability.. Endothelial barrier function was measured in HPAECs by monitoring transendothelial electrical resistance (TER) using an electric cell-substrate impedance sensing system, ECIS Z (Applied Biophysics). Endothelial permeability to macromolecules was determined using express permeability testing (XPerT) assay developed and routinely used by our group.

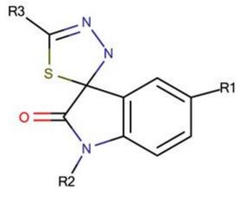
mRNA/protein expression. The mRNA and protein expression analysis of selected endothelial inflammation markers was carried out by quantitative real time PCR and western blot, respectively.

Acidosis. Cells were switched to low pH (6.5 or 6.8) media to examine the role of GPR68 in acidosis-induced endothelial permeability and inflammation.

GPR68 activation assay: GPR68 activation was analyzed by luciferase-based tango assay after cells were transfected with combination of Tango plasmids.

Animal studies: Vascular leak and inflammation induced by lipopolysaccharide (LPS from Escherichia coli) or heat-killed Staphylococcus aureus (HKSA) in C57BL/6 mice was evaluated by extravasation of intravenously injected Evans blue tracer into lungs and total cells/protein count in bronchoalveolar lavage samples.

Chemical structure and GPR68 inhibitory potencies of OGMs used in the study:



ID	R1	R2	R3	Phenotype	GPR68[μM]	LPA1[μM]
8345	Ethyl	H	Benz	Y	0.71	----
OGM	Ethyl	H	Napth	Y	0.16	8.7

References:

1. Xu, J., et al., *GPR68 Senses Flow and Is Essential for Vascular Physiology.* Cell, 2018. **173**(3): p. 762-775 e16.
2. Williams, C.H., et al., *Coupling metastasis to pH sensing G Protein-coupled receptor-68 through first in class inhibitor identified in an in vivo chemical genetic screen.* bioRxiv, 2019: p. 612549.

RESULTS

OGMs enhance endothelial barrier function

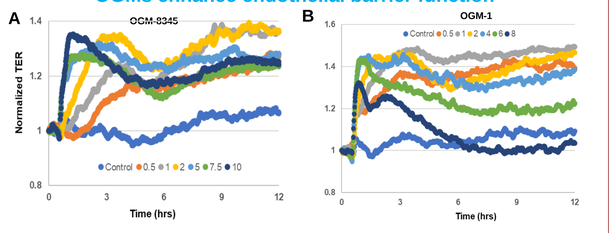


Figure 1. (A, B) OGMs enhance basal endothelial barrier function. HPAECs were exposed to increasing concentrations (0.5-10 μM) of OGM-8345 **(A)** or OGM-1 **(B)** and TER was monitored over time.

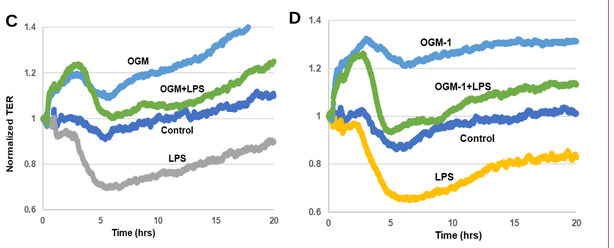


Figure 1. (C, D) OGMs protect against LPS-induced endothelial barrier disruption. Cells were pre-treated with 3 μM of OGM-8345 **(C)** or 1 μM of OGM-1 **(D)** for 30 min. followed by LPS (100 ng/ml) stimulation. Endothelial permeability was determined by monitoring TER over indicated time.

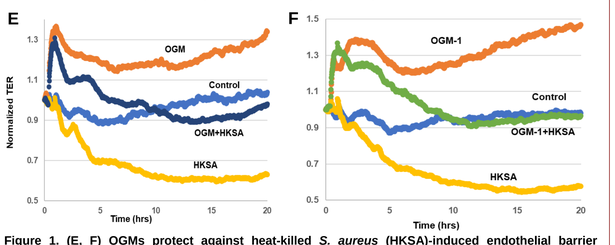


Figure 1. (E, F) OGMs protect against heat-killed *S. aureus* (HKSA)-induced endothelial barrier disruption. Cells were pre-treated with 3 μM of OGM-8345 **(E)** or 1 μM of OGM-1 **(F)** for 30 min. followed by HKSA stimulation. Endothelial permeability was measured by monitoring TER over indicated time.

GPR68 is ubiquitously expressed

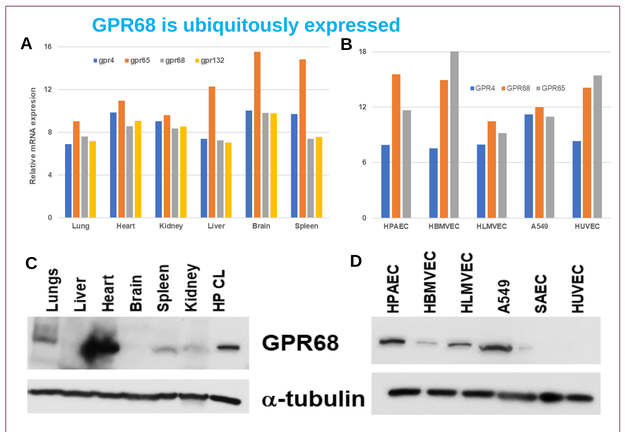


Figure 2. GPCRs mRNA and protein expression in various tissues and cell types . mRNA and protein expression levels of different GPCRs in indicated tissues **(A, C)** and cell types **(B, D)** were determined by qPCR and Western blot, respectively. HBMVEC – human brain microvascular endothelial cells, HLMVEC – human lung microvascular endothelial cells, SAEC – small airway epithelial cells, HUVEC – human umbilical vein endothelial cells, HP CL – HPAEC cell lysate.

OGMs inhibit LPS-induced inflammation

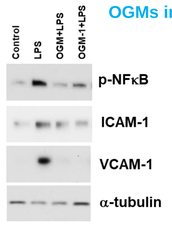


Figure 3. OGMs inhibit LPS-induced inflammation. HPAECs were pre-treated with 3 μM of OGM or 1 μM OGM-1 followed by LPS (50 ng/ml) stimulation for 6 h. Cells lysates were run on 8% SDS-PAGE and subjected for western blot analysis to detect protein levels of phospho-NF-κB, intercellular adhesion molecule-1(ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1). A-tubulin was used as a loading control.

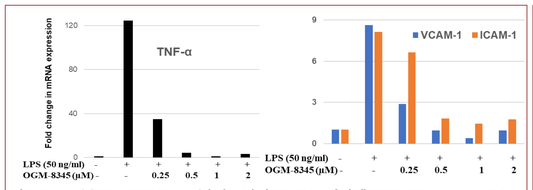


Figure 4 OGMs repress LPS-induced increase of inflammatory genes mRNA expression. Cells were pre-treated with indicated concentrations of OGM followed by LPS stimulation. RT-PCR was performed to measure the mRNA transcripts of indicated endothelial inflammation marker genes.

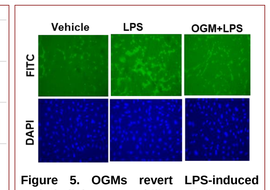


Figure 5. OGMs revert LPS-induced endothelial permeability. Cells were treated with LPS in the presence or absence of OGM-8345 and endothelial permeability was determined by XPerT assay.

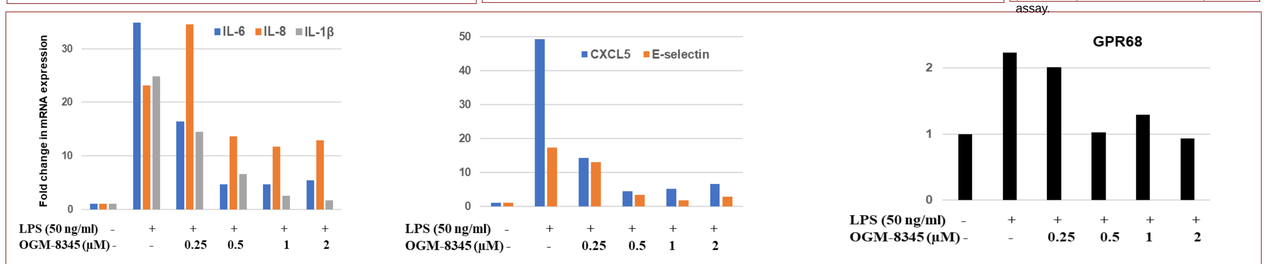


Figure 6. OGMs repress LPS-induced upregulation of inflammatory cytokines and GPR68. Cells were pre-treated with indicated concentrations of OGM-8345 for 30 min. followed by LPS stimulation. Changes in mRNA transcripts of inflammatory cytokines IL-6, IL-8, and IL-1β and CXCL5, E-selectin and GPR68 were determined by RT-PCR.

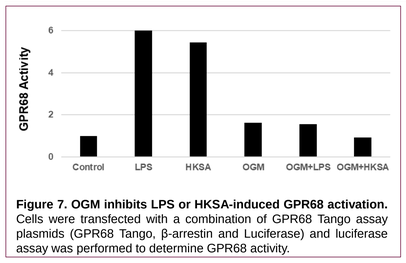


Figure 7. OGM inhibits LPS or HKSA-induced GPR68 activation. Cells were transfected with a combination of GPR68 Tango assay plasmids (GPR68 Tango, β-arrestin and Luciferase) and luciferase assay was performed to determine GPR68 activity.

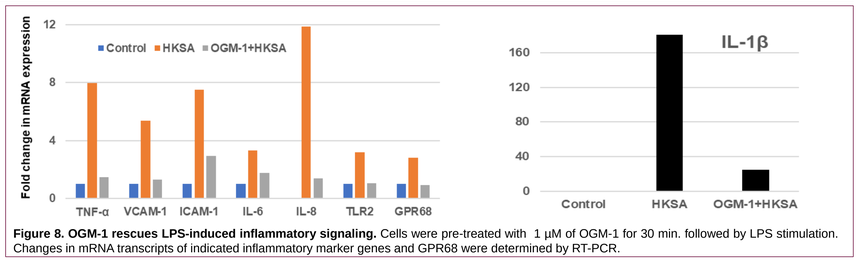


Figure 8. OGM-1 rescues LPS-induced inflammatory signaling. Cells were pre-treated with 1 μM of OGM-1 for 30 min. followed by LPS stimulation. Changes in mRNA transcripts of indicated inflammatory marker genes and GPR68 were determined by RT-PCR.

OGMs inhibit acidosis-intensified HKSA-induced inflammation

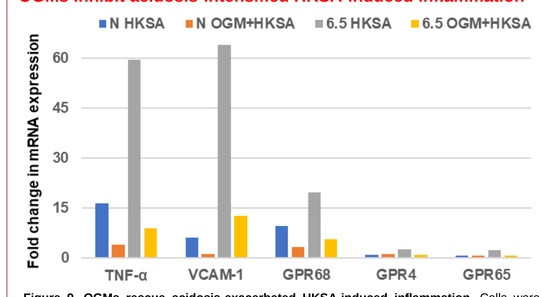


Figure 9. OGMs rescue acidosis-exacerbated HKSA-induced inflammation. Cells were either stimulated in normal or acidic pH 6.5 with HKSA in the presence or absence of OGM and RT-PCR analysis was carried out to determine mRNA levels of indicated genes.

SUMMARY AND CONCLUSION

- This study demonstrated potent endothelial barrier protective and anti-inflammatory effects of novel GPR68 inhibitors OGMs both in *vitro* and *in vivo*.
- Acidosis exacerbated inflammatory agonists-induced endothelial dysfunction that was rescued by OGMs.
- These results suggest a strong potential of OGMs to be developed as therapeutics against severe lung injuries.

OGMs ameliorate LPS or HKSA-induced ALI

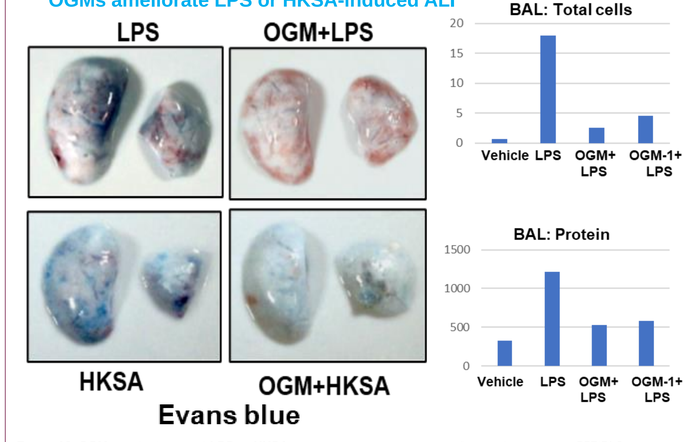


Figure 10. OGMs protect against LPS or HKSA-induced vascular leak and inflammation in vivo. C57/BL6 mice were exposed to OGMs (40 mg/kg, i.p.) immediately followed by LPS (1 mg/ml, i.t.) or HKSA ($2x10^8$ cells, i.t.). After 18 hrs, Evans blue extravasation assay was done to measure vascular leak. Bronchoalveolar lavage (BAL) was also collected to measure total cells and protein content.

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