Anti-oxidant Gene Expression in Airway Smooth Muscle and Epithelial Cells is Upregulated by Synthetic Secoisolaricesinol Diglucoside (LGM2605)

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Abstract:

Rationale: Exposure to the air pollutant ozone (O3) worsens pulmonary function and can lead to glucocorticoid insensitivity in asthmatics raising the importance of alternative or adjuvant treatment approaches. We previously showed that treatment with LGM2605, a synthetic anti-oxidant (originally derived from flaxseed) prevented O3-induced airway hyperreactivity and decreased airway inflammation in asthmatic rhesus macaques. LGM2605 increased expression of several anti-oxidant genes in the lung tissue of macaques, however, the main cellular target of this compound needed to be clarified.

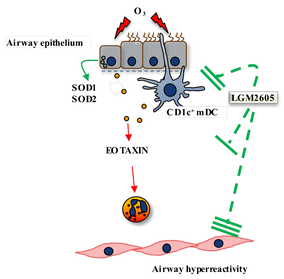
Methods: Here we evaluated the effect of LGM2605 on immortalized human airway epithelial cells (HBE1, obtained from the UC Davis Airway Epithelial Biobank), primary smooth muscles (hASM, a generous gift of Dr. Kenyon), and immortalized human alveolar cells (A549 cells). To mimic the effects of O3 exposure, cells were cultured and incubated with 0.05 mM tert-butyl hydroperoxide (TBHP) for 2 hours and were treated with 0, 12.5, 25, and 50 μM LGM2605 for 24 hours after which cells were harvested for RNA extraction. Expression of *Txnl1*, *Gsta4*, *Sod1*, *Eotaxin2* and *Sod2* was measured by qPCR.

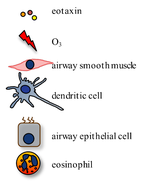
Results: TBHP significantly (p<0.05) increased the expression of *Txnl1*, *Sod1*, and *Sod2* in hASM but only *Sod2* in HBE1 cells. LGM2605 dose-dependently amplified the TBHP-induced expression of Txnl1, Sod1, and Sod2 in hASM, but not HBE1 cells indicating that hASM were a specific target of antioxidant gene modulation by LGM2605.

Conclusions: Our data suggest that airway smooth muscle is highly sensitive to oxidative stress as well as to the anti-oxidant effects of LGM2605 raising the potential of a novel treatment approach in asthma.

Introduction:

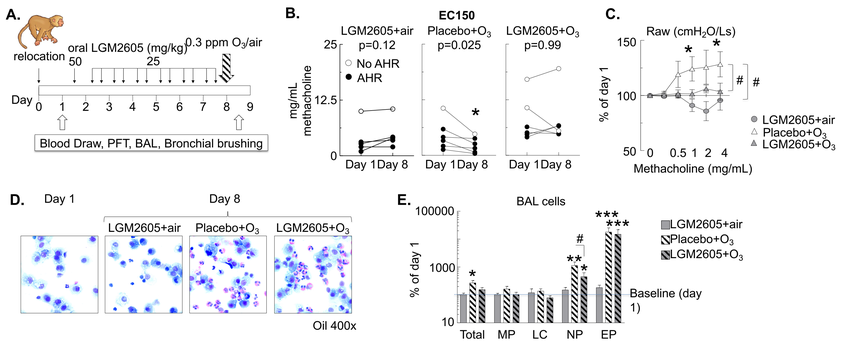
- Exposure to the air pollutant ozone (O_3) worsens pulmonary function and can lead to glucocorticoid insensitivity in asthmatics raising the importance of alternative or adjuvant treatment approaches.
- We previously showed that treatment with LGM2605, a synthetic anti-oxidant (originally derived from flaxseed) prevented O_3-induced airway hyperreactivity and decreased airway inflammation in asthmatic rhesus macaques.
- LGM2605 increased expression of several anti-oxidant genes in the lung tissue of macaques, however, the main cellular target of this compound needed to be clarified.





O_3 induces
- Oxidative stress
- Proinflammatory gene activation
- Airway smooth muscle activation
- Impairs the effects of glucocorticoids

Does anti-oxidant treatment directly affect epithelial cell and/or smooth muscle function?

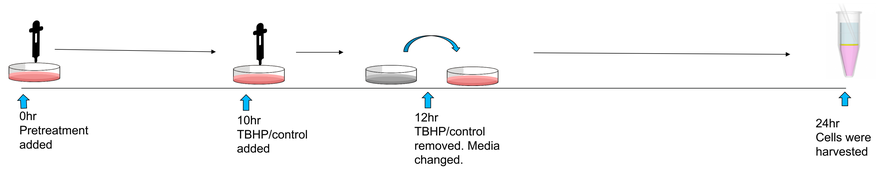


(A) Rhesus macaque study time-line: Baseline measurements were performed on recruited macaques following relocation indoors (day 1). Animals were given LGM2605 orally (as a small treat, in a peanut butter sandwich) daily for 7 days, then exposed to 0.3 ppm O_3 or air for 6.6 hours. 12 hours post exposure blood draw and bronchoscopy were repeated (day 8). BAL: bronchoalveolar lavage, PFT: pulmonary function test. Experimental groups: LGM2605+air, Placebo+O_3, and LGM2605+O_3. **(B)** Effective concentration of methacholine that raised airway resistance (Raw) by 150% (EC150) on days 1 and 8 (mg/mL). Mean±SEM of n=5-6, *p<0.05 (day 1 *vs.* day 8, Student's paired *t*-test) **(C)** Methacholine dose response (Raw; % of day 1; each macaque served as its own control). **(D)** Representative photomicrographs of Kwik-Diff stained cytospins from day 1 and day 8 BAL indicating neutrophil and eosinophil influx in O_3-exposed animals. **(E)** Macrophages (MP), lymphocytes (LC), neutrophils (NP), and eosinophils (EP) were differentially counted on cytospins (the absolute cell counts are expressed as % of day 1; each macaque served as its own control). (**C, E):** Mean±SEM of n=5-6; *p<0.05, **p<0.01, ***p<0.001 (day 1 *vs.* day 8, or vs. 0 mg/mL methacholine (C); Two-way ANOVA with Bonferroni's multiple comparisons) #p<0.05; between groups; (Two-way ANOVA).

Hypothesis:

LGM2605 promotes antioxidant gene activity in epithelial and smooth muscle cells (A549 and HBE1, hASM) and decreased inflammatory gene activity when exposed to oxidative stress using TBHP.

Methods:

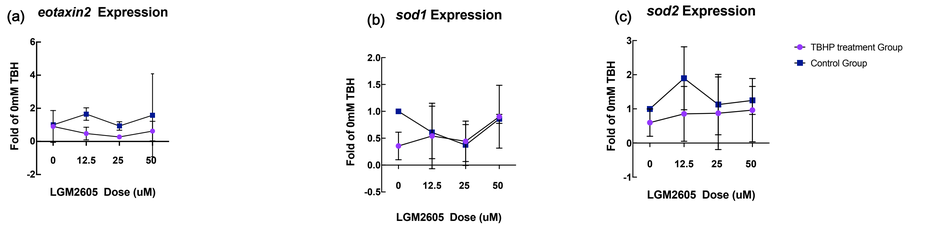


In order to create an adequate model to simulate the lung, we evaluated gene expression via qpcr in 3 different immortalized cells lines—A549, HBE1, hASM . We hypothesized that 1) Tert-butyl hydroperoxide (TBHP)—a good mimic for O3 would induce oxidative stress in these cells leading to increase antioxidant and proinflammatory genes and 2) LGM2605 would attenuate inflammation by augmenting the antioxidant gene expression in these cells. Although dexamethasone has been previously studied, we also evaluated the role of dexamethasone in decreasing inflammatory gene expression.

In the timeline above, A549, HBE1, and hASM cells were cultured and grown to 80% co-fluency prior to experiment. At hour 0 cells were treated with LGM2605 at varying concentrations of 0,12.5, 25, 50 uM. Or pretreated with dexamethasone at varying conditions of 0,10, 100nM. At hour 10, cells were exposed to 0.05mM TBHP vs control (water of the same volume) for 2 hours. At hour 12, the media was changed and cells were again treated with LGM2605 and dexamethasone at the same concentrations as avoe. Cells were harvested after 24 hours of LGM2605 treatment and underwent mRNA extraction and RT qPCR.

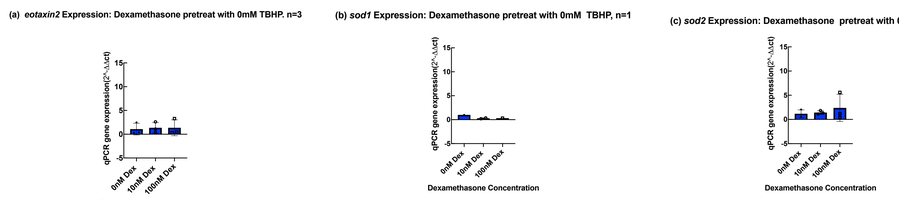
Results:

1) LGM2605 did not alter *eotaxin2, sod1 or sod2* in A549 cells



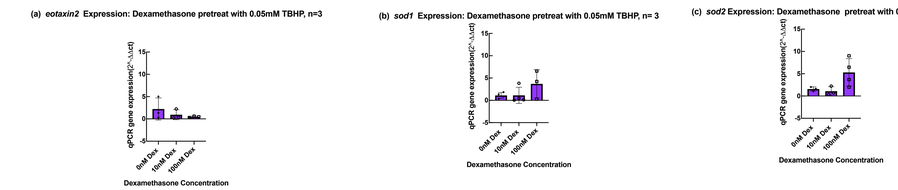
A549 cells treated with LGM2605 in increasing doses of 0, 12.5, 25, 50 μM. Treatment was followed by exposure of treatment group to 0.05mM TBHP vs control group treated with H2O. RNA was extracted and cells underwent RT qpcr, gene expression was calculated ΔΔCT and graphed as a fold of 0 mM TBHP. Mean ± SEM of n=6-11 a) *eotaxin2* expression is slightly higher in control group however is not significantly lower in TBHP treated group. b) *sod1* expression is unchanged between TBHP treatment and control group. c) *sod2* expression peaked in the control group at 12.5 μM LGM dose however there were no significant differences in *sod1* expression between treatment and control groups.

2) In A549 cells without exposure to TBHP, dexamethasone did not alter expression for *eotaxin2*, and *sod1* but but did augment s*od2* expression in a dose dependent manner.



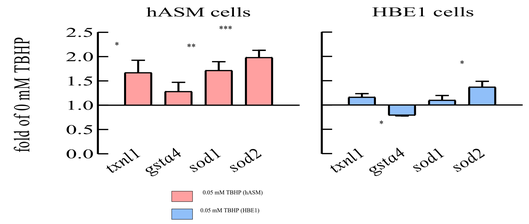
A549 cells were pretreated with dexamethasone (dex) at varying concentrations of 0,10, 100nM without any TBHP treatment. RNA was extracted and gene expression was quantified via RT qPCR. Gene expression here is shown as $2^{-\Delta\Delta CT}$ Mean ±SEM of n=1-3 a) *eotaxin2* expression in dex treated cells was not different across varying doses of dex. b) *sod1* expression was slightly decreased with increasing doses of dex. c) *sod2* expression increased in a dose dependent manner when treated with dex.

3) In A549 cells with exposure to TBHP, dexamethasone did decrease *eotaxin2* expression and augment *sod1* and *sod2* expression in a dose dependent manner .



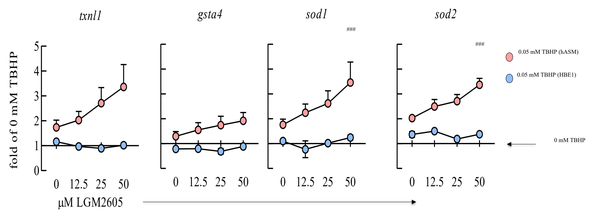
A549 cells were pretreated with dex at varying concentrations of 0,10, 100nM with TBHP treatment. RNA was extracted and gene expression was quantified via RT qPCR. Gene expression here is shown as $2^{-\Delta\Delta CT}$ Mean ±SEM, n=3 a) *eotaxin2* was increased at baseline in TBHP treated cells and decreased with increasing doses of dex concentration. b) *sod1* expression increased with increased dex concentration. c) *sod2* did not increase until dose of dex reached 100nM.

4) TBHP significantly (p<0.05) increased the expression of *Txnl1*, *Sod1*, and *Sod2* in hASM but only *Sod2* in HBE1 cells



hASM cells and HBE1 cells cultured for 24 hours with LGM2605 treatment, exposed to TBHP and were harvested at 24 hours. This is the effect of TBHP *on txnl1, gsta4, sod1,* and *sod2* antioxidant gene expression in hASM and HBE1 cells (qPCR). ΔΔCt expressed as fold of 0 mM TBHP. Mean±SEM of n=3-6 (hASM); n=2-3 (HBE1); *p<0.05, **p<0.01, ***p<0.001 (mM TBHP vs. 0.05 mM TBHP; Statistical analysis used was student's unpaired t-test;

5) LGM2605 dose-dependently amplified the TBHP-induced expression of *Txnl1*, *Sod1,* and *Sod2* in hASM, but not HBE1 cells



hASM cells and HBE1 cells cultured for 24 hours with LGM2605 treatment, exposed to TBHP and were harvested at 24 hours. Dose dependent effects of LGM2605 on TBHP-induced *txnl1, gsta4, sod1*, and *sod2* antioxidant gene expression. Mean±SEM of n=3-6 (hASM); n=2-3 (HBE1); #p<0.05, ##p<0.01, ###p<0.001 (vs. 0 mM TBHP; Statistical analysis used was Two-way ANOVA).

Future Direction:

- Increase sample size for dexamethasone treated cells in epithelial cells to further elucidate mechanism of action of dexamethasone in epithelial cells
- Compare dexamethasone and LGM2605 in hASM cells.
- Evaluate steroid sparing effect if any of LGM2605 in hASM

Summary:

1) LGM2605 did not alter *eotaxin2*, *sod1*, or *sod2* in A549 cells.
2) In A549 cells without exposure to TBHP, dexamethasone did not change expression for *eotaxin2* and *sod1* but augmented *sod2* expression in a dose dependent manner.
3) In A549 cells with exposure to TBHP, dexamethasone dose dependently decreased *eotaxin2* expression and dose dependently augmented *sod1* and *sod2* expression
4) TBHP significantly (p<0.05) increased the expression of *txnl1*, *sod1*, *sod2* in hASM but only *sod2* in HBE1 cells
5) LGM2605 dose dependently amplified the TBHP induced expression of *txnl1*, *sod1*, and *sod2* in hASM but not in HBE1 cells

Conclusions:

Airway smooth muscle is highly sensitive to oxidative stress and is amenable to anti-oxidant effects of LGM2605 raising the potential of a novel treatment approach in asthma.

Funding:

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