Online Data Supplement

Hydrogen Sulfide Inhibits Proliferation and Release of IL-8 from Human Airway Smooth Muscle Cells

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It could be postulated that the effect of methemoglobin upon DNA synthesis and IL-8 release is the result of another mechanism and not its H_2S scavenger activity. Methemoglobin is a form of hemoglobin, in which the iron in the heme group is in the Fe^{3+} (ferric) state, not the Fe^{2+} (ferrous) of normal hemoglobin. For almost 25 years now, it has been well documented that it is the ferric heme group of methemoglobin that scavenges H_2S [30;31]. In order to confirm that the action of methemoglobin upon ASM proliferation and IL-8 release was via its H_2S scavenger activity and not another mechanism, we reproduced the above experiments with ferrous stabilized human hemoglobin. Ferrous stabilized human haemoglobin had no effect upon either DNA synthesis or cell viability at 48 h (**Supplementary Figure 1A & B**). These results were duplicated at 72 h (data not shown).

At 48 h, ASM proliferation was induced by IL-1β (1 ng/ml) ($p < 0.05$), an effect that was diminished by NaSH ($p < 0.05$) (**Supplementary Figure 2A**). Methemoglobin (10 μM) increased DNA synthesis induced by IL-1β by twice that caused by IL-1β alone ($p < 0.01$). Addition of methemoglobin (10 μM) for 1 h prior to NaSH (100 μM) plus IL-1β (1 ng/ml) resulted in a return to the same level of DNA sythesis as observed with IL-1β alone (**Supplementary Figure 2A**). There was no effect on cell viability (**Supplementary Figure 2B**). IL-1β (1 ng/ml) increased IL-8 release to 1,500 pg/ml, which was inhibited by NaSH ($p < 0.001$) (**Supplementary Figure 2C**). Methemoglobin (10 μM) did not increase IL-1β-induced IL-8 release, and methemoglobin (10 μM) added for 1 h prior to either NaSH (100 μM) and IL-1β (1 ng/ml) did not decrease IL-8 release (**Supplementary Figure 2C**). These results were duplicated at 72 h (data not shown).

Having already shown that inhibition of the CSE enzyme did not induce proliferation (**Fig. 2A**), we examined the effect of exogenous H_2S upon CBS expression. The H_2S 'donor' compounds, NaSH or GYY4137 (100 μM), had no effect on CSE mRNA or protein expression (**Figs. 4B & Supplementary Figure 3**), further supporting the notion that endogenous H_2S production is solely dependent on CBS.

Supplementary Figure 1: Effect of ferrous stabilized human hemoglobin on ASM proliferation induced by FCS. Ferrous stabilized human hemoglobin had no effect upon cell proliferation induced by FCS. ASM cells were incubated with ferrous stabilized human hemoglobin (10 μM) for 1 h, NaSH (100 μM) was added for another 48 h. DNA synthesis (A) and cell viability (B) were subsequently measured by BrdU ELISA and MTT assay respectively. Bars represent mean ± SEM of 6 ASMC donors. *** $p < 0.001$.

Supplementary Figure 2: Effect of the H_2S 'donor' NaSH upon ASM proliferation and IL-8 release induced by IL-1β. NaSH inhibited cell proliferation induced by IL-1β. ASM cells were incubated with methemoglobin (10 μm) for 1 h, NaSH (100 μm) and IL-1β (1 ng/ml), was added for another 48 h in the presence or absence of 2.5 % FCS. DNA synthesis (A), cell viability (B) and IL-8 release (C) were subsequently measured by BrdU ELISA, MTT assay and Duoset ELISA respectively. Bars represent mean ± SEM of 6 ASMC donors. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Supplementary Figure 3: Effect of the H_2S 'donors' NaSH and GYY4137 upon CSE protein expression in human ASM cells. Exogenous H_2S had no effect on CSE protein expression. ASM cells were incubated with methemoglobin (10 μM) for 1 h, NaSH (A) or GYY4137 (B) (100 μM), was added for another 48 h. CSE and β-actin were detected by Western blotting. In panels C & D, changes in CSE protein expression were quantified by densitometry, normalised against β-actin expression and then expressed as the % change versus untreated controls. Bars represent mean ± SEM of 6 ASMC donors.