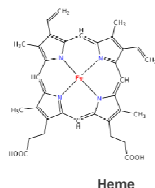


Abstract

Studies of heme oxygenase in keratinocytes are few despite it being an accessible target for pharmacological manipulation. We have used an immortalised human keratinocyte line to demonstrate the possibility of synergistically up-regulating keratinocyte heme oxygenase-1 (HO-1) as a novel means of treating inflammatory dermatoses such as acne, atopic dermatitis and psoriasis. HO-1 is the inducible rate-controlling enzyme that catalyses the conversion of heme to ferrous iron, bilirubin and carbon monoxide. HO-1 possesses anti-inflammatory, cytoprotective and immunosuppressive effects and hence limits tissue damage caused by reactive oxygen species. The transcriptional activator, Nrf2, is intimately involved in the regulation of HO-1 gene expression. Cytosolic Nrf2 is phosphorylated and translocated into the nucleus in response to activation of mitogen-activated protein kinases (MAPKs). Most known inducers of HO-1 including antioxidants such as curcumin and carnosol target protein phosphorylation cascades. Cobalt protoporphyrin IX (CoPP) and metal salts on the other hand appear, at least in part, to target Bach-1 by promoting its nuclear export. Bach-1 is a transcriptional repressor, heterodimers of which normally suppresses transcription of the HO-1 gene by binding to the stress response elements within the promoter region. Using ELISA to detect HO-1 levels in lysates from HEK001 cells grown for 72 h without inducer followed by 24 h with different concentrations of inducer, we found that CoPP at 0.78 to 3.12 μ M induced a >200 fold increase in HO-1 protein compared to baseline levels. Using the photosensitiser and heme precursor, aminolevulinic acid (ALA), the maximum increase in HO-1 production was 21 fold at 1 mM. When 100 μ M ALA was combined with 100 μ M cobalt chloride, the maximal increase in HO-1 production was 260 fold compared to baseline, 320 fold compared to 100 μ M cobalt chloride alone and 32 fold compared to 100 μ M ALA alone. When cobalt chloride was used alone the maximum induction of HO-1 protein production was 15 fold at 1 mM. We hypothesise that ALA and cobalt chloride affect different aspects of the HO-1 regulatory pathway and this results in a synergistic inducing effect as previously observed in hepatoma cells (Mitani et al. *Biochem J* 1993; 290: 819-25). Identifying other combinations of compounds that synergistically modulate aspects of HO-1 regulation represents a novel approach to the development of topical anti-inflammatories.

Background

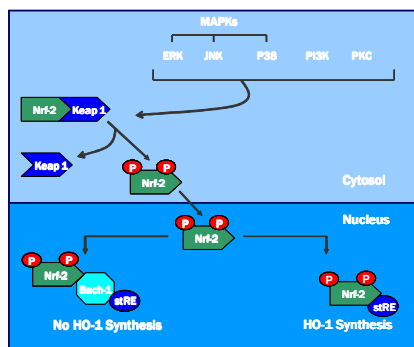
- Heme is a redox responsive molecule which is pro-inflammatory in high concentrations
- Heme-oxygenase-1 (HO-1) is the inducible rate-controlling enzyme that catalyses the conversion of heme into ferrous iron, bilirubin & carbon monoxide
- The products of heme catabolism exert a number of beneficial effects^{1,3}
 - Immunomodulatory
 - Anti-proliferative
 - Anti-inflammatory
 - Cytoprotective against oxidative stress
 - Promotion of wound healing
- Keratinocyte HO-1 represents a highly manipulable global regulator in the control of inflammatory dermatoses especially those in which T cells are implicated³



Objectives

- To assess the feasibility of synergistically up-regulating keratinocyte HO-1 using a combination of inducers, which target different aspects of the complex regulatory pathway (see Figure 1).
- In this work, we used the photosensitiser & heme precursor, δ -aminolevulinic acid (ALA), as an inducer of MAP kinases (JNK & p38)⁴ with cobalt chloride that acts via increased binding of a Nrf2-MafG heterodimer to stress response elements in the HO-1 promoter⁵. Cobalt protoporphyrin IX (CoPP) which targets Bach 1 & Nrf2 was used as a control⁶.

Figure 1. Regulation of HO-1



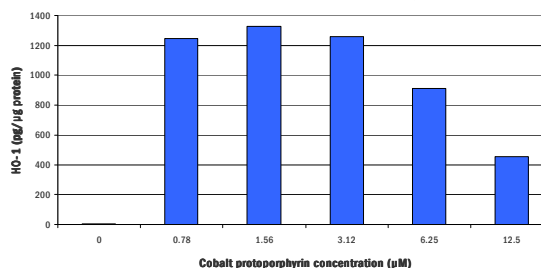
Methods

- Human epidermal keratinocytes (HEK001 cells) were cultured for 72h without inducer and exposed to a range of concentrations of inducer for 24h at 37°C in an atmosphere of 5%CO₂
- To detect synergy, ALA and cobalt chloride were each used at 10% of the concentration that induced HO-1 maximally on its own.
- HO-1 was extracted from the cells and assayed in duplicate using an ELISA sandwich immunoassay (Stressgen).

Results I

- As expected, CoPP was a potent inducer of HO-1 in HEK100 cells
 - 0.78 - 3.12 μ M CoPP increased HO-1 protein production over 200 fold compared to basal levels (Figure 2).
 - Higher concentrations were less effective.
- In contrast, the maximum increase in HO-1 protein production with ALA was 21 fold at 1 mM (data not shown).
- The poorest inducer was cobalt chloride; maximum induction of HO-1 was 15 fold at 1 mM (data not shown).

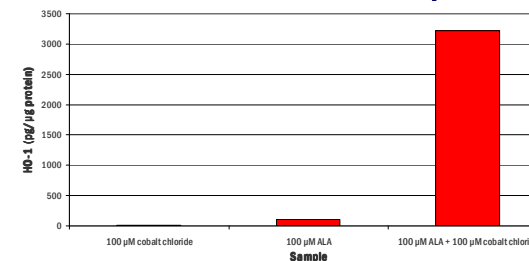
Figure 2. Effect of cobalt protoporphyrin on HO-1 induction in HEK001 keratinocytes



Results II

- When 100 μ M ALA was combined with 100 μ M cobalt chloride, the maximum increase in HO-1 production was
 - 260 fold compared to uninduced levels,
 - 320 fold compared to 100 μ M cobalt chloride alone
 - 32 fold compared to 100 μ M ALA alone (Figure 3).

Figure 3. Effect of 100 μ M ALA and 100 μ M cobalt chloride on HO-1 induction in HEK001 keratinocytes



Conclusions

- Although neither ALA nor cobalt chloride are very effective inducers of HO-1 on their own, in combination they are as effective as CoPP, albeit at higher concentrations. Our data confirm in human keratinocytes the results of Mitani et al⁷ using hepatoma cells and mRNA instead of protein.
- Synergy could be explained by each molecule affecting a different aspect of the HO-1 regulatory pathway and/or via incorporation of the metal into the porphyrin macrocycle
 - this latter mechanism depends on the recently confirmed non-specificity of mammalian ferrochelatase for divalent metal ions⁸.
- It may be possible to identify other synergistic pairs of compounds that target different aspects of the HO-1 induction pathway.
- This proof-of-principle study shows that synergistic up-regulation of keratinocyte HO-1 with small molecules delivered topically is a feasible means of increasing skin levels of this important target in inflammatory dermatoses such as acne, psoriasis, atopic dermatitis and rosacea.

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