

FINAL PROJECT REPORT

Date: 1st December 2015

Name:

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Project Title:

Regulation of Endothelial Cell Death by Hypothiocyanous Acid

Please copy the "Specific Objective(s)" statement, entered on your application form, in the space below.

Aim 1: Investigate the inhibition of activator and executioner caspases by HOSCN

We have shown that HOSCN potently inhibits apoptosis and caspase 3 activity in HUVEC. We will extend these studies and include caspases 8 and 9.

- (i) Cells will be exposed to increasing concentrations of HOSCN for varying time-frames. Caspase (3, 8 & 9) activity will be measured using a fluorescent enzyme assay²¹, and cell death will be measured using flow cytometry with Annexin-V and PI.
- (ii) Apoptosis is an active and dynamic process which does not occur simultaneously in all cells. We will monitor caspase 3 activity in real-time using live-imaging microscopy with NucView™ 488; cells will be incubated in an apoptosis-inducing buffer (Hanks' buffered saline solution, HBSS), with or without HOSCN and events will be recorded using time-lapse photography.

Aim 2: Investigate the induction of necroptosis by HOSCN

Given the multiple functions of caspase 8 in deciding cell fate (survival, apoptosis or necroptosis), it is likely that caspase 8 is pivotal in determining whether HOSCN-exposed cells survive, and if not, how they die.

- (iii) Dimerisation of caspase 8 is critical in determining the fate of cells and we will isolate multi-protein complexes to be probed for the presence of caspase 8, FADD and FLIP.
- (iv) We will use mass spectrometry to identify which of the 13 thiols of caspase 8 are oxidised; immunoprecipitated caspase 8 will be digested using trypsin and the resulting peptides analysed for changes indicative of oxidation.
- (v) We will monitor effects on members of the RIP kinase pathway; phosphorylation of RIP1, RIP3, and the downstream target, mixed lineage kinase domain-like protein (MLKL)³⁶ is essential for necroptosis³⁷ and we will examine this using Western blotting.
- (vi) Changes in cell morphology are an important determinant of cell death³⁸. Cells exposed to HOSCN undergo a form of cell death unlike that induced by other types of oxidative stress; cells remain attached to the substratum at distinct points, exhibiting membrane blebbing and eventual lysis (Fig. 2). We will document these events in real time using live-imaging microscopy.

Briefly describe how successful you were in achieving the stated objective(s). If the objective(s) was not achieved, explain why that is the case and describe what you did manage to achieve.

Caspase Activity & Cell Morphology

Overall progress on the project has been very good. At the six-month point I reported that the upstream activators of executioner caspase 3 (caspases 8 and 9) are also sensitive to inhibition by HOSCN but that caspase 3 is the most susceptible (refer to earlier report, Fig.1). I also showed, using flow cytometry, that cells exposed to HOSCN do not undergo classical apoptotic cell death at any level of exposure (refer to earlier report, Fig.2).

An apparently incongruous result, revealed using live imaging microscopy, was that caspase activity appeared to be induced at early time points during incubation of cells with HOSCN (refer to earlier report, Fig.3). However, apoptosis is typically induced in HUVEC after several hours of exposure to an inducer (serum deprivation, chemotherapy drugs, other oxidants) and characteristic morphological changes occur simultaneously. In contrast, in the presence of HOSCN, extensive morphological changes are observed within 30 min of oxidant exposure (at lethal levels) and the apparent caspase activity followed once cells were obviously dead. This suggests that the “caspase activity” we observed was artefactual and was caused by cleavage of the assay substrate after cell death, rather than actual enzymatic activity. Our previous extensive observations of this cell/oxidant system demonstrate that, at the time points concerned, cells are no longer viable, and since apoptosis is an active process requiring enzymatic (caspase) activity, it cannot occur after cell death. This is corroborated by the lack of any caspase activity detected in these cells using a well-established caspase activity assay developed in our lab. These live imaging experiments have enabled me to document the distinctive morphological changes that occur in endothelial cells exposed to lethal levels of HOSCN.

Effects on Cell Signalling

HOSCN selectively targets thiol (cysteine) groups on proteins but not all thiol-containing proteins are equal; some, like caspase 3, are much more sensitivity than others (like glutathione or GAPDH). Many cell signalling pathways are redox regulated (switched on/off by oxidation/reduction) and other groups have shown that, at non-lethal levels, HOSCN activates the NF- κ B survival pathway in endothelial cells. This, however, also induces the expression of adhesion molecules on the cell surface which contribute to inflammation and HOSCN is thus implicated in atherosclerosis and plaque development. I earlier reported that the PI3K/Akt survival pathway, an activator of NF- κ B, was activated by non-lethal levels of HOSCN, but was switched off soon after exposure of cells to lethal concentrations of HOSCN (refer to earlier report, Fig.5).

HOSCN has also been shown to activate mitogen-activated protein kinase (MAPK) signalling, possibly through inactivation of the protein tyrosine phosphatases (PTP) that negatively regulate MAPKs. I also showed that the JNK and p38 stress response pathways were activated by increasing levels of HOSCN; the higher the concentration of HOSCN, the more activation (phosphorylation) of these kinases that regulate the expression of genes involved in the oxidative stress response.

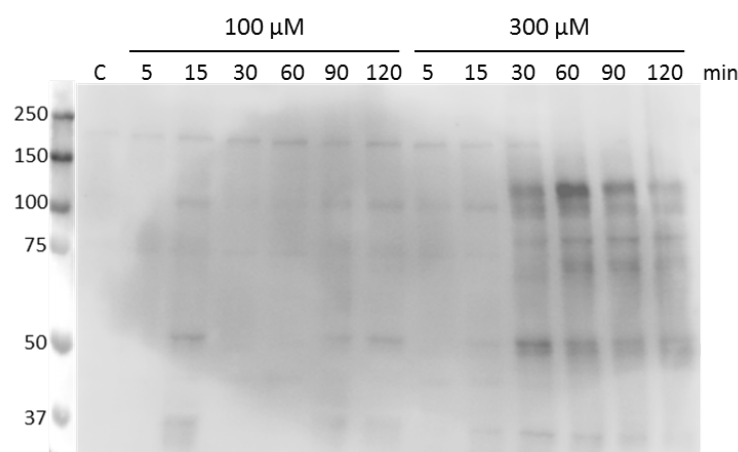


Fig. 1: Phosphorylation of global protein tyrosine phosphatases by HOSCN. There is an increase in on PTP phosphorylation (deactivation) at lethal levels of HOSCN (300 μ M). PTP phosphorylation was detected by Western blotting with an antibody against all PTPs.

More recently I have examined the effect of HOSCN on PTP in endothelial cells and my preliminary results suggest that a high concentration (300 μ M) results in their

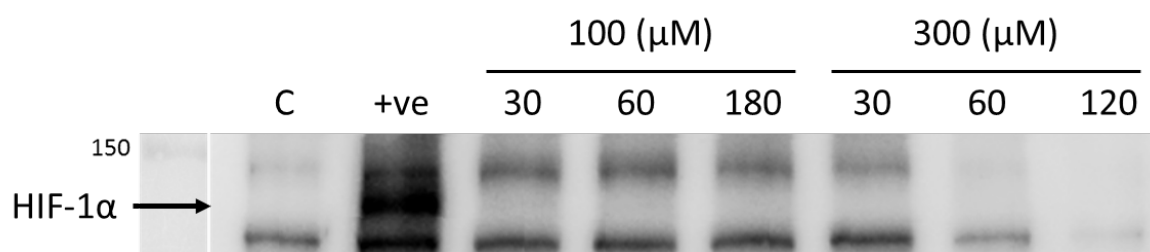


Fig. 2: HOSCN does not induce HIF-1 α stability. Western blotting revealed no band corresponding to HIF-1 α in cells treated with HOSCN at either high or low concentrations.

phosphorylation and inactivation (Fig. 1).

We had previously detected increased levels of the HIF-1 α transcription factor in cells exposed to HOSCN however has yet to be repeated (Fig. 2). One explanation may be that HIF-1 α is not stable when stored at -20 $^{\circ}$ C, as was the case with the cell lysates used here, and I will investigate this further using freshly-extracted proteins.

Effects on the Necroptosis Pathway

We have proposed that the form of cell death triggered by HOSCN is necroptosis and in my earlier report I showed that a key component of the necroptosis pathway, MLKL, was affected by exposure of cells to HOSCN; protein levels of MLKL were increased in cells exposed to a non-lethal concentration of HOSCN (100 μ M), whereas at a lethal dose (300 μ M), MLKL levels were dramatically reduced over time. More recently, in line with the work of others showing that MLKL becomes phosphorylated and localised to the cell membrane during necroptosis, I have discovered that MLKL becomes phosphorylated in cells undergoing HOSCN-induced death (Fig. 3). I was also able to use immunofluorescent microscopy to demonstrate aggregation of phospho-MLKL in these cells (Fig. 4). These

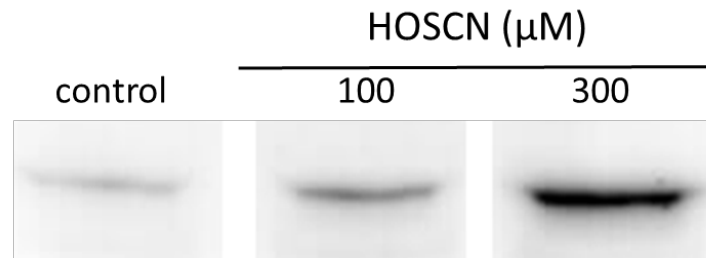


Fig. 3: HOSCN induces MLKL phosphorylation in endothelial cells exposed to a lethal level of oxidant.
Western blotting with antibodies against phospho-MLKL in whole lysate from cells exposed to HOSCN for 1h.

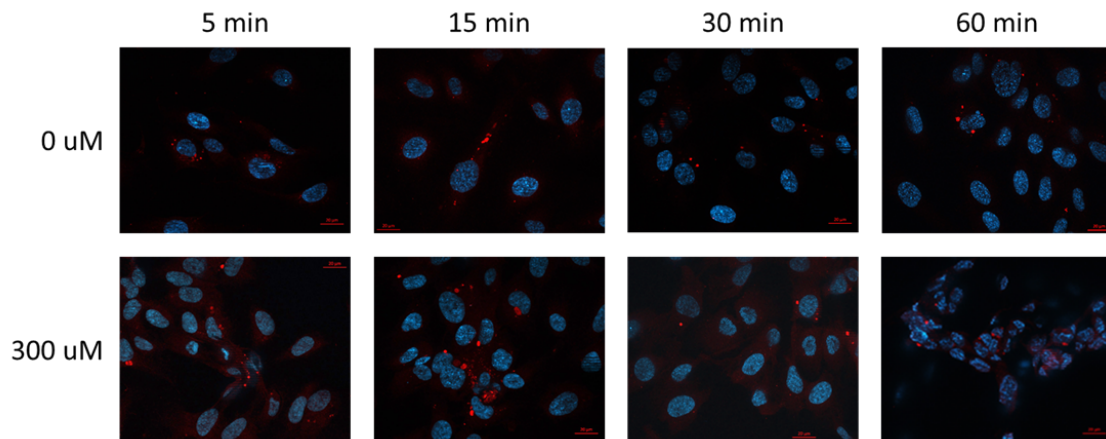


Fig. 4: Phosphorylated MLKL forms aggregates in endothelial cells exposed to HOSCN.
Immunofluorescent imaging using antibodies specific to phospho-MLKL (red) shows more accumulation of the phosphorylated protein than control cells, especially at early time points. Nuclei are shown in blue.

findings suggest that HOSCN may be the first identified naturally occurring molecule to induce necroptosis in cells.

Role of caspase 8

We have hypothesised that caspase 8 plays a central role in determining the fate of cells exposed to HOSCN and that the outcome depends on its interaction partners: “physiological levels of HOSCN selectively target and block caspase-mediated cell death; the apoptotic activity of caspases is lost and the survival pathways are stimulated (through NF- κ B and HIF-1 α); as the concentration of HOSCN increases, caspase 8 becomes fully inhibited and necroptosis proceeds (Fig. 5). This loss of function has important consequences for the cell; loss of caspase activity not only prevents apoptosis, leading to the survival of damaged cells with aberrant function, but also triggers the induction of necroptosis. Furthermore, since necroptosis is an inflammatory process, this results in further inflammation in a positive feedback loop. This capacity of HOSCN to override cell death regulation suggests that HOSCN may be unique in its ability to selectively inhibit caspases and dictate cell fate.

In order to characterise the mechanism(s) behind these events I am currently dissecting the functions of caspase in cells exposed to HOSCN. Preliminary results are encouraging and suggest that caspase 8 associates with several necroptosis-related proteins following exposure of cells to HOSCN; immunoprecipitation of caspase 8 from whole cell lysates and subsequent Western blotting has revealed interaction of caspase 8 with FLIP (predominantly the short

form, FLIP_S, which is associated with resistance to apoptosis), with RIP1 and RIP3, and with MLKL itself (Fig. 6).

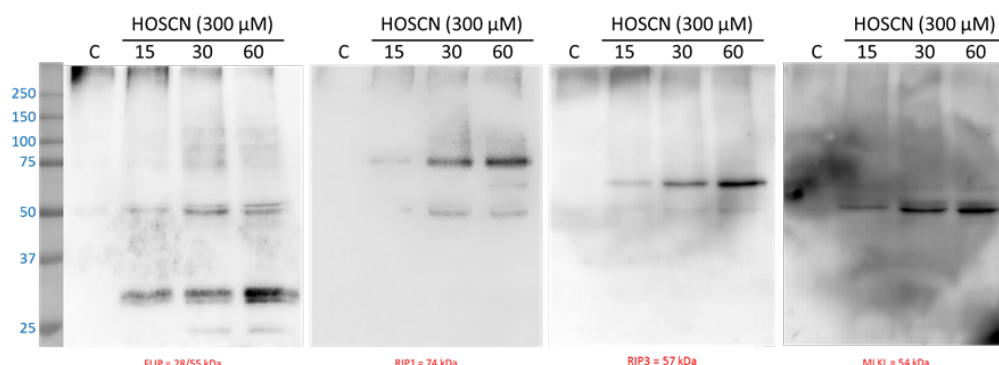


Fig. 6: Caspase 8 interacts with several necroptosis-related proteins after exposure to lethal levels of HOSCN. Caspase 8 was immunoprecipitated from whole cell lysates (after exposure of cells to 300 μ M HOSCN) and Western blotting was used to identify other proteins co-immunoprecipitated with it. Antibodies against FLIP, RIP1, RIP3 and MLKL were used to identify these proteins on a single membrane which was stripped of antibody after each.

Briefly describe any interesting outcomes which might not have been considered in your original objectives (if any).

Our initial objectives were primarily focussed on the mechanisms and regulation of cell death by HOSCN and, while this has revealed some very interesting outcomes, the non-lethal effects of HOSCN are also incredibly interesting. While it is exciting to propose HOSCN as the first identified small molecule capable of single-handedly inducing necroptosis in a physiological setting, it remains to be seen whether lethal concentrations of this oxidant would be generated and maintained in a physiological system; this will be investigated in future. I also plan to more fully investigate the interactions of caspase 8, following exposure to non-lethal levels of HOSCN, and to look in depth at the effects of HOSCN on MLKL (e.g. its ability to form oligomers as reported by others).

With regards to the non-lethal effects of HOSCN, uncovering the role of this oxidant as a signalling molecule is an exciting prospect. Many signalling pathways are redox-sensitive with key proteins becoming phosphorylated, resulting in activation of kinases and inactivation of their upstream regulators (e.g. PTP, serine/threonine phosphatases) in response to oxidative stress. These cascades control gene expression governing downstream events and ultimately affecting cell function.

I look forward to extending the current study to increase our understanding of this little-studied, and less understood, oxidant.