A pivotal role for NF-κB in the macrophage inflammatory response to the myeloperoxidase oxidant hypothiocyanous acid

Gary J. Pan[a,b*], Benjamin S. Rayner[a,c,*] Yunjia Zhang[a,c], David van Reyk[b] and Clare L. Hawkins[a,c,d†]

[a] *Heart Research Institute, 7 Eliza Street, Newtown, NSW 2042, Australia;* [b] *School of Life Sciences, University of Technology Sydney, PO Box 123, Broadway, NSW 2007, Australia;* [c] *Sydney Medical School, University of Sydney, NSW 2006, Australia;* [d] *Department of Biomedical Sciences, University of Copenhagen, Panum Institute, Blegdamsvej 3, Copenhagen N, DK-2200, Denmark.*

[*]These authors contributed equally to the work.

[†]**Corresponding author:** Prof. Clare Hawkins, Department of Biomedical Sciences, University of Copenhagen, Panum Institute, Blegdamsvej 3, Copenhagen N, DK-2200, Denmark. Ph: +45 35 33 70 05, Email: clare.hawkins@sund.ku.dk

ABSTRACT

Atherosclerosis is characterised by the infiltration of macrophages at sites of inflammation within the vessel wall and the release of myeloperoxidase (MPO), which forms hypochlorous acid (HOCl) and hypothiocyanous acid (HOSCN). HOCl is a damaging oxidant strongly implicated in the development of atherosclerosis. Preferential formation of HOSCN occurs under conditions where thiocyanate ions are elevated, as is the case in the plasma of smokers. HOSCN reacts selectively with thiols, which can result in a greater extent of enzyme inactivation leading to more extensive cellular damage than HOCl at susceptible sites; this may contribute to the increased risk of atherosclerosis seen in smokers. In this study, we show that the exposure of macrophages to HOSCN results in a time- and dose-dependent increase in the mRNA expression and release of pro-inflammatory cytokines and chemokines, including monocyte chemotactic protein 1, tumour necrosis factor alpha, and interleukins 6, 8 and 1β. Only at high oxidant concentrations (>200 μM) is a significant loss of cellular thiols and increased cell death observed. HOSCN-induced cytokine and chemokine expression and cell death were decreased on pharmacological inhibition of nuclear factor kappa B. These data highlight a potential mechanism by which the formation of HOSCN could promote inflammation and the development of atherosclerosis, in the presence of supra-physiological levels of the precursor thiocyanate, which are achievable by cigarette smoking.

Keywords: Macrophage; Atherosclerosis; Myeloperoxidase, Oxidative Stress, Inflammation, Cytokine

ABBREVIATIONS

BSA, bovine serum albumin; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; HOCl, hypochlorous acid; HOSCN, hypothiocyanous acid; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; IL-1β, interleukin 1 beta; IL-1R, interleukin-1 receptor; IL-6, interleukin 6; IL-8, interleukin 8; JC-1; tetraethyl-benzimidazolyl-carbocyanine iodide; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; MAPK, mitogen activated protein kinase; MCP-1, monocyte chemotactic protein; MPO, myeloperoxidase; NF-κB, nuclear factor κB; qPCR, quantitative real-time polymerase chain reaction; PMA, phorbol myristate acetate; PVDF, polyvinylidene fluoride; SCN^-, thiocyanate; TBST, Tris-buffered saline containing 0.1% (v/v) Tween-20; TLR, Toll-like receptors; TNFα, tumour necrosis factor α; TNFR, tumour necrosis factor receptors; TrxR, thioredoxin reductase; VCAM-1, vascular cell adhesion molecule 1; 18S, 18S ribosomal RNA; β2M, β2-microglobulin.

HIGHLIGHTS

- HOSCN upregulates cytokine and chemokine expression in THP-1 macrophages
- Expression of cytokines and chemokines is driven by NF-κB activation
- NF-κB activation by HOSCN leads to cell death in THP-1 macrophages

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease that is characterised by the accumulation and infiltration of leukocytes within the vessel wall, which trigger lipid deposition resulting in lesion formation and the progressive narrowing of affected blood vessels [1]. Macrophages comprise the largest subset of leukocytes within the lesion, and are responsible for accumulating excessive lipid resulting in “foam cell” formation, with macrophage cell death contributing to necrotic core formation, lesion destabilisation and ultimately thrombosis [1, 2]. In addition, macrophages release a battery of cytokines and chemokines, including monocyte chemotactic protein 1 (MCP-1), tumour necrosis factor alpha (TNFα), interleukin 6 (IL-6), interleukin 8 (IL-8) and interleukin 1β (IL-1β), which propagate disease by exacerbating inflammation and vascular cell dysfunction [3]. Tissue-resident macrophages (together with neutrophils) have also been implicated in the release of the peroxidase enzyme, myeloperoxidase (MPO) [4], which is present in elevated amounts in atherosclerotic tissue [5].

MPO is a heme enzyme that catalyses the reaction of halide and pseudo-halide ions with hydrogen peroxide (H_2O_2) to form hypohalous acids. Hypohalous acids are oxidants, and play an important role in the immune system by killing bacteria and other invading pathogens [6, 7]. The overproduction of hypohalous acids by MPO during chronic inflammation, has however, been strongly linked with the host tissue damage and the development of disease, particularly atherosclerosis [7, 8]. MPO is also recognised as both a risk factor for the development of coronary artery disease [9] and a prognostic factor determining patient outcome following cardiovascular events [10]. Under physiological conditions, MPO utilises predominantly chloride ions (Cl^-) to produce hypochlorous acid (HOCl), which is a potent and indiscriminate oxidant, that reacts rapidly with most biological molecules [7, 11]. MPO also utilises thiocyanate (SCN^-) to produce hypothiocyanous acid (HOSCN) [12]. SCN^- has a very high specificity for MPO (~730-fold higher than Cl^-) [13], and is therefore able to compete effectively with the more abundant Cl^- ions present under physiological conditions, which reduces the amount of HOCl formed [11, 14]. Unlike HOCl,

HOSCN is a selective oxidant, which reacts predominantly with free Cys or thiol (R-SH) residues [11, 12, 15]. Therefore, conditions that favour HOSCN production will result in an altered pattern and extent of cellular damage to that which occurs with HOCl (reviewed [11, 16]).

Currently, the role of HOSCN in the development atherosclerosis is unclear and controversial. The detoxification of cyanide on inhalation of cigarette smoke results in elevated plasma SCN^- levels [14], which is associated with a higher incidence of cardiovascular disease [17]. In smokers, it is reported that serum SCN^- levels correlate with an increased deposition of lipid and expression of markers of foam cell formation in the arterial wall [18, 19]. However, SCN^- supplementation of transgenic mice that are both genetically predisposed to develop atherosclerosis, and over-express human MPO, results in decreased in lesion formation [20]. Moreover, high plasma SCN^- levels have been associated with improved long-term survival in patients following myocardial infarction [21]. Similarly, comparison of the reactivity of HOCl and HOSCN in different cellular models gives mixed results (reviewed [16]). There are clear examples where increased targeting of susceptible thiol-containing proteins by HOSCN results in more extensive enzyme inactivation compared to HOCl, for example, GAPDH and other glycolytic enzymes [15, 22], protein tyrosine phosphatases [23, 24], caspases [25, 26], ATPases [27, 28], which can lead to a greater extent of cell death [25, 29]. However, the addition of SCN^- to enzymatic MPO/H_2O_2/Cl^- systems can also protect cells from HOCl-mediated cytotoxicity [30-32].

It has been shown previously that HOSCN can stimulate the increased expression of the cellular adhesion molecules E-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on exposure to human umbilical vein endothelial cells (HUVEC), which is attributed to the activation of redox-sensitive transcription factors, including nuclear factor κB (NF-κB) [33]. In light of this, and the conflicting data regarding the role of SCN^- in the pathogenesis of atherosclerosis, we performed experiments to assess the effect of HOSCN on the activation of pro-inflammatory signalling and survival pathways using a well-characterised human macrophage cell model, the THP-1 monocytic cell line, differentiated to macrophages by

exposure to phorbol myristate acetate (PMA). This is of importance as macrophages play an integral role in lesion development, and it is well established that exposure to HOSCN can perturb the redox environment in this cell type to promote cellular dysfunction [15, 22, 25].

MATERIALS AND METHODS

Materials and Reagents – All aqueous reagents were prepared using nanopure water, filtered through a four-stage milli-Q system. All reagents were from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise noted. HOSCN was prepared enzymatically with lactoperoxidase (LPO; from bovine milk: Calbiochem, Kilsyth, VIC, Australia) using LPO/H_2O_2/SCN^- as described previously [25, 34]. The concentration of HOSCN was determined by quantifying the consumption of 5-thio-2-nitrobenzoic acid (TNB) at 412 nm using a molar absorption coefficient ε of 14,150 M^{-1} cm^{-1} [35, 36]. Experiments were also performed with HOSCN that had been allowed to decompose (at 22 °C, in the dark, > 2 weeks; dHOSCN) to assess the contribution from decomposition products.

Cell culture and treatment – The human monocytic cell line THP-1 (TIB-2; ATCC, Manassas, Virginia), was cultured under sterile conditions in RPMI media supplemented with 10% (v/v) foetal bovine serum (Bovogen Biologicals, Keilor East, VIC, Australia), 2 mM L-glutamine (Lonza, Basel, Switzerland), 100 units/mL penicillin (Sigma-Aldrich), and 10 µg/mL streptomycin (Lonza) in 175 cm^2 cell culture flasks (Corning, NY, USA) at 37 °C in a humidified atmosphere of 5% CO_2 with routine passaging every 2 – 4 days. The monocytes were differentiated into macrophages by seeding at density of 1 x 10^6 cells/well in 12 well tissue culture plates in culture media containing 50 ng/mL PMA for 72 h [37]. Cells were washed in HBSS prior to treatment with patho-physiological HOSCN concentrations (0 – 250 µM) for 1 h at 37 °C in a humidified atmosphere of 5% CO_2. Following treatment, the cells were washed with HBSS and re-cultured in cell media for either 3, 6 or 24 h at 37 °C in a humidified atmosphere of 5% CO_2.

Assessment of cellular function – Cell viability was determined by the lactate dehydrogenase (LDH) release assay [38]. Quantification of total cellular thiols following treatment with HOSCN was assessed using the ThioGlo assay, using a reduced glutathione (GSH) standard curve [35] and was normalised to the cell protein concentration assessed by the BCA assay. Mitochondrial function was assessed by measuring alterations in mitochondrial membrane potential using the Mitoprobe JC-1 (tetraethyl-benzimidazolyl-carbocyanine iodide) assay kit (Life Technologies, Carlsbad, CA, USA) with flow cytometry as previously described [29]. Secretion of various cytokines / chemokines was quantified in clarified cell culture supernatants by ELISA, according to the manufacturer's instructions (Peprotech, Rocky Hill, NJ, USA).

RNA extraction and cDNA synthesis – RNA was extracted using ReliaPrep RNA Cell Miniprep System kits following the manufacturer's instructions (Promega, Madison, WI, USA). Quantity and yield of total RNA extracted was assessed using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA samples were normalised to 500 ng total RNA per reaction using RNase free water (Promega, Madison, WI, USA) and cDNA synthesised using an iScript cDNA synthesis kit following manufacturer's protocol (Bio-Rad, Hercules, CA, USA).

Quantitative real-time polymerase chain reaction (qPCR) – The expression of mRNA for the inflammatory cytokines were assessed by qPCR using the primer sequences outlined in Table 1, designed using the National Centre for Biotechnology Information (NCBI; Bethesda, MD, USA) nucleotide database and accompanying primer design software, coupled with iQ SYBR Green Supermix following manufacturer's protocol (Bio-Rad, Hercules, CA, USA). Cycling consisted of an activation step of 95 °C for 3 min, followed by denaturation at 95 °C for 30 s annealing at 60 °C for 30 s, and extension at 72 °C for 30 s repeated over 40 cycles, before a final denaturation at 95 °C for 2 min. Relative mRNA concentrations of the genes of interest were normalised against the

corresponding relative mRNA concentrations of the 18S ribosomal RNA (18S) and β2-microglobulin (β2M) house-keeping genes, with the resultant data expressed as a fold increase in the genes of interest over cells incubated in the presence of decomposed HOSCN [39].

SDS-PAGE and Western blotting – Nuclear and cytoplasmic cellular fractions were prepared using a NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, Waltham, MA, USA) before separation by SDS-PAGE and transfer to a polyvinylidene fluoride (PVDF) membrane using the iBlot 2 Dry Transfer System (Thermo Fisher). Membranes were blocked with 5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween-20 (TBST; 20 mM Tris-HCl, pH 7.4, 135 mM NaCl, 0.1% (v/v) Tween) for 1 h and then incubated with primary antibodies raised against the p65 subunit of NF-κB (sc-372; Santa Cruz Biotechnology, Dallas, TX, USA), total and phosphorylated p38 (8690, 4511; Cell Signaling), ERK1/2 (sc-93, Santa Cruz; 4370 Cell Signaling), JNK (9258, 4668; Cell Signaling) or the loading controls β-actin (sc-47778; Santa Cruz) and histone H3 (4499; Cell Signaling) diluted in 1% (w/v) BSA in TBST overnight at 4 °C. Results were visualised following incubation with goat anti-rabbit or mouse HRP (7074, 7076; Cell Signaling) secondary antibody, prior to washing and exposure to Western Lighting chemiluminescence reagents as per manufacturer's instructions (Perkin Elmer, Sydney, NSW, Australia). Membranes were imaged using an ImageQuant LAS4000 (GE Healthcare, Notting Hill, VIC, Australia) and band densitometry performed using ImageJ software (NIH, Bethesda, Maryland).

Statistical analyses – Statistical analyses were performed using GraphPad Prism software 7.0 (GraphPad Software, San Diego, USA) using one-way or two-way ANOVA with Dunnett's, Tukey's or Bonferroni's multiple comparison post-hoc test with $p < 0.05$ taken as significant as outlined in the Figure Legends.

RESULTS

HOSCN is consumed by macrophages resulting in loss of thiols and cell viability

Initial studies examined the consumption of HOSCN by THP-1 macrophages exposed to the HOSCN (250 μM) for 1 h at 37 °C by measuring the residual oxidant concentration in the cell media over the course of the incubation. Exposure of THP-1 macrophages to HOSCN resulted in the loss of ~ 70 % of the initial oxidant added to the cells within 15 min, which decreased by a further 10 % over the 1 h treatment time (Fig. 1A). A loss in HOSCN concentration was also observed on incubation of the oxidant in HBSS in the absence of cells, but this occurred to a lesser extent than that seen in the presence of cells (Fig. 1A). Treatment of THP-1 macrophages with HOSCN also resulted in a decrease in intracellular thiol levels compared to control cells, which was statistically significant following 1 h exposure with > 200 μM HOSCN (Fig. 1B).

The extent of LDH release from THP-1 macrophages following exposure to a range of HOSCN concentrations (0 – 250 μM) was assessed as a surrogate measure of cell death both immediately following oxidant exposure and following 24 h re-incubation in complete media in the absence of HOSCN. No evidence was obtained for cell death following 1 h HOSCN exposure, whereas significant LDH release, consistent with cell lysis, was apparent with ≥ 200 μM HOSCN following 24 h re-incubation in the absence of oxidant (Fig. 1C). The losses in intracellular thiols and viability were not seen in experiments performed with decomposed HOSCN.

The effect of HOSCN on mitochondrial membrane permeability was examined using flow cytometry with the probe JC-1. However, no significant change in the ratio of red : green JC-1 fluorescence was observed on exposure of the THP-1 macrophages to HOSCN, in contrast to experiments performed with the mitochondrial uncoupling agent, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which was used as a positive control (data not shown). This contrasts with previous work with other cell types (e.g. [29]).

HOSCN upregulates pro-inflammatory cytokines and chemokines

Exposure of THP-1 macrophages to increasing concentrations of HOSCN (0 – 250 μM) resulted in a dose- and time-dependent increase in the mRNA expression of MCP-1, IL-6, IL-8, TNFα and IL-1β measured at 3 h (open bars), 6 h (grey bars) and 24 h (black bars) post-treatment by qPCR (Fig. 2). With IL-1β and the chemokines MCP-1 and IL-8, the most pronounced increase in mRNA expression was apparent 24 h post-HOSCN treatment, whereas with cytokines IL-6 and TNFα, a greater extent of mRNA expression was seen 6 h post-HOSCN treatment (Fig. 2). The effect of HOSCN treatment on the ability of THP-1 macrophages to secrete MCP-1, IL-6, IL-8 and TNFα was also assessed. A significant increase in the concentration of each cytokine / chemokine in the cellular supernatant compared to the non-treated control cells was observed 24 h after exposure of the THP-1 macrophages to HOSCN (250 μM) by ELISA. This is consistent with increased expression and secretion of MCP-1, IL-6, IL-8 and TNFα by THP-1 macrophages exposed to HOSCN (Fig. 3).

HOSCN-induced upregulation of cytokine / chemokine expression is dependent on NF-κB

The effect of HOSCN treatment on the activation of MAPK signalling cascades and the transcription factor NF-κB was examined as these pathways are redox-sensitive [40] and known to promote cytokine and chemokine release [41]. Western blotting studies to assess the extent of phosphorylation of ERK1/2, p38 and JNK as a measure of MAPK activation were performed on THP-1 macrophages exposed to HOSCN (0 – 250 μM) for 15 min. However, high basal MAPK protein phosphorylation was seen in the untreated control cells, which did not alter with HOSCN treatment (data not shown). This may be associated with the addition of PMA to the cells to promote macrophage differentiation, which makes it difficult to assess the effect of HOSCN on MAPK signalling. In contrast, increased nuclear translocation of the p65 subunit of NF-κB was apparent on treatment of macrophages with HOSCN (250 μM) for 1 h (Fig. 4A), consistent with activation of this transcription factor.

To examine whether NF-κB played a role in the elevated cytokine and chemokine expression, experiments were performed to examine MCP-1, IL-6, IL-8, TNFα and IL-1β expression in THP-1 macrophages that had been pre-incubated with the specific inhibitor BAY117085 (20 μM) for 30 min prior to exposure HOSCN. This resulted in a reduction in the mRNA expression of all of the cytokines and chemokines, which was statistically significant in the case of IL-8, TNFα and IL-1β (Fig. 4B). Pre-treatment of the THP-1 macrophages with BAY117085, also significantly attenuated cell death (Fig. 4C) as measured by LDH release at 24 h post-exposure to HOSCN (250 μM).

DISCUSSION

Macrophages play an integral role in promoting inflammation and the formation of lesions within the arterial wall, which together drive the development of atherosclerosis [1]. Lesions are enriched with enzymatically-active MPO, which co-localises with macrophages [5], suggesting that these cells will be key targets for MPO-derived oxidants. It is well established that HOCl causes significant disruption to macrophage function, including perturbing intracellular Ca^{2+}, depleting antioxidants, and inactivating enzymes, which culminate in both necrotic and apoptotic cell death [25, 42, 43]. The presence of SCN^- promotes the formation of HOSCN by MPO, which can also disrupt macrophage function, by selectively targeting thiol-dependent enzymes [15, 22, 25]. In this study, we show for the first time that HOSCN can upregulate the expression of pro-inflammatory cytokines and chemokines in macrophages via activation of the transcription factor NF-κB, which also plays a role in promoting cell death.

This could be a pathway that contributes to exacerbation of inflammation in the arterial wall, and accelerated lesion development. The concentrations of HOCl and HOSCN formed *in vivo* in a chronic inflammatory setting are not known. The concentration of HOCl produced *in vivo* can be estimated as ca. 200 – 500 μM from calculations based on either the activation of a circulating

concentration of human neutrophils [44] or from the reported levels of MPO present in human lesions [5], though higher mM doses have been reported at inflammatory sites [45]. The concentration of HOSCN produced *in vivo* will be dependent on the availability of the precursor SCN^-. It is noted that the concentrations at which HOSCN stimulates the activation of NF-κB and cytokine / chemokine release (> 100 μM) are likely only to be formed in the presence of supra-physiological amounts of SCN^-, which are generally only achievable with supplementation or on cigarette smoking [14].

The THP-1 macrophages consumed HOSCN over a period of 1 h, which is a similar time course to previous studies with murine J774A.1 macrophage-like cells, though the total extent of oxidant consumption differed, with the THP-1 macrophages consuming 80% of the initial HOSCN after 1 h, compared with 30% in the experiments with the murine cells [25]. The greater oxidant consumption seen with the THP-1 macrophages did not appear to result in more extensive depletion of intracellular thiols or cell death compared to the J774A.1 cells under comparable treatment conditions. This may reflect that HOSCN is more readily metabolised by THP-1 macrophages compared to J774A.1 cells. Thus, thioredoxin reductase (TrxR) has been implicated as a key pathway responsible for the cellular metabolism of HOSCN, with inhibition of this enzyme shown to sensitise mammalian cells to HOSCN-mediated toxicity [30]. In this study, the expression and activity of TrxR in the THP-1 macrophages in the presence and absence of HOSCN was not assessed, though J774A.1 cells have been reported to have reduced TrxR activity compared to other macrophage cell lines [46]. That concentrations of HOSCN > 100 μM are required to see activation of NF-κB may reflect the point at which this antioxidant defensive pathway becomes overwhelmed. Alternatively, there may be differential reactivity of HOSCN with cell membrane components, including extracellular membrane receptors, which could also vary on THP-1 macrophages compared to J774A.1 cells.

We demonstrate that exposure of THP-1 macrophages to HOSCN results in the increased

expression of pro-inflammatory cytokines (IL-6, TNFα, IL-1β) and chemokines (MCP-1, IL-8) in a dose- and time-dependent manner (Fig. 2), which is concomitant with the release of active forms of these signalling molecules by the macrophages (Fig. 3). MCP-1 and IL-8 promote the migration of leukocytes and activation of the endothelium, which together can contribute to lesion development by increasing the uptake of low-density lipoprotein (LDL) [47]. The cytokines IL-6, TNFα and IL-1β are critical mediators of both acute and chronic inflammation [41] and have also been implicated as strong independent risk factors for the development of cardiovascular disease [48]. The cytokines IL-6, TNFα and IL-1β are reported to signal through type 1 cytokine receptors, whereas the chemokine IL-8 interacts with G protein-coupled receptors (GPCRs) [41], suggesting that HOSCN could potentially influence multiple pro-inflammatory signalling cascades relevant to lesion formation in atherosclerosis.

The increased expression of cytokines and chemokines is attributed to the HOSCN-mediated activation of NF-κB, as pre-treatment with BAY117085 prevents the increased cytokine and chemokine expression and the associated loss in cell viability (Fig. 4), though the ability of this inhibitor to prevent the translocation of the p65 subunit of NF-κB was not directly measured in this study. NF-κB is regulated by IκB proteins, which bind to the protein subunits and mask their DNA binding domains. The activity of the IκB proteins, including IκBα, is controlled by phosphorylation by upstream IκB kinases (IKK) [49]. BAY117085 is an irreversible inhibitor of IκBα phosphorylation [50], suggesting that HOSCN acts on the canonical (classical) pathway of NF-κB activation, leading to phosphorylation of IκBα and subsequent release and nuclear translocation of NF-κB [51]. These data support previous studies in HUVEC exposed to HOSCN, where increased expression of E-selectin, ICAM-1 and VCAM-1 is attributed to activation of NF-κB [33].

In light of the previous studies showing targeting of thiols in macrophages exposed to HOSCN [15, 25], the mechanism involved in the activation of NF-κB is likely to be related to alterations in the cellular redox environment. Reactive oxygen species (ROS) are known to

influence the activation of NF-κB via the canonical signalling pathway, which is characterised by binding of inflammatory molecules, including cytokines, to Toll-like receptors (TLR), tumour necrosis factor receptors (TNFR) and interleukin-1 receptors (IL-1R) [40, 49, 52]. Exogenously added oxidants such as H_2O_2, can regulate NF-κB activation by inducing the phosphorylation of IκBα, or by influencing the activity of IKK (reviewed [49]). It has also been demonstrated that the extracellular Cys-rich domains of TNFR are sensitive to oxidation by externally generated oxidants [53], which can promote the activation of NF-κB [54]. This could be important in THP-1 macrophages exposed to HOSCN, particularly as the increased expression of cytokines and chemokines is observed under treatment conditions where no intracellular thiol loss is apparent.

In this study, it was not possible to assess whether HOSCN increased the phosphorylation of the MAPK proteins ERK1/2, p38 or JNK, owing to the relatively high extent of phosphorylation of these proteins, when assessed following 1 h incubation of the macrophages in the presence and absence of oxidant. However, evidence for MAPK activation has been reported in previous experiments with J774A.1 macrophages and HUVEC exposed to HOSCN [23, 33, 55]. The significant basal level of MAPK phosphorylation seen in the THP-1 macrophages may be related to the pre-treatment of the cells with PMA to promote differentiation. This is a limitation of the current study, as PMA is an activator of protein kinase C, which is also reported to influence NF-κB activation (reviewed [51]). However, HOSCN clearly increases the extent of nuclear translocation of the NF-κB p65 subunit over and above that seen in the control, non-treated cells. Similarly, a transcriptional upregulation of cytokine and chemokine expression and secretion is not seen to the same extent in control cells, which is analogous to the activation of NF-κB and cell adhesion molecule upregulation seen in HUVEC exposed to HOSCN [33].

Exposure of THP-1 macrophages to HOSCN for 1 h did not cause a significant extent of cell death, when viability was determined immediately after oxidant treatment, in agreement with previous studies with murine macrophages [15, 25]. However, cell death reflected by increased

leakage of LDH, was observed with ≥ 200 μM HOSCN, following re-incubation of the macrophages in the absence of oxidant for 24 h. Again, this could reflect the concentration at which the TrxR activity within the macrophages becomes saturated. In this study, the specific pathway responsible for cell death was not examined, though a role for NF-κB is apparent, as pre-treatment of the cells with BAY117085 mitigated the cytotoxicity induced by HOSCN (Fig. 5). NF-κB is an important regulator of programmed cell death, by both apoptotic and necrotic pathways (reviewed [56]). Binding to the TNFR in particular, triggers the activation of a complex series of signalling cascades that can determine cell fate by either promoting survival or death by programmed apoptosis and necrosis [56]. Evidence for cell death by both apoptotic and necrotic pathways was apparent in previous work with murine macrophages exposed to HOSCN, which was associated with increased mitochondrial release of cytochrome *c* [25]. However, this is consistent with HOSCN inducing changes in mitochondrial membrane permeability, which is not seen in analogous experiments with THP-1 macrophages.

In summary, we provide evidence for a critical role of NF-κB as a mediator of both cell survival and pro-inflammatory signalling on exposure of human THP-1 macrophages to HOSCN. This is a novel pathway by which HOSCN could act to promote inflammation within the vascular setting in the presence of elevated levels of SCN^-, and is particularly significant given the strong association between elevated circulating levels cytokines, including IL-1β, IL-6 and TNFα, and risk of developing atherosclerosis [48]. These results highlight a role for HOSCN as an inducer of stress-related, survival, signalling cascades, which can activate both repair and adaptive pathways, or promote apoptotic signalling and necrosis. Low concentrations of HOSCN may therefore have a protective function, but high amounts can promote cellular dysfunction. Overall, these data help to rationalise the accelerated development of atherosclerosis seen in smokers, and provide a greater understanding as to how SCN^- could potentially modulate the development of disease during chronic inflammation.

ACKNOWLEDGEMENTS

Funding: This work was supported by the Australian Research Council through the Future Fellowships Scheme [FT120100682].

REFERENCES

[1] P. Libby, Inflammation in atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 32(9) (2012) 2045-51.

[2] I. Tabas, Macrophage apoptosis in atherosclerosis: consequences on plaque progression and the role of endoplasmic reticulum stress, Antioxid. Redox Signal. 11(9) (2009) 2333-9.

[3] K.J. Moore, F.J. Sheedy, E.A. Fisher, Macrophages in atherosclerosis: a dynamic balance, Nat. Rev. Immunol. 13(10) (2013) 709-21.

[4] S. Sugiyama, Y. Okada, G.K. Sukhova, R. Virmani, J.W. Heinecke, P. Libby, Macrophage myeloperoxidase regulation by granulocyte macrophage colony- stimulating factor in human atherosclerosis and implications in acute coronary syndromes, Am. J. Pathol. 158(3) (2001) 879-91.

[5] A. Daugherty, J.L. Dunn, D.L. Rateri, J.W. Heinecke, Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions, J. Clin. Invest. 94(1) (1994) 437-44.

[6] S.J. Klebanoff, A.J. Kettle, H. Rosen, C.C. Winterbourn, W.M. Nauseef, Myeloperoxidase: a front-line defender against phagocytosed microorganisms, J. Leukocyte Biol. 93(2) (2013) 185-98.

[7] M.J. Davies, C.L. Hawkins, D.I. Pattison, M.D. Rees, Mammalian heme peroxidases: from molecular mechanisms to health implications, Antioxid. Redox Signal. 10(7) (2008) 1199-234.

[8] S.J. Klebanoff, Myeloperoxidase: friend and foe, J. Leukocyte Biol. 77 (2005) 598-625.

[9] R. Zhang, M.L. Brennan, X. Fu, R.J. Aviles, G.L. Pearce, M.S. Penn, E.J. Topol, D.L. Sprecher, S.L. Hazen, Association between myeloperoxidase levels and risk of coronary artery disease, J. Am. Med. Assoc. 286(17) (2001) 2136-42.

[10] M.L. Brennan, M.S. Penn, F. Van Lente, V. Nambi, M.H. Shishehbor, R.J. Aviles, M. Goormastic, M.L. Pepoy, E.S. McErlean, E.J. Topol, S.E. Nissen, S.L. Hazen, Prognostic value of myeloperoxidase in patients with chest pain, N. Engl. J. Med. 349(17) (2003) 1595-604.

[11] D.I. Pattison, M.J. Davies, C.L. Hawkins, Reactions and reactivity of myeloperoxidase-derived oxidants: differential biological effects of hypochlorous and hypothiocyanous acids, Free Radic. Res. 46(8) (2012) 975-95.

[12] T.J. Barrett, C.L. Hawkins, Hypothiocyanous Acid: benign or deadly?, Chem. Res. Toxicol. 25(2) (2012) 263-73.

[13] C.J. van Dalen, M.W. Whitehouse, C.C. Winterbourn, A.J. Kettle, Thiocyanate and chloride as competing substrates for myeloperoxidase, Biochem. J. 327(2) (1997) 487-92.

[14] P.E. Morgan, D.I. Pattison, J. Talib, F.A. Summers, J.A. Harmer, D.S. Celermajer, C.L. Hawkins, M.J. Davies, High plasma thiocyanate levels in smokers are a key determinant of thiol oxidation induced by myeloperoxidase, Free Radic. Biol. Med. 51(9) (2011) 1815-22.

[15] D.T. Love, T.J. Barrett, M.Y. White, S.J. Cordwell, M.J. Davies, C.L. Hawkins, Cellular targets of the myeloperoxidase-derived oxidant hypothiocyanous acid (HOSCN) and its role in the inhibition of glycolysis in macrophages, Free Radic. Biol. Med. 94 (2016) 88-98.

[16] B.S. Rayner, D.T. Love, C.L. Hawkins, Comparative reactivity of myeloperoxidase-derived oxidants with mammalian cells, Free Radic. Biol. Med. 71 (2014) 240-55.

[17] Z. Wang, S.J. Nicholls, E.R. Rodriguez, O. Kummu, S. Horkko, J. Barnard, W.F. Reynolds, E.J. Topol, J.A. DiDonato, S.L. Hazen, Protein carbamylation links inflammation, smoking, uremia and atherogenesis, Nature Med. 13(10) (2007) 1176-84.

[18] C.E.O. Scanlon, B. Berger, G. Malcom, R.W. Wissler, Evidence for more extensive deposits of epitopes of oxidized low density lipoproteins in aortas of young people with elevated serum thiocyanate levels, Atherosclerosis 121(1) (1996) 23-33.

[19] T.P. Botti, H. Amin, L. Hiltscher, R.W. Wissler, A comparison of the quantitation of macrophage foam cell populations and the extent of apolipoprotein E deposition in developing

atherosclerotic lesions in young people: high and low serum thiocyanate groups as an indication of smoking, Atherosclerosis 124(2) (1996) 191-202.

[20] P.E. Morgan, R.P. Laura, R.A. Maki, W.F. Reynolds, M.J. Davies, Thiocyanate supplementation decreases atherosclerotic plaque in mice expressing human myeloperoxidase, Free Radic. Res. 49(6) (2015) 743-9.

[21] P.E. Nedoboy, P.E. Morgan, T.J. Mocatta, A.M. Richards, C.C. Winterbourn, M.J. Davies, High plasma thiocyanate levels are associated with enhanced myeloperoxidase-induced thiol oxidation and long-term survival in subjects following a first myocardial infarction, Free Radic. Res. 48(10) (2014) 1256-66.

[22] T.J. Barrett, D.I. Pattison, S.E. Leonard, K.S. Carroll, M.J. Davies, C.L. Hawkins, Inactivation of thiol-dependent enzymes by hypothiocyanous acid: role of sulfenyl thiocyanate and sulfenic acid intermediates, Free Radic. Biol. Med. 52(6) (2012) 1075-85.

[23] A.E. Lane, J.T. Tan, C.L. Hawkins, A.K. Heather, M.J. Davies, The myeloperoxidase-derived oxidant HOSCN inhibits protein tyrosine phosphatases and modulates cell signalling via the mitogen-activated protein kinase (MAPK) pathway in macrophages, Biochem. J. 430(1) (2010) 161-9.

[24] N.L. Cook, C.H. Moeke, L.I. Fantoni, D.I. Pattison, M.J. Davies, The myeloperoxidase-derived oxidant hypothiocyanous acid inhibits protein tyrosine phosphatases via oxidation of key cysteine residues, Free Radic. Biol. Med. 90 (2016) 195-205.

[25] M.M. Lloyd, D.M. Van Reyk, M.J. Davies, C.L. Hawkins, HOSCN is a more potent inducer of apoptosis and protein thiol depletion in murine macrophage cells than HOCl or HOBr., Biochem. J. 414(2) (2008) 271-80.

[26] S.M. Bozonet, A.P. Scott-Thomas, P. Nagy, M.C. Vissers, Hypothiocyanous acid is a potent inhibitor of apoptosis and caspase 3 activation in endothelial cells, Free Radic. Biol. Med. 49(6) (2010) 1054-63.

[27] N.L. Cook, H.M. Viola, V.S. Sharov, L.C. Hool, C. Schoneich, M.J. Davies, Myeloperoxidase-derived oxidants inhibit sarco/endoplasmic reticulum Ca^{2+}-ATPase activity and perturb Ca^{2+} homeostasis in human coronary artery endothelial cells, Free Radic. Biol. Med. 52(5) (2012) 951-61.

[28] M. Arlandson, T. Decker, V.A. Roongta, L. Bonilla, K.H. Mayo, J.C. MacPherson, S.L. Hazen, A. Slungaard, Eosinophil peroxidase oxidation of thiocyanate - Characterization of major reaction products and a potential sulfhydryl-targeted cytotoxicity system, J. Biol. Chem. 276(1) (2001) 215-24.

[29] M.M. Lloyd, M.A. Grima, B.S. Rayner, K.A. Hadfield, M.J. Davies, C.L. Hawkins, Comparative reactivity of the myeloperoxidase-derived oxidants hypochlorous acid and hypothiocyanous acid with human coronary artery endothelial cells, Free Radic. Biol. Med. 65 (2013) 1352-62.

[30] J.D. Chandler, D.P. Nichols, J.A. Nick, R.J. Hondal, B.J. Day, Selective metabolism of hypothiocyanous acid by mammalian thioredoxin reductase promotes lung innate immunity and antioxidant defense, J. Biol. Chem. 288(25) (2013) 18421-8.

[31] Y. Xu, S. Szep, Z. Lu, The antioxidant role of thiocyanate in the pathogenesis of cystic fibrosis and other inflammation-related diseases, Proc. Natl. Acad. Sci. U.S.A. 106(48) (2009) 20515-9.

[32] B.A. Wagner, K.J. Reszka, M.L. McCormick, B.E. Britigan, C.B. Evig, C.P. Burns, Role of thiocyanate, bromide and hypobromous acid in hydrogen peroxide-induced apoptosis, Free Radic. Res. 38(2) (2004) 167-75.

[33] J.G. Wang, S.A. Mahmud, J. Nguyen, A. Slungaard, Thiocyanate-dependent induction of endothelial cell adhesion molecule expression by phagocyte peroxidases: a novel HOSCN-specific oxidant mechanism to amplify inflammation, J. Immunol. 177(12) (2006) 8714-22.

[34] C.L. Hawkins, D.I. Pattison, N.R. Stanley, M.J. Davies, Tryptophan residues are targets in hypothiocyanous acid-mediated protein oxidation, Biochem. J. 416(3) (2008) 441-52.

[35] C.L. Hawkins, P.E. Morgan, M.J. Davies, Quantification of protein modification by oxidants, Free Radic. Biol. Med. 46(8) (2009) 965-88.

[36] P. Eyer, F. Worek, D. Kiderlen, G. Sinko, A. Stuglin, V. Simeon-Rudolf, E. Reiner, Molar absorption coefficients for the reduced Ellman reagent: reassessment, Anal. Biochem. 312(2) (2003) 224-7.

[37] W. Chanput, J.J. Mes, H.J. Wichers, THP-1 cell line: an in vitro cell model for immune modulation approach, Int. Immunopharmacol. 23(1) (2014) 37-45.

[38] R.T. Dean, W. Hylton, A.C. Allison, Induction of macrophage lysosomal enzyme secretion by agents acting at the plasma membrane, Exp. Cell Biol. 47(6) (1979) 454-62.

[39] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29(9) (2001) e45.

[40] Y.M.W. Janssen-Heininger, B.T. Mossman, N.H. Heintz, H.J. Forman, B. Kalyanaraman, T. Finkel, J.S. Stamler, S.G. Rhee, A. van der Vliet, Redox regulation of signal transduction: Principles, pitfalls and promises, Free Radic. Biol. Med. 45(1) (2008) 1-17.

[41] M.D. Turner, B. Nedjai, T. Hurst, D.J. Pennington, Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease, Biochim. Biophys. Acta. 1843(11) (2014) 2563-82.

[42] Y.T. Yang, M. Whiteman, S.P. Gieseg, Intracellular glutathione protects human monocyte-derived macrophages from hypochlorite damage, Life Sci. 90(17-18) (2012) 682-8.

[43] Y.T. Yang, M. Whiteman, S.P. Gieseg, HOCl causes necrotic cell death in human monocyte derived macrophages through calcium dependent calpain activation, Biochim. Biophys. Acta. 1823(2) (2012) 420-9.

[44] A.J. Kettle, C.C. Winterbourn, Assays for the chlorination activity of myeloperoxidase, Meth. Enzymol. 233 (1994) 502-12.

[45] A.B. Weitberg, S.A. Weitzman, M. Destrempes, S.A. Latt, T.P. Stossel, Stimulated human phagocytes produce cytogenetic changes in cultured mammalian cells., N. Engl. J. Med. 308 (1983) 26-30.

[46] J.D. Chandler, B.J. Day, Biochemical mechanisms and therapeutic potential of pseudohalide thiocyanate in human health, Free Radic. Res. 49(6) (2015) 695-710.

[47] R.E. Gerszten, E.A. Garcia-Zepeda, Y.C. Lim, M. Yoshida, H.A. Ding, M.A. Gimbrone, Jr., A.D. Luster, F.W. Luscinskas, A. Rosenzweig, MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions, Nature 398(6729) (1999) 718-23.

[48] M. Cesari, B.W. Penninx, A.B. Newman, S.B. Kritchevsky, B.J. Nicklas, K. Sutton-Tyrrell, S.M. Rubin, J. Ding, E.M. Simonsick, T.B. Harris, M. Pahor, Inflammatory markers and onset of cardiovascular events: results from the Health ABC study, Circulation 108(19) (2003) 2317-22.

[49] M.J. Morgan, Z.G. Liu, Crosstalk of reactive oxygen species and NF-kappaB signaling, Cell Res. 21(1) (2011) 103-15.

[50] J.W. Pierce, R. Schoenleber, G. Jesmok, J. Best, S.A. Moore, T. Collins, M.E. Gerritsen, Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo, J. Biol. Chem. 272(34) (1997) 21096-103.

[51] B. Hoesel, J.A. Schmid, The complexity of NF-kappaB signaling in inflammation and cancer, Mol. Cancer 12 (2013) 86.

[52] H.J. Forman, M. Torres, Redox signaling in macrophages, Mol. Aspects Med. 22(4-5) (2001) 189-216.

[53] S. Dominici, L. Pieri, A. Paolicchi, V. De Tata, F. Zunino, A. Pompella, Endogenous oxidative stress induces distinct redox forms of tumor necrosis factor receptor-1 in melanoma cells, Ann. N. Y. Acad. Sci. 1030 (2004) 62-8.

[54] H.Z. Ozsoy, N. Sivasubramanian, E.D. Wieder, S. Pedersen, D.L. Mann, Oxidative stress promotes ligand-independent and enhanced ligand-dependent tumor necrosis factor receptor signaling, J. Biol. Chem. 283(34) (2008) 23419-28.

[55] J.G. Wang, S.A. Mahmud, J.A. Thompson, J.G. Geng, N.S. Key, A. Slungaard, The principal eosinophil peroxidase product, HOSCN, is a uniquely potent phagocyte oxidant inducer of endothelial cell tissue factor activity: a potential mechanism for thrombosis in eosinophilic inflammatory states, Blood 107(2) (2006) 558-65.

[56] Y. Fan, J. Dutta, N. Gupta, G. Fan, C. Gelinas, Regulation of programmed cell death by NF-kappaB and its role in tumorigenesis and therapy, Adv. Exp. Med. Biol. 615 (2008) 223-50.

Table 1: Primer sequences used for qPCR

Gene	Forward sequence	Reverse sequence	NCBI Reference Sequence
18S	5′ - GAG GAT GAG GTG GAA CGT GT-3′	5′ - TCT TCA GTC GCT CCA GGT CT-3′	NM_022551.2
β2M	5′ -AGA TGA GTA TGC CTG CCG TG-3′	5′ - GCG GCA TCT TCA AAC CTC CA-3′	NM_004048.2
MCP-1	5′ - TTG GGT TTG CTT GTC CAG GT-3′	5′ - AGC CAC CTT CAT TCC CCA AG-3′	X14768.1
IL-8	5′ - TCT GCA GCT CTG TGT GAA GG-3′	5′ - TTC TCC ACA ACC CTC TGC AC-3′	NM_000584.3
TNF-α	5′ - AAC CTC CTC TCT GCC ATC AA-3′	5′ - CCA AAG TAG ACC TGC CCA GA	NM_000594.3
IL-6	5′ - CCA GAG CTG TGC AGA TGA GT-3′	5′ - AGC TGC GCA GAA TGA GAT GA-3′	M54894.1
IL-1β	5′ - CAG GCT GCT CTG GGA TTC TC-3′	5′ - GTC CTG GAA GGA GCA CTT CAT-3′	NM_000576

FIGURE LEGENDS

Figure 1 – HOSCN is consumed by THP-1 macrophages resulting in loss of intracellular thiols and viability. (A) THP-1 macrophages (1 x 10^6 cells) were exposed to HOSCN (250 μM) for 15 min - 1 h at 37 °C with the concentration of HOSCN in supernatant (HBSS) determined in the presence (solid line) and absence of cells (dotted line) using the TNB assay. (B) Intracellular thiol levels following 1 h incubation of THP-1 macrophages with HOSCN (25 – 250 μM; black bars) compared to untreated cells (white bar) and decomposed HOSCN (De; grey bar) using the Thioglo assay. (C) Cell viability was determined by LDH release on exposure of THP-1 macrophages to HOSCN (0 – 250 μM) or decomposed HOSCN (De) for 1 h (white bars) and at 24 h post exposure to HOSCN (black bars). Data represent 3 independent experiments performed in triplicate. *, ** and *** show a significant ($p < 0.05$, 0.01, and 0.001, respectively) difference compared to non-treated controls using either a one-way ANOVA with Dunnett's post-hoc test or two-way ANOVA with Bonferroni *post-hoc* test.

Figure 2 – Exposure of THP-1 macrophages to HOSCN increases inflammatory cytokine and chemokine mRNA expression. THP-1 macrophages (1 x 10^6 cells) were exposed to HOSCN (0 – 250 μM) or decomposed HOSCN (De) for 1 h at 37 °C before determination of mRNA expression of (A) MCP-1, (B) IL-6, (C) IL-8, (D) TNFα and (E) IL-1β, following re-incubation in complete media for 3 h (white bars), 6 h (grey bars) or 24 h (black bars) by qPCR. Data are normalised to the expression of 18S and β2M house-keeping genes, and represented as the fold change compared to the decomposed HOSCN (De) treatment for 3 individual experiments performed in triplicate. *, **, *** and **** show a significant ($p < 0.05$, 0.01, 0.001, 0.0001, respectively) difference compared to respective non-treated controls using a two-way ANOVA with Tukey's *post-ho*c test.

Figure 3 – Exposure of THP-1 macrophages to HOSCN increases inflammatory cytokine and chemokine secretion. THP-1 macrophages (1 x 10^6 cells) were exposed to HOSCN (250 μM) for 1

h at 37 °C before determination of (A) MCP-1, (B) IL-6, (C) IL-8 and (D) TNFα in the cellular supernatants following re-incubation in complete media 24 h by ELISA. Data represent 3 individual experiments performed in triplicate. * and ** show a significant ($p < 0.05$ and 0.01, respectively) difference compared to respective controls by a one-tailed t-test.

Figure 4 – HOSCN-induced THP-1 macrophage cytokine/chemokine expression and cell death occurs through NF-κB activation. (A) The extent of cytosolic (white bars) and nuclear (black bars) accumulation of the p65 subunit of NF-κB was assessed following 1 h exposure to 250 μM HOSCN; inset: representative images of Western blot images analysed. (B) The extent of THP-1 macrophage mRNA expression of MCP-1, IL-6, IL-8, TNFα and IL-1β was assessed by qPCR with (grey bars) or without (black bars) pre-treatment with BAY117085 (20 μM for 30 min). (C) THP-1 cell death as indicated through the extent of LDH release at 24 h following exposure to 250 μM HOSCN with and without pre-incubation of cells with BAY117085 (20 μM for 30 min). Data represent at least 3 individual experiments performed in triplicate. * and *** show a significant ($p < 0.05$, and 0.001, respectively) difference in the presence and absence of NF-κB inhibition by two-way ANOVA with Bonferroni's *post-hoc* test.

Figure 1

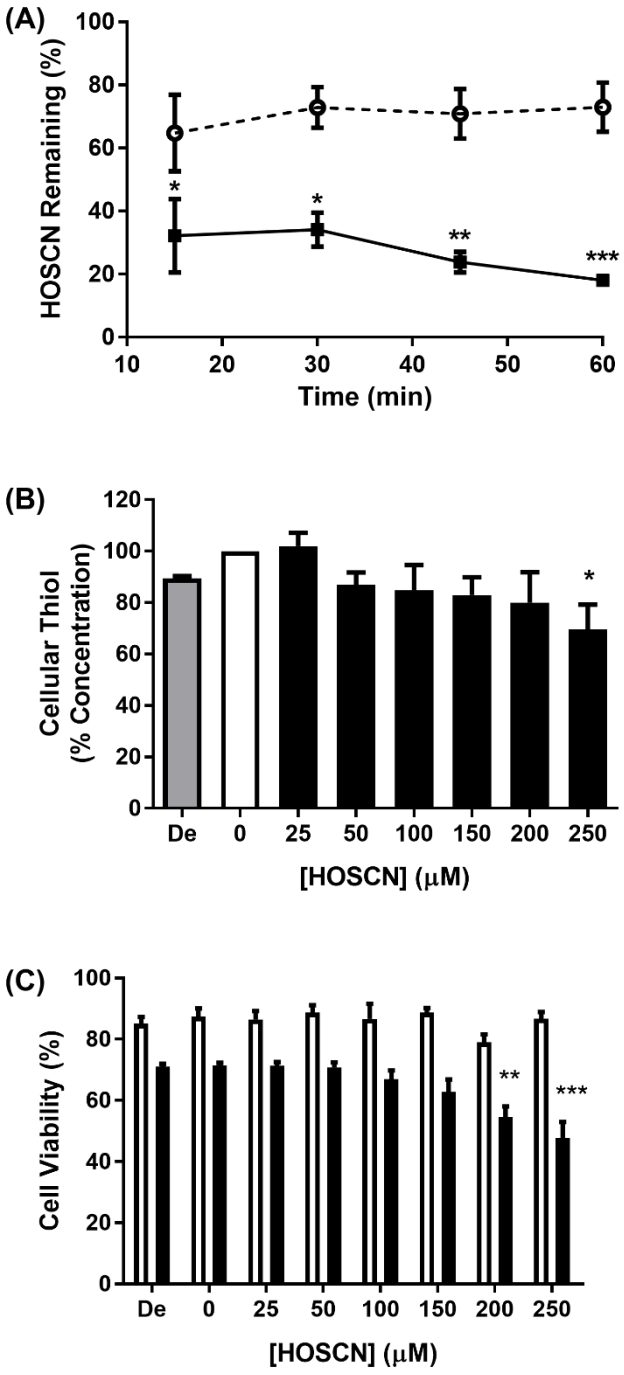

(A)
HOSCN Remaining (%)
100
80
60
40
20
0
10
20
30
40
50
60
Time (min)
*
*
**
***
(B)
Cellular Thiol
(% Concentration)
120
100
80
60
40
20
0
De
0
25
50
100
150
200
250
[HOSCN] (μM)
*
(C)
Cell Viability (%)
100
80
60
40
20
0
De
0
25
50
100
150
200
250
[HOSCN] (μM)
**
***

Figure 2

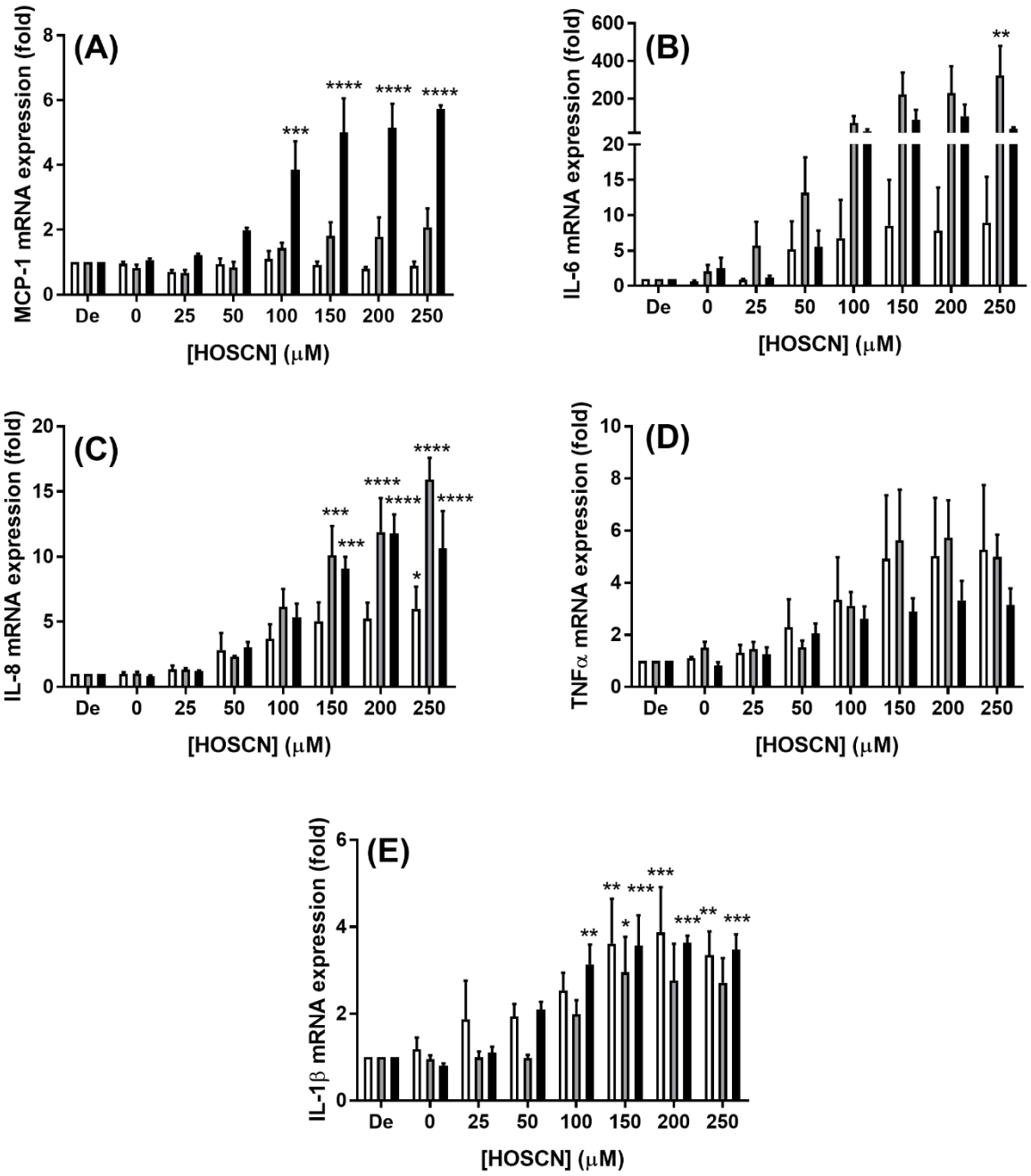

(A)
MCP-1 mRNA expression (fold)
[HOSCN] (μM)
De 0 25 50 100 150 200 250
(B)
IL-6 mRNA expression (fold)
[HOSCN] (μM)
(C)
IL-8 mRNA expression (fold)
[HOSCN] (μM)
(D)
TNFα mRNA expression (fold)
[HOSCN] (μM)
(E)
IL-1β mRNA expression (fold)
[HOSCN] (μM)

Figure 3

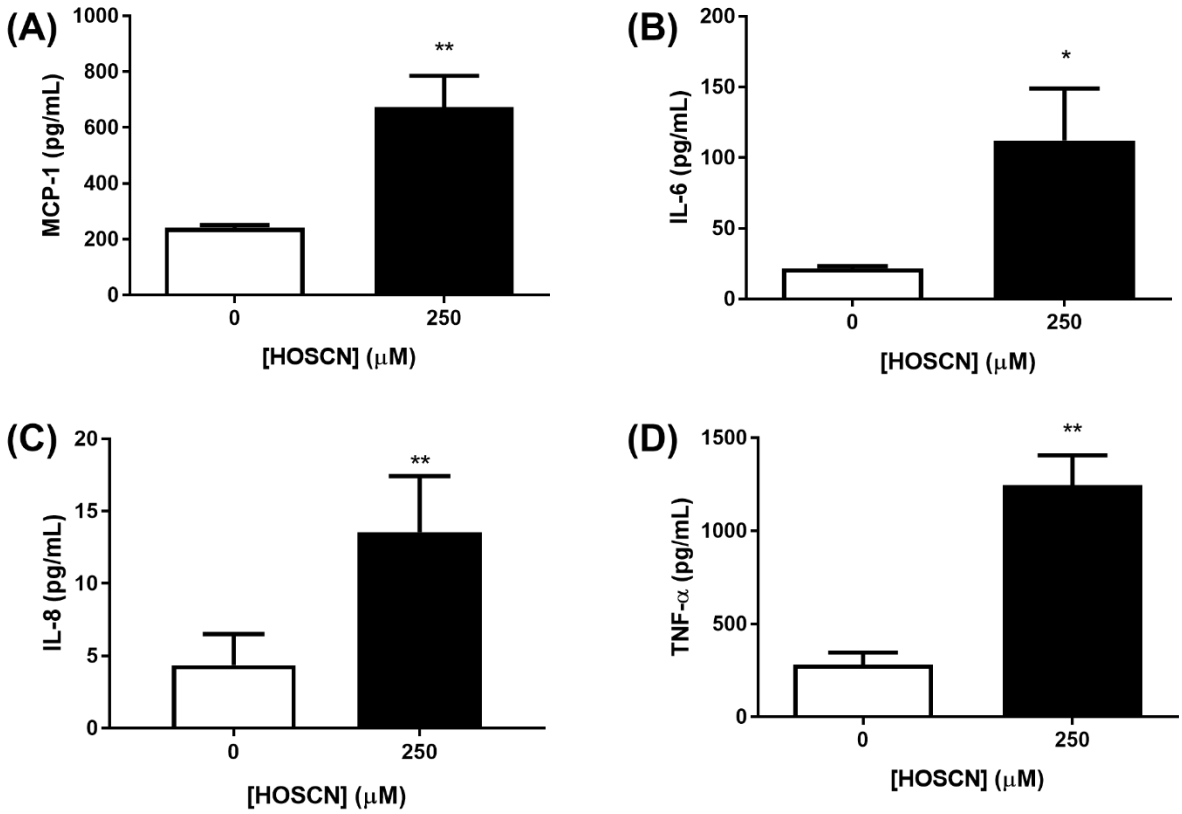
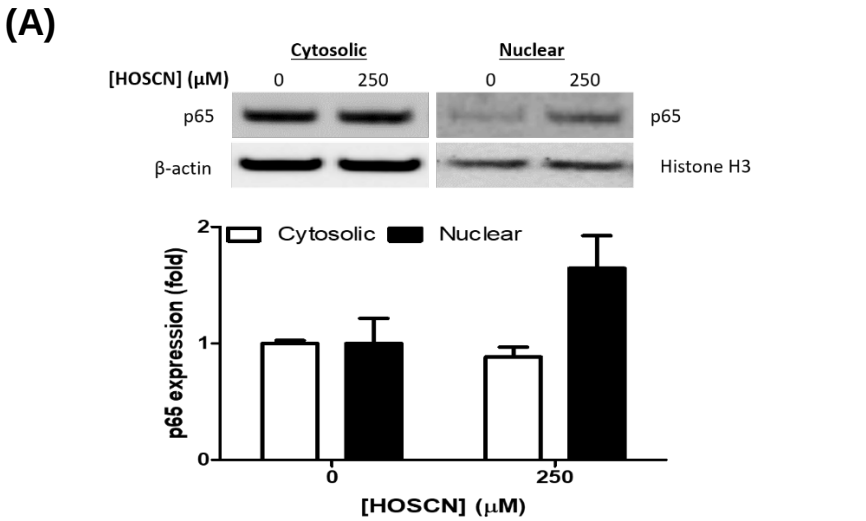
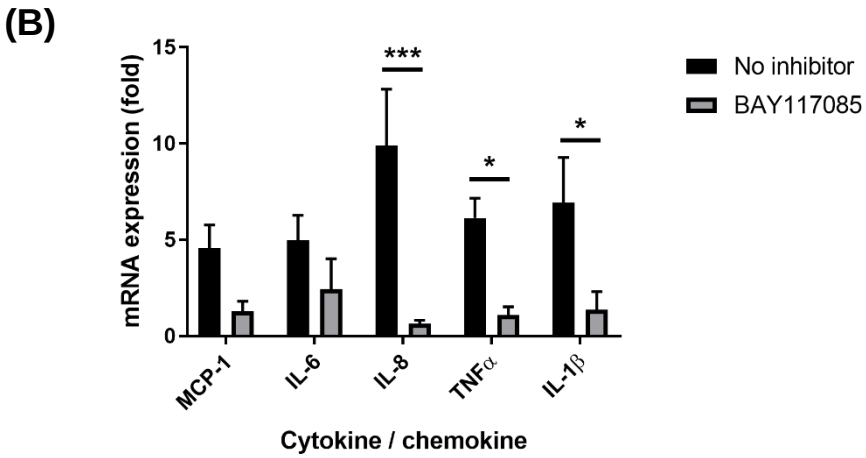
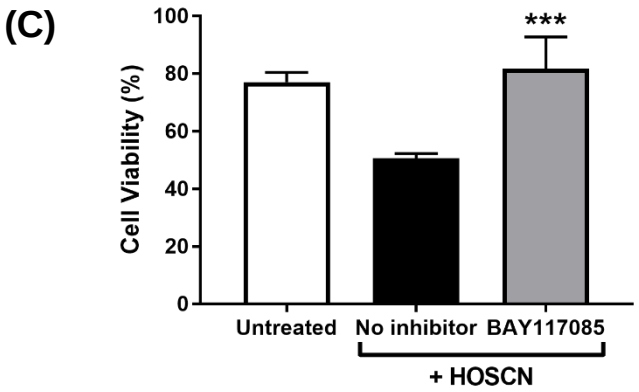

(A)
MCP-1 (pg/mL)
1000
800
600
400
200
0
**
0
250
[HOSCN] (μM)
(B)
IL-6 (pg/mL)
200
150
100
50
0
*
0
250
[HOSCN] (μM)
(C)
IL-8 (pg/mL)
20
15
10
5
0
**
0
250
[HOSCN] (μM)
(D)
TNF-α (pg/mL)
1500
1000
500
0
**
0
250
[HOSCN] (μM)

Figure 4


(A)
Cytosolic
Nuclear
[HOSCN] (μM)
0
250
0
250
p65
β-actin
p65
Histone H3
Cytosolic
Nuclear
p65 expression (fold)
2
1
0
0
250
[HOSCN] (μM)


(B)
mRNA expression (fold)
15
10
5
0
***
*
*
No inhibitor
BAY117085
MCP-1
IL-6
IL-8
TNFα
IL-1β
Cytokine / chemokine


(C)
Cell Viability (%)
100
80
60
40
20
0
***
Untreated
No inhibitor
BAY117085
+ HOSCN