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Review

Hypochlorous acid generated in the heart following acute ischaemic injury promotes myocardial damage: a new target for therapeutic development

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ABSTRACT

Acute myocardial infarction (AMI) results from an occlusive thrombus physically blocking a coronary vessel supplying the heart with oxygenated blood. Myocardial ischaemia leads to enhanced oxidative stress and irreversible damage to the affected myocardium. In response to myocardial ischaemia, cardiac myocytes undergo a cascade of signalling events including the recruitment of inflammatory cells such as neutrophils. Activated neutrophils cause interstitial fibrosis and promote secondary cellular damage through unregulated production of potent oxidants such as hypochlorous acid (HOCl), which in turn may play a role in promoting cardiac remodelling and subsequent cardiac dysfunction. Understanding the molecular mechanisms underlying the recruitment and activation of inflammatory cells following myocardial infarction and subsequent impact of inflammatory processes on the myocardium is important due to the potential for these processes to promote heart failure. This review highlights key changes to signalling proteins subsequent to myocardial inflammation after AMI, and will focus discussion on oxidant species generated in the myocardium during inflammation and how these oxidants impact on signal transduction cascades in cardiac myocytes and finally discusses the development of inhibitors that diminish oxidant production by activated neutrophils by targeting neutrophil myeloperoxidase.

KEYWORDS: ischaemia, inflammation, neutrophil, myeloperoxidase, heart failure

INTRODUCTION

Pathogenesis of acute myocardial infarction

Clinical overview

Ischaemic heart disease (IHD) is a clinical term used to indicate that blood flow to the heart through the coronary arteries is insufficient to supply the heart muscle cells (termed cardiac myocytes) with oxygen and nutrients and is summarised as "inadequate coronary perfusion relative to myocardial demands" [1]. Acute myocardial infarction (AMI) is the final manifestation of a progressive coronary artery disease (CAD) disturbing the blood flow to the level or threshold of severity or duration that can affect the function or viability of cardiac myocytes [2]. Heart attack can be broadly defined as heart tissue damage (death) due to a period of sufficient ischemia (blood flow loss) with or without the restoration of blood flow to the affected myocardium (termed ischemia reperfusion (I/R) injury) [1].

Major improvement in the prevalence and prognosis of IHD in the last 50 years is largely related to the modulation of disease (atherosclerosis) risk factors

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rather than developing pharmacological approaches to treatment. In this regard, clinicians have recently started to evaluate the importance of risk factors for IHD with a different vision. For example, one focus is an understanding of the metabolic syndrome involved in changing the pathology of the heart before CAD reaches levels that can affect the blood flow through the coronary system [3]. A deep understanding of the factors that are linked to IHD along with careful exploration of the information obtained from experimental models is critical to optimise research outcomes in terms of developing new pharmacological agents.

Myocardial inflammation following AMI

Neutrophil infiltration

Polymorphic neutrophils (PMN) are the innate front-line response to infections and/or damaged tissue and are highly specialised for the destruction of microorganisms through mechanisms involving phagocytosis or release of anti-microbial agents [4]. After AMI necrotic myocytes secrete cytokines such as tumour necrosis factor-α (TNFα) and a range of interleukins (IL), including the neutrophil-activating factor IL-8 [5]. It has been shown that recruitment of neutrophils to the site of ischaemic damage occurs within 24 h of myocardial infarction [6]. This inflammatory response ultimately establishes a platform for healing; neutrophils secrete collagen and fibrin to form scar tissue through a process of fibrosis. In addition, apoptotic neutrophils promote inflammatory cascades that stimulate cardiac repair [7] suggesting that neutrophils play key roles in all phases of myocardial inflammation.

Target necrotic cells become coated by opsonins (generally complement and/or specific antibodies), which in turn bind to specific receptors on the neutrophil surface to increase the level of activation and stimulate phagocytosis [8, 9]. The membrane of the phagocyte invaginates and incorporates the target cell or debris into an intracellular phagosome. This is accompanied by a burst of oxygen consumption that corresponds to production of cytotoxic reactive oxygen species (ROS) within the phagosome [8]. In addition, enzymes released by neutrophil degranulation, such as myeloperoxidase (MPO), have the potential to generate potent oxidants in the extracellular milieu [10]; chief amongst these oxidants is hypocholorous acid (HOCl).

Prognostic implications of neutrophils and MPO

Peripheral leukocyte counts have important prognostic implications as elevated neutrophil levels (neutrophilia) are associated with a poor prognosis in CAD [10-13]. Unregulated inflammatory responses derived from the activation of neutrophils have been described following AMI [14-16] and in patients with peripheral neutrophilia [17]. Furthermore, plasma concentrations of MPO post-myocardial infarction can predict patient mortality [18].

Activated neutrophils both produce and release ROS and other low-molecular weight fatty acid derivatives such as leukotrienes that can potentially damage healthy cells and tissues [19, 20]. While measurement of MPO levels may have a prognostic relationship with CAD, MPO fails to provide incremental information for patients when compared to sensitive troponin [21], the current gold standard for assessing AMI in the clinical setting. However, a recent review still argues for MPO as a protagonist of inflammation and cardiovascular disease [22].

Interestingly, the extent of neutrophilia may be an indicator of post-AMI congestive heart failure [17]. Elevation of neutrophil levels in the coronary circulation is an independent risk factor for acute coronary events including unstable angina and myocardial infarction. This is potentially related to the ability of MPO-derived HOCl to activate proteolytic enzymes secreted by activated neutrophils and to elevate expression of adhesion factors [23-25] that further increase neutrophil adhesion to the vascular endothelium. This phenomenon is regulated by taurine, the physiological antagonist of HOCl [26, 27]. Collectively these data suggest that neutrophil-derived MPO and HOCl may contribute to pathological changes associated with IHD.

Impact of myeloperoxidase-H_2O_2-halide system on the myocardium after AMI

The available evidence suggests that MPO contributes to cardiovascular disease by generating ROS, which are implicated in endothelial dysfunction,

plaque rupture, and ventricular remodelling post-AMI [28-31]. Furthermore, elevated MPO is associated with progression of heart failure [32, 33]. Thus, a clinical study by Mocatta *et al.* [18] revealed that plasma MPO levels were increased in patients after AMI (MPO median: 55 ng/ml in AMI group vs. 39 ng/ml of control) and that an above-median MPO level was an independent marker of 5-year mortality (above-median MPO mortality: 21% vs. below-median MPO mortality: 10%, $p = 0.001$) [18]. Indeed, it has been suggested that pharmacological inhibition of polymorphonuclear leukocyte activation (including the modulation of MPO activity) may benefit patients with AMI [34].

Circulating MPO levels are low in AMI patients, suggesting that the MPO detected is derived from neutrophil degranulation [35]. Also, MPO differs both structurally and functionally from other peroxidases present in eosinophils [36, 37] and therefore, the presence of MPO or markers of MPO activity in the myocardium (such as 3-chlorotyrosine) is representative of activated neutrophils. Although MPO-positive granules are also enclosed within human monocytes, the number of granules in monocytes is fewer than within neutrophils.

MPO - mechanism of action

Myeloperoxidase primarily oxidises peroxidase substrates to radical intermediates. Hydrogen peroxide (H_2O_2) is an essential requirement for the MPO system as peroxide is utilised by the enzyme to oxidise halide and pseudo-halide ions, thereby generating the corresponding hypohalous acids (Figure 1). The sources of H_2O_2 may vary but in the setting of inflammation, H_2O_2 is commonly produced from superoxide radical anion ($O_2^{-\bullet}$), which is generated by activated phagocytes during the "respiratory burst". The enzyme responsible for $O_2^{-\bullet}$ production is a membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The main function of NADPH oxidase is to catalyse the one-electron reduction of oxygen to $O_2^{-\bullet}$ consuming the cofactor NADPH in the process (equation 1).

$$NADPH + O_2 \rightarrow O_2^{-\bullet} + NADP^+ + H^+ \quad (1)$$

Under normal physiological conditions, $O_2^{-\bullet}$ equilibrates with its protonated form, the per-hydroxyl radical ($HO_2^{\bullet}$) with dissociation constant p*K*a = 4.8. Thus, at physiological pH, the radical is mostly present as the non-protonated form $O_2^{-\bullet}$ [8, 38]. Although $O_2^{-\bullet}$ mainly reacts as a reductant, it can also act as an oxidant where it accepts electrons and is converted to H_2O_2. The latter reaction is favourable at lower pH including the acidic conditions encountered in the myocardium after AMI. However, reaction between $O_2^{-\bullet}$ and superoxide dismutase (SOD) catalyses the formation of $O_2^{-\bullet}$ and H_2O_2 through the interaction of two $O_2^{-\bullet}$ molecules via a dismutation process where one $O_2^{-\bullet}$ molecule is reduced and another is oxidised simultaneously. Soluble enzyme systems like xanthine oxidase represent an alternate source of ROS in ischaemia/reperfusion injury yielding both H_2O_2 and $O_2^{-\bullet}$.

The haem protein MPO forms two main complexes to generate hypohalous acids upon reaction with $O_2^{-\bullet}$ and H_2O_2 [39]. In the presence of excess H_2O_2 the haem iron forms complex I that catalyses the two-electron reduction of a halide ion (*e.g.*, Cl^- or Br^-) to form the corresponding hypohalous acid while the enzyme cycles to reform resting MPO (Figure 1).

Complex I also reacts with H_2O_2, leaving an inactive complex II that does not react further with halide ions. Nonetheless, MPO complex II can be reduced to either MPO complex I or the rest-state MPO with an additional $O_2^{-\bullet}$ molecule. Therefore, $O_2^{-\bullet}$ maintains MPO in an active form in the presence of excess H_2O_2, optimising MPO-dependent hypohalous acid production and potentiating oxidative damage at inflammatory sites. On this basis SOD is considered a physiological regulator for MPO activity [40-42]. The halide and pseudo-halide ions predominantly oxidised by MPO are chloride ion (Cl^-), bromide ion (Br^-) and thiocyanate ion (SCN^-), which generate hypochlorous acid (HOCl), hypobromous acid (HOBr) and hypothiocyanous acid (HOSCN), respectively. These potent oxidants produced by MPO under physiological conditions react with a wide range of biological target molecules resulting in the oxidation of thiol residues and/or methionine residues within amino acids, halogenation of tyrosine residues and depletion of tissue antioxidants and their cofactors [43-46].

Notably, MPO has widely varying specificity constants for these anions, with relative values for

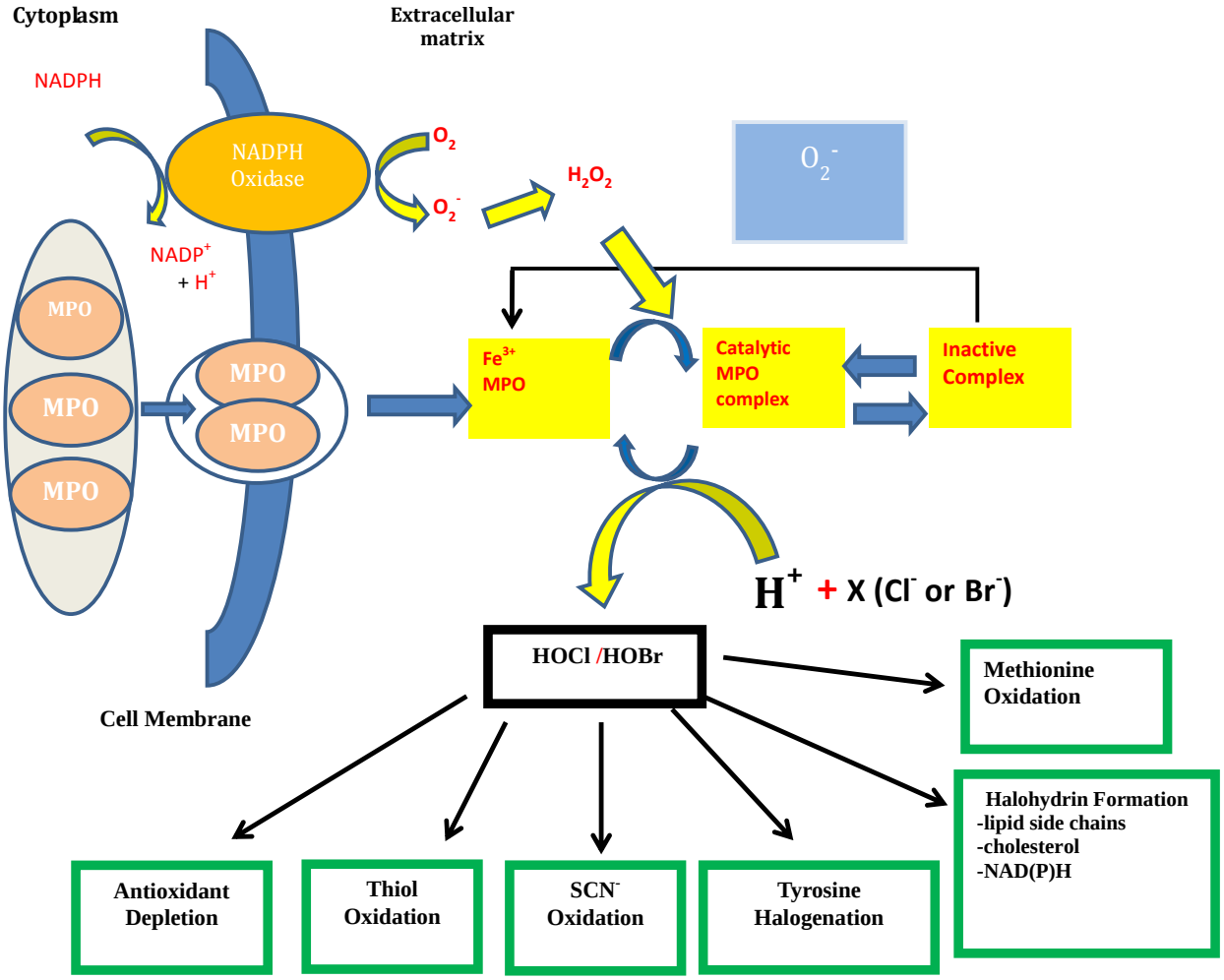

Figure 1. A summary scheme to illustrate the MPO/H_2O_2/Cl enzymic system. The schematic illustrates the catalytic subunits involved in the production of the various hypohalous acids indicated. The concentration of hypohalous acid produced will be dependent on the level of the concentration of the halide in the local environment and the extent of infiltrating cells (containing the enzyme MPO) and their level of activation.

Cl^-, Br^- and SCN^- being 1:60:730, respectively. However, Cl^- is present at the highest concentration in plasma (100–140 mM) compared to the concentration of Br^- (20–100 µM) and SCN^- (20–250 µM) [47], and this impacts on the kinetics of their reactions with MPO. Notably, diet and smoking can modulate circulating SCN^- concentrations [46]. The preponderance of Cl^- results in approximately 45% of the H_2O_2 consumed by MPO being used for the formation of HOCl. Therefore, HOCl is one of the most biologically important components of MPO-mediated cell damage during the inflammatory response. Neutrophil-derived HOCl is known to play an integral part in the innate immune system due to its antibacterial properties [48]. Yet, unregulated production of this potent oxidant may lead to uncontrolled tissue damage and determination of a suitable surrogate biomarker to indicate this damage has been the focus of much recent research [49-52].

The p*K*a of HOCl is 7.53 and therefore HOCl exists as an equilibrium mixture of the acidic and conjugate base (hypochlorite ion; OCl^-) at physiological pH. However, HOCl begins to dominate within a phagosome or the ischaemic heart where the pH is lower due to acidosis [53]. Under these conditions, the molecule reacts with

the abundant Cl^- to generate molecular chlorine (Cl_2), which reacts with oxidisable protein amino acid groups such as sulfhydryl groups, iron-sulfur centres, sulfur-ether groups, haem moieties and unsaturated fatty acids [54].

In humans, circulating PMNs are present at 1.5-6 x 10^6 cells/mL, and at this density activated cells generate between 0.08 to 0.48 mM H_2O_2 per hour [41]. Another potentially relevant source of H_2O_2 is activated monocytes. Notably, the enhanced rates of H_2O_2 production by cells are generally matched by increased rates of consumption, so that the steady-state level remains relatively low and does not impact on cell viability. However, in patients with coronary artery disease, plasma MPO concentrations range from 0.36–0.52 μM [55] and the rate constant for the reaction between MPO and H_2O_2 is ~10^7 $M^{-1}s^{-1}$ [56] suggesting that MPO competes with other proteins for H_2O_2 generated within the phagocyte and that local tissue concentrations of HOCl may be significantly elevated during an acute inflammatory response. Following AMI mononuclear cells are recruited to the affected myocardium, therefore HOCl concentrations are likely to be markedly elevated and HOCl may make a significant contribution to the oxidants produced within the affected myocardial tissues.

Neutrophils secrete MPO when activated and ~45% of H_2O_2 consumed in the MPO system is converted into HOCl, making HOCl a major source of oxidative stress in the period following AMI. Proteins are the major component of most biological systems and the rate constant for the reaction of HOCl with amino acid side chains is ~10-1000 times greater than reactions with lipids and some antioxidants [48]. In particular, HOCl is highly reactive toward cysteine (thiol) [56] and methionine [57] moieties. In addition, HOCl induces two-electron halogen-transfer reactions with amine groups, predominantly with lysine and arginine yielding reactive chloramines [58]. The impact of protein oxidation includes unfolding and conformational changes affecting both the protein structure and function.

Protein chloramines contain reactive nitrogen-chlorine bonds that mediate secondary oxidation reactions. In addition, HOCl reacts with amide groups on protein backbones, particularly sulfated glycosaminoglycans in the extracellular matrix [59] forming reactive chloramides that share many properties with chloramines including reactivity with other amino acids [58, 60]. Substitution reactions can take place between HOCl and histidine residues to yield short-lived N-chlorinated products. Other residues susceptible to oxidation by HOCl include tryptophan and tyrosine (the latter can also be chlorinated) and HOCl reacts with phospholipids at either the lipid groups or with unsaturated fatty acid side chains [58]. Potential biological marker compounds of HOCl-mediated damage include methonine sulfoxide, cysteic acids and di-tyrosine formation; however, these may be produced by other oxidants thus impacting on the specificity of these biomarkers [43, 49]. However, considering that the predominant product of MPO activity is HOCl, the presence of neutrophils and MPO within tissue can be considered as indirect surrogate markers for the presence/production of HOCl. The reaction of HOCl with biological molecules may result in loss of membrane transport and interruption of the electron transport chain, depletion of adenylate energy reserves and inhibited DNA synthesis.

Oxidative stress and protein damage during myocardial inflammation

Mitochondria are the primary physiological source of ROS, which are mostly produced by leakage of electrons from the redox reactions that comprise the electron transport chain to generate $O_2^{-\bullet}$ [58]. The superoxide radical anion contributes to the formation of many other ROS through various pathways – some involving H_2O_2 [58]. Another source of $O_2^{-\bullet}$ is the NADPH oxidase system that plays an important role in mediating the effects of angiotensin in vascular smooth muscle cells and contributes to ventricular myocyte growth signalling [61]. A further free radical species produced from vascular endothelium is nitric oxide ($^{\bullet}NO$). Its physiological role is in stimulating vasodilation and protection of the endothelium but excess $^{\bullet}NO$ can also contribute to the formation of bio-harmful peroxynitrite through rapid combination with $O_2^{-\bullet}$, which either alters or eliminates $^{\bullet}NO$ bioactivity on the physiological time-scale.

In biological systems, ROS play multiple roles with both beneficial and damaging activities reported.

An example of beneficial roles of ROS is their participation in angiotensin signalling system resulting in promotion of cell growth [58]. However, cytotoxic levels of ROS (typically inducing oxidative stress) can cause cellular apoptosis and necrosis. An imbalance may result from the over-production of free radicals or an inability to detoxify these ROS or repair the resulting oxidative damage. Once formed, ROS rapidly react with lipids, proteins and other biomolecules, causing changes and/or irreversible damage in the target molecule and disturbing physiological function [58, 62].

Unregulated ROS formation may be responsible for expanding myocardial injury post-AMI [63]. Free radicals produced during ischaemia reperfusion insult cause micro-vascular damage, which enhances neutrophil aggregation. This is followed by micro-vessel plugging by activated neutrophils that further impairs blood flow to the ischaemic zone. On the other hand, ROS may cause damage during AMI by stimulating changes to intracellular Ca^{2+} concentration and distribution [64]. Thus, ROS can affect and reverse the normal Na^{+}-Ca^{2+} exchange channels and play a role in the intracellular calcium increase [65]. ROS can also influence intracellular Ca^{2+} release as well as oxidise membrane lipids thereby increasing Ca^{2+} permeability; the concentration gradient favours flow of Ca^{2+} from extracellular space into the myocytes [66]. Subsequently, the increased Ca^{2+} concentration within myocytes leads to ATP depletion, increased mitochondrial ROS production and mitochondrial dysfunction.

Signalling cascades central to pathological changes in cardiac tissue after AMI

Acute damage to myocardial tissues post-AMI includes the activation of various signalling pathways, which stimulate cellular responses involving protein synthesis, enlargement of cells, cardiac fibrosis and cell death. Two well-characterised pathways include the G protein-coupled receptor (GPCR) and the mechanical stress induced pathways (Figure 2). In response to a pathological stimulus such as cardiac pressure overload and hypoxia, hormones and vasoactive factors such as angiotensin-II (Ang II), endothelin-1 (ET-1) and noradrenaline (NE) are released and act on GPCR to further activate the heterotrimeric G protein Gαq and its downstream signal transduction cascade [67-69].

The relationship between Gαq activation and cardiac growth has been reported in the literature. In animal studies, transgenic mice over-expressing Gαq developed pathological cardiac hypertrophy and showed premature death [70, 71]. In contrast, a study by Akhter *et al.* in 1998 reported that mice lacking G proteins in cardiac myocytes showed a blunted response against pressure overload in the absence of cardiac hypertrophy [72]. Among the ligands that interact with GPCR, Ang II plays a significant role in developing cardiac hypertrophy [73, 74] (Figure 2).

Angiotensin II is the principal vasoactive substance of the rennin-angiotensin system which acts in the cardiovascular system via vasoconstriction, aldosterone release and cell growth. It has been shown that blocking formation of Ang II attenuates pressure overload-induced hypertrophy in animal models and humans via inotropic effects that likely depend on endothelin receptor activation [75]. The predominant endothelin in the heart is ET-1, however, results from clinical trials of ET-1 antagonists have been largely disappointing with cardiac remodelling persisting in patients with heart failure [76].

The activation of GPCR by Ang II and the associated hypertrophy is related to activation of mitogen-activated protein kinases (MAPKs) [77, 78]. MAPKs participate in many cellular events including proliferation, cell death and acute hormonal responses. MAPKs are activated by a series of protein kinases in a cascade that involves at least two upstream kinases [79].

There are three main subfamilies of MAPKs, each differentiated based primarily on the terminal kinase in the cascade; extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinase (JNKs) and p38-MAPKs [79]. All three MAPKs are activated in response to GPCR agonists and mechanical stress as well as pressure overload in the heart [80]. Interestingly, authentic HOCl can stimulate mitogen-activated protein kinase pathways in endothelial cells [81] and this may involve ERK activation via the epidermal growth factor receptor [82]. To date a role for this HOCl-activated signalling pathway in the myocardium is unclear although it is anticipated that this signal cascade will be activated given that neutrophils are

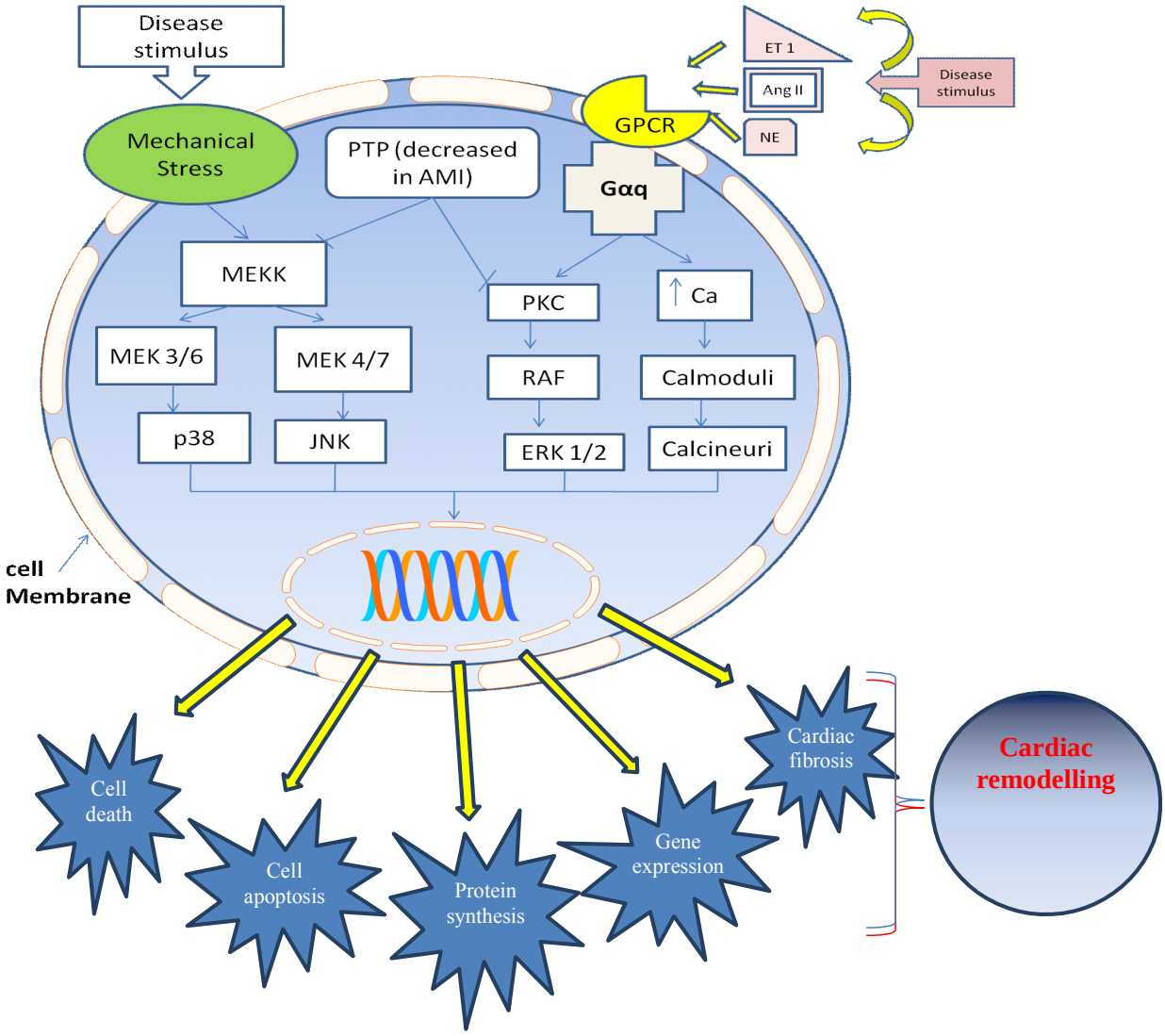

Figure 2. A schematic overview of signalling pathways, cellular response and cardiac function implicated in mediating pathological hypertrophy of heart (schematic figure adapted from the data presented in reference [80] and shows the key pathways that are relevant to HOCl-mediated activation). *Abbreviations:* Ang II = angiotensin II; ERK = extracellular signal-regulated kinase; ET-1 = endothelin-1; GPCR = G protein-coupled receptor; JNK = c-Jun amino-terminal kinase; MEKK = MAPK kinase kinase; MEK = MAPK kinase; NE = noradrenalin; PKC = protein kinase C; PTP = protein tyrosine phosphatase.

recruited to the damaged myocardium after AMI and are likely to produce HOCl.

Post-AMI fibrosis may also be related to p38 regulation of Ca^{2+} signal-mediated lipid accumulation through modulating vitamin D receptor expression [83] although such a relationship requires further substantiation in myocytes. Furthermore, p38 is an important component of stress response pathways [84] and is activated during ischaemia [80]. Activation of P38 by phosphorylation has also been implicated as an inducer of apoptosis [80]. Four isoforms of p38 (α, β, δ and γ) are known although only p38α and p38β are present in the human heart [85]. Extensive studies have been undertaken to identify the role of p38 in pathological cardiac hypertrophy but the results have been contradictory; p38 seems to have no effect on heart growth in some studies while other data suggests

that its presence is essential for the pathological hypertrophy [80].

Whether or not ERK1/2 is a critical mediator of cardiac remodelling is unclear. For example, the phosphorylation of the amino acid Thr188 in the sequence of ERK2 is implicated in developing cardiac hypertrophy [86]. Notably, Ang II, ET-1 and NE, all activate ERK1/2 and such activation is reportedly essential for cardiac hypertrophy [87]. Consistent with this notion, mutation of Raf-1, a signal molecule downstream of Gαq and upstream of p38 and ERK1/2, attenuates cardiac hypertrophy in mice [88]. On the other hand, transgenic mice over-expressing cardiac specific MEK1, which is the immediate upstream signal molecule for ERK1/2 activation, developed a physiological hypertrophy that was characterised by enhanced cardiac function and no interstitial fibrosis [89]; notably, MEK1 does not activate the MAPKs JNK or p38. These data implicate ERK1/2 in the pathogenesis of cardiac myocyte hypertrophy, whereas p38 activation is associated with cardiac hypertrophy primarily through stimulating fibrosis. Establishing precisely how HOCl activates ERK1/2 and p38 after AMI and what role this plays in cardiac remodelling subsequent to AMI requires further investigation.

Signal transduction is largely affected by phosphorylation of tyrosine, threonine and serine residues in mammalian cells [90]. Protein phosphorylation is generally reversible and is governed by the opposing activities of protein kinases and protein phosphatases (*e.g.*, protein tyrosine phosphatases, PTPs) [91]. The three subclasses of PTPs include: dual-specificity PTPs, low-molecular weight PTPs and tyrosine-specific PTPs. Tyrosine-specific PTPs only act to dephosphorylate at tyrosine within proteins. Dual-specificity PTPs are a heterogeneous group of protein phosphatases capable of dephosphorylating at serine and threonine residues in addition to tyrosine residues within the one substrate. Whereas, similar to tyrosine-specific PTPs, the family of low-molecular weight-PTPs primarily show specificity towards phosphotyrosine. Irrespective of specificity, all PTPs share an identical catalytic motif with a conserved cysteine residue in the active site [92, 93].

Protein phosphorylation and cell signalling after AMI

Several molecules released from necrotic cells are known to elicit inflammation including uric acid, purine metabolites ADP and even DNA when it is released into the cell cytoplasm and not sequestered in the nuclei [94]. Leukocytes have specialised receptors that recognise these molecules and trigger a signalling cascade that activates transcription factors such as NFκB, resulting in the production of an array of cytokines (*e.g.*, TNFα), which are involved in the inflammatory response as part of the innate immune response [94]. The cytokine TNFα, primarily sourced from macrophages and vascular endothelial cells, is an important mediator of inflammation as it increases expression of leukocyte adhesion molecules on the vascular endothelium. This process aids in the recruitment of other leukocytes including neutrophils. Moreover, TNFα participates in wound healing reactions by inducing fibroblast proliferation and thereby increasing collagen synthesis. In this sense, TNFα expressed during inflammation establishes a platform for healing by stimulating fibrosis.

Interestingly, PTP inhibitors (pervanadate and phenylarsine oxide) suppress NFκB nuclear translocation in transformed cell lines (U-937 and Jurkat) and primary fibroblasts (MRC-5 and REF) [95]. Alternately, alkaline phosphatase inhibition with nicotinamide [96] can attenuate phosphorylation of NFκB's inhibitory protein, regulate NFκB activation and abrogate inflammation [97]. Also, chloramines derived from the reaction between HOCl and amine groups within amino acids are known to stabilise IκB by increasing its resistance to degradation and thereby enhancing the inhibition of NFκB activity [57]. Macrophage derived HOSCN also inhibits protein tyrosine phosphatases and modulates cell signalling via the mitogen-activated protein kinase (MAPK) pathway [46]. These findings demonstrate that HOCl and other hypohalous acids produced by MPO can affect a broad range of cellular phosphatases and this may stimulate errant phosphorylation of a range of MAPK that will subsequently impact on cellular signalling in the damaged myocardium after AMI.

HOCl modification of cellular phosphatases

The available data implicates a role for cellular redox state in controlling PTP activity by either

reversible or irreversible oxidation of key catalytic cysteine residues [98-102]. The physiological role for PTP is to catalyse the degradation of its preferred substrate(s) by binding the electrophilic phosphorus atom with its thiolate nucleophile in the active site [103]. This results in the transfer of the phosphate group from the substrate to associated PTP enzyme and forms a thiol-phosphate intermediate species [104]. The catalytic cysteine residue of PTP has a pKa value of 5.5 and exists predominantly as thiolate under physiological pH, which increases its susceptibility towards oxidation. Potent oxidants and thiol-directed agents were shown to induce rapid phosphorylation of tyrosine in treated cells by inhibiting PTP activity [101, 102].

Reaction rates of selected amino acids with reagent HOCl at physiological pH indicate that thiol-containing amino acids were the most reactive, with the order of reactivity determined as Cys> Met > His > Ser >Leu [62, 105, 106]. In light of this, it was demonstrated that inactivation of PTP1B by oxidants resulted in the oxidative conversion of the catalytic cysteine into sulfinic acid (Cys-SO_2H), which was partially reversed by reducing agents such as hydroxylamine (NH_2OH) providing evidence for the presence of other oxidised forms of the catalytic cysteine (*e.g.*, sulfenic acid (Cys-SOH)). This has led to the proposal of a model of reversible oxidation/reduction of active cysteine residues where the essential active site of PTP is the specific target of oxidants that can regulate the enzymatic activity [107].

HOCl modulates intracellular signal transduction via MAPK

Mitogen-activated protein kinases (MAPKs) exist in all eukaryotic organisms and control diverse cellular processes including proliferation, differentiation, migration through a large number of downstream molecules [108] and apoptosis despite its somewhat simple cascade pathway [109]. Establishing the intracellular regulators of MAPKs is a current area of research focus. In particular, phosphatases play a role in controlling MAPK signalling. One study showed that phosphorylation of MAPK was markedly influenced by individual cellular phosphatases that were selectively depleted by siRNA [110]. Thus, phosphatases are capable of diverse modes of MAPK regulation. In particular, PTPs have been regarded as key regulators of MAPKs, which are vulnerable to ROS modification of catalytic cysteine residues. For example, endogenous production of ROS during Jurkat T cell activation enhances MAPK activity [111] and this may be due to a concomitant ROS-mediated decrease in PTP activity.

It has been suggested that activation of ERK and p38 is executed by phosphorylation of specific tyrosine and threonine residues [86, 112, 113]. In particular, ERKs act on their respective substrates mainly when they are phosphorylated at both regulatory tyrosine and threonine residues in the activation loop and the enzyme is quite inactive when unphosphorylated [114]. Whether regulation of MAPK is beneficial in the ischaemic myocardium and how oxidants such as HOCl may play a role in activating MAPKs in the heart after AMI also remains unclear.

Both ERK and p38 MAPKs have been implicated in the regulation of cardiac gene expression, cardiac myocyte apoptosis, hypertrophy and cardiac remodelling [80, 115-117]. Many studies have employed cardiac specific transgenic and knockout mouse models to elucidate the putative role for p38-MAPK in the pathologies of the heart. This includes a report indicating that cardiac response to pressure overload in a mouse model of dominant-negative form of p38α (*dn*; a mutation that removes the activation domain in a transcription factor leading to reduced levels of gene activation) showed significantly less myocardial fibrosis [118]. In the same study, neither *dn*-p38α nor *dn*-p38β transgenic models showed myocyte hypertrophy in response to pressure overload. In yet another study, transgenic mice with the *dn* form of MEK3 and MEK6, the immediate upstream regulators of p38, exhibited developed heart failure that again was not associated with cardiac hypertrophy [115]. Overall, the available data suggest that the p38-MAPK plays a role in pathological cardiac hypertrophy through a mechanism that includes interstitial fibrosis and loss of myocardial contractility. Furthermore, the p38 and ERK1/2 MAPK pathways may play opposing roles in the regulation of cardiac contractility and selective regulation of these pathways may be necessary to provide therapeutic benefit [119].

The ERK family of proteins phosphorylate a range of cytosolic and nuclear substrates and activation of ERK1/2 in the G protein-coupled receptor pathway is essential for protein synthesis in isolated cardiac myocytes [87]. Therefore, ERK induction may be necessary to compensate for decreased muscle cell number whereas the role of p38 in the recovery of heart after AMI is not so significant. Nonetheless, the importance of a balance between ERK and p38 in post-AMI cardiac remodelling and recovery is yet to be fully elucidated.

Importantly, as MAPK and PTP families are redox sensitive (see above) then the excessive production of HOCl in the myocardium after AMI can potentially modulate their signalling pathways. Indeed all PTP sub-types are typically susceptible to oxidation by various oxidants where the oxidation leads to inactivation of the enzyme [46]. In contrast, oxidation of MAPKs and the consequent Cys modification can activate the enzymes [120] leading to opposing effects on the converging pathways that regulate protein phosphorylation. Thus, neutrophils recruited to the myocardium immediately after AMI may release HOCl that stimulates the over-phosphorylation of MAPKs, which results in the promotion of myocyte apoptosis in the region surrounding the infarct core.

Pharmacologic inhibition of the MPO-H_2O_2-halide system

In light of the potential detrimental effects of MPO-mediated HOCl production in the myocardium post-AMI, researchers have focused much attention on the design of pharmacological inhibitors of MPO. Investigations have focussed on the inhibition of the MPO-halogenation cycle via competitive substrate inhibition. Compound I of MPO is unique due to its high two-electron reduction potential, enabling oxidization of halides (X^-), as well as pseudohalides [46] to their respective hypo(pseudo-)halous acids (HOX). However, a myriad of substrates (RH) can donate a single electron to compound I to yield an intermediate compound II, without the porphyrin π-cation radical, that is unable to react with (pseudo-)halides [121]. Compound II is further reduced to the ferric state by an additional one-electron transfer process by natural reductants. Physiological reducing substrates for compound I and compound II include urate, ascorbate, nitric oxide, nitrite, serotonin, superoxide, tyrosine and both phenol and indole derivatives [56, 122, 123]. Although transformation of compound I to compound II bypasses (pseudo-) halous acid production, free radicals are also produced when physiological substrates reduce compound II to ferric MPO. Therefore, the identification of substrates that abrogate hypo(pseudo-)halous acid production without concomitant release of free radicals are of paramount importance in curbing the deleterious effects of MPO *in vivo*.

2-Thioxanthines

Recently, 2-thioxanthines have been identified as suicide substrates of MPO-compound I [124]. The thioxanthine class of inhibitors are transformed to strongly oxidising radicals that react with the haem prosthetic group of MPO and irreversibly modify the haem moiety. Hence, 2-thioxanthines are able to prevent HOCl production without subsequent release of free radicals. Two thioxanthine compounds, 3-[(4-fluorophenyl) methyl]-2-thioxo-7H-purin-6-one (TX2) and 3-[[(2R)-tetrahydrofuran-2-yl]methyl]-2-thioxo-7H-purin-6-one (TX4) are documented for their ability to inhibit chlorination reactions involving HOCl produced by MPO, by a mechanism that precludes conversion of the activated MPO-compound I to compound II [124]. Furthermore, 3-isobutyl-2-thioxo-7H-purin-6-one (TX1) reacts directly with MPO-compound I with a rate constant sufficiently large (6.8×10^5 $M^{-1}s^{-1}$) to effectively compete with the reaction between MPO and chloride (2.5×10^4 $M^{-1}s^{-1}$) [125]. In an inflammatory model of peritonitis, treatment with 3-(tetrahydrofuran-2-ylmethyl)-2-thioxo-7H-purin-6-one (TX3) decreased the ratio of 3-chlorotyrosine/ parental tyrosine and inhibited formation of glutathione sulphonamide [126] suggesting it may be a useful inhibitor of MPO activity. Despite some promise as an efficacious antioxidant/MPO inhibitor that is known to prevent two-electron oxidation events mediated by HOCl, further research is required to completely identify the therapeutic potential of 2-thioxanthines in pre-clinical models of acute and chronic inflammation.

Indole derivatives

Research that focused on exploiting the atypical thermodynamic properties of MPO resulted in the selection of a tryptamine derivative

5-fluorotryptamine, as a reversible MPO inhibitor [127]. This aromatic one-electron reducing agent binds to the hydrophobic pocket within the distal haem cavity of the homodimer and is oxidised efficiently by MPO-compound I. The metabolically stable halide at position 5 of the indole ring renders 5-fluorotryptamine an efficient reversible inhibitor [127]. However, the indole moiety is prone to cleavage between positions C-2 and C-3 during substitution by electron-withdrawing groups and this promotes its metabolism *in vivo*. Employing *in silico* structure-based docking to screen active compounds, researchers designed a series of 3-(aminoalkyl)-5-fluoroindole analogues with potential additional interactions with the haem moiety in MPO [128]. These compounds showed improved efficacy for inhibiting MPO by acting as MPO-compound I (oxidisable) substrates that promote the accumulation of MPO-compound II, thereby shifting MPO from its halogenation cycle to the peroxidase cycle; that is inhibiting HOCl production while promoting H_2O_2 consumption, which may have multiple beneficial effects during inflammation.

Specificity for MPO may be an issue for these compounds as these inhibitors are structurally similar to serotonin (5-hydroxytryptamine, 5HT) and inhibit 5HT uptake via the SERT transporter at nM levels [128, 129]. The recent design and synthesis of 3-alkylindole derivatives featuring potent affinity for the active site of MPO but not for SERT may overcome this shortcoming [128] and may prove useful in future structure-function approaches to the design of enhanced MPO inhibitors. For example, kinetic studies with several 3-alkylindole derivatives of highest MPO inhibitory potency showed that these molecules rapidly react with compound I, while reacting very slowly with compound II [128, 129]. Notably, toxicological tests with the 3-alkylindole derivatives indicate that these drugs are tolerated during acute exposures prompting several investigations using *in vivo* models of acute I/R injury, as well as models of chronic inflammatory disorders to evaluate the therapeutic efficiency of this class of inhibitors [128, 130-131].

The ferulic acid derivative INV-315

A low-molecular weight inhibitor of MPO (INV-315) was recently synthesised and validated by Rajagopalan, S. and colleagues [132]. Dietary administration of INV-315 inhibited MPO-mediated nitrotyrosine formation in a mouse model of atherosclerosis and improved endothelial function [132]. The cardio-protective effects of high-density lipoprotein (HDL) are proposed to be abrogated via MPO-mediated oxidation of ApoA-1, a major apolipoprotein of HDL, resulting in decreased reverse cholesterol efflux from cells through an impaired recognition by the membrane receptor ABCA1 [133]. Interestingly, mice treated with INV-315 showed enhanced cholesterol efflux, possibly due to inhibition of HDL oxidation via attenuated MPO activity. However, no evidence of modification of lipoprotein oxidation in response to INV-315 was demonstrated in this study [132]. Chronic administration of INV-315 was associated with reduced $CD11b^{+}$ $Ly6G^{low}$ $7/4^{hi}$ monocytes in atherosclerotic lesions. A decrease in this subset of monocytic cells has previously been associated with favourable end-points including regression of atherosclerotic lesions and macrophage accumulation [134]. Additional evidence of MPO inhibition has been attributed to a decreased adherence of inflammatory leukocytes in response to stimulation with TNFα and a concomitant reduction in IL-6 [132]. Presently further research is required to provide additional evidence for the specificity of these inhibitors including *in vivo* functionality and regulation of MPO-mediated modifications to HDL in acute and chronic models of inflammation.

Nitroxides

Nitroxides are generally cell-permeable stable free radicals that have been documented widely for their antioxidant properties and relatively low toxicity [135]. For example, the hydrophilic cyclic nitroxide TEMPOL inhibits oxidative damage *in vitro* and *in vivo* in a dose-dependent manner [136-138]. Nitroxides are also susceptible to one-electron reduction by antioxidants such as vitamin E [139], in which subsequent recycling occurs via the corresponding oxammonium cation ($TPNO^{+}$) and hydroxylamine derivatives (TPNOH). However, nitroxides are eventually consumed by radicals (in bimolecular coupling processes) and/or metabolised [140-142].

Several potential mechanisms have been described to identify the underlying protection

provided by nitroxides. Previously, TEMPOL has been shown to react with $O_2^{\bullet-}$, yielding O_2 and H_2O_2, functionally mimicking SOD [143] which itself, cannot cross the cell membrane. However, a more recent study has indicated that nitroxide compounds are not efficient SOD mimetics at physiological pH and are unlikely to surpass SOD isoenzymes [144], which are abundant in most physiological compartments. Notably, it is unclear what impact TEMPOL will have on $O_2^{\bullet-}$ degradation in the acidic environment of the infarcted myocardium. TEMPOL is also reported to compete with H_2O_2 for redox reactive metals such as Fe(III) and Cu(II), and can accept electrons from mitochondrial electron transport chain proteins, thereby inhibiting Fenton-type chemistry and diminishing physiological sources of ROS [137, 145]. The ability of TEMPOL to protect animals from inflammatory injury, typically associated with increased $^{\bullet}NO$ production, leads to investigations aimed at probing possible interactions of cyclic nitroxides with $^{\bullet}NO$-derived oxidants. For example, TEMPOL inhibits MPO-mediated RNase and protein nitration [146, 147], possibly via its rapid reaction with $NO_2^{\bullet}$ and $CO_3^{\bullet-}$. Notably, $NO_2^{\bullet}$ is recycled to NO_2^- following the transformation of TEMPOL to $TPNO^+$, the latter may be recycled to TEMPOL by reacting with H_2O_2 and $O_2^{\bullet-}$, producing O_2 [144, 148].

Although nitroxides were previously identified as substrates for MPO-compounds I and II [149], the importance of such reactions under pathophysiological conditions has largely been overlooked. *In vitro* reports have documented that nitroxides are rapidly oxidised by MPO-compound I, via one-electron transfer to the porphyrin π-cation radical in the presence of physiological concentrations of Cl^-, to yield compound II [149, 150]. Nitroxides are poor substrates for MPO-compound II and direct scavenging reactions between HOCl and nitroxides are not kinetically favoured [150]. Therefore, metabolism of nitroxides by MPO results in the accumulation of compound II and subsequent inhibition of the MPO-H_2O_2-Cl^- enzymic system.

Despite initial optimism, supplementing with nitroxides has, for the most part failed to inhibit MPO *in vivo*. For example, supplementing TEMPOL or other more hydrophobic derivatives (modification at the 4-position of the TEMPO structure) in the carrageenan-induced inflammatory model attenuated all indexes of inflammation studied, including decreased nitration and oxidation of proteins; however, no inhibition of MPO activity was evident [151]. Interestingly, TEMPOL behaved in a similar manner to colchicine, a classical inhibitor of neutrophil chemotaxis [152]. The local concentration of MPO is proportional to the extent of neutrophil recruitment to the inflamed site, indicating that TEMPOL most likely acted primarily by inhibiting neutrophil migration rather than inhibiting MPO enzyme activity *per se*. Although the mechanisms by which nitroxides inhibit neutrophil chemotaxis remain unclear, it is possible that nitroxides, like other antioxidants, reduce the oxidative stimuli that regulate neutrophil chemotaxis [153]. Interestingly, it may be possible to alter the structure of TEMPOL to enhance its activity toward MPO *in vivo* [149] while retaining its antioxidant and anti-inflammatory activity. For example, recent studies have shown that tetraethyl substituted nitroxides at the 2 and 6 positions of TEMPO retained their ability to inhibit HOCl production by MPO, yet showed longer plasma and cellular half-lives than the corresponding tetramethyl derivative [154].

CONCLUSION

As discussed above, the consequence of AMI is the progression of cell hypoxia to cell injury and cell death. The consequences of AMI-mediated ROS production include altered cellular ion balance and signalling cascades that are the primary sources of damage during and after the ischaemic insult. As cardiac muscle cells of adult humans are largely incapable of proliferating, interstitial fibrosis replaces the dying myocardium, which ultimately stiffens the heart and exposes the patient to the risk of post-AMI congestive heart failure.

Typically, inflammation accompanies AMI and subsequent remodelling of the heart. During this period, not only tissues within the infarct zone are affected but also those within the area at risk are endangered. Tissue necrosis of this area leads to a larger interstitial fibrosis that takes place within the myocardium, which in turn leads to poorer prognosis and recovery of the patient who survived from AMI. The present review describes oxidative

damage by MPO-derived HOCl and the potential for minimising post-AMI complications by ameliorating HOCl-induced oxidative insult using cellular mediators such as inhibitors of MPO and antioxidant supplements that reduce the local concentration of HOCl in tissue. Some aspects of the inflammatory response are essential for myocardial repair including tissue fibrosis and the laying down of scar tissue, although uncontrolled myocardial remodelling can also lead to heart failure. Whether specific inhibition of MPO can yield a therapeutic advantage after AMI without affecting beneficial remodelling of the damaged myocardium is not clear and further studies assessing inhibitors of MPO and their impact on signalling cascades within the affected myocardium following AMI are warranted.

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CONFLICT OF INTEREST STATEMENT

All authors have no conflicts to declare.

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