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Review Article

Effects of 4-hydroxynonenal on vascular endothelial and smooth muscle cell redox signaling and function in health and disease [☆]Sarah J. Chapple ¹, Xinghua Cheng ¹, Giovanni E. Mann ^{*}

Cardiovascular Division, British Heart Foundation Centre of Research Excellence, School of Medicine, King's College London, 150 Stamford Street, London SE1 9NH, U.K.

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ABSTRACT

4-hydroxynonenal (HNE) is a lipid hydroperoxide end product formed from the oxidation of n-6 polyunsaturated fatty acids. The relative abundance of HNE within the vasculature is dependent not only on the rate of lipid peroxidation and HNE synthesis but also on the removal of HNE adducts by phase II metabolic pathways such as glutathione-S-transferases. Depending on its relative concentration, HNE can induce a range of hormetic effects in vascular endothelial and smooth muscle cells, including kinase activation, proliferation, induction of phase II enzymes and in high doses inactivation of enzymatic processes and apoptosis. HNE also plays an important role in the pathogenesis of vascular diseases such as atherosclerosis, diabetes, neurodegenerative disorders and *in utero* diseases such as pre-eclampsia. This review examines the known production, metabolism and consequences of HNE synthesis within vascular endothelial and smooth muscle cells, highlighting alterations in mitochondrial and endoplasmic reticulum function and their association with various vascular pathologies.

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Abbreviations: Akt, Protein kinase B; AR, Aldose reductase; ARE, Antioxidant response element; AP-1, Activator protein-1; ATF6, Activating transcription factor 6; BH₄, Tetrahydrobiopterin; BAEC, Bovine aortic endothelial cells; BLMVEC, Bovine lung microvascular vein endothelial cells; BPAEC, Bovine pulmonary arterial endothelial cells; BTB, Broad complex Tramtrack and Bric-brac domain; CHOP, C/EBP-homologous protein; CREB, cAMP response element-binding protein; EGFR, Epidermal growth factor receptor; eIF2 α , Eukaryotic translation initiation factor 2 α ; Elk1, ETS domain-containing protein; eNOS, Endothelial nitric oxide synthase; EpRE, Electrophile response element; ER, Endoplasmic reticulum; ERAD, Endoplasmic reticulum assisted degradation; ERK1/2, Extracellular signal-regulated kinase 1/2; FAK, Focal adhesion kinase; FAP, Familial amyloidotic polyneuropathy; GCLC, Glutamate cysteine ligase catalytic subunit; GCLM, Glutamate cysteine ligase modifier subunit; GS-DHN, Glutathionyl-1,4 dihydroxynonenol; GSH, Glutathione; GS-HNE, HNE-conjugates; GST, Glutathione-S-transferase; GTPCH, Guanosine triphosphate cyclohydrolase I; HASMC, Human aortic smooth muscle cells; HCSMC, Human coronary smooth muscle cells; HERP, Homocysteine inducible ER protein; HMEC, Human microvascular endothelial cells; HO-1, Heme oxygenase-1; HNE, 4-hydroxynonenal; Hsp-70/72/90, Heat shock proteins-70/ -72/ -90; HUVEC, Human umbilical vein endothelial cells; iNOS, Inducible nitric oxide synthase; IRE1, Inositol requiring enzyme 1 IRE1; IVR, Central intervening region; JNK, c-jun N-terminal kinase; Keap1, Kelch-like ECH-associated protein 1; MASMC, Mouse aortic smooth muscle cells; MEK1/2, Mitogen activated protein kinase kinase 1/2; MMP-1/2, Matrix metalloproteinase-1/ -2; MPEC, Mouse pancreatic islet endothelial cells; NAC, N-acetylcysteine; NF κ B, Nuclear factor kappa B; NO, Nitric oxide; NQO1, NAD(P)H quinone oxidoreductase; Nrf2, Nuclear factor-E2-related factor 2; oxLDL, Oxidized low density lipoprotein; PCEC, Porcine cerebral endothelial cells; PDGF, Platelet-derived growth factor; PDI, Protein disulfide isomerases; PERK, Protein kinase-like endoplasmic reticulum kinase; 15d-PGJ₂, 15-deoxy-Delta (12,14) prostaglandin-J₂; PKC, Protein kinase C; PUFAs, Polyunsaturated fatty acids; RASMC, Rat aortic smooth muscle cells; ROS, Reactive oxygen species; RVSMC, Rat vascular smooth muscle cells; SMC, Smooth muscle cell; tBHP, Tert-butylhydroperoxide; TKR, Tyrosine kinase receptor; UPR, Unfolded protein response; xCT, cystine/glutamate amino acid transporter.

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^{*} Correspondence to: Cardiovascular Division, BHF Centre of Research Excellence, School of Medicine, Franklin-Wilkins Building, Rm. 3.01, 150 Stamford Street, London SE1 9NH, UK. Tel.: +44 20 7848 4306; fax: +44 20 7848 4500.

E-mail address: giovanni.mann@kcl.ac.uk (G.E. Mann).

¹ The authors contributed equally to this work.

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HNE production in the vasculature

The oxidation and subsequent β -cleavage of n-6 polyunsaturated fatty acids (PUFAs) by H^+ abstracting agents (e.g. hydroxyl radicals, $OH\cdot$) yields a variety of lipid hydroperoxide products [1]. Of these, the alkenal 4-hydroxy-2-trans-nonenal (HNE) is considered one of the most abundant and bioactive species [1,2] and has been studied extensively both in the context of physiological as well as pathological vascular events. HNE can form adducts with DNA [3,4] but is also highly reactive with phospholipids (containing PUFAs such as linoleic and arachidonic acid) and nucleophilic amino acids (e.g. cysteine, histidine and lysine residues, [5]), making lipid membranes particularly vulnerable to HNE modification. Furthermore, HNE Michael additions can induce further cross-linking and/or other HNE conjugates, with roles in physiology and pathophysiology [6]. In addition to the plasma membrane, specific organelles, such as the

mitochondria and endoplasmic reticulum (ER), are highly susceptible to HNE-induced damage, with propagating lipid peroxidation ultimately resulting in organelle dysfunction and loss of redox homeostasis Fig. 1.

It is estimated that free HNE concentrations in the plasma of healthy individuals range between 0.3 and 0.7 μM [7]. HNE levels increase significantly in plasma and tissues during aging [8] and in diseases associated with oxidative stress such as atherosclerosis and diabetes [9]. As a lipid peroxidation derived product, HNE is highly associated with the generation of reactive oxygen species (ROS), and hence levels of HNE and HNE conjugates in plasma, tissues and urine are frequently used as markers of oxidative stress. In disease states, levels of HNE within the low micromolar range ($\sim 1\text{--}20 \mu\text{M}$) have been reported, although conceivably far higher concentrations ($\sim 100 \mu\text{M}$) may be achieved in discrete areas such as the plasma membrane [1].

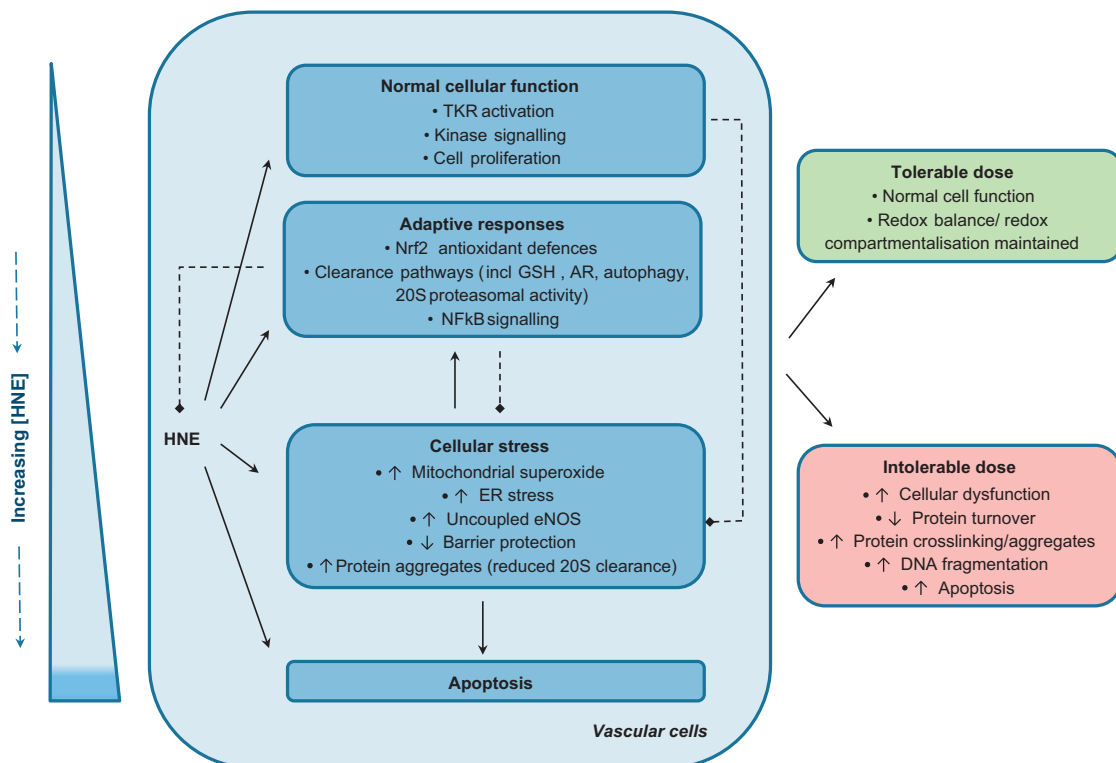


Fig. 1. Hormetic modulation of vascular cell function in response to HNE. Whilst low physiological levels of HNE can directly target specific cellular pathways such as tyrosine kinase receptor (TKR) activation and downstream kinase signaling, exerting positive effects on cell function such as cell proliferation, progressively increasing concentrations of HNE can be detrimental. Adaptive responses in phase II metabolism largely through activation of the redox-sensitive Nrf2 serve to neutralize HNE protein adduction and have a potential role in preventing off-target effects of HNE, prolonged organelle stress and loss of function. Chronic exposure to pathological concentrations of HNE found in disease states or which may accumulate in discrete cellular regions or organelles are associated with cellular stress and dysfunction, characterized by loss of function of enzymatic systems, cellular damage and activation of pro-inflammatory and ultimately pro-apoptotic signaling.

In vitro incubation of vascular cells with linoleic and arachidonic acid directly increases HNE formation [10], with HNE also known to accumulate indirectly within vessels via macrophage infiltration. Cholesterol consumption reportedly increases HNE synthesis [11], with HNE inducing low-density lipoprotein oxidation and increased uptake by macrophages [12], and HNE accumulation within atherosclerotic plaques [13].

Metabolism of HNE in vascular cells

The half-life of HNE is relatively short within cells, with HNE rapidly removed by phase II reactions, allowing for excretion of water soluble conjugates [14]. HNE modified proteins can also be removed by autophagic and proteasomal degradation pathways [15–17]. Although HNE can form Michael adducts directly with the abundant cellular antioxidant glutathione (GSH), HNE conjugation is accelerated by enzymatic systems [14]. HNE conjugation is catalyzed by enzymes including glutathione-S-transferases (GST), alcohol dehydrogenases and aldehyde dehydrogenases, leading to the formation of glutathionyl HNE (GS-HNE), 1,4-dihydroxynonene and 4-hydroxy-2-nonenic acid, respectively. Among them, GST mediated conjugation of HNE to glutathione (GSH) accounts for approximately 60% of the total degradation in vascular cells [18,19]. The GST isoform GST-4 displays a high selectivity for α,β unsaturated aldehydes and overexpression of GST-4 has been shown to protect endothelial cells from HNE-mediated apoptosis [20]. In human endothelial and vascular smooth muscle cells (SMCs), the primary HNE-glutathione conjugate can be further converted to glutathionyl-1,4 dihydroxynonene (GS-DHN) by aldose reductase (AR, AKR1B1), a member of the aldo-keto reductase superfamily and the rate-limiting enzyme involved in metabolizing glucose via the polyol pathway [18]. These metabolites may then be exported for secondary degradation [21].

In hepatocytes, the half-life of 100 μM HNE is only 5 min [22]. Srivastava et al. reported that in human endothelial cells incubated with 5 μM HNE, 95% of the compound was metabolized after 30 min [18]. Following addition of 25 μM HNE to bovine aortic endothelial cells (BAEC), intracellular HNE levels peak at 4 h and decline by 50% after 6 h [23]. In contrast, vascular smooth muscle cells may be more resistant to HNE modification. Hill et al. reported that exposure of primary rat aortic SMCs to HNE concentrations below 50 μM fails to induce widespread cellular accumulation of HNE protein adducts, with adducts only apparent after at least 30 min incubation with 50 μM HNE [15]. Thus, clearance is faster in SMCs than endothelial cells, with complete removal of HNE adducts in SMC treated with 50 μM HNE occurring within ~8 h [15].

The rate of HNE metabolism is critically dependent on cellular levels of GSH and relevant enzyme activity, reflecting the antioxidant capacity of different cells [24,25]. SMCs are known to induce rapid synthesis of intracellular GSH from cysteine or extracellular GSH [26], and basal GSH levels are markedly higher in SMCs compared with endothelial cells [27]. Furthermore, SMCs exposed to HNE promote the induction of aldose reductase (AR) activity and expression, increasing GS-HNE metabolite clearance and promoting cell survival [28,29]. Collectively, the enhanced induction of these clearance pathways within SMCs may account for the apparent increased sensitivity of endothelial cells to HNE.

Downstream effects of HNE production on cellular function

Although the majority of HNE is rapidly removed from cells, approximately 1–8% of HNE may remain bound to functional amino acid groups including cysteine, histidine and lysine and thereby continue to exert cellular effects [19]. Among them,

cysteine residues display the highest reactivity with HNE at their C-3 electrophilic center [30]. Effects of HNE on endothelial and smooth muscle cells are summarized in Tables 1 and 2, respectively. For a more comprehensive overview of the biochemistry and general cell signaling of lipid peroxidation products we refer readers to existing reviews [14,31,32]. In biological systems, lipid peroxidation products have been referred to as 'second messengers' of free radicals, as they are more stable and diffuse easily to affect distant targets enabling participation in a diverse number of cellular events. Cellular effects of HNE may evoke both damaging and hormetic cytoprotective actions. Whilst high concentrations of HNE can inhibit cellular processes inducing widespread cellular damage ultimately resulting in apoptosis [1,33], a range of relatively low concentrations of HNE produced in response to mild oxidative challenge appear to modulate normal physiological processes including upregulation of endogenous antioxidant defense pathways [34,35].

Targeted signaling induced by HNE within the vasculature

Instead of a mere by-product of oxidative stress, both reactive oxygen species and resulting lipid hydroperoxide products (e.g. HNE) are capable of modifying nucleophilic residues and are now recognized as signaling agents participating in a wide array of cellular pathways. Key pathways targeted by HNE in vascular cells include tyrosine kinase receptor (TKR) activation and modulation of transcriptional activity.

Enzyme and receptor targets of HNE signaling

A classical signaling pathway known to be induced by HNE is the activation of TKRs, with limited data also supporting their activation in vascular endothelial and SMCs. HNE (0.1 μM) is reported in a human endothelial cell line to modify and induce autophosphorylation of the epidermal growth factor receptor (EGFR) which is associated with activation of intrinsic tyrosine kinase activity and implicated in cell proliferation/differentiation [36]. The formation of HNE protein adducts with sulfhydryl groups, lysine and histidine is a probable mechanism accounting for the activation of TKRs triggering pleiotropic regulatory pathways. In bovine aortic endothelial cells, Natarajan et al. also demonstrated protein tyrosine phosphorylation is responsible for the activation of phospholipase D (PLD) by HNE, although the concentration of HNE (50 μM) in their treatments may be somewhat higher than reported pathophysiological levels [37]. Activation of PLD is recognized as a trigger to generate the intracellular lipid messenger phosphatidic acid and the latter is associated with diverse cellular functions including reorganization of the cytoskeleton, intracellular membrane transport and cell signaling which is reviewed elsewhere [38].

Interestingly, modulation of intracellular thiol levels, either by supplementing with a thiol rich compound (N-acetyl-L-cysteine) or treatment with a GSH chelating compound (L-buthionine-(S,R)-sulfoximine, BSO), attenuates or enhances PLD activation by HNE respectively, implicating a potential competitive role of intracellular reactive free thiols in modulating PLD activation [39]. These findings highlight the importance of basal redox status in determining the various physiological or pathological consequences of HNE exposure. In SMCs relatively low concentrations of HNE (1–2.5 μM) also lead to platelet-derived growth factor (PDGF) receptor activation, preceding metalloproteinase-1 and extracellular signal-regulated kinase 1/2 (ERK1/2) activation [40]. HNE activation of extracellular matrix degrading matrix metalloproteinases-1 and -2 [40–42] is essential for SMC proliferation [43], as is the activation of ERK1/2 [43,44] which occurs within 5 min of HNE stimulation.

Table 1
Effects of HNE and 15d-PGJ₂ on endothelial cells.

Cell type	Treatment	Conc.* (μmol/L)	Time	Main findings	References
RTK and MAPK activation					
HEC	HNE oxLDL	0.1	3 h	HNE (0.1 μM) and oxLDL (200 μg/ml) ↑ EGFR activation	[36] [49]
BLMVEC	HNE	10–100	2 h	↑ Phosphorylation of ERK, JNK, p38 MAPK	
MPEC	HNE	20	0–2 h	↑ p-JNK within 30 min, ↔ p-p38, p-ERK1/2	[20]
BLMVEC	HNE	25	30 min	↑ MAPK activation	[88]
BPAEC	HNE	50	30 min	↑ Phospholipase D activation via protein tyrosine phosphorylation	[37,141]
HUVEC	15d-PGJ ₂	10	2 h	↑ Phosphorylation of MAPK JNK (2 h)	[142]
Transcriptional activity					
HUVEC	HNE	5	12 h	↑ HO-1 and NQO1 via Nrf2, protects HUVEC from tBHP attack	[55]
HUVEC	HNE	1–10	12 h	↔ NFκB activation, ↓ IL-8 and ICAM-1 production in a concentration dependent manner indicating ↓ inflammatory responses	[10]
HUVEC	15d-PGJ ₂	2.5	2–8 h	↑ Nrf2 nuclear accumulation, ↑ HO-1, ↑ NQO1, ↑ adducts with Keap1 at cysteines residues in IVR region, which may mediate shear induced Nrf2 activation	[143]
BAEC	15d-PGJ ₂	2	16 h	↑ GSH, HO-1, ↑ Keap1 adduct formation	[144]
HUVEC	15d-PGJ ₂	5	24 h	↑ GSH, ↑ GCLC, ↑ GCLM, ↑ resistance to oxidative stress which relies on <i>de novo</i> GSH synthesis, ARE responsible for GCLC induction, Cys273 and Cys278 of Keap1 conjugated by 15d-PGJ ₂	[52,145]
Oxidative stress and protein adduct clearance					
BAEC	HNE	5	1 h	Modifies thioredoxin-1 at cys-73 and ↓ activity, ↑ ROS, ↓ GSH	[90]
HAEC	HNE	5	24 h	Protects HAEC from 6-hydroxydopamine induced cell death	
BAEC	HNE	25	4 h	Proposed ↑ proteasomal degradation of GTPCH and Hsp90	[23]
Cellular dysfunction					
BLMVEC	HNE	10–100	2 h	↑ Endothelial permeability; ↑ Michael adducts formation, actin fiber remodeling via phosphorylation of ERK, JNK, p38 MAPK	[49]
BLMVEC	HNE	25	30 min	Induces actin rearrangement, ↓ GSH, ↑ cell adhesion and surface integrins; ↓ tyrosine phosphorylation of FAK, ↑ MAPK activation, ↑ Michael adducts with ↑ focal adhesion & adherens junctional proteins, reversed by pretreatment NAC	[88]
BAEC	HNE	5	1 h	↑ Monocyte adhesion	[90]
BAEC	HNE	10	5 d	↑ Membrane phospholipid perturbation, ↑ prostacyclin, ↑ monocyte migration	[146]
BAEC	HNE	10	6–8 h	↓ Junctional communication	[87]
BAEC	HNE	25	4 h	↑ ROS, ↓ NO, GSH, ↑ apoptosis involving caspase-3 activation which can be attenuated by BH ₄ supplementation. ↓ BH ₄ by ↓ GTPCH leading to ↑ eNOS uncoupling, ↓ HSP90 leading to ↓ eNOS phosphorylation, proposed ↑ proteasomal activity and proteasomal degradation of BH ₄ and HSP 90	[23]
Mitochondrial function					
BAEC	HNE	5–10	4–16 h	↑ GSH and HO-1 at 16 h, ↑ ROS from mitochondria after 4 h	[71]
HUVEC	15d-PGJ ₂	2.5	24 h	↑ GSH, ↑ complex I activity in cell extracts, dependent on <i>de novo</i> protein synthesis in response to ROS but independent of GSH depletion	[94]
BAEC	15d-PGJ ₂	10	16 h	↑ HO-1, ↓ mitochondrial membrane potential ↑ Nrf2 nuclear accumulation, blocked IBTP, a mitochondrial-targeted thiol reactive compound.	[53]
ER stress					
HUVEC	HNE	25	30 min – 2 h	↑ Protein adduction (HSP90, HSP70, PDI), ↑ xBP-1 splicing, ↑ PERK, p-eIF2α, and ATF6 translocation; ↑ Grp78 and HERP, ↑ ICAM-1, cell adhesion, TNF-α, IL-6, and IL-8; activation of endothelial cells mediated by ↑ ER stress, noting depletion of GSH cannot ↑ ER stress	[107]
HMEC	HNE	20	24 h	↑ PERK and ATF6; ↑ p-IRE1 and p-eIF2α; ↑ ER stress, which is prevented by pretreatment with NAC	[108]
HMEC	HNE/ oxLDL	0–25 (HNE)/ 200 μg/ml oxLDL	14–18 h	Forms adducts with PDI, ↓ PDI activity and ↓ cell viability. ↑ CHOP and xBP1s mRNA as indicators of ↑ ER stress. Loss of PDI activity and reduced viability prevented by NAC.	[110]
Apoptosis					
HUVEC	HNE	10–50	3 h	↑ Cell death and ↓ growth capacity but this effect is affected by serum in the medium	[147]
HUVEC	HNE	10	12 h	↑ Apoptosis, ↓ protein synthesis	[10]
PCEC	HNE	1–50	3 h	↑ Chromosomal aberrations and micronuclei formation	[113]
BAEC	HNE	25	4 h	↑ ROS, ↓ NO, ↓ GSH, ↑ apoptosis involving caspase-3 activation which can be attenuated by BH ₄ supplementation. ↓ BH ₄ resulting from ↓ GTPCH and ↓ HSP90 levels leads to ↑ eNOS uncoupling and ↓ eNOS phosphorylation. Proposed ↑ proteasomal activity towards GTPCH and HSP 90 underlies impaired NO production	[23]
MPEC	HNE	40	8 h	↑ p-JNK leading to ↑ p53 and Bax expression and ↑ apoptosis. Abolished by GST4 overexpression	[20]
HUVEC	15d-PGJ ₂	10	2–16 h	↑ ROS (1 h), ↑ phosphorylation of MAPK and JNK (2 h), ↑ p53 expression and phosphorylation (8 h), ↑ caspase associated apoptosis	[142]

Cell type abbreviations: MPEC, mouse pancreatic islet endothelial cells; HEC human endothelial cell line CRL-1998; BAEC, bovine aortic endothelial cells; BLMVEC, Bovine lung microvascular vein endothelial cells; BPAEC, bovine pulmonary arterial endothelial cells; HMEC, human microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; PCEC, porcine cerebral endothelial cells.

Although in SMCs, ERK1/2 appears to be the principal kinase pathway activated by physiological concentrations (0.1–1 μM) of HNE, presumably indirectly via activation of upstream mitogen-

activated protein kinase 1/2 (MEK 1/2), several other kinase pathways are HNE sensitive. In SMCs, HNE (1 μM) promotes rapid protein kinase B (Akt) [41], p38 MAPK [42] as well more latent

Table 2
Effects of HNE on vascular smooth muscle cells.

Cell type	[HNE] $\mu\text{mol/L}$	Time	Findings	References
RTK, MAPK and PI3K/Akt activation				
MASMC	0.1	10–90 min	In young but not aged animals \uparrow p-ERK1/2, \leftrightarrow p-p38, p-Jnk	[44]
RASMC	1	5 min	\uparrow p-ERK1/2	[43]
HCSMC	10	10 min	\uparrow p-PDGFR, \uparrow p-ERK1/2	[40]
MASMC	1	5–120 min	\uparrow p-ERK1/2 that was maximal within 60 min, \uparrow p-p38 maximal within 30 min and p-JNK reduced after 30 min of HNE treatment	[42]
RASMC	1	5–120 min	Dose-dependent \uparrow Akt activity	[41]
RASMC	1	4 h	\uparrow PKC activation, abolished by AR inhibition	[45]
Proliferation				
RASMC	0.1–1	12–24 h	Dose-dependent \uparrow MMP-2 activity, mRNA and protein expression, which was dependent on Akt activation	[41]
MASMC	1	24 h	\uparrow MMP-2 expression and activity which was dependent on HNE-induced ERK activation	[42]
MASMC	0.1–10	24 h	0.1 μM HNE \uparrow cell density in cells from young but not aged mice, \uparrow ERK1/2 activation and downstream cyclin D1 mRNA expression. Higher doses 1–10 μM HNE \downarrow cell density	[44]
RASMC	0.1–10	24 h	0.1 μM HNE \uparrow cell survival in cells from young but not aged mice. Aged cells showed \uparrow ROS generation in response to HNE, with NAC \uparrow survival in aged cell populations	[45]
HCSMC	2–20	24 h	HNE and HNE conjugates (e.g. GS-HNE) $< 1 \mu\text{M}$ \uparrow cell proliferation, with higher doses \downarrow cell density. Aldose reductase activity required for cell survival/proliferation. Inhibition of HNE conjugate removal by RLIP76 transporter \uparrow cell growth	[40]
RASMC	1	48–72 h	Dose-dependent \uparrow MMP-1 requiring HNE-induced PDGFR and downstream ERK1/2 activation	[43]
RASMC	2.5	48 h	\uparrow Proliferation	[74]
HASMC	2.5	72 h	\uparrow Cell proliferation, dependent on aldose reductase activity	[29]
Transcriptional activity				
HASMC	1	0.5–6 h	30 min following HNE exposure \uparrow I κ B α phosphorylation without inducing I κ B degradation. \uparrow NF κ B DNA binding was also increased from 30 min of HNE stimulation	[72]
RASMC	1	4 h	\uparrow NF κ B and AP-1 activation	[45]
RASMC	1	12 h	\uparrow NF κ B activity but no change in AP-1, c-jun or CREB activity. NF κ B activation was dependent on mitochondria derived superoxide mediated Akt activation which and proceeds MMP-2 expression	[41]
RVSMC	0–5	24 h	Dose-dependent \downarrow NF κ B activation, with 50 μM HNE shown to prevent LPS/IFN induced proteasomal I κ B α degradation. Accordingly, HNE dose-dependently (0–5 μM) \downarrow LPS/IFN induced nitrite production, with doses $> 12.5 \mu\text{M}$ \downarrow iNOS expression	[73]
RASMC	1–10	3 h	\uparrow Elk1, c-jun, CHOP and AP-1 activity, \uparrow c-jun and c-fos mRNA	[74]
RASMC	2.5	1–2 h	\uparrow c-fos and c-jun expression, \uparrow AP-1 DNA binding	[43]
MASMC	20	0–5 h	\uparrow Nrf2 nuclear accumulation and downstream HO-1, Prx1 and A170 mRNA, absent in cells derived from Nrf2 knockout mice	[54]
Phase II metabolism				
HASMC	0.05	30 min	\uparrow Aldose reductase metabolized GSH conjugates	[29]
RASMC	2.5	8–12 h	\uparrow Aldose reductase mRNA (8 h) and protein (12 h) expression	[43]
RASMC	1	5 min	\downarrow Cellular thiol content	[43]
RVSMC	5–10	0–24 h	\uparrow Aldose reductase mRNA (7 h), protein (12 h) and activity (12–24 h)	[28]
RASMC	0.1–10	24 h	HNE and HNE conjugates (e.g. GS-HNE) $< 1 \mu\text{M}$ \uparrow cell proliferation, with higher doses \downarrow cell density. Aldose reductase activity required for cell survival/proliferation. Inhibition of HNE conjugate removal by RLIP76 transporter \uparrow cell growth	[45]
Oxidative stress and protein adduct clearance				
HASMC	1	0–12 h	\uparrow 8-isoprostane induced following 9 h HNE treatment	[72]
RASMC	50	30 min	\uparrow Autophagy but not proteasomal HNE degradation	[15]
RVSMC	50	4 h	\downarrow LPS/IFN induced proteasomal I κ B α degradation	[73]
RBASMC	0–3000	4.5 h	\downarrow Recognition and clearance of LDL with increasing [HNE]	[12]
Cellular dysfunction				
PBMS	10–100	0 min	\downarrow Carbachol induced relaxation	[148]
RVSMC	0–5	24 h	HNE dose-dependently (0–5 μM) \downarrow LPS/IFN induced nitrite production, with doses $> 12.5 \mu\text{M}$ \downarrow iNOS expression	[73]
Mitochondria				
RASMC	1–30	10–60 min	HNE dose-dependently \uparrow ROS production measured by DCF fluorescence with inhibitors of mitochondria abolishing HNE-induced superoxide generation	[96]
RASMC	1	30 min	\uparrow Mitochondria derived superoxide	[41]
RASMC	20	20–180 min	\downarrow Oxygen consumption rate (OCR), \leftrightarrow extracellular acidification rate (ECAR)	[97]
Apoptosis				
RASMC	1–30	24 h	HNE $> 10 \mu\text{M}$ \uparrow apoptosis, with 30 μM HNE also increasing necrosis	[44,96]
RVSMC	5–15	6 h	HNE dose-dependently \uparrow apoptosis, with AR inhibition \uparrow HNE-induced apoptosis	[28]
HASMC	0–100	6 h	100 μM HNE induced DNA fragmentation	[72]

Cell type abbreviations: HCSMC, human coronary smooth muscle cells; HASMC, Human aortic smooth muscle cells; RVSMC, rat vascular smooth muscle cells; RASMC, rat aortic smooth muscle cells; RBASMC, rabbit aortic smooth muscle cells; MASMC, mouse aortic smooth muscle cells; PBMS, pig bladder muscle strips.

protein kinase C (PKC) [45] activity, whereas c-jun N-terminal kinase (JNK) signaling appears to be suppressed [42,44].

The downstream effects of Akt, p38, PKC and JNK activation in SMC function in response to low concentrations of HNE are not well characterized, and the predominant activation of ERK is somewhat surprising given the well characterized induction of cysteine rich PKC in non-vascular cell types [46–48]. As highlighted in Table 2, studies assessing kinase activation in SMC following challenge with pathological concentrations of HNE (~10 μ M) are limited. In contrast, *in vitro* studies with endothelial cells have largely used rather high HNE concentrations (> 10 μ M) to demonstrate upregulation of JNK, ERK1/2 and/or p38 [20,49]. Interestingly, both HNE and its conjugates (e.g. GS-HNE) appear to be bioactive and participate in cell signaling [45]. Furthermore, based on proliferative responses of SMCs to a range of HNE concentrations, it is notable that low 'signaling' doses (~1 μ M) of HNE elicit proliferative or 'pro-survival' responses in SMCs, whereas stimulation with higher and pathological levels of HNE (~10 μ M) cause apoptosis in both endothelial and SMCs (see Apoptosis Section).

Transcriptional targets of HNE

HNE can also induce the activation of a number of transcription factors. Due to its integral role as a mediator of oxidative stress, the best described role of HNE mediated transcriptional regulation is its activation of nuclear factor-E2-related factor 2 (Nrf2) antioxidant defenses. Biphasic effects of HNE are characterized by an initial decrease in GSH levels over 1–2 h and a later rebound upregulation of GSH (12–24 h) and other antioxidant enzymes, including heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase (NQO1), glutamate cysteine ligase catalytic subunit (GCLC), glutamate cysteine ligase modifier subunit (GCLM) and the cystine/glutamate amino acid transporter (xCT), all of which enhance cellular defenses against further oxidative stress [34,50]. These cytoprotective enzymes possess an antioxidant response element (ARE)/electrophile response element (EpRE) in their promoter region which binds Nrf2, a key transcription factor regulating redox homeostasis [34,51]. Lipid peroxidation derivatives significantly increase nuclear accumulation of Nrf2 [35,52–54] and knockdown of Nrf2 with siRNA almost completely abolishes the adaptive upregulation of GSH by HNE [55], highlighting the importance of this transcriptional pathway in HNE triggered cellular responses.

Nrf2 is a member of the CNC ('cap n collar') family of transcription factors which possesses a b-Zip binding motif [56]. Nrf2 deficient mice are more susceptible to vascular damage due to diminished glutathione levels and an impaired compensatory induction of GSH synthesis (Chan & Kwong, 2000), highlighting a fundamental role for Nrf2 in antioxidant defenses against oxidative stress. Activation of Nrf2 is mainly regulated by its cytosolic inhibitor Kelch-Like ECH-Associated Protein 1 (Keap1), which under basal conditions targets Nrf2 for ubiquitin-dependent proteasomal degradation [57–59]. Keap1 is a cysteine rich protein that serves as a key redox sensor [60]. Modification of cysteine residues on Keap1 by electrophiles, particularly Cys 151 in the BTB (Broad complex Tramtrack, and Bric-brac) domain and Cys 273 and Cys 288 in the central intervening region (IVR) domain, suppress Keap1 mediated degradation of Nrf2 [61,62]. Although the exact residues modified by HNE have yet to be determined, Michael addition of HNE to Keap1 has been demonstrated [52]. When the level of Nrf2 exceeds the sequestration capacity of Keap1, excess Nrf2 can accumulate in the nucleus where it initiates transcription [56].

In addition to the genes mentioned above, the Nrf2 inducible enzymes aldo-keto reductase (AR) [63,64] and GSTs including GST4 [65,66] are critical in neutralizing HNE in vascular cells. Increased levels of GSH can detoxify HNE conjugated proteins such

as protein disulfide isomerases (PDIs), heat shock protein 72 (Hsp72) and restore their function [67]. Interestingly, knockdown of Nrf2 results in increased HNE accumulation in tissues of aged (> 24 months) mice [68], and in aged humans elevated HNE levels correlate with diminished Nrf2 activity [30,69]. However, the mechanisms underlying loss of Nrf2 function during aging remain to be elucidated. Because activation of cytoprotective pathways could be enhanced by HNE treatment, moderate doses of HNE may thus exert beneficial effects on cell survival, overcoming its initial detrimental damage. In SMCs, HNE challenge increases AR activity, mRNA and protein expression [28,29], which is associated with increased cell survival [45].

Moreover, Chen et al. reported that pre-treatment of human arterial endothelial cells with 5 μ M HNE for 24 h significantly improved cell survival following oxidative stress induced by 6-hydroxydopamine [70]. Consistent with these findings, 5 μ M HNE markedly reduced tert-butyl hydroperoxide-induced cytotoxicity in human umbilical vein endothelial cells, which was accompanied by increased expression of HO-1 and NQO1 and activated Nrf2/ARE signaling [55]. In addition, pretreatment of 15-deoxy-Delta (12,14) prostaglandin J₂ (15d-PGJ₂), another peroxidation product with a highly reactive electrophilic center that shares common functions with HNE, also enhanced cell resistance to oxidative stress via a similar mechanism [71], indicating hormetic actions of HNE in endothelial cells.

Surprisingly, whilst the transcriptional effects of HNE in endothelial cells seem to be predominantly confined to Nrf2, in SMCs the effects of HNE appear to be more diverse. In SMCs relatively low concentrations of HNE (~1 μ M) also promote I κ B α phosphorylation, leading to rapid (30 min) nuclear factor kappa B (NF κ B) DNA binding, with NF κ B activation maintained for at least 12 h and promoting SMC proliferation [41,45,72]. In contrast, higher concentrations of HNE (5–50 μ M) inhibit I κ B- α degradation via the proteasome [73], consistent with reduced proliferation observed in response to higher concentrations of HNE [44,45]. In contrast to SMCs, HNE (10 μ M) fails to modulate NF κ B signaling in endothelial cells [10]. Furthermore, other transcription factors and transcriptional activators including activator protein-1 (AP-1), cAMP response element-binding protein (CREB) and ETS domain-containing protein (Elk1) are acutely (1–4 h), albeit transiently, activated in SMCs in response to HNE [43,45,74] but similar reports for endothelial cells are to our knowledge lacking. Whether HNE directly activates redox sensitive transcription factors such as AP-1, or whether this occurs as a consequence of perturbations within the cell redox environment remains to be established.

Restoration and clearance pathways

In addition to inducing the expression of HNE-responsive GSTs (e.g. GST4) and aldo-keto reductases (e.g. AR) to metabolize HNE (see Section 2), export of GS-HNE conjugates in many cell types predominantly via the non-ABC transporter RLIP76 is also known to modulate HNE-induced cell signaling [75]. Chemical or antibody based inhibition of RLIP76 has been shown to prevent GS-DHN removal and to potentiate low dose HNE-induced SMC proliferation [45]. These findings highlight a specific signaling rather than cytotoxic role for low physiological concentrations of HNE and its conjugates in the vasculature, a finding confirmed in many other cell types [76]. The export of HNE conjugates by RLIP76 can be saturated, with purified RLIP76 shown to have a K_m ~2.5 μ M for GS-HNE conjugates [77]. Exposure to high levels of HNE, exceeding the capacity of RLIP76, may be a critical factor mediating the hormetic actions of HNE. Indeed, inhibition of RLIP76 *in vitro* has been shown to induce HNE accumulation and activation of JNK mediated apoptosis [78]. In RLIP76^{-/-} mice, HNE and GS-HNE metabolites accumulate in tissues [79] and, whilst the activity of

RLIP76 in aging has yet to be determined, increased RLIP76 autoantibodies have been found in other vascular diseases and are implicated in metabolic syndrome [78,80].

Failure to induce HNE clearance can lead to the accumulation of HNE protein adducts, which below a certain threshold can be removed via proteasomal or autophagic degradation pathways to restore redox balance. In the cytosol, the proteasome largely consists of a 20S catalytic core, capped by regulatory subunits 19S (PA700) and/or 11S (PA28) subunits [81]. The 20S core is capped at either end by a 19S subunit, collectively termed the 26S proteasome, and has been shown to be key for normal turnover of cellular proteins in an ubiquitin-dependent manner. Oxidized proteins are a poor substrate for the 26S proteasome, but detachment of the 19S regulatory subunits allows oxidized proteins to be degraded by the 20S proteasome independent of ubiquitin conjugation [81]. It is generally accepted that whilst mild HNE-induced protein oxidation stimulates 20S proteasomal clearance, extensively oxidized and cross-linked proteins are more resistant to proteasome degradation leading to the accumulation of oxidatively modified and cytotoxic protein aggregates [17]. The proteasome itself may also be a target for HNE adduction, with HNE at concentrations as low as 10 μ M shown to directly bind specific subunits within the 20S proteasomal catalytic core inhibiting their activity [82] and potentially exacerbating HNE-modified protein accumulation.

In endothelial cells, Vieira et al. reported that exposure to 50–200 μ g/ml oxLDL induced protein adduct formation which was replicated by 1 μ M HNE treatment [83]. Protein oxidation coincided with elevated proteolysis peaking within 3–5 h and was inhibited by the proteasomal inhibitors LLnL and PSI and partly dependent on the previous ubiquitination of substrates [83]. Whitsett et al. also proposed that increased proteasomal degradation in response to HNE (25 μ M) underlies loss of guanosine triphosphate cyclohydrolase I (GTPCH) in bovine aortic endothelial cells [23]. In SMCs, there is limited information on the effects of HNE on proteasomal activity and autophagy, however proteasomal activity appears limited following exposure to pathological concentrations of HNE [15], perhaps due to extensive protein crosslinking. Studies by Hill et al., demonstrate that treatment of rat aortic SMCs with 50 μ M HNE induces widespread protein-adduction, with inhibitors of autophagy (3-MA) but not the proteasome (lactacystin) further increasing the presence of adduct, suggestive of an active autophagic response in SMCs to remove HNE-adducts [15]. In other studies, HNE (50 μ M) was shown to prevent I κ B proteasomal degradation in response to LPS/IFN treatment, with similar results obtained using the proteasomal inhibitor MG115 [73]. Collectively, these findings support an increase in HNE clearance pathways as a necessary step to prevent widespread and aberrant HNE modifications, ultimately resulting in cellular dysfunction and activation of pro-apoptotic signaling pathways.

More recently, in addition to increasing phase II metabolism, a role for Nrf2 has been implicated in the induction of proteasomal genes in response to oxidative stress. Hydrogen peroxide and a range of dietary Nrf2 inducers have been shown to increase the expression of 20S proteasomal and immunoproteasomal subunits, resulting in a rebound increase in oxidized protein degradation capacity [51,84]. Although, as reviewed elsewhere [51], not all Nrf2 inducers have the capacity to modulate proteasomal gene expression, it is likely given the known role of HNE in modulating proteasomal activity that HNE may indeed be able to do so. Studies monitoring the effects of HNE on proteasomal activity in normal and Nrf2 deficient cells have yet to be conducted to confirm this, but the effects of HNE on proteasomal/autophagic clearance remains a potential factor influencing the transition from physiological to pathological HNE-induced cellular events.

Aberrant HNE modifications and misguided targets of lipid peroxidation

Whilst relatively low doses of HNE can orchestrate cell signaling events, higher concentrations of HNE appear to modify a further set of target proteins, inhibiting or dysregulating previously functional cellular processes and organelle functions. In particular, endothelial cells, which form the primary vascular interface for potentially oxidized circulating components, appear to be highly susceptible to HNE induced damage [10,85]. HNE can exert a range of pathophysiological effects, including interfering with the synthesis and release of vasoactive mediators, breakdown of the endothelial barrier function and inducing a pro-inflammatory phenotype within the vessel wall.

Loss of vasoprotection

Although not directly reactive with nitric oxide (NO), HNE is able to reduce bioavailability of the key vasoactive agent NO via modulation of nitric oxide synthase (NOS) activity. In BAEC treated with HNE, Whitsett et al. showed an elevation of peroxynitrite and uncoupled endothelial nitric oxide synthase (eNOS) with reduced tetrahydrobiopterin (BH₄) levels and eNOS phosphorylation, resulting from a loss of guanosine triphosphate cyclohydrolase I (GTPCH) and heat shock protein 90 (HSP90) [23]. Reduced GTPCH and HSP90 induced by HNE can be reversed by proteasomal inhibition, suggesting accelerated proteasomal degradation of these proteins following HNE adduction [23,86]. However, as no additional studies were conducted to assess whether these proteins are adducted by HNE or whether HNE is modulating proteasomal activity, the mechanism mediating these effects remains uncertain. In SMCs, HNE also inhibits the production of nitrite from NO through modulating gene expression, decreasing expression of inducible nitric oxide synthase (iNOS) following inhibition of NF κ B activation [73].

Elevated plasma levels of HNE also damage endothelial barrier function [10,87] due to impaired cell–cell communication and inhibition of membrane associated enzymes [85]. Treatment of endothelial cells with HNE increases Michael additions to adhesion and adherens junction proteins, reduces surface integrin expression and phosphorylation of focal adhesion kinase (FAK) and redistributes tight junction proteins, leading to impaired focal adhesion and intercellular gap formation [88]. Intracellular F-actin arrangement may also be remodeled by HNE, contributing to gap formation and barrier dysfunction [49]. Notably, all these changes can be inhibited by pre-treatment of cells with the thiol protectant N-acetylcysteine, indicating an underlying redox sensitive mechanism [49,88]. It has been suggested that sulfhydryl groups (e.g. Cys374 on actin regulating polymerization) are potential targets of HNE addition [89]. Moreover, lipid peroxidation products induce inflammatory responses and increase the adhesion of monocytes to endothelial cells [88,90], with HNE also reported to modify LDL leading to increased uptake and reduced clearance of oxLDL from SMCs [12]. As discussed in Section 6, these processes may be active contributors towards the development of vascular diseases.

Organelle specific effects—mitochondrial dysfunction

Mitochondria are a major energy source in cells and generate reactive oxygen radicals (e.g. superoxide which can be rapidly dismutated to hydrogen peroxide) via the electron respiratory chain, and thereby not only contribute to cell signaling but also to the development of disease [91]. Mitochondrial membranes are rich in protein thiols which make them potential targets for lipid peroxidation products [92]. Similar to other non-vascular cell types, reviewed elsewhere [31], HNE is reported to increase cellular generation of reactive oxygen species in both endothelial and SMCs, with

superoxide generated by the mitochondria the predominant source. Landar et al. reported that in BAECs, HNE (5 μ M) evokes an increase in mitochondrial reactive oxygen species generation measured by DCF fluorescence [71]. However, in our studies, acute treatment with HNE (20 μ M) fails to induce superoxide generation in human umbilical vein endothelial cells measured by lucigenin and L-012 chemiluminescence. Although no studies have yet determined the effects of mitochondrial inhibitors in endothelial cells in response to HNE evoked superoxide generation, rotenone, a complex I inhibitor, can inhibit 15-deoxy-Delta (12,14) prostaglandin-J₂ (15d-PGJ₂) induced mitochondrial ROS generation [93]. Similarly, in BAEC treated with 15d-PGJ₂ for an extended time period, mitochondrial membrane potential is reduced [53] with elevated complex I activity [94], perhaps suggestive of mitochondrial uncoupling providing a feedback mechanism in mitochondria to combat sustained HNE stress [95]. In SMCs, HNE has been shown to dose-dependently increase ROS generation, with inhibition of mitochondrial complex I preventing HNE-induced superoxide generation [44,96]. Interestingly, exposure of SMCs to relatively high (20 μ M) HNE concentrations fails to induce any change in O₂ consumption [97], indicating that rather than exerting a direct effect on the respiratory chain HNE may be modulating clearance of reactive oxygen species by GSH or other antioxidant pathways.

Although yet to be investigated, it is worthwhile highlighting the role that mitochondria may play in HNE induced activation of the Nrf2 defense pathway. Using immunofluorescence, Lo et al. have shown that cytosolic Keap1 is closely associated with mitochondria [98]. Modulation of the mitochondrial redox status in endothelial cells has been proposed as a necessary step in the activation of Nrf2 signaling by lipid peroxidation products [53] and shear stress [99], although a specific role for mitochondria in HNE-induced Nrf2 activation in endothelial or SMCs has yet to be established. Addition of a mitochondrial targeted thiol reactive compound 4-iodobutyl-triphenylphosphonium abolishes hemin and 15d-PGJ₂ induced Nrf2 signaling [53] without interfering with Nrf2-Keap1 dissociation, indicating an intermediate role for mitochondrial thiols. Interestingly, using modified 15d-PGJ₂ specifically targeted to mitochondria, Diers et al. found that the modified compound caused profound mitochondrial reactive oxygen species generation and apoptosis, with minimal induction of HO-1 or adaptive increases in GSH. These findings suggest that both cytosolic and mitochondrial modifications induced by lipid peroxidation products are required for activating Nrf2 [100]. Moreover, modulation of redox regulating enzymes rather than altered ROS generation in mitochondria may be responsible for the activation of Nrf2/ARE signaling, as overexpression of mitochondrial thiol reductive enzyme thioredoxin 2 (Trx2) can abrogate the induction of this pathway [101]. Although HNE-induced modification of Trx2 has yet to be determined, inhibition of thioredoxin 1 by HNE conjugation has been previously reported [90] in aortic endothelial cells, and may thus serve as a mechanism for the activation of Nrf2 by HNE.

Organelle specific effects - endoplasmic reticulum (ER) stress

ER stress and the subsequent unfolded protein response (UPR) are emerging as key mechanisms determining the fate of cells and may contribute to the development of atherosclerosis, diabetes and neurodegenerative disorders. Oxidative stress (e.g. from mitochondria), lipid peroxides such as HNE and elevation of intracellular Ca²⁺ are known activators of ER stress [102]. In eukaryotic cells, the ER regulates chaperone-assisted protein folding, which requires tight regulation of its internal redox environment to prevent mis-directed disulfide bond formation and accumulation of misfolded proteins [103]. ER stress, induced by excessive accumulation of misfolded proteins, results in activation of the UPR to (i) transiently reduce ER load and induce chaperones to

enhance correct folding capacity, (ii) degrade misfolded proteins via proteasomal and autophagy ER assisted degradation (ERAD) pathways and (iii) induce antioxidant responses to restore redox homeostasis and reduce ER stress [102,104]. If a balanced folding capacity cannot be achieved, induction of pro-apoptotic signaling (e.g. C/EBP-homologous protein, CHOP transcription) triggers programmed cell death. The UPR is activated by the disassociation of Grp78 (BiP) from ER transmembrane UPR effectors and its association with accumulated misfolded proteins, allowing activation of transmembrane ER effectors [105] inositol requiring enzyme 1 (IRE1), RNA-dependent protein kinase-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6), and down-stream signaling cascades including the phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α) and xBP-1 splicing [102,106].

Similar to mitochondria, ER proteins are also thiol abundant and targets of lipid peroxidation products [107]. Whilst not affecting intracellular Ca²⁺, incubation of endothelial cells with 20 μ M HNE for 24 h significantly elevates phosphorylation of IRE1 and eIF2 α , which is effectively inhibited by pre-treatment with NAC, indicating increased ER stress by HNE is thiol dependent [107,108]. Moreover, depleting GSH alone with tert-butylhydroperoxide (tBHP) cannot trigger ER stress [107], implicating a direct modification of ER proteins by HNE. Liquid chromatography-mass spectrometry analysis has shown that chaperones including Grp78, protein disulfide isomerases (PDIs), and heat shock proteins including HSP90 and HSP70 are predominantly conjugated by HNE (25 μ M, 30 min) in human umbilical vein endothelial cells [107]. It is presently unclear whether HNE Michael addition via lysine residues to Grp78 is alone sufficient to trigger UPR activation, but it is likely that HNE adduction to PDI may trigger activation. PDI is a key ER-resident enzyme catalyzing the formation and rearrangement of disulfide bonds, and thus determines protein unfolding capacity in ER. Activity of PDI can be inhibited by HNE in a concentration dependent manner due to cysteine modifications [67], leading to accumulation of unfolded proteins and consequential ER stress [109,110]. Fortunately, PDI modification is reversible by physiological concentrations of GSH [67] allowing for eventual resolution of ER stress.

Secondary to ER stress, activation of the UPR by HNE is evidenced by the upregulation of ER regulators and chaperones (e.g. Grp78 and homocysteine inducible ER protein (HERP)) to enhance overall protein folding capacity [107]. It worth noting that Nrf2 has been identified as a direct downstream signaling target activated by PERK phosphorylation [111], suggesting that HNE-induced Nrf2 activation may also occur secondary to ER stress as well as a consequence of cytosolic or potentially mitochondrial oxidation. Depletion of Nrf2 renders cells more susceptible to ER stress, and Nrf2 activation in response to ER stress activators has been shown to be critical for cell survival [109,111]. These findings highlight that, in addition to enhancing protein folding capacity, restoration of redox homeostasis through activation of Nrf2 mediated induction of GSH related genes, phase II enzymes, antioxidant enzymes and potentially 20S proteasomal gene expression and oxidized protein clearance, is necessary for cellular recovery from HNE stress. Together these Nrf2-mediated responses prevent continued ER stress and activation of apoptotic signaling cascades. Unfortunately, information concerning ER stress in SMCs is extremely limited and to our knowledge there are no reports on whether HNE is capable of inducing ER stress in SMCs. However, induction of ER stress in SMCs by non-lipid peroxides has been reviewed [112] and, as highlighted in this review, a causative relationship between ER stress and apoptosis is lacking in SMCs. Notably, ER stress has important implications for diseases such as atherosclerosis and diabetes in which it has actively been assigned a role in disease pathogenesis.

Apoptosis

Failure to remove HNE adducts following exposure to pathological concentrations of HNE results in cellular dysfunction and oxidative damage in organelles such as mitochondria and the ER in vascular cells. In human endothelial cells, pathological concentrations of HNE increase caspase activation and induce apoptosis [23]. Similarly in SMCs, HNE has been shown to increase mitochondrial generation of reactive oxygen radicals and induce apoptosis [96], which was augmented by AR inhibition and reduced HNE clearance [28]. DNA adduction and subsequent fragmentation has also been reported to occur following HNE treatment of both endothelial and SMCs [72,113].

In most studies, pathological concentrations of HNE are required to initiate apoptosis, with the hormetic effects of HNE highlighted in the proliferation responses of SMCs. Whilst low physiological levels of HNE (0.1–1 μ M) dose-dependently increase protein synthesis and SMC proliferation, exposure to higher concentrations $> 1 \mu$ M progressively inhibit protein synthesis and induce cell apoptosis. It has recently been suggested that detoxification of HNE through GSH phase II reactions may represent an important buffering system within cells to limit and create specificity in lipid peroxide signaling by preventing aberrant HNE modifications [31]. Indeed, the capacity of cells to remove HNE and HNE-damaged proteins (via GSTs, AR, RLIP76, proteasome, autophagy, UPR etc.) clearly plays a critical role in determining cell fate. Moreover, in vascular diseases in which cells may be exposed chronically to elevated levels of HNE, it could be argued that HNE-induced apoptosis can occur not only as a result of accumulative damage but as a loss of normal cell signaling (e.g NO production, TKR activation and downstream kinase signaling) inducing progressive endothelial dysfunction ultimately resulting in programmed cell death.

Role of HNE in vascular diseases

Lipid peroxide induced damage is widely thought to be an active contributor to a number of vascular diseases, including, atherosclerosis, neurodegenerative disorders, diabetes and pregnancy diseases such as pre-eclampsia and gestational diabetes. Unlike reactive oxygen species, lipid peroxides are generally more stable allowing them to diffuse through membranes and affect distant organelles or tissues including fetal tissues following transplacental transfer [1,114,115]. Indeed, HNE-modified oxLDL accumulates in atherosclerotic plaques [13,116], with HNE adducts and associated peroxides also reported pancreatic beta cell islets [117], neuronal tissue [118] and in circulating cord blood [119]. Notably, and as highlighted below, whilst HNE accumulation in many vascular disease models is well documented, evidence directly linking HNE to cellular dysfunction is in most cases associative rather than causal, underlining the need for further *in vitro* studies to assess the specific effects of HNE on cellular organelles.

In utero diseases

Lipid peroxides including HNE have been extensively implicated in the pathogenesis of *in utero* diseases such as pre-eclampsia through the induction of pro-inflammatory signaling cascades [120] in the maternal as well as fetal vasculature. Moreover, as reviewed elsewhere [119,120], enhanced HNE formation appears to correlate with alterations in fatty acid metabolism and co-insides with impairments in antioxidant defenses, with these changes ultimately resulting in systemic vascular dysfunction. Commonly, central to the role of HNE in disease is the dysregulation of organelles such as mitochondria and the ER. Whilst PE appears to have a vast inflammatory component associated with its etiology, markers of ER stress and mitochondrial dysfunction have been detected. Activation of UPR effector pathways is clearly evidenced in the

placentas of PE pregnancies [121], although no causative link has yet been made to suggest that ER stress occurs secondary to lipid peroxidation. Furthermore, ER stress remains to be assessed in the fetal vasculature, although markers of mitochondrial dysfunction are found in fetal endothelial cells [122] and are well documented in the placenta to co-inside with markers of oxidative stress [123].

Atherosclerosis

In atherosclerosis and cardiac disorders, there are indications that HNE-induced mitochondrial dysfunction and ER stress may play a key role in disease pathogenesis through the modulation of vascular cells. In spontaneously hypertensive rats, HNE adducts to mitochondrial NADP⁺-isocitrate dehydrogenase and reduced enzymatic activity have been reported and shown to enhance cardiac hypertrophy [124]. Furthermore, *in vitro* oxLDL has been shown to lead to endothelial HNE accumulation and the induction of ER stress through the inhibition of PDI activity, consequent downstream effector signaling (e.g xBP-1 splicing) and endothelial caspase activation and apoptosis [110]. *In vivo*, endothelial ER stress in vessel regions associated with low or disturbed shear stress have also been reported [125,126] and even to precede loss of Nrf2 [126]. ER stress appears to have a negative effect on endothelial cell stability [125], which may in part account for plaque susceptibility to rupture through ER stress-mediated apoptosis, promoting loss of the endothelial layer covering the plaque. Without direct confirmation that HNE can induce ER stress within SMCs, it is more difficult to assess whether this vascular cell type can also be destabilized during atherosclerosis as a consequence of oxLDL/HNE accumulation.

Diabetes

ER stress has also been implicated in diabetic pathologies, including in beta cell death [102,127] and vascular dysfunction in diabetic retinopathy [128]. Whilst there is little information regarding the potential role of lipid peroxides in inducing ER stress within pancreatic beta cells, ER stress markers have been found in the retinas of human diabetic patients [128] and may be as a consequence of oxidative stress or elevated HNE production. *In vitro* human retinal capillary pericytes treated with oxLDL or HNE exhibit markers of ER stress and mitochondrial dysfunction [128], with ER stress also found in human retinal Muller cells following oxLDL or HNE exposure [129]. Although not well characterized in diabetes, it is likely that the vascular endothelium may also be affected, since treatment of healthy human endothelial cells with HNE induces ER stress [107]. Similarly, mitochondrial dysfunction and the resulting elevation of reactive oxygen species generation has been implicated in the pathogenesis of diabetes [50], and there is evidence to support HNE-induced mitochondrial dysfunction in vascular diabetic pathologies. In diabetic rat hearts HNE adducts formed with complex II subunit SDHA have been shown to result in reduced mitochondrial respiration, with insulin administration able to prevent HNE adduction and loss of mitochondrial function [130]. Conversely, in diabetic skeletal muscle biopsies reduced mitochondrial respiration has been detected with the absence of HNE accumulation in tissue, suggesting that in diabetes mitochondrial dysfunction may be induced independently from lipid peroxide generation [131].

Neurodegenerative diseases

In neurodegenerative diseases associated with amyloid, α -Synuclein or transthyretin protein deposition such as Alzheimer's disease, Parkinson's disease or familial amyloidotic polyneuropathy (FAP), indirect associations have been made between HNE-induced oxidative stress and downstream ER stress/UPR activation or mitochondrial dysfunction. HNE colocalises with Lewy body

protein accumulations present within brain tissues, such as in Parkinson's disease [118], indirectly implicating HNE in the pathogenesis of these diseases. In addition, HNE has been shown to directly form Michael additions with proteins such as α -Synuclein implicated in familial Parkinson's [132], however the oligomers formed, whilst toxic, are not fibrillar implying that HNE alone cannot account of the formation of Lewy bodies. In Parkinson's patients, the accumulation of HNE [118] and markers of ER stress such as the upregulation of UPR target HERP have been reported in Lewy bodies present within the substantia nigra [133]. Whilst not providing a definite link, in retinal Muller glial cells HNE has been shown to induce ER stress *in vitro* [129] in agreement with the possibility that in the brain HNE-induced ER stress may be a cause rather than consequence of neuronal injury. In FAP, use of antioxidant scavengers was able to reduce HNE accumulation and markers of ER stress in stomach tissue and reduce transthyretin accumulation [134], suggestive of an active role of redox regulation in transthyretin deposition. Conversely, transthyretin has been shown to directly activate ER stress, though whether this is through HNE accumulation remains unknown [135]. Furthermore, following neuronal injury such as in stroke, HNE concentrations are increased in plasma and positively correlate with homocysteine concentrations [136]. Whilst not directly implicating HNE in the pathogenesis of stroke, spontaneously hypertensive stroke prone rats exhibit higher circulating levels of HNE [136] and HNE has also been shown form Michael additions to various adhesion molecules inducing breakdown of endothelial barrier function, which may have important implications for blood-brain barrier integrity [88]. Consistent with a role for HNE in inducing mitochondrial dysfunction, Sompol et al. have reported that in an animal model of Alzheimer's disease, HNE accumulation is associated with reduced mitochondrial membrane potential in neuronal cells [137]. As overexpression of MnSOD or use of a SOD mimetic was able to partially restore mitochondrial function, it is likely that HNE modulates mitochondrial superoxide output either directly by adduction to the respiratory chain or indirectly through inhibition of antioxidant proteins such as Trx2.

Concluding remarks and future research perspectives

Bioactive lipid peroxides such as HNE are increasingly becoming recognized as exerting hormetic actions within the vasculature (Fig. 1). Low physiological levels of HNE appear to positively modulate the cell cycle and proliferation of vascular cells [44] and, by inducing non-toxic protein oxidation, can induce long-term cytoprotection through the activation of Nrf2 antioxidant defenses protecting against later oxidant insults [55]. Indeed, oxLDL, containing 0.5 μ M free HNE, and 5 μ M HNE alone have been shown in human endothelial cells to upregulate a variety of Nrf2-dependent genes with a similar transcriptional profile to laminar shear stress [138], arguably the ultimate vasoprotective stimulus exerted in the vasculature. In endothelial cells, there is limited information concerning the effects of physiological concentrations of HNE (< 1 μ M) on endothelial function, which may have important implications for regulation of basal transcriptional activity and thus merits further investigation. Progressively higher concentrations of HNE, extending into the pathological range, are well documented to exert opposing effects. At high doses, HNE induces widespread oxidation of cellular proteins [1], particularly those located within cholesterol rich membranes or in organelles with a high thiol content such as the mitochondrial or the ER [71,107]. Modification of such proteins can not only induce a pro-inflammatory phenotype or disrupt endothelial barrier function [88], but can also precipitate further oxidative stress by disrupting protein folding and potentially mitochondrial function, which if not counteracted by Nrf2 antioxidant defenses ultimately results in apoptosis [10,71,107]. Further studies

using both physiological and pathological HNE concentrations will enhance our understanding of the extent and effects of HNE adduction to mitochondrial or ER associated proteins in vascular endothelial and SMCs, and will have important implications for targeting endogenous antioxidant defense pathways to prevent or limit the progression of vascular diseases.

Levels of HNE within tissues are dependent not only on the rate of fatty acid oxidation and thus HNE production, but also on its removal by phase II metabolic pathways. Key to the removal of HNE is its conjugation to the abundant thiol GSH, which in vascular cells is primarily mediated via GST-4 [20]. Whilst GSH conjugation is essential for removal of HNE, importantly some HNE conjugates such as GS-DHN produced by AR are also bioactive and may perhaps help to prolong the biological actions of short-lived HNE following low-dose HNE stimulation [45]. Interestingly, whilst modulation of some HNE degradation pathways such as the HNE-conjugate transporter RLIP76 appear to have a clear role in disease pathologies such as in atherosclerotic lesions or metabolic syndrome [78,80], others such as AR are less clearly defined. Thus, when considering redox modulating therapies, there is a need to consider both the hormetic effects of HNE in vascular pathologies and other potential off-target effects following HNE exposure.

Whilst studies clearly demonstrate short-term inhibition of AR is able to upregulate low dose HNE proliferative effects within vascular cells, AR activity is also critical in promoting cell survival following aberrant HNE modifications in response to higher HNE doses [45]. Moreover, in diseases such as diabetic retinopathy associated with HNE accumulation [139], AR activity is reportedly higher [140], presumably in part to increase HNE clearance and combat oxidative stress. Whilst elevated AR activity may be of benefit in removing HNE conjugates, increased AR activity also increases glucose metabolism to sorbitol, resulting in osmotic stress and cytotoxicity [140]. In such instances where it is clear that dampening AR activity may be of overall benefit in terms of restoring redox homeostasis via relieving glucose-induced cytotoxicity, increasing HNE degradation by other means may provide an alternative strategy to alleviate HNE-induced oxidative injury.

In conclusion, HNE is capable of modulating both survival and proapoptotic pathways within vascular endothelial and SMCs. Aging or disease pathologies associated with HNE accumulation may be due to both elevated HNE synthesis and/or decline in phase II metabolic pathways required to remove HNE, inducing aberrant HNE protein oxidation and resulting in vascular dysfunction. Whilst HNE can form widespread protein adducts, specific organelles including the plasma membrane, mitochondria and ER clearly more susceptible to HNE modification, and thus the convergence of HNE concentration and activation of clearance pathways within these organelles may be the critical determinant as to whether cells can induce adaptive antioxidant defenses via Nrf2 to counteract HNE modifications or ultimately undergo apoptosis. Targeting of endogenous antioxidant defenses such as Nrf2-linked genes to remove HNE adducts may be of therapeutic benefit, but considerations concerning the effects on parallel metabolic pathways should be considered when pursuing this strategy.

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