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Review

Lipid peroxidation and cell cycle signaling: 4-hydroxynonenal, a key molecule in stress mediated signaling✪

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Role of lipid peroxidation products, particularly 4-hydroxynonenal (4-HNE) in cell cycle signaling is becoming increasingly clear. In this article, recent studies suggesting an important role of 4-HNE in stress mediated signaling for apoptosis are critically evaluated. Evidence demonstrating the modulation of UV, oxidative stress, and chemical stress mediated apoptosis by blocking lipid peroxidation by the α-class glutathione S-transferases (GSTs) is presented which suggest an important role of these enzymes in protection against oxidative stress and a role of lipid peroxidation products in stress mediated signaling. Overexpression of 4-HNE metabolizing GSTs

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Abbreviations: AP-1, the transcription factor activator protein; CAT, catalase; CBAs, chain-breaking antioxidants; CU-OOH, cumene hydroperoxide; $cPLA_2$, cytosolic phospholipase A_2; FA-OOH, fatty acid hydroperoxides; HLE B-3 cells, human lens epithelial cells; 4-HNE, 4-hydroxynonenal; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; 13*S*-HPODE, 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid; GSH, glutathione (reduced form); GPxs, glutathione peroxidases; GS-HNE, glutathione conjugate of 4-HNE; GST, glutathione *S*-transferase; K562 cells, human erythroleukemia cells; LOOH, lipid hydroperoxide; MAPK, mitogen activated protein kinases; NFκ-B, nuclear factor kappa B; PC-OOH, phosphatidylcholine hydroperoxide; PL-OOH, phospholipid hydroperoxides; PUFA, polyunsaturated fatty acids; RLIP76, 76 kDa Ral-binding GTPase activating protein (RalBP1); ROS, reactive oxygen species; SAPK/JNK, stress activated protein kinase/c-Jun N-terminal kinase; SOD, superoxide dismuatase; t-BHP, *tert*-butyl hydroperoxide.

(mGSTA4-4, hGSTA4-4, or hGST5.8) protects cells against 4-HNE, oxidative stress (H_2O_2 or xanthine/xanthine oxidase), and UV-A mediated apoptosis by blocking JNK and caspase activation suggesting a role of 4-HNE in the mechanisms of apoptosis caused by these stress factors. The intracellular concentration of 4-HNE appears to be crucial for the nature of cell cycle signaling and may be a determinant for the signaling for differentiation, proliferation, transformation, or apoptosis. The intracellular concentrations of 4-HNE are regulated through a coordinated action of GSTs (GSTA4-4 and hGST5.8) which conjugate 4-HNE to GSH to form the conjugate (GS-HNE) and the transporter 76 kDa Ral-binding GTPase activating protein (RLIP76), which catalyze ATP-dependent transport of GS-HNE. A mild stress caused by heat, UV-A, or H_2O_2 with no apparent effect on the cells in culture causes a rapid, transient induction of hGST5.8 and RLIP76. These stress preconditioned cells acquire ability to metabolize and exclude 4-HNE at an accelerated pace and acquire relative resistance to apoptosis by UV and oxidative stress as compared to unconditioned control cells. This resistance of stress preconditioned cells can be abrogated by coating the cells with anti-RLIP76 antibodies which block the transport of GS-HNE. These studies and previous reports discussed in this article strongly suggest a key role of 4-HNE in stress mediated signaling.

It has been known for a long time that reactive oxygen species (ROS) and the lipid peroxidation products formed due to oxidative stress are cytotoxic. Recent studies suggest that both, ROS and lipid peroxidation products, are also involved in the intracellular signaling mechanisms which determine the cell's final fate. Intracellular levels of ROS may be crucial for determining whether the cells undergo differentiation, proliferation, or apoptosis because it has been shown that at low concentrations, H_2O_2 induces mitosis in fibroblasts, while at relatively higher concentrations, it causes a transient cell growth arrest by inducing the expression of gadd45, gadd153, and adapt15 genes (Crawford *et al.*, 1996). Even further higher concentrations permanently damage the cells perhaps due to the combined effects of apoptosis and necrosis (Chen & Ames, 1994; Davies, 2000). Likewise, it has been shown that while higher levels of superoxide anions (O_2^-) are toxic, low levels of O_2^- elicit cellular proliferation in a variety of cells including fibroblasts, amnion cells, and colonic epithelial cells (Murrell *et al.*, 1990; Ikebuchi *et al.*, 1991; Craven *et al.*, 1986). Interestingly, lipid peroxidation products, particularly 4-HNE, also affect signaling mechanisms in a concentration dependent manner. It has been shown that while low levels of 4-HNE promote proliferation (Ruef *et al.*, 1998; Cheng *et al.*, 1999), at higher concentrations it induces differentiation and apoptosis (Dianzani *et al.*, 1999; Cheng *et al.*, 1999; 2001a; 2001b; Kruman *et al.*,1997; Soh *et al.*, 2000). Since O_2^- and H_2O_2 are known to cause membrane lipid peroxidation through Fenton reaction ($H_2O_2 + Fe^{2+} = {}^{\bullet}OH + Fe^{3+}$), it is conceivable that O_2^- and H_2O_2 mediated cellular signaling may indeed be transduced at least in part, through lipid peroxidation products. This idea finds support from the studies demonstrating that lipid hydroperoxides (LOOH) and 4-HNE *per se* can affect intracellular signaling mechanisms (Leonarduzzi *et al.*, 2000; Uchida *et al.*, 1999).

It is possible that the stress mediated signaling for apoptosis in general may involve lipid peroxidation products because most of the stress causing agents, e.g., oxidants, heat shock, UV irradiation, and serum starvation etc., invariably induce lipid peroxidation. Recent studies in our laboratory strongly support this idea and suggest that stress mediated cellular signaling is at least in part due to lipid peroxidation products, 4-HNE in particular (Yang *et al.*, 2001; Cheng *et al.*, 2001a; 2001b). These studies show that stress (UV, heat, oxidative, or chemical) mediated signaling in various cells can be modulated by manipulating the lipid peroxidation levels resulting from the stress. These studies also suggest

that the α-class GSTs may be the major determinants of the intracellular concentrations of lipid peroxidation products and that these enzymes can modulate stress mediated signaling. In this review, we have summarized these studies against the back drop of our current understanding of the role of lipid peroxidation products in signaling processes. Furthermore, we have evaluated the physiological roles of GSTs, and a newly discovered transporter RLIP76 (Awasthi *et al.*, 2000; 2001), in regulating the intracellular concentrations of 4-HNE.

REACTIVE OXYGEN SPECIES AND LIPID PEROXIDATION

In aerobic organisms, ROS including O_2^-, H_2O_2, hydroxyl radical ($^{\bullet}OH$), and nitric oxide/peroxynitrate ($NO/ONOO^-$) are continually generated in cells *via* various endogenous and exogenous sources such as mitochondrial electron transport chain, biotransformation of various xenobiotics and drugs, inflammation, UV and ionic irradiation, and nitric oxide synthetase (Ames *et al.*, 1993; Beckman & Koppenol, 1996). ROS are toxic and are known to be involved in the etiology of age-related disease such as Alzheimer's disease, cataract, atherosclerosis, Parkinson's disease (Bhuyan *et al.*, 1986; Witztum, 1994; Markesbery & Lovell, 1997; Yoritaka *et al.*, 1996), etc. To detoxify ROS, mammalian cells have developed elaborate defense mechanisms. Oxidative stress occurs in the cells as a consequence of an imbalance between the pro-oxidant/antioxidant systems (Sies, 1985). Oxidative stress causes damage to cellular macromolecules such as nucleic acids, proteins, and lipids. Among these targets, the peroxidation of lipids is particularly more damaging because the formation of lipid peroxidation products leads to a facile propagation of free radical reactions. Abstraction of a hydrogen atom from the polyunsaturated fatty acid (PUFA) moiety of membrane phospholipids initiates the process of lipid peroxidation (Fig. 1). The resulting alkyl radical may rearrange to a more stable conjugated diene, which enters the autocatalytic lipid peroxidation cascade. Phospholipid hydroperoxides (PL-OOH) and fatty acid hydroperoxides (FA-OOH) constitute the major portion of the lipid peroxidation products and can propagate lipid peroxidation chain reactions. The fatty acid carbon chain may also be spontaneously cleaved (β-scission) during lipid

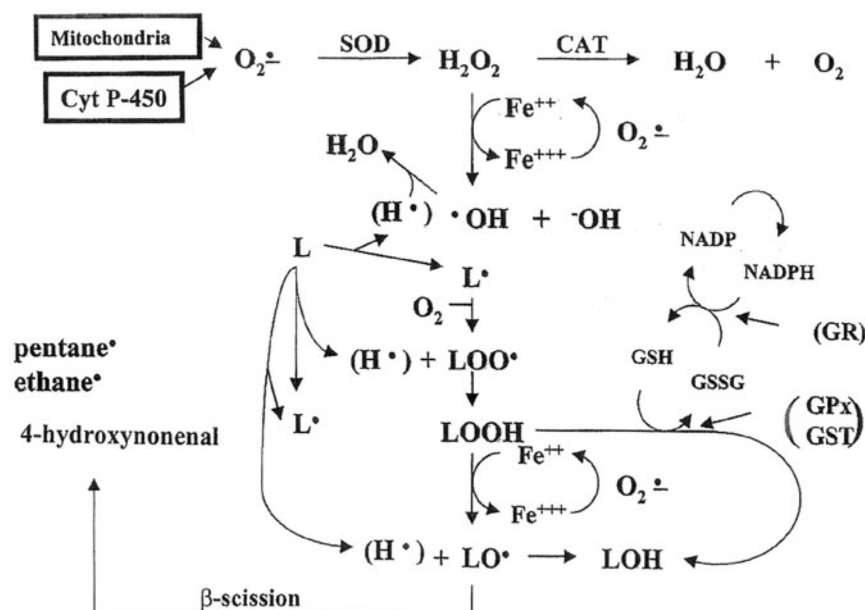

Figure 1. Lipid peroxidation, an autocatalytic chain process.

peroxidation, yielding a variety of highly reactive compounds, including pentane and ethane radicals, and the α,β-unsaturated aldehydes. In particular 4-HNE, the major α,β-unsaturated aldehyde formed by the degradation of both ω-3 and ω-6 PUFA during lipid peroxidation is fairly stable and present in relatively higher amounts in biological membranes under the conditions of oxidative stress (Esterbauer *et al.*, 1991).

DEFENSE AGAINST INTRACELLULAR LIPID PEROXIDATION

In mammalian cells, there are two tiers of antioxidant defense mechanisms against ROS mediated lipid peroxidation. Low molecular mass compounds which act primarily against peroxyl radicals involved in radical propagation provide first line of defense against lipid peroxidation. These compounds referred as "chain-breaking antioxidants (CBAs)" can terminate the propagation of free radical mediated reactions and interrupt the autocatalytic chain reaction of lipid peroxidation (Acworth *et al.*, 1997). The main cellular CBAs include α-tocopherol, ascorbic acid, glutathione (GSH), uric acid, carotenoids, ubiquinone, and polyphenols, etc. (Acworth *et al.*, 1997). Among these, GSH is particularly important because it also serves as the substrate for the two major antioxidant enzyme systems, glutathione peroxidases (GPxs) and GSTs.

The antioxidant enzymes constitute the second line of defenses which provide a variety of primary and secondary defenses against oxidative stress. Primary antioxidant enzymes are mainly preventive and these enzymes such as superoxide dismuatase (SOD), catalase (CAT), and GPxs can decompose ROS and prevent the damage to cellular constituents and initiation of lipid peroxidation. Secondary defenses typically involve excision or repair of any lesions caused by ROS. In the event of ROS induced lipid peroxidation, secondary defense enzymes are involved in the removal of LOOH to terminate the autocatalytic chain of lipid peroxidation and protect membranes. GPxs and GSTs which catalyze GSH-dependent reduction of LOOH (PL-OOH and FA-OOH) through their peroxidase activity are the major secondary defenses to guard against ROS-induced lipid peroxidation. So far, four selenium-dependent GPx isoenzymes have been characterized in mammalian tissues (Awasthi *et al.*, 1975; Chu *et al.*, 1993; Takahashi *et al.*, 1987; Ursini *et al.*, 1985). Among these enzymes, GPx-1, GPx-2, and GPx-3 are primarily involved in the reduction of H_2O_2 and FA-OOH, whereas GPx-4 displays activity towards PL-OOH and cholesterol hydroperoxides (Thomas *et al.*, 1990).

ROLE OF GLUTATHIONE *S*-TRANSFERASES IN THE REGULATION OF LIPID HYDROPEROXIDE LEVELS

In addition to selenium-dependent GPxs, the selenium-independent GPx activity of the α-class GSTs is also involved in the reduction of LOOH. A GPx activity designated as GPx-II was first described in rat liver (Lawrence & Burk, 1976) and was later shown to be catalyzed by GSTs (Prohaska & Ganther, 1976). Subsequently, it was shown that in human liver, the GPx-II activity was not expressed by all isoenzymes of the cytosolic GSTs but was confined only to the α-class GSTs (Awasthi *et al.*, 1980; Singhal *et al.*, 1992). Comprehensive reviews on GSTs are available (Mannervik & Danielson, 1988; Hayes & Pulford, 1995; Awasthi *et al.*, 1994) and a table of α-class GSTs with relevance to lipid peroxidation products is provided (Table 1) with their currently used designations (Mannervik *et al.*, 1992).

Earlier studies (Awasthi *et al.*, 1980) used the organic hydroperoxides such as cumene hydroperoxide (CU-OOH) and *tert*-butyl hydroperoxide (t-BHP) as the substrates for

GPx-II activity of GSTs and provided no information on the physiological role of GSTs in the GSH-dependent reduction of the LOOH generated during lipid peroxidation. Studies in our laboratory have shown that human α-class GST isoenzymes can effectively reduce both FA-OOH and PL-OOH (Fig. 2) through their selenium-independent GPx activity (Singhal *et al.*, 1992; Zhao *et al.*, 1999; Yang *et al.*, 2001). The α-class GST isoenzymes of other mammalian species also display this activity. For example, the α-class GSTs isolated from rat skin catalyze the reduction of cholesterol hydroperoxides (Hiratsuka *et al.*, 1997) and the α-class GSTs of rat and mouse liver can utilize lipid peroxidation products as substrates (Zimniak *et al.*, 1994; Yang *et al.*, 2002a; 2002b). The GPx activity of human α-class GSTs towards PL-OOH has been confirmed by studies with the recombinant GST isoenzymes, hGSTA1-1 and hGSTA2-2 (Zhao *et al.*, 1999; Yang *et al.*, 2001). More importantly, recent studies in our laboratory have demonstrated that hGSTA1-1 and hGSTA2-2 can reduce PL-OOH present in biological membranes which strongly suggest that these enzymes can arrest membrane lipid peroxidation *in situ* (Yang 2001; 2002a; 2002b). Thus contrary to earlier suggestions (Tan *et al.*, 1984), the α-class GSTs can reduce membrane PL-OOH without requiring phospholipase A_2 mediated release of FA- OOH from the peroxidized membrane phospholipids.

The contribution of the α-class GSTs in the reduction of PL-OOH in various human and rat tissues has been measured by immunoprecipitation studies using highly specific polyclonal antibodies against the α-class GSTs (Yang *et al.*, 2001; 2002b). These studies indicate that in liver and testes of normal humans and rats, about 50–60% of total GPx activity towards PL-OOH is contributed by the α-class GSTs. It appears that a higher expression of the α-class GSTs in these tissues provides these vital organs an added protection against ROS induced lipid peroxidation. In the liver of GPx-1 knockout mice, GPx activity towards H_2O_2 is decreased by about 90% but there is no significant decrease in the GPx activity towards PL-OOH. Kinetic studies using recombinant α-class GSTs and the GSTs purified from human lung and human lens epithelial cells show that K_m of these enzymes for PL-OOH is in the range of 30–50 μM which falls within the range of estimated physiological concentrations of PL-OOH, particularly during the oxidative stress (Singhal *et al.*, 1992). These findings further reaffirm that the α-class GSTs play a key role in regulating lipid peroxidation by terminating the autocatalytic chain of lipid peroxidation.

Table 1. GST isoenzymes involved in detoxification of lipid peroxidation products

Enzyme name*	Species	Class	Preferred substrate**
hGSTA1-1 and hGSTA2-2	human	Alpha	PL-OOH, FA-OOH
hGSTA4-4	human	Alpha	4-HNE
hGST5.8	human	Alpha	4-HNE
mGSTA4-4	mouse	Alpha	4-HNE
rGSTA4-4	rat	Alpha	4-HNE

*In this nomenclature (Mannervik *et al.*, 1992), lowercase letter identifies species while uppercase letter identifies the class (alpha). Nomenclature of hGST5.8 is based on its pI value of 5.8. mGSTA4-4, hGST5.8, and rGSTA4-4 are immunologically similar but distinct from hGSTA1-1, hGSTA2-2, hGSTA3-3 and hGSTA4-4. **PL-OOH, phospholipid hydroperoxides; FA-OOH, fatty acid hydroperoxides; 4-HNE, 4-hydroxynonenal.

ROLE OF GLUTATHIONE *S*-TRANSFERASES IN THE REGULATION OF 4-HYDROXYNONENAL LEVELS

In addition to the cationic α-class GSTs (GSTs A1-1, A2-2, and A3-3), a subgroup of the anionic α-class GSTs present in human (hGST5.8) (Singhal *et al.,* 1994a; 1994b), rat (rGSTA4-4) (Alin *et al.*, 1985; Stenberg *et al.*, 1992), and mouse (mGSTA4-4) (Medh *et al.*, 1991; Zimniak *et al.*, 1992; 1994) can utilize 4-HNE as the preferred substrate and conjugate it to GSH with high efficiency. In humans, at least two GST isoenzymes with high affinity for 4-HNE have been described. A hGST isoenzyme immunologically similar to mGSTA4-4 with higher specific activity towards 4-HNE was first described in our laboratory (Singhal *et al,*. 1994a; 1994b; 1995; 1999). This enzyme is temporarily designated as hGST5.8 (according to its pI value) since its complete primary structure is still not known and it has not been cloned yet perhaps due to its extremely low constitutive abundance in cDNA libraries (Cheng *et al.*, 2001a). Another human enzyme hGSTA4-4 with high affinity for 4-HNE has been cloned (Hubatsch *et al.*, 1998). Subsequent studies have shown that hGSTA4-4 and hGST5.8 are immunologically distinct not only from other human GSTs but also from each other and are expressed in a tissue specific manner (Cheng *et al.*, 2001c). Both, hGST5.8 and mGSTA4-4, display much lower GPx activity towards PL-OOH as compared to that of hGSTA1-1 and hGSTA2-2 (Zimniak *et al.*, 1994; Singhal *et al.*, 1994a; 1994b). However, the catalytic efficiency of these enzymes for the conjugation of 4-HNE to GSH is higher than the efficiencies of most of the GST isoenzymes for xenobiotics. This observation along with their low K_m for 4-HNE strongly suggest that these enzymes play an important role in the regulation of the intracellular levels of 4-HNE.

In vitro studies with cell lines strongly suggest that GSTsA4-4 and hGST5.8 are the major determinants of the intracellular concentrations of 4-HNE (Cheng *et al.*, 1999; 2001a). Even though 4-HNE can be reduced by aldehyde dehydrogenase and aldose reductase (Hartley *et al.*, 1995; Srivastava *et al.*, 1995), majority of cellular 4-HNE is metabolized through its conjugation by reaction catalyzed by GSTs (Srivastava *et al.*, 1998). Overexpression of mGSTA4-4 leads to a dramatic decrease in the levels of 4-HNE in human erythroleukemia cells (Cheng *et al.*, 1999) due to its conjugation to GSH. The conjugate of 4-HNE (GS-HNE) must be transported out of cells to sustain GST-mediated conjugation of 4-HNE because the conjugate inhibits GSTs. GS-HNE is transported from cells by ATP-dependent primary active transport similar to other GSH-conjugates (Awasthi *et al.*, 1994; 1998a; 1998b; 2000). Our studies with various cell lines and erythrocytes indicate that in humans, majority (about 2/3) of GS-HNE transport is catalyzed by RLIP76 (Awasthi *et al.*, 2000; 2002; Sharma *et al.*, 2001; 2002), a previously described Ral binding protein (Jullien-Flores *et al.*, 1995). These studies

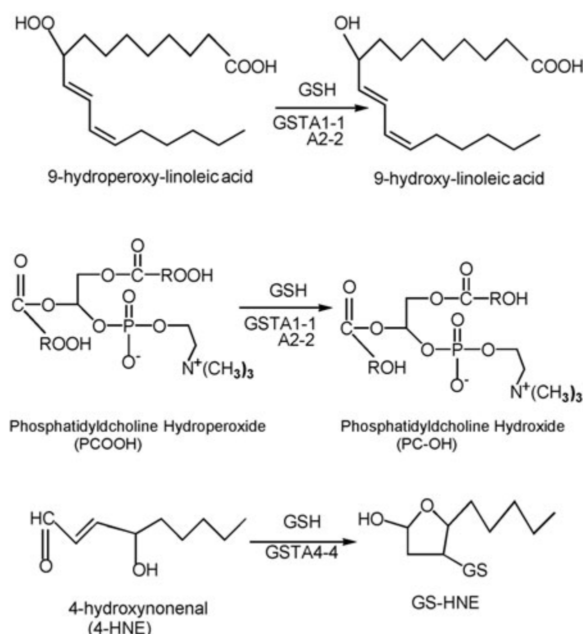

Figure 2. Lipid peroxidation products are substrates of GSTs.

(Singhal *et al.*, 2003, Awasthi *et al.*, 2003a; 2003b) show that the multidrug resistance protein (MRP1) accounts for only about 1/3 of GS-HNE transport which is consistent with reports that MRP1 also mediates transport of GS-HNE (Renes *et al.*, 2000). We have shown that a coordinated action of GSTs and RLIP76 (Fig. 3) is the major determinant of 4-HNE concentration in cells. During oxidative stress, heat shock, or UV irradiation, which cause increased 4-HNE levels in cells, a rapid but transient induction of hGST5.8 and RLIP76 occurs, which strongly suggests that both these proteins play an important role in the regulation of the intracellular levels of 4-HNE (Cheng *et al.*, 2001a). Cells with induced hGST5.8 and RLIP76, transport GS-HNE at a several fold higher rate as compared to the controls which further confirms the role of these proteins in regulations of cellular concentrations of 4-HNE. Manipulating the intracellular concentrations of these proteins has provided useful information on the role of 4-HNE in signaling (Cheng *et al.*, 1999; 2001a; Yang *et al.*, 2001) and some of these studies are discussed in detail later in this article.

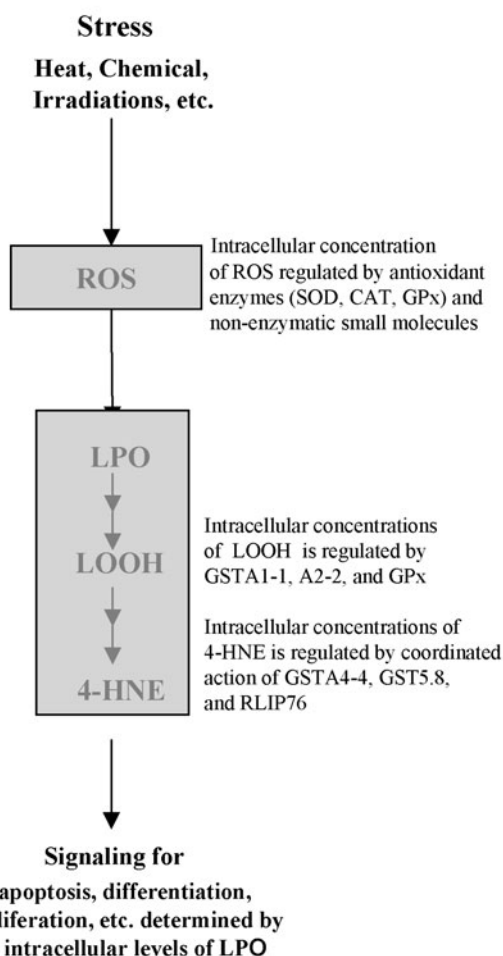

Figure 3. Lipid peroxidation and stress-mediated signaling.

ROLE OF LIPID PEROXIDATION PRODUCTS IN SIGNALING

4-HNE and LOOH have been extensively studied for their possible roles in signaling (see for review Esterbauer *et al.*, 1991; Dianzani *et al.*, 1999). Review of all these studies is beyond the scope of this article and only some of these studies which implicate LOOH and 4-HNE in signaling are reviewed briefly.

Lipid hydroperoxides and signaling

It has been shown that membrane PL-OOH can activate PKC activity, which in turn promotes cytosolic phospholipase A_2 ($cPLA_2$) phosphorylation and its translocation to membrane, possibly through mitogen activated protein kinases (MAPK). Thus, PL-OOH can increase the hydrolytic activity of $cPLA_2$ without marked changes in intracellular free Ca^{2+} concentration (Rashba-Step *et al.*, 1997; Suzuki *et al.*, 1997). PL-OOH can also stimulate the interleukin-1-induced nuclear factor kappa B (NFκB) activation in a human endothelial cell line as suggested by studies showing that overexpression of GPx-4, which preferentially reduces PL-OOH, leads to the inhibition of the above signaling (Brigelius-Flohe *et al.*, 1997). PL-OOH separated from oxidized low density lipoprotein shows platelet-activating factor-like activity, which can stimulate neutrophil adhesion and smooth muscle cell proliferation (Heery *et al.*, 1995). Low levels of FA-OOH including 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and 13*S*-hydroperoxy-9*Z*,11*E*-octa-

decadienoic acid (13*S*-HPODE) are required for cyclooxygenase and 5- or 15-lipoxygenase activity (Schnurr *et al.*, 1996; Foley, 2001). Yang *et al.* (2001; 2002a) have shown that PL-OOH can induce apoptosis in human erythroleukemia (K562) and human lens epithelial (HLE B-3) cells through a sustained activation of stress activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and caspase 3.

4-Hydroxynonenal and signaling

4-HNE, a relatively stable end product of lipid peroxidation, is a potent alkylating agent which can react with a variety of nucleophilic sites in DNA and protein, generating various types of adducts (Esterbauer *et al.*, 1991). Its role in signaling mechanisms has been suggested for quite some time (Barrera *et al.*, 1996). Lately, numerous studies from different laboratories using a variety of cell lines have shown that 4-HNE activates SAPK/JNK (Parola *et al.*, 1998; Uchida *et al.*, 1999; Cheng *et al.*, 2001b), a member of MAPK family which is involved in apoptosis (Seger & Krebs, 1995). In hepatic stellate cells, 4-HNE activates JNK through direct binding and not by phosphorylation (Parola *et al.*, 1998), while in other cells, 4-HNE appears to activate JNK through the redox-sensitive MAPK kinase cascade (Uchida *et al.*, 1999). It has been suggested that 4-HNE-induced JNK activation promotes its translocation in the nucleus where JNK-dependent phosphorylation of c-Jun and the transcription factor activator protein (AP-1) binding take place (Camandola *et al.*, 2000; Cheng *et al.*, 2001b), leading to the transcription of a number of genes having AP-1 consensus sequences in their promoter regions. JNK activation by 4-HNE also leads to the activation of caspase 3 (Soh *et al.*, 2000; Cheng *et al.*, 2001b).

4-HNE has also been shown to modulate the expression of various genes, including PKC βII, c-myc, procollagen type I, aldose reductase, c-myb, and transforming growth factor β1 (Poli & Schaur, 2000). Although the majority of studies show that 4-HNE is pro-apoptotic, it can also stimulate cell proliferation at relatively lower intracellular concentrations (Ruef *et al.*, 1998; Cheng *et al.*, 1999). In HL-60 cells, physiological concentration of 4-HNE inhibits proliferation and induces a granulocyte-like differentiation (Barrera *et al.*, 1996). Several studies suggest that the intracellular concentration of 4-HNE may differentially affect the signal for proliferation, differentiation, and apoptosis (Cheng *et al.*, 1999; 2001a; 2001b). A simplified version of the proposed role of lipid peroxidation products in stress mediated signaling is outlined in Fig. 4. The hypothesis stems from the studies

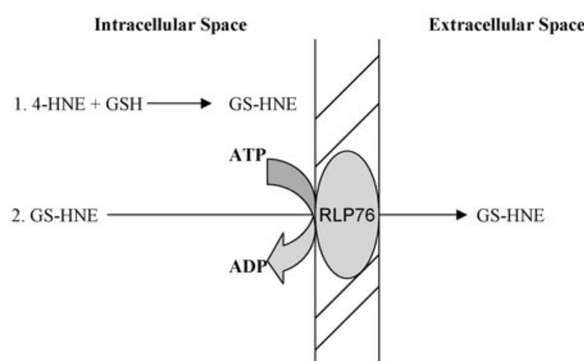

Figure 4. Regulation of 4-HNE concentration by coordinated action of GSTs and RLIP76.

1. Reaction catalyzed by hGST5.8 (Singhal *et al.*, 1994), hGSTA4-4 (human) (Hubatsch *et al.*,1998), mGSTA4-4 (mouse) (Zimniak *et al.*, 1994), and rGSTA4-4 (rat) (Stenberg *et al.*, 1992). **2**. ATP-dependent transport catalyzed by RLIP76 (Awasthi *et al.*, 2000; Cheng *et al.*, 2001a).

which suggest that both, PL-OOH and 4-HNE mediate cell cycle signaling. Perhaps stress mediated signaling for apoptosis is transduced through lipid peroxidation products because recent studies suggest apoptosis caused by UV, oxidative stress, and chemical agents can be modulated by manipulating the expression of GSTA1-1, GSTA2-2, GSTA4-4, hGST5.8, and RLIP76, which regulate the intracellular concentrations of LOOH and

4-HNE (Cheng *et al.*, 2001a; 2001b; Yang *et al.*, 2001; 2002a).

GLUTATHIONE *S*-TRANSFERASES CAN MODULATE ROS-INDUCED SIGNALING FOR APOPTOSIS BY CONTROLLING LIPID PEROXIDATION

Role of lipid hydroperoxides in signaling for apoptosis

Studies in our laboratory (Yang *et al.*, 2001) have shown that transfection of K562 cells with the α-class GSTs, hGSTA1-1/A2-2 results in about 10-fold higher GPx activity towards PL-OOH, while the activities of other antioxidant enzymes such as CAT, SOD, peroxidase activity towards H_2O_2 remain unaltered. Intracellular lipid peroxidation under physiological conditions as well as under the conditions of oxidative stress is dramatically decreased in the transfected cells as compared to the empty vector transfected control or wild type cells. Upon treatment with 30 μM H_2O_2, hGSTA2-2 transfected cells show a transient activation of JNK, which quickly returns to the basal levels. H_2O_2 does not cause caspase 3 activation under these conditions in the transfected cells and these cells do not undergo apoptosis. In contrast, the wild type and empty vector-transfected cells show a persistent activation of JNK and caspase 3 and these cells undergo apoptosis as measured by characteristic DNA laddering (Fig. 5). Since hGSTA1-1 and hGSTA2-2 display no detectable activity towards H_2O_2 , this protective effect of GSTs against H_2O_2-mediated apoptosis must be exerted through their GPx activity and reduction of PL-OOH/FA-OOH. This is suggested by the studies showing that phosphatidylcholine hydroperoxide (PC-O-OH) treatment results in a marked apoptosis in the wild type and vector-transfected cells but the transfected cells are protected from PC-OOH mediated apoptosis (Fig. 5). These studies strongly suggest that at least a part of H_2O_2-induced signaling for JNK, caspase 3 activation, and apoptosis must be transduced through PL-OOH or their downstream products, particularly, 4-HNE. Unpublished studies in our laboratory show that overexpression of hGSTA1-1 or hGSTA2-2 protects various cell types from UV-A induced apoptosis. Likewise, hGSTA1-1/hGSTA2-2 transfected cells are resistant to apoptosis caused by the presence of oxidative stress causing agents such as xanthine/xanthine oxidase, adriamycin, and naphthalene. HLE B-3 cells show a persistent activation of JNK and caspase and undergo apoptosis in the presence of naphthalene in medium. However, hGSTA1-1 transfected HLE B-3 cells neither show JNK or caspase activation nor undergo apoptosis under similar conditions of naphthalene treatment (Yang *et al.*, 2002a). These studies suggest that lipid peroxidation products, PL-OOH or their downstream products, are involved in ROS-mediated signaling for apoptosis. Furthermore, these studies provide crediable evidence that lipid peroxidation products may be a common factor in the mechanisms of the signaling for apoptosis by oxidative stress, chemical agents, and UV irradiation.

Effect of 4-hydroxynonenal levels on signaling

The role of 4-HNE in signaling has been examined in K562 cells transfected with *mGSTA4* (Cheng *et al.*, 1999). The transfected cells had about 5-fold higher GST activity toward 4-HNE as compared to the controls and 4-HNE content of the transfected cells (5.9 pmol/5 $\times$ 10^7 cells) was found to be only about 10% of that observed in the empty vector transfected or wild types cells (62.9 pmol/5 $\times$ 10^7 cells). Antioxidant enzymes (CAT, SOD, and GPx) were not affected by *mGSTA4* transfection. Surprisingly, the *mGSTA4* transfected cells had about 50% higher growth rate as compared to the wild type or vector transfected cells suggesting that lowering the levels of 4-HNE in these cells

promoted proliferation. Promotion of proliferation at low intracellular levels of 4-HNE has also been observed in other cell lines. HLE B-3 cells transfected with hGSTA4-4 resulting in lower levels of intracellular 4-HNE grow at levels of 4-HNE has been observed at Ruef *et al.* (1998). Increasing concentrations of 4-HNE in the medium differentially affected the *mGSTA4*-transfected and empty vector-transfected cells. Exposure of 20 μM

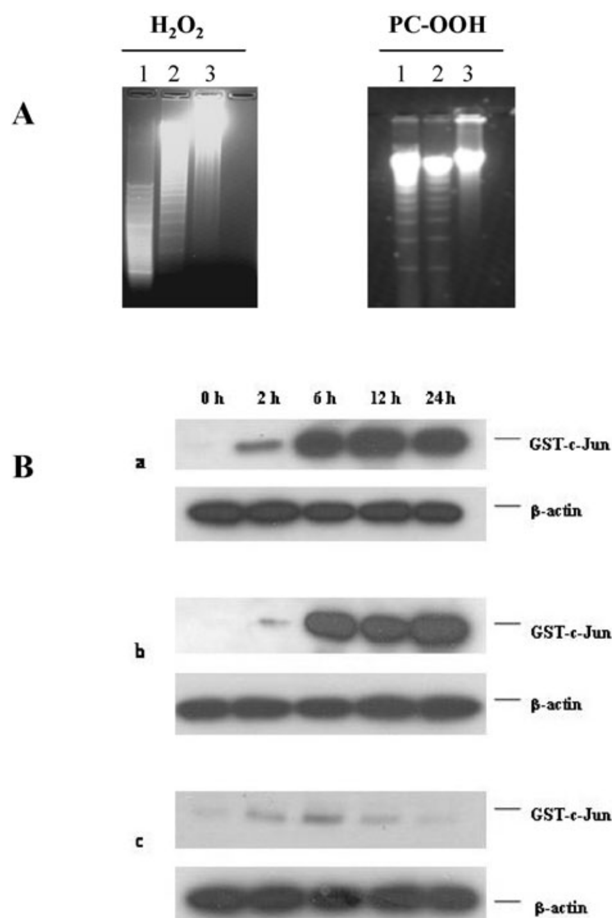

Figure 5. Effects of hGSTA2-2 over-expression on H_2O_2 induced apoptosis and JNK activation in K562 cells.

Panel A: wild-type, vector-transfected, and *hGSTA2*-transfected K562 cells were treated with 30 μM H_2O_2 for 48 h or 40 μM PC-OOH for 8 h in RPMI complete medium. After the incubations, genomic DNA was extracted and electrophoresed on 2% agarose gel. Lanes 1, 2, 3 in all panels represent the wild-type, vector-transfected, and *hGSTA2*-transfected K562 cells, respectively. Apoptosis was examined by the appearance of characteristic DNA laddering. **Panel B**: cells were incubated with 30 μM H_2O_2 for the indicated times. Cell extracts containing 250 μg proteins from the wild-type (a), vector-transfected (b), and *hGSTA2*-transfected (c) cells were incubated overnight with 2 μg of GST-c-Jun (1-89) fusion protein. After extensive washing, the kinase reaction was performed in the presence of 100 μM of cold ATP. Phosphorylation of c-Jun at Ser63 was detected by Western blot analysis using Phospho-c-Jun (Ser63) antibody. β-Actin expression was shown to confirm same amount of protein incubated with c-Jun (reproduced from Yang *et al.*, 2001, with permission from *Journal of Biological Chemistry*).

a rate about 50% faster than the wild type or vector transfected cells (unpublished observations). Likewise, the promotion of proliferation of aortic smooth muscle cells at very low 4-HNE to the wild type or empty vector transfected K562 cells resulted in a marked erythroid differentiation while the cells over expressing mGSTA4-4 and having lower

intracellular levels of 4-HNE did not undergo such differentiation suggesting a role of 4-HNE in signaling for differentiation. Exposure of the wild type or vector transfected K562 cells to even higher concentration of 4-HNE (>20 μM) in the medium resulted in apoptosis while the cells transfected with *mGSTA4* were resistant to apoptosis under these conditions. More importantly, the cells transfected with *mGSTA4* also showed resistance to H_2O_2 induced apoptosis, which implied that the signaling for H_2O_2 induced apoptosis was conveyed through 4-HNE. Since the transfection with *mGSTA4* did not affect the antioxidant enzymes such as CAT, GPx, SOD, the apoptotic effect of H_2O_2 could be blocked by the over expression of *mGSTA4* only if 4-HNE was directly involved in signaling. The protective effect of the induction of the 4-HNE metabolizing enzyme, hGST5.8 against oxidative stress mediated apoptosis is also observed in a variety of cell lines (e.g., HL-60, H69, HLE B-3, RPE, H-226) of human origin (Cheng *et al.*, 2001a). These studies strongly support the idea of the involvement of 4-HNE in oxidative stress mediated signaling for apoptosis and that it is not limited to a specific cell type but appears to be a generalized phenomenon.

There is strong evidence that a sustained activation of c-Jun N-terminal kinase (JNK) precedes apoptosis (Uchida *et al.*, 1999). It has been shown that the prolongation of TNF-α-induced JNK activation by incubating the cells with TNF-α in the presence of cycloheximide, actinomycin D, or orthovanadate leads to apoptosis. A sustained activation of JNK achieved by inhibiting the expression of MKP-1, a dual specific phosphatase that inactivates JNK, has been shown to potentiate TNF-α induced apoptosis (Guo *et al.*, 1998). A sustained activation of JNK is also observed in H_2O_2-induced apoptosis in K562 and HL-60 cells and these cells can be protected from apoptosis by blocking JNK activation (Cheng *et al.*, 2001a; 2001b). Studies in our laboratory suggest a specific role of 4-HNE in the activation of JNK and caspase -3 in several cell lines of diverse origin. We have shown that the wild type or empty vector transfected HL-60 cells undergo apoptosis within 2 h when 20 μM 4-HNE is included in the medium. Cells transfected with *mGSTA4* having an enhanced capacity to metabolize 4-HNE leading to reduced intracellular levels of 4-HNE do not undergo apoptosis even after a prolonged period of exposure with 20 μM 4-HNE treatment. 4-HNE induces apoptosis in the wild type or vector-transfected cells which is preceded by a sustained activation of JNK and an increase in AP-1 binding within 2 h. In *mGSTA4*-transfected cells, these effects of 4-HNE are significantly delayed which strongly suggests a role of 4-HNE in these processes. 4-HNE treated wild type cells show caspase -3 activation within 2 h while a detectable activation of caspase -3 is seen in *mGSTA4*-transfected cells only after 8 h. Together, these studies strongly suggest a key role of 4-HNE in the events upstream to the activation of JNK and caspases.

ROLE OF RLIP76 AND hGST5.8 IN REGULATION OF 4-HYDROXYNONENAL LEVELS AND STRESS-MEDIATED SIGNALING

If 4-HNE is involved in stress mediated signaling for apoptosis, lipid peroxidation and consequently 4-HNE formation may be enhanced in stressed cells. Many of the agents that induce apoptosis cause oxidative stress and subsequent lipid peroxidation and apoptosis subsequent to H_2O_2, UV, or drug (e.g. doxorubicin) exposure is usually accompanied with lipid peroxidation. Cells exposed to a transient and mild heat or oxidative stress show an induction of the mechanisms responsible for maintaining the intracellular levels of 4-HNE (Cheng *et al.*, 2001a). Various human cell lines of diverse origin when exposed to either a mild heat, oxidative stress, or UV-A show an increased formation of

4-HNE. Apoptosis is not observed under such mild stress conditions and the initial adaptive response of these cells includes a rapid but transient induction of hGST5.8 (the enzyme catalyzing the formation of the glutathione conjugate of 4-HNE) and RLIP76 (which catalyzes ATP dependent transport of GS-HNE) even prior to any noticeable induction of HSP70 or other antioxidant enzymes. This suggests that the maintenance of the basal "physiologic" levels of 4-HNE is crucial to cells. The cells exposed to mild stress initially show a transient increase in 4-HNE levels and activation of JNK both of which quickly return to basal levels. The stress preconditioned cells with induced hGST5.8 and RLIP76 can exclude intracellular 4-HNE by forming and transporting its glutathione conjugate (GS-HNE) out of cells at a rate several times faster than that observed in controls. The stress preconditioned cells are relatively more resistant to H_2O_2, oxidative stress, or 4-HNE induced JNK activation, AP-1 DNA binding, caspase -3 activation and eventual apoptosis as compared to controls (Cheng *et al.*, 2001a). Our unpublished studies show that control K562 cells undergo apoptosis when irradiated with UV-A for 30 min. In contrast, the cells exposed to UV-A irradiation for 5 min and allowed to rest for 2 h show induction of RLIP76 and hGST5.8 and acquire relative resistance to UV-A induced apoptosis as compared to the control cells (Fig. 6). Likewise, cells preconditioned with mild UV-A exposure acquire resistance to apoptosis due to oxidative stress caused by including xanthine/xanthine oxidase in the medium. This resistance of stress-preconditioned cells to H_2O_2, xanthine/

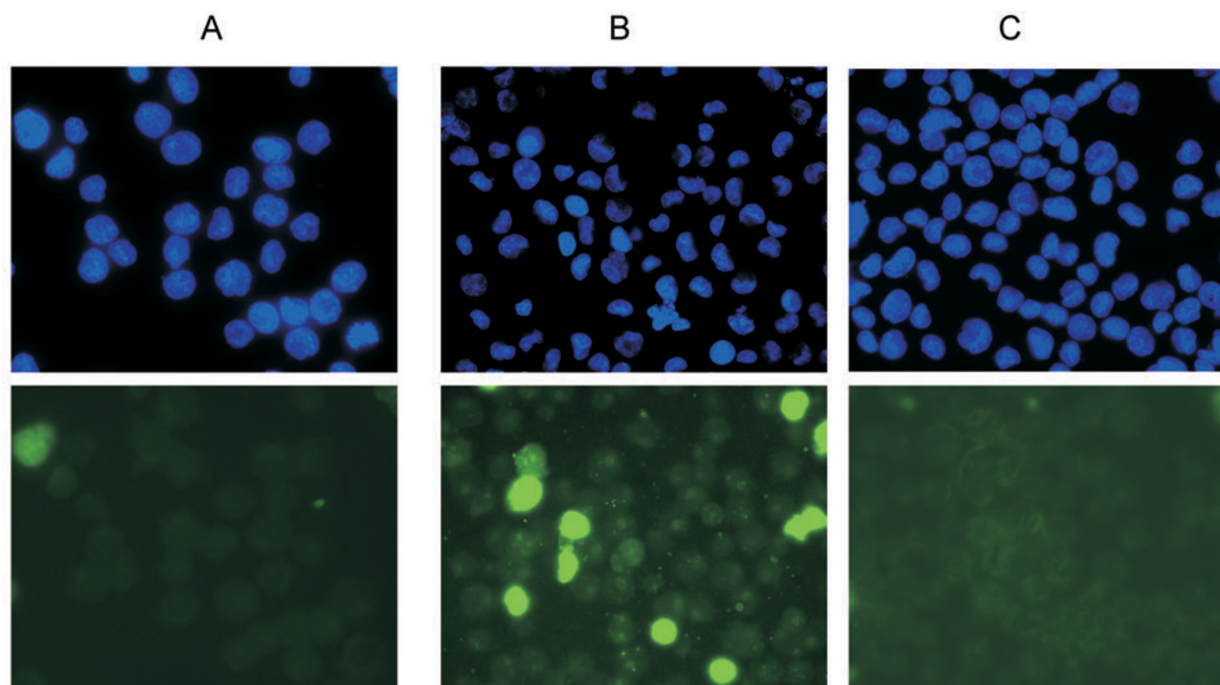

Figure 6. Effects of UV-A preconditioning on apoptosis induced by further UV-A irradiation.

K562 cells were irradiated with UV-A (365 nm) for 5 min at 3 mW/cm^2 and were allowed to rest for 2 h. After preconditioning, these cells were exposed to 30 min UV-A and the activation of caspases was examined by incubating with 10 μM of CaspACE FITC-VAD-FMK *in situ* marker (Promega). The slides was mounted with mounting medium containing DAPI (1.5 μg/ml) and observed under fluorescent microscopy (Olympus, Japan). All the pictures were taken at ×400 magnification. **Panel A**: control cells without any treatment; **panel B**: control cells irradiated for 30 min UVA; **panel C**: 5-min UV-A preconditioned cells irradiated for 30 min. All the upper slides were stained with DAPI and all the lower slides were stained with FITC.

xanthine oxidase, UV-A, or 4-HNE induced apoptosis correlates with their ability to metabolize and extrude GS-HNE at an accelerated rate as compared to the control cells. These results indicate that accelerated exclusion of 4-HNE from cells provides protection against H_2O_2, UV-A, and 4-HNE induced apoptosis and strongly suggest that a role of 4-HNE in stress mediated signaling for apoptosis.

If the stress preconditioned cells acquired resistance to 4-HNE and oxidative stress induced apoptosis by accelerated elimination of 4-HNE from cells through an increased formation and transport of GS-HNE, one would expect that this resistance could be overcome by blocking the transport of GS-HNE. Consistent with this expectation, the stress preconditioned cells can be made sensitive to H_2O_2 and 4-HNE mediated apoptosis by coating the cells with anti-RLIP76 IgG which blocks the transport of GS-HNE. Coating with preimmune IgG has no effect on GS-HNE transport and the stress-preconditioned cells remain resistant to H_2O_2 and 4-HNE mediated apoptosis and the efflux of GS-HNE from cells is not affected. However, coating the stress preconditioned cells with anti-RLIP76 IgG results in blockage of GS-HNE efflux and the cells undergo apoptosis when treated with 4-HNE or H_2O_2 the (Cheng *et al.*, 2001a). These results provide strong evidence that 4-HNE mediates signaling for apoptosis in cells stressed with oxidants or heat. More importantly, these results also demonstrate that RLIP76 mediates the transport of GS-HNE and is one of the major determinants for intracellular concentration of 4-HNE. This is in contrast to earlier suggestions that MRP1 is the major (Pulaski *et al.,* 1996) efflux pump for GSH-conjugates of endo- and exogenous electrophiles (Ishikawa, 1992). Further studies are needed to elucidate the role of various transporters which catalyze the ATP-dependent transport of GSH-conjugates.

The maintenance of the intracellular concentration of 4-HNE appears to be crucial for cell cycle signaling. Our studies suggest that cells respond to stress by inducing mechanisms for elimination of the excess 4-HNE formed due to stress and this response is even quicker than the induction of heat shock proteins and antioxidant enzymes. Even a slight increase in 4-HNE levels influences cell cycle signaling. Exposure of cells to $42^{o}C$ for 30 min, 50 μM H_2O_2 for 20 min, or UV-A exposure for 5 min, results only in a 50% increase in the steady state levels of 4-HNE concentrations above its basal levels. Yet there is transient activation of JNK that quickly subsides as the cells acquire the capacity to exclude 4-HNE at accelerated rates and bring 4-HNE concentrations to basal "physiologic" levels. These studies suggest that there is a narrow window of the basal levels of 4-HNE, which is tightly controlled by factors facilitating its metabolism and exclusion from cells. In case of a sustained stress and over production of 4-HNE the protective functions of GST5.8 and RLIP76 are overwhelmed, the intracellular concentrations of 4-HNE rise, and the cell in desperation calls for death signal. At low 4-HNE concentrations cells (at least some type, e.g., aortic smooth muscle, and K562 erythroleukemia cells) show proliferation. It is possible that there is a narrow range of "physiological" 4-HNE concentration within the cells. The cells tend to undergo differentiation, apoptosis when 4-HNE concentrations rise above this range. On the other hand, at 4-HNE concentrations below this range, the cells tend to proliferate. This idea is supported by unpublished studies in our laboratory which show that transfection of HLE B-3 cells with *hGSTA4* results in lower 4-HNE concentrations and a faster growth rate of these cells and that it is accompanied with the activation of ERK which is involved in cell proliferation. This intriguing hypothesis on the role of 4-HNE in regulating cell cycle signaling needs substantiation through further studies. Observed resistance to oxidative stress of the cells preconditioned with mild transient stress raises some interesting questions. Can the beneficial effects of

exercise or that of ancient yogic practices such as *Pranayam* (controlled deep breathing) be attributed to mild oxidative stress? Likewise, can the reported beneficial effects of various Chinese and Indian herbal medicines be attributed to mild oxidative stress due to the metabolism of their constituents by cytochrome P-450 system? It may be worthwhile to explore these possibilities.

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