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Review

4-Hydroxy-2,3-nonenal as a signal for cell function and differentiation*

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4-Hydroxy-2,3-nonenal (HNE) is a biologically active aldehydic end product of oxidative decomposition of ω-3 and ω-6 polyunsaturated fatty acids of membrane phospholipids, a process referred to as lipid peroxidation. HNE has been detected in several experimental and clinical conditions in which oxidative stress has been reported to occur and several authors have suggested that HNE and related 4-hydroxy-2,3-alkenals (HAKs) of different chain length may act not only as toxic and mutagenic mediators of oxidative stress-related injury but also as biological signals in normal and pathological conditions. In this paper we will review the literature supporting the concept that HNE and HAKs may act as signal molecules able to modulate biological events such as chemotaxis, signal transduction, gene expression, cell proliferation and cell differentiation.

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Abbreviations: HNE, 4-hydroxy-2,3-nonenal; HAKs, 4-hydroxy-2,3-alkenals; HHE, 4-hydroxy-2,3-hexenal; HOE, 4-hydroxy-2,3-octenal; HUE, 4-hydroxy-2,3-undecenal; PLC, phospholipase C; $NO^{\bullet}$, nitric oxide or nitric monoxide; FCS, foetal calf serum; PDGF, platelet-derived growth factor; DAG, diacylglycerol; AP-1, activator protein-1; NF-κB, nuclear factor kappa B; HSC, hepatic stellate cells; JNKs, c-Jun amino-terminal kinases; PMNL, polymorphonuclear leukocytes; CDK, cycline-dependent kinase(s); Me_2SO, dimethylsulfoxide.

4-HYDROXYALKENALS: ORIGIN, REACTIVITY, METABOLISM AND SIGNIFICANCE IN PHYSIOPATHOLOGICAL CONDITIONS

Oxidative decomposition of ω-3 and ω-6 polyunsaturated fatty acids of membrane phospholipids (i.e. lipid peroxidation) was first described by chemists as a mechanism of lipid deterioration and is now known as a major mechanism of elementary cellular lesions elicited by oxidative stress (Dianzani, 1993).

Lipid peroxidation leads to the production of several carbonylic compounds including saturated, 2,3-*trans*-unsaturated and 4-hydroxy-2,3-*trans*-unsaturated aldehydes (see for a review: Esterbauer *et al.*, 1991). Among these aldehydic end products of lipid peroxidation 4-hydroxy-2,3-alkenals (HAKs) and, in particular, 4-hydroxy-2,3-nonenal (HNE), have been suggested as putative molecular mediators responsible for many of the biological and toxic effects detected during the course of this process (Dianzani, 1982; Esterbauer, 1985). In this connection, HNE (i.e. the most abundant and the best characterized HAK) has been detected *in vivo* in several experimental and clinical conditions of disease, including atherosclerosis, liver fibrosis, Alzheimer's disease, to name such a few (see for a review: Poli & Parola, 1997). However, lipid peroxidation may also occur under physiological conditions, particularly in non-rapidly proliferating cells (Dianzani, 1993). In the last decade, it has become evident that HNE is a normal constituent of plasma as well as of many cells and tissues in mammalians at concentrations ranging from 0.2 to 2.8 μM (Esterbauer *et al.*, 1991). Concentrations of HNE detected in different tissues represent the steady state level between the amount of aldehyde produced and that catabolized in the cells. Enzyme metabolizing aldehydes (aldehyde dehydrogenase, aldehyde reductase, alcohol dehydrogenase and glutathione-S-transferase) have been reported to be responsible for the modulation of steady state aldehyde concentration (Canuto *et al.*, 1994; Hartley *et al.*, 1995). Studies on the activity of these enzymes have been performed in normal tissues, including liver (Canuto *et al.*, 1994; Hartley *et al.*, 1995), kidney (Ullrich *et al.*, 1994) and retina (Singhal *et al.*, 1995), as well as in hepatoma cell lines (Canuto *et al.*, 1994; Grune *et al.*, 1994), in cholestatic liver (Leonarduzzi *et al.*, 1995), in rat liver undergoing chemical carcinogenesis by diethylnitrosamine (Canuto *et al.*, 1989) and in HL-60 human leukemic cells (Barrera *et al.*, 1996a). From these studies two main concepts arise: first, biological effects of HNE and HAKs are strictly related to HNE metabolism in a given cell type, epithelial cells like normal hepatocytes being much more effective in removing these compounds than cells of mesenchymal origin; second, in most tumour tissues and cell lines a marked increase in aldehyde dehydrogenase and aldehyde reductase activities has been reported which seems to be related to the degree of neoplastic deviation (Canuto *et al.*, 1994; Grune *et al.*, 1994).

Biological and toxic effects of HNE largely depend on its ability to react with sulphydryl groups of low relative molecular mass thiols (i.e. glutathione) as well as to form HNE-protein adducts by interacting with the sulphydryl group of cysteine or with the ε-amino group of lysine and the nitrogen atom of imidazole ring of histidine by a Michael type reaction (Esterbauer *et al.*, 1991; Waeg *et al.*, 1996). The peculiar molecular structure of HNE and of the HAKs lead first to an addition to the C2=C3 double bond followed by cyclization and formation of a rather stable hemiacetal structure (Esterbauer *et al.*, 1991; Waeg *et al.*, 1996).

It is relevant to note that cytotoxic effects have been reported mainly in the presence of relatively high concentrations of these aldehydes (usually 10^{-5} M and higher) either in cell lines in culture, isolated hepatocytes or in subcellular fractions (see for review: Dianzani, 1982; 1998; Esterbauer *et al.*, 1991). In

this review we would like to analyze those biological effects which have been reported to occur at concentrations compatible with those found in normal conditions or in conditions of mild to moderate oxidative stress (usually concentrations ranging from 10^{-8} M to 10^{-5} M). Moreover, we will emphasize the concept that HNE may have a role in physiopathological conditions by acting as a signal molecule able to modulate relevant biological events such as chemotaxis, cell signaling, gene expression, cell proliferation and cell differentiation.

HAKs AND CHEMOTAXIS

Historically, the first demonstration for a physio-pathological role of HAKs as chemotactic and chemokinetic molecules, particularly for neutrophils, has been provided by Curzio and coworkers in a series of paper published between 1982 and 1987 (Curzio *et al.*, 1982; 1983; 1985; 1986a; 1987). Curzio and coworkers, using the classic model of Boyden's chamber, showed that HAKs possess chemotactic activity towards rat neutrophils and induce oriented migration and morphological polarization in a range of concentrations between 10^{-12} M to 10^{-6} M, depending on the chain length of the aldehyde. HNE was found to be active at concentrations around 10^{-6} M but the most effective aldehyde in these experiments was 4-hydroxy-2,3-octenal (HOE) which elicited maximal chemotactic activity at 10^{-11} M. Other HAKs have been described to elicit both oriented migration (chemotaxis) and random migration (chemokinesis) of rat neutrophils, including 4-hydroxy-2,3-hexenal (HHE), 4-hydroxy-2,3-undecenal (HUE) and the non-natural aldehydes 4-hydroxytetradecenal, 4-hydroxypentadecenal and 4-hydroxyheptadecenal (Curzio *et al.*, 1982; 1983; 1985; 1986a). Concerning human neutrophils, HNE (active range 10^{-8} -10^{-6} M) has been shown to stimulate random migration (chemokinesis) whereas a chemotactic effect was observed only in some human neutrophil preparations (Curzio *et al.*, 1990). Concentrations of HAKs higher than 10^{-5} M decreased progressively leukocyte mobility and this phenomenon may be related to a HAKs-induced block in cytoskeletal proteins (as shown in the past for tubulin) or to a dose-dependent enhancement of HAKs cytotoxicity (Dianzani, 1982; 1998; Esterbauer *et al.*, 1991). Results concerning chemoattraction by HNE have been confirmed by Schaur and coworkers in recent years (Schaur *et al.*, 1994; Schaur & Curzio, 1995): these authors, by using an acute and aseptic *in vivo* experimental model of inflammation have been able to show that HNE can act as chemoattractant also *in vivo*.

These early studies have been performed mostly in rat and human neutrophils. More recently, HNE has been shown to be able to stimulate chemotaxis also towards human monocyte-macrophage cell populations isolated from peripheral blood of healthy volunteers. HNE elicited its maximal chemotactic effect at a concentration of 2.5 μM (Müller *et al.*, 1996).

Concerning the mechanism of action of HNE and, more generally, of HAKs, the feature at present is still unclear. All the reported effects on either oriented or random migration of polymorphonuclear leukocytes are not usually reproduced by alkanals and alkenals of corresponding chain length, indicating that the hydroxyl group in position C4 associated with the double bond between C2 and C3 is structurally and functionally relevant. In this connection, it is interesting to note that the CH=CH-CHO grouping is also present in the C12 chemotactic aldehyde 12-oxododeca-5,8,10-trienoic acid, which is a major lipoxygenase metabolite formed in neutrophils from arachidonic acid (Glasgow *et al.*, 1986).

In order to understand the basis for chemotactic action of HAKs, HNE has been compared to known steps for well characterized chemoattractants and these experiments

have pointed out that HNE seems to act in a peculiar way. HNE has the peculiarity to stimulate, at very low concentrations, the activity of phospholipase C (PLC) in plasma membranes isolated from rat neutrophils or other cells (Rossi *et al.*, 1990; 1994). Activation of PLC is a characteristic step in chemotaxis stimulated by formylmethionyl peptides, such as the model compound formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe) and then it may be reasonable to think that HNE may lead to activation of chemotaxis using this pathway. However, fMet-Leu-Phe acts on defined plasma membrane receptors and polymorphonuclear leukocytes (PMNL) treated with fMet-Leu-Phe still were able to respond to HNE: this lack of deactivation (seen also with PMNL stimulated by HNE and then still able to respond to fMet-Leu-Phe) suggests that HNE does not act on the receptor system responsive to fMet-Leu-Phe. Preliminary experiments have been performed in order to identify the presence of a putative receptor for HNE (Curzio *et al.*, 1994). Using [^{3}H]HNE no apparent binding of HNE has been detected on neutrophil plasma membranes, whereas Scatchard analysis has revealed the possible existence of a yet undefined cytosolic receptor(s) which bind(s) HNE obeying to the laws of the agonist-receptor interactions and apparently behaving like a medium affinity receptor.

Chemoattractants (such as fMet-Leu-Phe) usually also induce an increase in the activity of NADPH oxidase, resulting in an increased generation and release of superoxide anion, a phenomenon usually referred to as "respiratory burst" (Babior *et al.*, 1973; Babior & Peters, 1981). HNE, which can affect respiratory burst evoked by fMet-Leu-Phe, is devoided of any direct effect on this parameter (Di Mauro *et al.*, 1995; Dianzani, 1998). Activation of inflammatory cells by chemoattractants may also frequently result in the generation of $NO^{\bullet}$ which can react quickly with superoxide anion to form peroxynitrite: once again, HNE differs from other chemoattractants since inactivates $NO^{\bullet}$ synthesis from L-arginine (Di Mauro *et al.*, 1995). Whatever the mechanism involved, it is relevant to emphasize that HNE (and possibly other aldehydes) has been detected in inflammatory exudate (Curzio *et al.*, 1986b) and at inflammatory site (Schaur *et al.*, 1994; Schaur & Curzio, 1995), and then it may play a role as mediator in the inflammatory process. Moreover, in addition to its direct chemotactic effect on neutrophils and monocyte-macrophages, HNE has been recently found to be able to contribute to the inflammatory response also by stimulating the synthesis of monocyte chemotactic protein-1 (MCP-1) and then to promote recruitment of circulating monocyte to damaged liver in a model of acute hepatic injury (Marra *et al.*, 1999).

HNE, CELL PROLIFERATION AND PROLIFERATION-RELATED GENE EXPRESSION

In rapidly growing tissues such as testis, bone marrow, intestinal epithelium, regenerating liver and in highly proliferating neoplastic cells, the level of lipid peroxidation is consistently low (Dianzani, 1993). These observations suggested the idea that some lipid peroxidation products, particularly those provided with the highest biological activity, may be involved in the control of cell proliferation. Most of the data actually available have been obtained by using cell cultures. However, HNE treatment of cultured cells presents some difficulties: a) HNE easily reacts with the thiol groups of serum amino acids and proteins, thus is not completely available for the cells; b) aldehyde-metabolizing enzymes rapidly remove HNE added to the cell. To evaluate HNE disappearance in the medium, preliminary experiments have been performed by HPLC When 10 μM HNE was added to RPMI medium containing 10% fetal calf serum (FCS), 40% of HNE disappeared within ten minutes and after 1 h HNE concen-

tration was about 5 μM. In cell cultures (K562 human erythroleukemic cells) 10 μM HNE disappeared completely in 45 min in the presence of 10% FCS, whereas in cell cultures without FCS HNE disappeared within 60 min (Fig. 1).

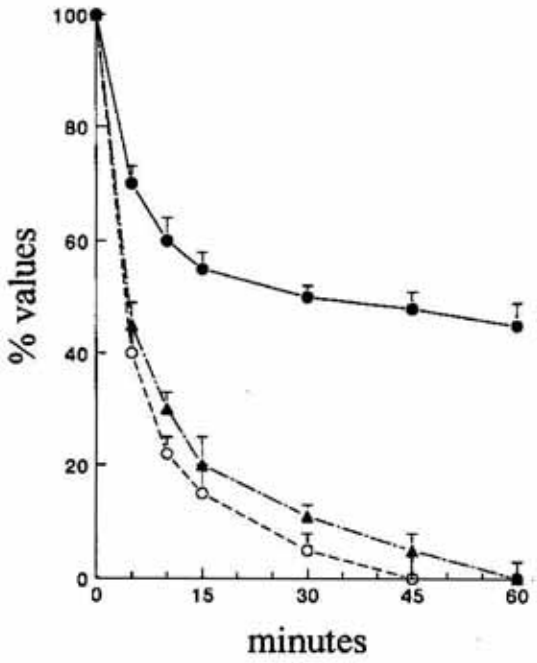

Figure 1. HNE disappearance in cell culture.

10 μM HNE was added to RPMI medium containing 10% FCS (●), to K562 cell culture in RPMI medium without FCS (▲) or to K562 cell culture in RPMI medium containing 10% FCS (○). Results are expressed as percent values.

The high reactivity of HNE with serum protein led us to perform experiments in the absence of FCS. Using cultured leukemic cells HNE was added at different concentrations for 1 h without FCS. At the end of this period, the cells were washed and resuspended in medium with 10% FCS. This experimental procedure has been used to analyze the antiproliferative effect of HNE on K562 and HL-60 (derived from a human acute myeloid leukemia) cells. HNE, at concentrations ranging from 1 to 10 μM, inhibited cell proliferation, DNA synthesis and ornithine decarboxylase activity in a dose dependent way (Barrera *et al.*, 1991a).

To further explore the mechanisms by which HNE exerted its antiproliferative action, the expression of *c-myc* and *c-myb*, two oncogenes involved in the control of cell proliferation, have been analyzed after HNE treatment in K562 and HL-60 cells. The aldehydes down modulated *c-myc* and *c-myb* expression from 1 to 6 h after a single treatment (Fazio *et al.*, 1992; Barrera *et al.*, 1994; 1996b). Moreover, run-on transcription analysis demonstrated that the early and rapid decline of *c-myc* gene expression can be ascribed to a transcriptional block of the third exon, whereas the subsequent decrease of the steady state level of *c-myc* RNA depended on post-trascriptional mechanisms (Fazio *et al.*, 1992). Both duration and intensity of *c-myc* expression inhibitions, after HNE treatment, were dose-dependent in K562 as well as in HL-60 cells. These observations suggested that the inhibitory effect may be increased by prolonging the time of cell exposure to aldehydes. Thus we performed a set of experiments in which the cells were repeatedly treated with 1.0 μM HNE at intervals of 45 min for several hours (from 8 to 12 treatments) (Barrera *et al.*, 1991b). By this experimental procedure it has been observed that cell growth was more affected by increasing the exposure time (Barrera *et al.*, 1991b; 1991c). Moreover, ten repeated treatments with 1.0 μM HNE (7.5 h of exposure to the aldehyde) strongly inhibited *c-myc* and *c-myb* expression in HL-60 cells and the inhibition lasted for several hours, much more than after a single treatment (Barrera *et al.*, 1994; 1996b). In these experiments also *N-ras* and *c-fos* expression has been evaluated. However, their remained unchanged, indicating the HNE acts on specific genes. In another set of experiments the cell cycle distribution of HL-60 cells after HNE treatment has been studied. We found that 24 h and 48 h after ten repeated treatments with 1.0 μM HNE, a marked increase in the proportion of G_0 to G_1 cells occurred, demonstrating that cell cycle progression of HL-60 cells was affected (Barrera *et al.*, 1996c). These results

lead us to investigate the HNE action on the cyclins and the cyclin-dependent kinases (CDKs), which control the cell cycle progression. Each cyclin/CDK combination appears to regulate specific events in the cell cycle; cyclin E/CDK2 is involved in G_1/S phase transition (Dulić *et al.*, 1992) and cyclin B/cdc2 in G_2/M (Pines & Hunter, 1990). The complex cyclin A/CDK2 is important for the S phase progression (Walker & Maller, 1991) and cyclin A/cdc2 for S/G_2 transition (Pagano *et al.*, 1992). D-type cyclins, by binding to CDK4, appear to be relevant for the regulation of G_1 phase progression (Sherr, 1993). In HL-60 cells after HNE treatments (single treatment with 1.0 and 10.0 μM HNE and repeated treatments with 1.0 μM HNE) both mRNA and protein contents of cyclins D1, D2 and A were down-regulated until 24 h, while cyclins B and E as well as protein kinases CDK2 and CDK4 were not affected by the aldehyde (Pizzimenti *et al.*, 1998). Since cyclins D1, D2 and A are involved in G_1 phase progression, these results are in agreement with previous observations indicating an accumulation of HL-60 cells in G_0/G_1 phase and a reduction of cells in S phase 24 and 48 h after HNE treatment (Barrera *et al.*, 1996c). It is noteworthy that the inibition of gene expression (see Fig. 2) lasted for several hours after disappearance of aldehyde in the medium. Thus HNE seems to act as a signal that elicits a sequence of events leading to the final cell response.

The antiproliferative action of HNE and other HAKs has also been studied in the model of human hepatic stellate cells (hHSC) in primary culture. hHSC are known to proliferate rapidly and to synthesize extracellular

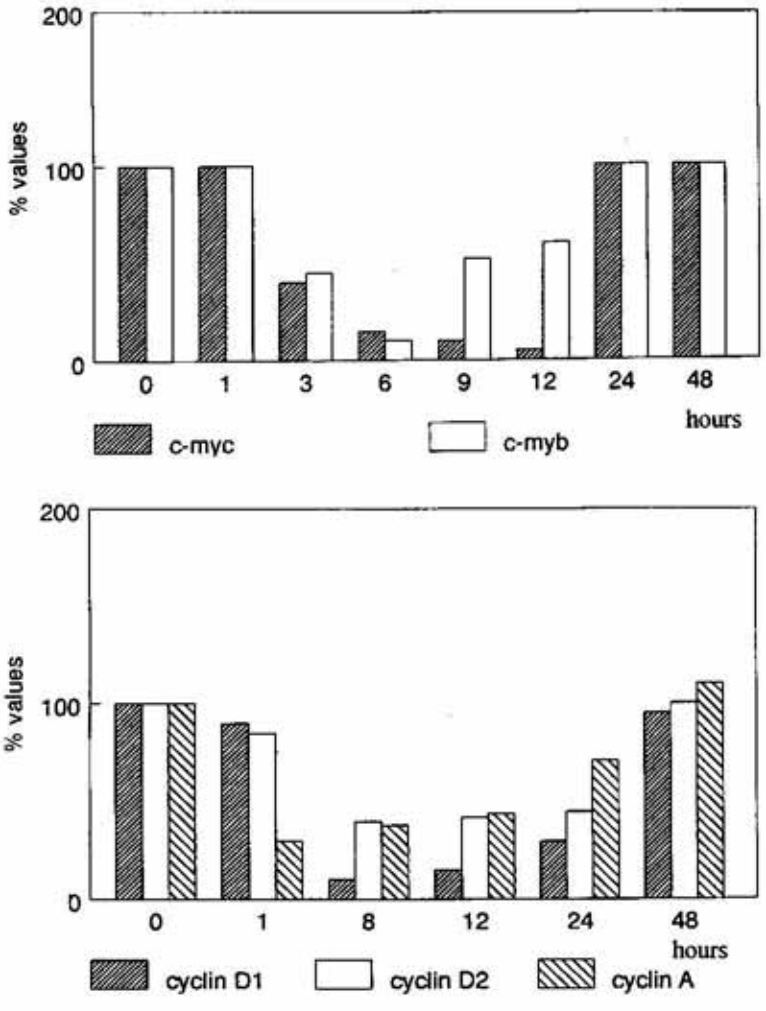

Figure 2. Expression of proliferation-related genes in HL-60 cells repeatedly treated (ten times) with 1 μM HNE.

Results are expressed as percent values referred to densitometric analysis of mRNA levels

matrix proteins during the course of chronic liver damage, being then the key cells in the development of liver fibrosis (Friedman, 1993; Pinzani, 1995) where oxidative damage and aldehyde generation have been reported to occur (see for a review: Poli & Parola, 1997). In our experiments, a single treatment with HNE inhibited basal cell proliferation in a dose-dependent way, starting from concentrations of 50 μM; these inhibitory effects were strictly related to the cytotoxicity of these relatively high HNE concentrations. Interestingly, lower doses of HNE (i.e. 1.0 μM) were able to practically abolish the proliferative response to the potent mitogenic stimulus represented for these cells of mesenchymal origin by platelet-derived growth factor (PDGF) (Robino *et al.*, 1998). Preliminary results indicate that HNE may bind to the PDGF receptor and inhibit the autophosphorylation on tyrosine residues of the receptor itself, a phenomenon which results in the suppression of the PDGF-mediated mitogenic signaling (Robino *et al.*, 1998). A very similar effect (i.e. HNE-induced inhibition of mitogen-activated cell proliferation) has also been reported for human T lymphocytes exposed to HNE before addition of the mitogenic stimulus represented by phyto-hemoagglutinin (PHA) (Cambiaggi *et al.*, 1997).

HNE, CELL SIGNALING AND GENE EXPRESSION

In the last 15 years several authors have provided evidence that HNE and HAKs of different chain length may act as mediators able to modulate signal transduction and to affect the expression of genes not involved directly with cell cycle. From a historical point of view, the first direct report in this field is the one of Paradisi and coworkers (Paradisi *et al.*, 1985), showing a peculiar biphasic, dose dependent and time-dependent effect of HNE on adenylate cyclase. This paper was later followed by the demonstration of an analogous effect of HNE and other HAKs on phosphatidylinositol-4,5-diphosphate hydrolase (PLC) (Rossi *et al.*, 1990; 1994). Both enzymes are regulated by specific membrane G-proteins and are strongly stimulated by concentrations of HNE ranging from 0.1 to 1 μM; the stimulation starts immediately after HNE addition to isolated plasma membranes and lasted for about 10 min; maximal stimulation of adenylate cyclase occurred with 1 μM HNE whereas PLC was maximally stimulated in the presence of 0.1 μM HNE. Data actually available concerning the adenylate cyclase system suggest that HNE is able to interact specifically with Gi (G-inhibitory protein), and then the stimulatory effect of HNE may be related to an inactivation of Gi which is based on a mechanism different from the classic one elicited by the pertussis toxin (Dianzani, 1998). Conversely, HNE did not affect the response of adenylate cyclase to glucagon (Paradisi *et al.*, 1985), cholera toxin and forskolin, indicating that HNE does not interact either with the glucagon receptor or with Gs (G-stimulatory protein) or the catalytic subunit (Dianzani, 1998). As a result of adenylate cyclase stimulation, HNE should elicit an increase in the production of cAMP and this might influence the activity of cAMP-dependent protein kinases (PKAs).

In the case of PLC activation by HNE it is reasonable to assume that HNE may again affect G proteins-mediated modulation of enzymatic activity. It is interesting to note that both adenylate cyclase and PLC were affected by all tested HAKs in the same way (stimulation at low doses and inhibition at 10 μM concentrations or higher), whereas corresponding saturated or unsaturated aldehydes lacking the hydroxy group in C4 were completely uneffective. As a result of HNE-mediated activation of PLC, an increased production of inositol trisphosphate and of diacylglycerol (DAG) should occur within the cells. DAG is known to act by stimulating several protein kinase C isoforms and indeed when hepatocytes were exposed to low concentrations of

HNE, a slight but significant increase in protein kinase C activity was observed (Pronzato *et al.*, 1990; 1993). In addition, very recently it has been shown that HNE may differentially modulate the expression of different PKC isoforms (Marinari and Poli, personal communication).

In connection with the described effects of HNE on PLC, another mechanism by which HNE may affect intracellular signal transduction has been identified quite recently in isolated hepatocytes (Carini *et al.*, 1996). Addition of micromolar concentrations of HNE (effective range 0.1–1.0 μM) to isolated rat parenchymal cells was found to cause an early and transient increase in cytosolic Ca^{2+} concentrations, followed by a more pronounced and progressive elevation. Such a late effect was prevented by Ca^{2+} chelation by EGTA or by the addition of $GdCl_3$, an agent known to block the activity of store operated Ca^{2+} channels in hepatocyte plasma membranes. Interestingly, both the early transient and late increases in cytosolic Ca^{2+} were completely inhibited by the PLC inhibitor U73122. When HNE was added to the cells 5 min after the emptying of intracellular Ca^{2+} stores by thapsigargin, the aldehyde caused a further increase in Ca^{2+} accumulation, once again prevented by $GdCl_3$. The authors concluded that HNE causes Ca^{2+} inflow across $GdCl_3$-sensitive Ca^{2+} channels and that the mechanism responsible for such an effect was triggered by the emptying of intracellular Ca^{2+} stores likely resulting from HNE-mediated stimulation of PLC. In addition, the authors stated that HNE may be able to interfere also with the channel protein(s) or with the mechanism regulating capacitative Ca^{2+} inflow.

Recently, it has also been shown that HNE can activate transcription factor activator protein 1 (AP-1) but, interestingly, not the redox sensitive trancription factor nuclear factor kappa B (NF-κB) in cells of the macrophage lineage (Camandola *et al.*, 1997). The activation of AP-1 suggests a possible way by which HNE may modulate cell proliferation and differentiation.

Modulation of effectors in signal transduction may result in modulation of gene expression and, indeed, in the last 10–15 years several examples of HNE-mediated stimulation of gene expression have been provided by different research groups. One line of research has outlined the ability of HNE to stimulate heat shock protein gene expression (Cajone & Bernelli-Zazzera, 1988; 1989; Cajone *et al.*, 1989). In particular, these studies have provided evidence that HNE is able to specifically induce in hepatocytes and hepatoma cells the transcription of a subset of heat shock proteins (hsp) of 31 kDa and 70 kDa, presumably by acting on the heat shock factor (HSF) which is supposed to bind to the heat shock element (HSE) to promote hsp protein synthesis.

Another example of HNE-mediated stimulation of gene expression is the one reported by the group of Azzi, concerning the stimulation of the transcription and expression of aldose reductase in A7r5 rat vascular smooth muscle cells (Spycher *et al.*, 1996; 1997). HNE has been found to be effective in the range of 1.00–10.0 μM, and the induction of aldose reductase by HNE and by higher doses of hydrogen peroxide has been considered as a novel response to oxidative stress (Spycher *et al.*, 1997). Similarly, it has been shown that HNE can induce glutathione *S*-transferase P (placental isoform belonging to the π class of glutathione *S*-transferases) as an adaptative cellular defense mechanism against oxidative injury (Fukuda *et al.*, 1997).

In addition, 10.0 μM HNE has been shown to elicit increased transcription of TGFβ1 mRNA as well as to increase the synthesis and the release of the same cytokine by cultured human U937 promonocytic cell line and by murine macrophage J774-A1 cell line (Leonarduzzi *et al.*, 1997).

The last line of research to be mentioned in this paragraph is the one that has shown that

HNE and other HAKs of different chain length are able to stimulate the synthesis of procollagen type I in human hepatic stellate cells (hHSC, i.e. the key cells in liver fibrosis) and, very recently, has explained how this phenomenon is modulated. These studies originated from a series of experiments which have provided evidence for the generation of HNE in different experimental models of liver fibrosis (Parola *et al.*, 1992a; 1992b; 1996a). A causative role between HNE and HAKs generation from one side and excess deposition of extracellular matrix was suggested either by the protective role exerted by vitamin E pretreatment (a procedure which is known to prevent the spreading of lipid peroxidation and then the generation of aldehydic end products) (Parola *et al.*, 1992a; 1992b) as well as by the evident association between HNE generation, infiltration of monocyte/macrophage cell populations and collagen deposition (Parola *et al.*, 1992a; 1996a). Addition of HNE at concentrations compatible with those occurring *in vivo* (1.0 μM) to hHSC cultured for 24–48 h in a serum- and insulin-free medium (SFIF), in order to make the cells quiescent and to avoid unspecific binding of HNE to serum proteins, resulted in an early and very significant increase in either mRNA transcription for procollagen α1(I) gene and the synthesis of the protein (Parola *et al.*, 1993). mRNA for this protein was already upregulated 1 h after HNE addition and the increased transcription lasted up to 6 h. This effect was specific, since only the gene for procollagen type I was affected, whereas procollagen type III and fibronectin were unchanged. This is relevant, since during the development of hepatic fibrosis the equilibrium between collagen type I and III exhibits a typical shift towards an uncontrolled rise in collagen type I synthesis and secretion (Friedman, 1993). The stimulation of procollagen type I synthesis in conditions of stimulation of lipid peroxidation and generation of HNE and HAKs was confirmed either by stimulating hHSC in culture with the pro-oxidant stimulus ascorbate/iron (Parola *et al.*, 1993) or by exposing hHSC to human neutrophils activated by fMet-Leu-Phe, a procedure which elicited superoxide anion-mediated increase in lipid peroxidation (Casini *et al.*, 1997). In both cases vitamin E pretreatment of hHSC resulted in an almost complete prevention of HNE-induced collagen type I synthesis.

In order to try to understand the mechanism(s) leading to this biological effect, it has become clear that not only HNE but all the HAKs tested having chain length of 6, 8, and 11 carbon atoms (HHE, HOE as well as HUE) were equally able to significantly stimulate procollagen type I gene expression (Parola *et al.*, 1996b). Moreover, nonanal and 2-nonenal were unable to affect gene expression, suggesting once again that the peculiar structure of HAKs (hydroxy group in C4) is responsible for this biological effect.

Since biological effects of HNE are known to depend on its ability to form adducts with proteins by interacting with either sulphydryl groups or amino groups of lysine and histidine, monoclonal antibodies specific for HNE-histidine adducts have been employed (Waeg *et al.*, 1996) in order to analyze morphologically and in terms of molecular biology the effects of HNE on hHSC. HNE, at doses ranging from 1.0 to 10.0 μM, led to an early generation of nuclear HNE-protein adducts that, by means of immunofluorescence applied to confocal laser microscopy, were observed in the nuclear compartment of hHSC as soon as 5 min after HNE addition, reaching maximal values of fluorescence after 30 min (Parola *et al.*, 1996c; 1998). HNE-protein adducts of 46, 54 and 66 kDa were detected and p46 and p54 isoforms of c-Jun amino-terminal kinase (JNK) were identified as HNE targets. HNE not only led to nuclear translocation of JNK isoforms (JNK1 and JNK2) but also resulted in their activation. In addition, JNK activation seemed related to the direct interaction between HNE and critical histidine residues in JNKs since upstream kinases were not activated and JNK isoforms translocated into the

nuclei were not phosphorylated (Parola *et al.*, 1998). Moreover, JNK activation by HNE was followed by increased AP-1 binding activity, due to the generation of mainly Jun-Jun homodimers, and by increased transcription of *c-jun* proto-oncogene (Fig. 3). In this connection, Jun-Jun AP-1 homodimer formation and JNK activation have been shown to be relevant and necessary steps for increased transcription of human procollagen type I gene expression in HSC (Armendariz-Borunda *et al.*, 1994; Chen & Davis, 1998). Furthermore, HSC were found to be extremely sensitive to HNE for they lack in HNE-metabolizing enzymatic activities (Parola *et al.*, 1996c; 1998). These data, in addition to those previously reported for HNE as chemotactic stimulus and as an agent able to stimulate TGFβ1 synthesis, suggest that HNE generation in chronic diseases may have a relevant role in sustaining chronic inflammation and excess deposition of extracellular matrix components.

HNE AND CELL DIFFERENTIATION

Several substances that inhibit cell proliferation also induce the onset of differentiation in leukemic cells when maintained for several hours in the culture medium. Our previous results demonstrated that HNE is able to inhibit cell growth in K562 and HL-60 cells. However, unlike other differentiating agents, such as dimethylsulfoxide (Me_2SO) and retinoids,

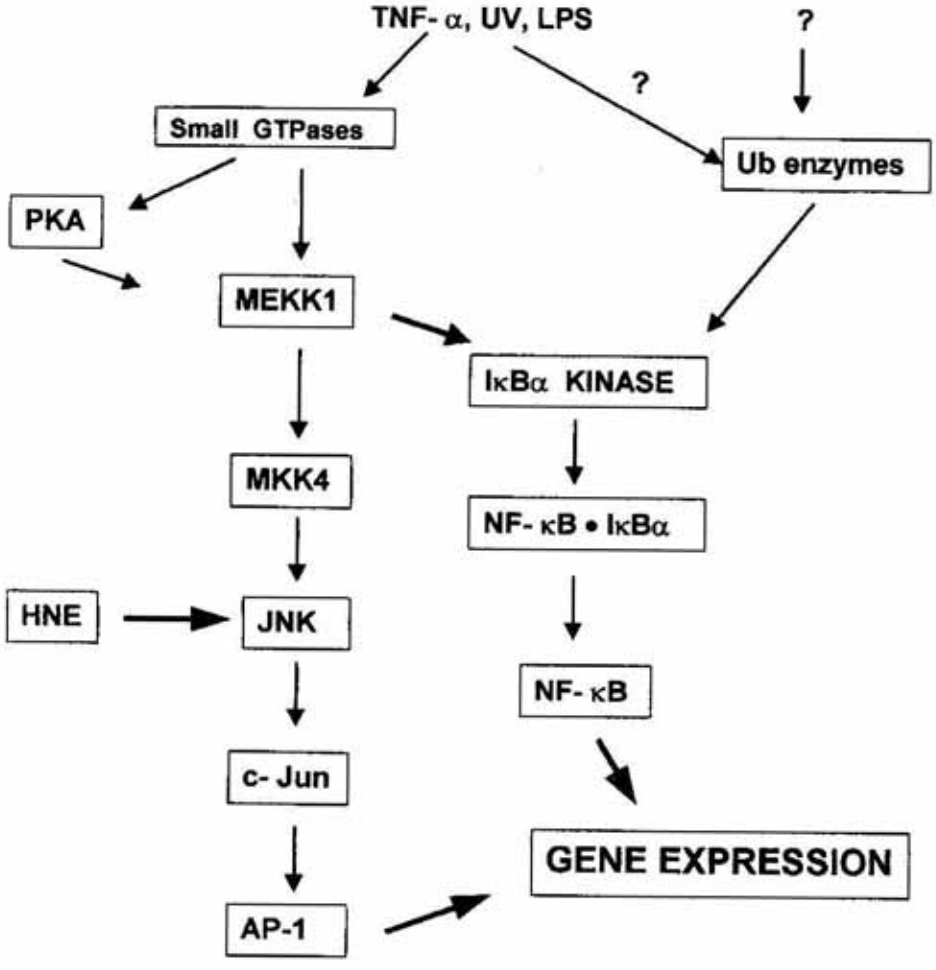

Figure 3. Classical signaling pathway for JNK, AP-1 and NF-κB and the action of HNE.

HNE activates a JNK/c-jun/AP-1 signaling pathway in human hepatic stellate cells without recruiting upstream kinases by directly interacting with JNKs.

HNE is highly unstable in culture medium. It has been previously reported that when K562 cells were treated with a single dose of 1.0 or 10.0 μM HNE they showed a marked, but transient, increase in γ-globin gene expression, suggesting that a stimulus towards differentiation has occurred (Fazio *et al.*, 1992). By using the repeated treatment procedure (see before), resulting in a longer exposure of the cells to HNE, the aldehyde effect on cell differentiation has been deeply investigated in HL-60 cell model. HL-60 cells can be induced to differentiate along the granulocyte or the monocyte-macrophage lineage, depending on the inducers used. Me_2SO is a common inducer of HL-60 cells towards the granulocytic lineage (Collins *et al.*, 1978), whereas 12-*O*-tetradecanoylphorbol 13-acetate leads the cells towards a monocyte/macrophage phenotype (Rovera *et al.*, 1979). After repeated HNE treatments, HL-60 cell differentiation was evaluated by assaying the phagocytic activity, the chemiluminescence production and the expression of differentiation-associated surface antigens CD11b, CD67 and CD36. The data were compared with those obtained by exposing cells to dimethylsulfoxide (Me_2SO) for 7.5 h (same overall time of aldehyde treatment) or the whole length of the experiment (5 days) (Barrera *et al.*, 1991c; 1996c). In HNE-treated cultures, the proportion of phagocytic cells gradually increased since day 2 up to a maximum of 35% at day 5. In Me_2SO-cultures the increase in phagocytic cells was negligible after a short treatment (7.5 h) whereas on continuous exposure the fraction of phagocytic cells progressively increased since day 2 up to a maximum of 63% at day 5. The expression of CD11b (leukocyte integrin subunit that occurs on the surface of both human granulocytes and monocyte/macrophages) and CD67 (a granulocyte specific antigen) increased in cell treated with HNE or continuously exposed to Me_2SO, whereas CD36 (monocyte specific antigen) was expressed at low level in the presence of both agents. These data and the morphological analysis indicated that HNE, like Me_2SO, can induce granulocytic differentiation in HL-60 cells. However, HNE appeared more effective than Me_2SO if we compare the length of exposure to the inducers; moreover, its induction pathway is, at least in part, different. With Me_2SO, growth inhibition and induction of differentiation were chronologically associated whereas with HNE growth inhibition directly preceeded the switch of differentiation.

CONCLUSIONS

4-Hydroxy-2,3-nonenal is endogenously produced in conditions of oxidative stress leading to peroxidation of membrane lipids as well as (at much more lower levels) in normal non-proliferating cells and by activated leukocytes in the inflammatory site. Moreover, HNE has been found in plasma and inflammatory exudates as well as in association with relevant pathologic conditions, including atherosclerosis, degenerative diseases of nervous system (Alzheimer and Parkinson diseases, amyothrophic lateral sclerosis) and liver fibrosis. Data actually available strongly suggest that HNE may contribute to the development of relevant physio-pathological conditions by acting as a molecular mediator for relevant cell functions such as chemotaxis, cell signaling, gene expression, cell proliferation and differentiation.

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