4-hydroxynonenal and cell cycle

Giuseppina Barrera, Stefania Pizzimenti, Stefano Laurora, Federica Briatore, Cristina Toaldo and Mario Umberto Dianzani*

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Department of Experimental Medicine and Oncology, University of Turin, Torino, Italy

Abstract.

Lipid peroxidation is very low in proliferating cells and tumours and it might have a role in the regulation of cell proliferation and differentiation by acting through its products. 4-hydroxynonenal (HNE) has been proposed as a mediator of lipoperoxidation effects. It has been demonstrated that HNE can inhibit cell growth and induce differentiation in different leukemic cell lines. The onset of differentiation, induced by HNE, was accompanied by a reduction of c-myc expression. In HL-60 cells, HNE induced the accumulation of cells in the G0/G1 phase of the cell cycle. Cell cycle progression is regulated by three protein classes, the cyclins, the cyclin-dependent kinases (CDKs), and the CDK inhibitors (CKIs). In HL-60 cells, HNE decreased the expression of cyclin D1, D2 and A and caused an increase of p21 (the most important CKI) expression, whereas it did not affect CDK expressions. Since cyclins D/CDK2 and cyclin A/CDK2 phosphorylate pRB, HNE caused an increase of hypophosphorylated pRb. Hypophosphorylated pRb binds and inactivates the E2F transcription factors. Band-shift experiments demonstrated that HNE caused a decrease of “free” E2F, as well as an increase of pRb (and pRB family members) bound to E2F with consequent repression of the transcription.

Keywords: 4-hydroxynonenal, cell cycle, cyclins, E2F, pRb, p53, tumor cells 1.

Introduction

Several experimental findings demonstrated that lipid peroxidation is very low or null in rapidly proliferating cells including tumour cells [17]. Some unique features of tumour metabolism may account for this characteristic, such as the low function of the microsomal drug metabolizing system, the increase of cytosolic antioxidants and the decrease of membrane peroxidizable fatty acids. This last point has been carefully examined in our laboratories and experiments have been performed to study the lipid peroxidation in hepatoma cells after enrichment of cell membranes with arachidonic acid [11]. Data

obtained demonstrated that tumour cells enriched with arachidonic acid become more susceptible to peroxidative stimuli. Moreover, these experiments demonstrated a link between lipid peroxidation and proliferation rate. In fact, after enrichment with arachidonic acid and stimulation of lipid peroxidation, a reduction of cell proliferation occurred in several different cell lines [31]. Among the products of lipid peroxidation, 4-hydroxynonenal (HNE) has been considered responsible for many biological effects consequent to peroxidation induction [6,18]. In the last few years, the action of HNE has been demonstrated in the regulation of cell growth, differentiation and apoptosis in different cell lines and in the modulation of signalling pathways and gene expressions (Table 1). HNE inhibits cell proliferation and induces differentiation in different cellular models at concentrations near to those detected in non-proliferating cells (about 1 μM), whereas it induces apoptosis at higher concentrations (from 1 to 30 μM), even though some of the concentrations reported are compatible with those detected under pro-oxidant conditions. The first demonstration of an effect of HNE on gene expression was provided by our study on the c-myc expression in K562 human erythroleukemic cells [1,20]. The inhibition of c-myc expression has been observed also in other cell lines as well as the murine erythroleukemic cells (MEL) [38] and the human myeloblastic leukaemia cells HL-60 [3]. In this last line it has been demonstrated that c-myc expression inhibition after HNE treatment was dose- and time-dependent. In fact, the repeated treatment of the cell suspension with 1 μM HNE (to maintain the cells in presence of aldehyde for at least 7.5 hours) provoked an inhibition of c-myc expression more pronounced and longer than that provoked by a single treatment with 10 μM HNE [3]. Moreover, in HL-60 cells the repeated treatment with 1 μM HNE, provoked the induction of a granulocytic-like differentiation [2].

Table 1 HNE effects on cell proliferation, differentiation and apoptosis Effects Cells

Cell cycle block in G0/G1 Growth inhibition	HL-60 (1 μM HNE, RT) [4] HL-60 (1 μM HNE, RT) [2] K562 (10 μM HNE, ST) [20] Human T cells (0.05–1 μM HNE, ST) [10] MEL (1 μM HNE, RT) [38] U937 (1 μM HNE, RT) [37]

	SaOS2 (1 *μ*M HNE, RT) [9]
Induction of differentiation	HL-60 (1 *μ*M HNE, RT) [2] K562 (1 *μ*M HNE, ST) [20] K562 (20 *μ*M HNE, ST) [13] MEL (1 *μ*M HNE, RT) [38] SaOS2 (1 *μ*M HNE, RT) [9]
Apoptosis induction	Human epithelial cells (30 *μ*M HNE, ST) [16] Swiss 3T3 fibroblasts (20 *μ*M HNE, ST) [28] Sympathetic neurons (1 *μ*M HNE, ST) [8] HL-60 (20 *μ*M HNE, ST) [14] CaCo2 (1 *μ*M HNE, ST + 10 TGF *β*-1 ng/ml) [48] K562 (20 *μ*M HNE, ST) [13]

Some HNE effects on cell proliferation, differentiation and apoptosis, reported in the last few years. HL-60: human promyelocytic leukaemia; K562: human erythroleukemia; MEL: murin leukaemia; U937: human promonocytic leukaemia; SaOS2: human osteosarcoma cell line; CaCo2: human colon adenocarcinoma. RT: repeated treatments (1 *μ*M HNE was added at regular intervals of time – 45 min – up to 10 treatments); ST: single treatment (HNE, at the indicated concentrations, was added to the cells once).

2. HNE and cell cycle regulation

The induction of differentiation of HL-60 cells correlated with a marked increase in the proportion of G0/G1 cells, 24–48 hours from the beginning of experiments, indicating that HNE affects cell cycle progression of HL-60 cells [4]. These results led us to investigate the effect of HNE on the expression of the three protein classes that regulate cell cycle progression: cyclins, cyclin-dependent kinases (CDKs) and the CDK inhibitors (CKIs).

Each cyclin/CDK combination appears to regulate specific events in the cell-cycle [33]. D-type cyclins binding to CDK4 appear to be important for the regulation of the G1 phase progression [39]. Cyclin E/CDK2 is involved in the G1/S phase transition [19] and cyclinB/cdc2 in G2/M [35]. The complex cyclin A/CDK2 is important for the S phase progression [42] and cyclin A/cdc2 for the S/G2 transition [34]. Therefore, the progression in the cell cycle is regulated not only by the activity of cyclin/CDK complexes but also by the amount of the different inhibitors able to control cyclin-activated kinase activities (CKIs) [25]. Two structurally defined classes of CKIs have been identified [22]. The first class of CKIs inhibits G1-specific cyclin D/CDK4/6-kinase activity (INK4) (p15INK4B, p16INK4A, p18INK4C and p19INK4D). The second class includes p21waf1 and p27kip1 which inhibit multiple CDKs. The p21 acting in CDK1, CDK2, CDK4 and CDK6, has therefore been described as an universal CKI [45]. In HL-60 cells, we demonstrated that the expression of cyclin D1, D2, and to a minor extent cyclin A, were consistently decreased after 10 repeated treatments with 1 μM HNE, whereas the expression of other cyclins and the cyclin dependent kinases (CDKs) were unaffected [37]. The decrease of cyclin expression has been detected, by RT-PCR and western blot, at 8 and 24 hours from the beginning of experiment. The analysis of p21 expression, performed by RT-PCR and western blot, in HNE-treated HL-60 cells revealed that the amount of this cyclin/CDK inhibitor was strongly increased with respect to untreated cells [5]. No variation was found for p16INK4A, p18INK4C, p19INK4D and p27KIP1 expression. p15INK4B expression was undetectable even at 35 amplification cycles and did not appear in HNEtreated cells. These data were consistent with the G0/G1 accumulation and the low level of cells in the S phase. Induction of p21 in response to various stimuli results in the further inhibition of cyclin/CDK complexes and G1 arrest. The increase of p21 expression, together with the decrease of G1 and S cyclin expression, may induce cell cycle arrest by inhibiting pRb/E2F pathway. Retinoblastoma protein (pRb) represents the most important substrate of CDK4/2 activity which is up-regulated by cyclins D and A (both inhibited by HNE) and down-regulated by p21 (increased by HNE) [7]. The antiproliferative stimuli lead to the hypophosphorylation of Rb, while on the contrary, the induction to proliferation leads to the hyperphosphorylation of pRb. Hypophosphorylated pRb binds to and inactivates the members of transcriptional factor family E2F, whereas the phosphorylation of pRb leads to the activation of E2F transcriptional activity [15]. In mammalian cells, E2F is a heterodimeric transcription factor composed of one of six E2F- family proteins bound to one of the two known DP family members (these proteins are required for the high-affinity, sequence-specific DNA binding

of E2F) [21,26]. E2F has binding sites in the promoters of many genes that are involved in cell-cycle progression such as c-myc, cdc2, cyclin A, DNA polymerase alpha, thymidine kinase, c-myb [41,32] and p21 [23]. E2F proteins induce the transcription of genes that regulate the S phase entry [46,40]. However, pRB-bound E2F may also repress gene transcription [43]. Among the E2F family proteins, in HL-60 cells, the free E2F activity is sustained mainly by E2F4 that represents the member of the E2F family expressed at the highest level in this cell line [26]. In HL-60 control cells that are undergoing cell cycle, the pRb is almost completely hyperphosphorylated (45(, and HNE treatment induces a progressive loss of phosphate starting from 6 to 24 hours [5] without affecting the pRb total content. This result suggests that the amount of pRb bound to E2F could be increased after HNE treatment. However the amount of pRB/E2F complex depends not only on the pRb phosphorylation but also on the net amount of E2F protein available for the binding. After HNEtreatment, E2F4 protein levels decreased, whereas the E2F1 protein content did not change [5]. This HNE effect was similar to that displayed by other well known differentiation inducers. Both E2F1 and E2F4 bind pRb. Moreover, the amount of hypophosphorylated pRb increased after HNE treatment, whereas the amount of pRb partners was regulated differently by HNE. To determine whether the HNE treatment can affect the amount of the complex formed by pRb with the E2F1 or E2F4, we performed immunoprecipitation with anti-pRb antibodies followed by Western blot using anti-E2F1 or anti-E2F4 antibodies. The results demonstrated that the pRb/E2F1 complex increased after HNE treatment, whereas pRb/E2F4 was slightly reduced. These results seem to reflect both the increase of pRb/E2F binding due to hypophosphorylation of pRb and the reduction of free E2F4, available for the pRb/E2F complex [5].

We further investigated the amount of free E2F and the E2F complexed with pRb family members on the P2 c-myc promoter in HL-60 cells by electrophoretic mobility shift assay experiments (EMSA). Briefly, nuclear extracts of control and HNE-treated cells (10 repeated treatments with 1 μM HNE) were incubated with a 32P-labeled oligonucleotide probe, corresponding to the E2F binding site of the c-myc P2 promoter. The reaction products were separated on a polyacrylamide gel run for 3 hours. Since E2F-DNA binding complexes are composed of different E2F family members interacting with several different proteins including pRB, the identification of the proteins present in the E2F complexes was performed by the superschift experiments, with specific antisera. HNE treatments induced a progressive reduction of the amount of free E2F bound to DNA and a relative increase of E2F complexes at higher molecular weights with repressive activity.

The superschift analysis demonstrated the high molecular weight complexes were composed of E2F4 bound to pRb and confirmed that the DNA-binding activity of E2F family members, in HL-60 cells, is sustained chiefly by the E2F4 protein. On the whole, these results demonstrated that HNE can inhibit HL-60 cell proliferation by affecting the pRB/E2F pathway [5].

However, the question arises if the effects displayed by HNE in leukemic cell models can be extended to other tumour cells. As illustrated in Table 1, HNE inhibits cell proliferation not only in leukemic but also in solid tumors, where it can induce differentiation [9] or apoptosis [48] or both events [9]. The key protein which controls the cell cycle arrest and apoptosis pathway is p53. p53 is a nuclear phosphoprotein induced in response to cellular stress, such as DNA damage from radiation or alkylating agents, and binds DNA in a sequence-specific manner to activate the transcription of a number of genes including p21, which blocks cell cycle progression [25], and BAX, a proapoptotic gene that forms mitochondrial pores leading to cytosolic release of cytochrome C, which activates caspases and leads to apoptosis [49]. p53 is the most frequently altered tumour suppressor gene in a wide spectrum of human cancer [24], with a mutated frequency of up to 50% [12]. In HL-60 cells the p53 is deleted [44], so the p21 induction after HNE treatment is a p53-independent effect. Recently, two p53 related genes were discovered: TP73 [27] and TP63 [47] which encode proteins with remarkable sequence homology to p53, suggesting that they are also involved in the regulation of cell growth and apoptosis [30]. To expand our study on the HNE effect on cell cycle control, we tested the HNE effects in a solid tumour cell model (SK-N-BE neuroblastoma cells), which have p53 wild-type and express the p53 family proteins, p73 and p63. These cells were treated with 10 repeated treatments with 1 μM HNE at the beginning of experiment (day 1) and after 2, 4 and 7 days. At the same days, other cells were treated with 10 μM retinoic acid, used as positive control for the differentiation induction. Results obtained demonstrated that HNE inhibited SK-N-BE growth without inducing differentiation. The reduction of cell proliferation by HNE was accompanied by the reduction of the proportion of S-phase cells and by the induction of apoptosis (about 35% of apoptotic cells at 72 hours) detected by DAPI staining and flow-cytometric analysis. The p53 expression (detected by RT-PCR and western blot) was strongly increased in HNE-treated cells at 24, 48, 72 and 96 hours. Whereas the increase of p73 expression was more evident at 72 hours and the increase of p63 expression at 48 hours [29].

Table 2 HNE effects on cell cycle

Inhibition of the expressions of cyclin D1, D2 and A [36]
Induction of p21 expression [5]
Reduction of E2F4 expression [5]
Reduction of pRb phosphorylation [5]
Reduction of E2F “free” bound to DNA [5]
Increase of E2F/pRb complexes bound to DNA [5]
Inhibition of S phase progression [4]
Granulocytic differentiation induction [4]
Apoptosis induction (in solid tumors) [29]
Increase of p53 expression (when expressed) [29]

Summary of the HNE effects on cell cycle. These effects have been obtained by using the repeated treatment protocol (10 repeated treatments at intervals of 45 min with 1 μM HNE).

3. Conclusion

Taken together, our findings (summarized in Table 2) show that HNE can modulate the simultaneous expression of many different genes involved in the control of cell proliferation, differentiation and apoptosis at a concentration near to those found in non-proliferating cells. However, the effect displayed by the same HNE concentration on cell survival or death is different, according to the cell type. The different responses of the tumour cells to micromolar (from 1 to 20 μMHNE) HNE concentrations (i.e. induction of differentiation or apoptosis pathway) may depend on the metabolic and genetic characteristics of the target cells.

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