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Effect of 4-hydroxynonenal on Antioxidant Capacity and Apoptosis Induction in Jurkat T Cells

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4-Hydroxynonenal (HNE) is one of the major end products of lipid peroxidation and may have either physiological or pathological significance regulating cell proliferation. We studied some biochemical effects of HNE, at various concentrations (0.1–100 μM), on Jurkat T cells incubated thereafter for 24, 48 and 72 h. HNE at low concentrations significantly enhanced the proliferation index, whereas at higher concentrations progressively blocked cell proliferation. Caspase 3 activity increased significantly at HNE concentrations between 1 and 10 μM and decreased at higher concentrations. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rd) increased progressively with HNE concentrations, particularly GSH-Px. Glucose-6-phosphate dehydrogenase (G6PDH) showed a different pattern, increasing at low HNE (1–5 μM) concentrations and rapidly declined thereafter. These results show that HNE may induce growth inhibition of Jurkat T cells and regulate the activity of typical antioxidant enzymes. Furthermore, the protective effect of doubling the foetal calf serum still points out the risk that cultured cells undergo oxidative stress during incubation.

Keywords: Antioxidant enzymes; Caspase 3; HNE; Oxidative stress; Proliferation

INTRODUCTION

4-Hydroxynonenal (HNE), a lipophylic, alkenal α–β unsaturated, is one of the major end products of lipid peroxidation. HNE concentrations between 0.28 and 2.8 μM are likely to occur in many cells and tissues and depending upon its concentration, may have either physiological or pathological significance, acting as a biological signal able to modulate signal transduction, gene expression and cell proliferation, via adenylate cyclase activation, stimulation of guanylate cyclase, phospholipase C[1,2] and caspases.[3] At concentrations in the range of 1–20 μM, occurring in tissues in response to oxidative stress, HNE inhibits DNA and protein synthesis, stimulates phospholipase A_2 and inhibits c-myc expression. Moreover, due to its high reactivity, HNE, by binding to proteins and enzymes, can induce conformational changes or molecular aggregation,[4,5] thus causing selective alterations in cell signalling, protein and DNA damage, cytotoxicity and apoptosis. At high concentrations (100 μM), HNE causes acute cytotoxic effects, apoptosis and cell death by inhibiting mitochondrial respiration and DNA, RNA and protein synthesis. Involvement of HNE in the induction of apoptosis has been documented in different cell type as neurons,[6] alveolar macrophages,[7] endothelial cells,[8] human leukaemic cell line[9] and Jurkat T cells.[3,10] The apoptotic process is though to be under the control of caspases (Cysteine-requiring Aspartate Protease).[11] Caspase 3 is considered the main executioner caspase and it is one of the critical enzymes of apoptosis, in particular in HNE induced apoptosis.[8,12] In the present study, we have studied the effects of HNE on cell proliferation, differentiation and death, in Jurkat T lymphoma cells. To further investigate the cytotoxic effect of HNE, caspase 3 levels have been included in our analysis.

During the last decade, we have been involved in clarifying mechanisms of action triggered by ozone when this gas is briefly mixed with human blood.[13] Ozone, solubilized in the water of plasma, reacts

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with a variety of biomolecules (hydrosoluble antioxidants, –SH groups and polyunsaturated fatty acids [PUFA] bound to albumin)[13,14] and generates reactive oxygen species (ROS), mainly hydrogen peroxide and a variety of lipid oxidation products (LOP), in particular 4-hydroxynonenal (HNE), able to activate multiple biochemical and immunological pathways in blood cells.[15–20] Prolonged treatments with low ozone levels induce an up-regulation of antioxidant enzymes (SOD, GSH-Px, GSH-Rx and GSH-Tr) as well as G6PDH in erythrocytes.[21–24] In a previous article,[25] we have demonstrated that Jurkat T cells, exposed to increasing ozone concentrations, are able to stimulate the enzymatic activity of typical antioxidant enzymes in a dose-dependent manner, as an adaptive response to oxidative stress.

The present study was undertaken to further understand the biochemical and metabolic effects of HNE, one of the main aldehydes formed during ozone-induced lipid peroxidation,[26] on the enzymatic activity of human lymphoma Jurkat T cells. In addition, the relevance of a higher concentration of foetal calf serum (FCS) in comparison to standard values was analysed.

MATERIALS AND METHODS

Cell Culture

Jurkat human lymphoma T cells (ECACC, UK) were cultured in RPMI 1640 medium containing 20% of heat-inactivated FCS, supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Sigma Company). Cells were incubated at 37°C in air, 5% CO_2 atmosphere at concentrations between 2×10^5 and 1×10^6 cells/ml and adjusted to a final concentration of 4×10^5 before HNE treatment.

Cell Treatments

HNE (Sigma Chemical Company) was added to Jurkat human T cells (4×10^5 cells/ml) at different concentrations (0.1, 0.5, 1, 5, 10, 50, 100 μM) and incubated for 24, 48 and 72 h. 2% DMSO (Sigma Chemical Company) was used as differentiation inducer for the same time incubation in a different set of experiments. Untreated cells were considered as control.

Cell Proliferation and Cell Viability

Cell proliferation was evaluated by a colorimetric immunoassay (Boehringer Mannheim, Mannheim, Germany) based on BrdU incorporation. Briefly, after 24, 48 and 72 h of incubation at 37°C in air–CO_2 (5%) and 100% humidity, cells were labelled with BrdU for 3 h (10 UI/well). The cells were then fixed, anti-BrdU-POD antibody was added and the immune complexes were detected by the subsequent substrate reaction. The proliferative index (PI) was obtained by calculating the ratio between treated cells and the unstimulated ones, after subtraction of the corresponding blanks.

Cell viability was assayed by the Trypan blue exclusion technique and light microscope observation. Viable cells were counted with a haemocytometer. Viability was represented as the percentage of number of live cells/number of total cells.

Measurement of Caspase 3

Caspase 3 was determined using a Caspase 3 Assay Kit, Colorimetric (Sigma). The assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in the release of the p-nitroaniline (pNA). Briefly, after 24, 48 and 72 h of incubation at 37°C in air–CO_2 (5%) and 100% humidity, the cells, previously treated with HNE at different concentrations, with DMSO or untreated (control), were washed and lysed, cell lysates were then incubated with the substrate at 37°C overnight. The concentration of the pNA released from the substrate was calculated from the absorbance values at 405 nm, from a calibration curve. A caspase 3 positive control, an inhibitor-treated cell lysates control (for measuring the non-specific hydrolysis of the substrate) and a reagent blank, were also prepared. The specific activity was measured in U/10^6 cells. One unit is the amount of enzyme that will cleave 1.0 μmol of the substrate Ac-DEVD-pNA per minute at pH 7.4 at 25°C.

Biochemical Determinations

Glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd) and superoxide dismutase (SOD) were measured on cell lysate with spectrophotometric Randox test kits (Randox Laboratories Limited, U.K.) and reported as U/10^6 cells. Briefly, GSH-Px was measured according to Paglia and Valentine's method;[27] GSH-Rd according to Melissinos' method;[28] SOD activity was determined, by the inhibition of p-iodonitrotetrazolium (INT) reduction due to the O_2^- generated by the combination of xantine and xantine oxidase. Intracellular glucose-6-phosphate dehydrogenase (G6PDH) was detected on cell lysate, with a Sigma spectrophotometric kit (Sigma Diagnostic), according to a modification of the spectrophotometric method of Komberg and Horecker and Lohr and Waller[29,30] and expressed as U/10^{12} cells.

Statistical Analysis

Results were expressed as the mean ± the standard deviation of the mean (SD) and the data were analysed using the Student's *t*-test. *p* values less than 0.05 (*), 0.01 (**) and 0.001 (***) were considered significant.

RESULTS

Evidence for the Influence of HNE on Cell Proliferation

Figures 1 and 2 showed respectively, the influence of the progressively increased concentration (from 0.1 up to 100 μM) of HNE on cell viability and proliferation index. Untreated cells (control) grow in almost exponential fashion steadily throughout the incubation period lasting 24, 48 and 72 h. HNE addition started to have a clear inhibitory effect already at 10 μM becoming highly significant at 50 and 100 μM, with an IC_{50} of 38.70, 42.34 and 44.17 μM at 24, 48 and 72 h, respectively. Lowest HNE concentrations (0.1–1 μM) significantly enhanced the PI. Moreover, even 5 μM induced an increase between 48 and 72 h incubation, probably because HNE quickly undergoes degradation[31] and the inhibitory effect disappears. On the other hand, the PI reduction was strikingly visible at concentrations of 50 and 100 μM, although, at the same concentrations, 60–70% of the original cells survived but they did not proliferate, similarly to DMSO treated cells (72%). DMSO is one of the most studied differentiation inducers on leukaemic cells, suggesting a hypothetical block of proliferation and induction of cell differentiation.

Release and Determination of Caspase 3

Caspase 3 in our study showed a maximum of activation at 24 h, thus decreasing at 48 and 72 h of incubation. Caspase activity in DMSO treated cells was, as we expected, significantly higher compared to the basic level in the control, at all times of incubation, although we observed a similar decrease of activity after 24 h of incubation. These data supported the hypothesis that the decrease in caspase activity was due rather to the time in culture than the differentiation process.

In comparison to untreated cells, we measured a highly significant increase throughout the three days of incubation at HNE concentrations from 1 to 10 μM and in DMSO treated cells, whereas HNE concentrations of 50 and 100 μM almost completely blocked caspase activity (Fig. 3). It appears that an increased amount of caspase 3 in cell samples tested with 5 and 10 μM may have induced cell apoptosis in particular during the first 24 h of incubation. The decreased protease activity at high HNE concentrations may be due either to the concomitant cell differentiation or to an excessive ROS production, which can block apoptosis causing cell death or to a more complex toxic effect of HNE.

Does HNE Influence Cellular Enzymatic Activities?

Our previous data have shown that Jurkat T cells, after a brief exposure to ozone, displayed a dose-dependent modification of the activity of typical antioxidant enzymes.[25] Because ozone causes lipid peroxidation and the release of HNE, it appeared worthwhile to investigate the direct effect of this

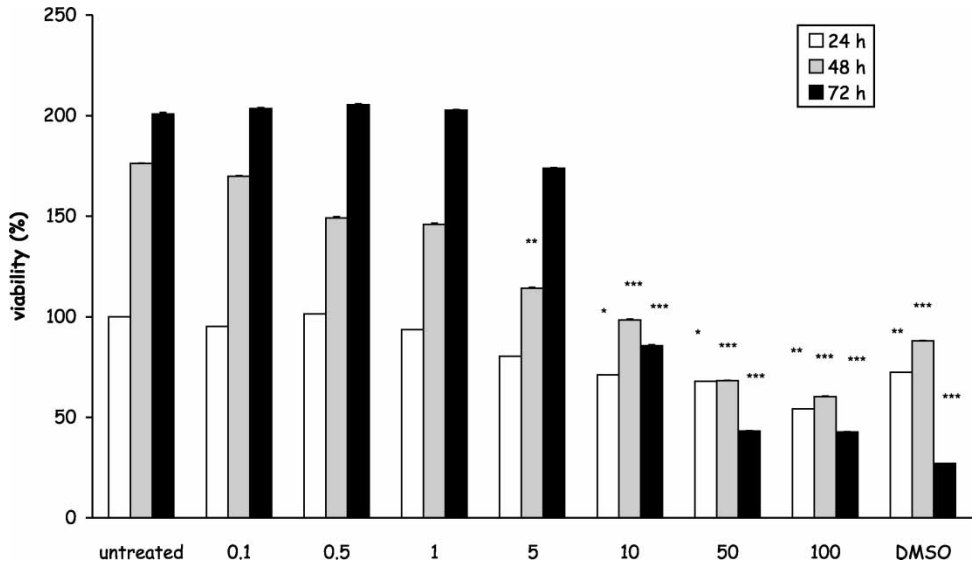

FIGURE 1 The diagram shows that viability (expressed as a percentage of the control) of Jurkat T cells during incubation times of 24, 48 and 72 h varies in relation to the HNE concentrations (from 0.1 to 100 μM). Cells were either untreated (control) or treated with DMSO. Values are means and SD of six different experiments.

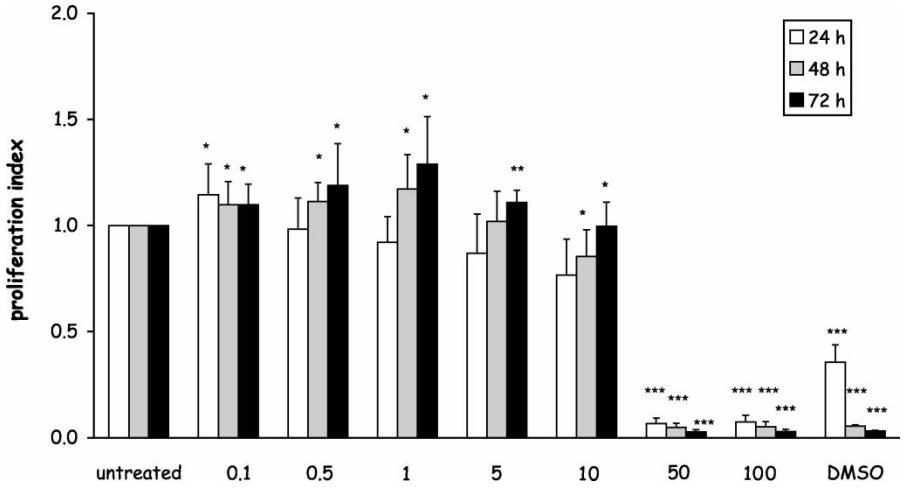

FIGURE 2 The diagram shows the proliferation index of untreated, DMSO treated and HNE treated (from 0.1 to 100 μM) Jurkat T cells after 24, 48 and 72 h of incubation. Symbols are the same of Fig. 1. Values are means and SD of six different experiments.

compound. Interestingly, results reported in Figs. 4–7 were similar to those observed after ozone exposure.[25]

Both control and samples treated with low HNE concentrations showed that during prolonged incubation the specific activity of enzymes progressively decreased with the lowest level at 72 h. This happened particularly for SOD, GSH-Px and GSH-Rd and implied a reduced activity of enzymes during intense cell proliferation. However, when HNE was present at concentrations from 10 to 100 μM (Figs. 4–6), a highly significant increase of enzymatic activities was observed particularly for GSH-Px. DMSO treated cells showed an increased enzymatic activity, particularly evident at 72 h, similarly to cells treated with HNE 10–100 μM. The pattern of enzymatic activity of G6PDH was quite different (Fig. 7) and resembled our previous results with ozone: it appeared that activity slightly increased between 24–48 h over basic levels only at low HNE concentrations (1 and 5 μM, $p < 0.05$). The toxic HNE concentrations (50 and 100 μM) almost totally inhibited the enzymatic activity. G6PDH activity in DMSO treated cells was not significantly different from the control.

DISCUSSION

We have examined the effect of HNE from physiological levels (0.1, 0.5 and 1 μM)[31] to progressively more toxic concentrations (5, 10, 50 and 100 μM) on Jurkat T cells incubated up to 72 h. By comparing the behaviour of HNE treated cells with

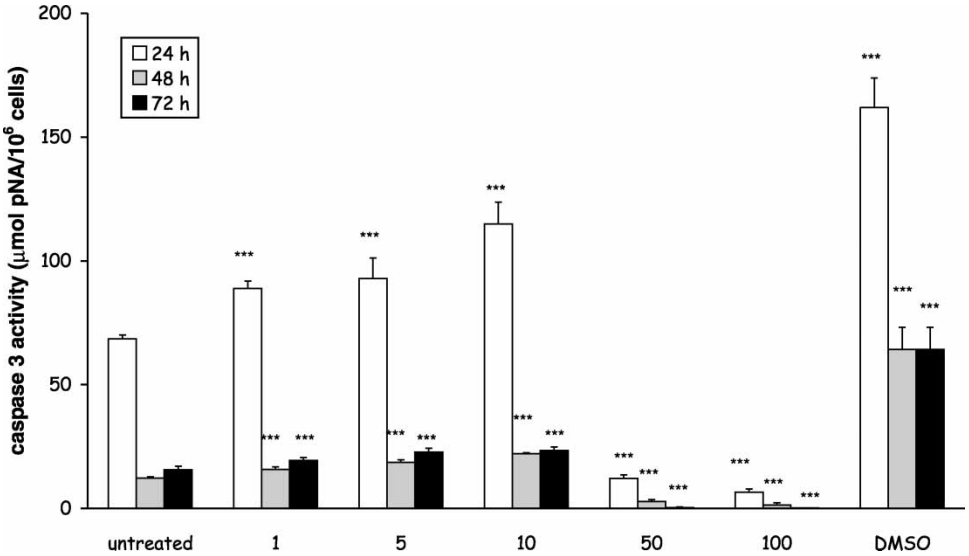

FIGURE 3 The diagram shows caspase 3 activity, measured after 24, 48 and 72 h of incubation, in relation to HNE concentrations (from 1 to 100 μM). The release of caspase 3 was significantly influenced by low levels of HNE (1 to 10 μM) and inhibited by high HNE concentrations (50–100 μM). Symbols are the same of Fig. 1. Values are means and SD of six different experiments.

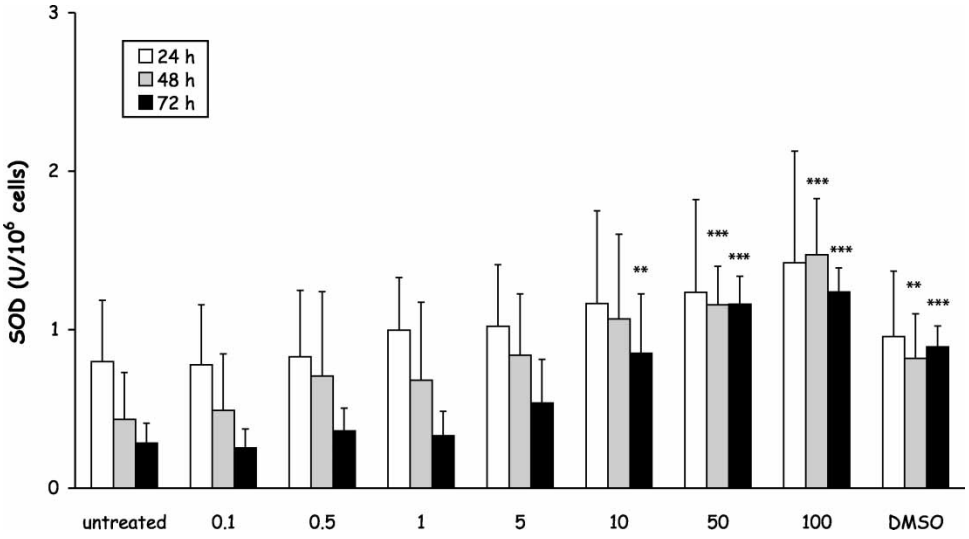

FIGURE 4 The diagram shows that Jurkat T cells displayed an increased SOD activity in relation to the progressive increase of HNE concentrations (from 0.1 to 100 μM) after 24, 48 and 72 h of incubation. Symbols are the same of Fig. 1. Values are means and SD of six different experiments.

either a control and DMSO treated cells, it has been possible to clearly confirm that low levels of HNE (from 0.1 to 1 μM) can be stimulatory because the PI became significantly higher than the control. Despite the increase of the PI, cell number was unmodified. The PI became supranormal, even at a HNE concentration of 5 μM, when cells were in the third day of incubation because most likely the toxic effect of HNE had disappeared. Interestingly, at this concentration, the enzymatic activity of both GSH-Px and G6PDH was significantly improved to cell advantage because both enzymes increase resistance to HNE- and H_2O_2-induced cell death.[32] HNE concentrations above 10 μM, had an inhibitory effect, blocking almost totally proliferation of Jurkat T cells, but 60–70% of the original cells remained viable, similarly to DMSO treated cells. As it is known,[33,34] DMSO inhibits cell proliferation and allows cell differentiation. Indeed HNE induced differentiation has been already demonstrated in HL-60 cells[34] and murine erythroleukemia cells,[37] with an action comparable to DMSO.[38,39] Furthermore, it has been demonstrated that DMSO induces cell cycle arrest[35] and apoptosis, modifies p53 protein, down regulates Bcl-2 and releases cytochrome *c* from mitochondria.[36]

The activity of caspase 3, one of the key executioners of apoptotic cell death was measured to discriminate whether the inhibitory effect on proliferation of HNE at high concentrations was due to apoptosis or to a block of proliferation and induction of cell differentiation. Our results showed

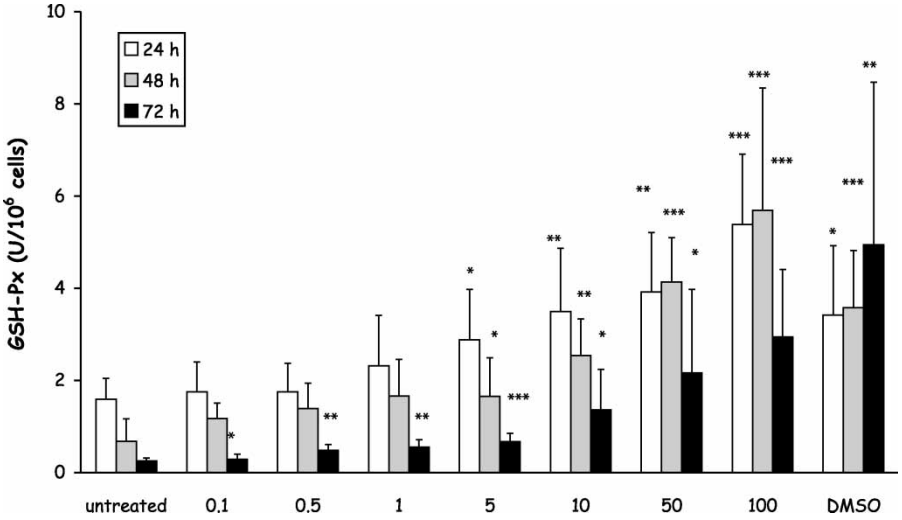

FIGURE 5 The diagram shows that Jurkat T cells display an increased GSH-Px activity in relation to the progressive increase of HNE concentrations (from 0.1 to 100 μM) after 24, 48 and 72 h of incubation. The maximum activity was reached in the first 48 h of incubation. Symbols are the same of Fig. 1. Values are means and SD of six different experiments.

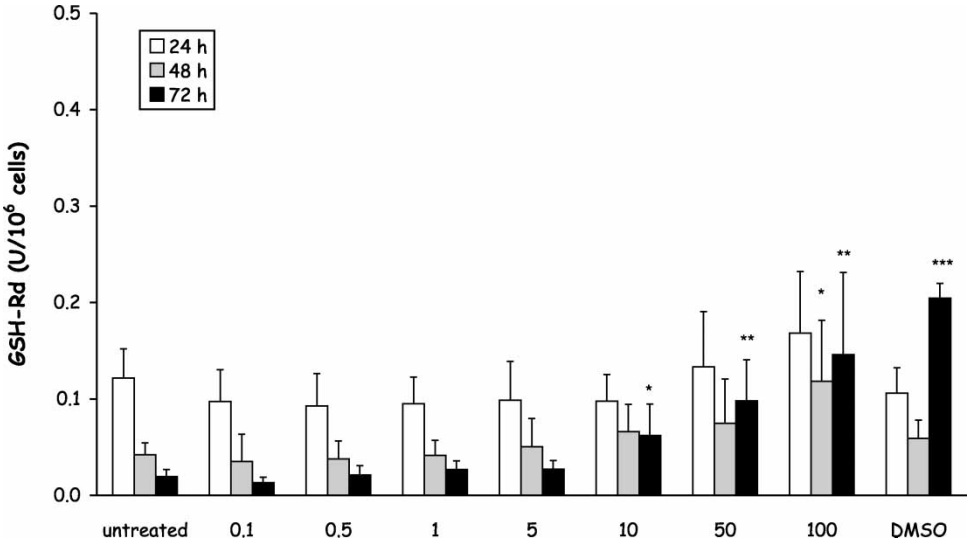

FIGURE 6 The diagram shows that Jurkat T cells displayed an increased GSH-Rd activity in relation to the progressive increase of HNE concentrations (from 0.1 to 100 μM) after 24, 48 and 72 h of incubation. A significant increase was reached after 72 h of incubation. Symbols are the same of Fig. 1. Values are means and SD of six different experiments.

that HNE displayed different effects on caspase 3 activity. As it has already been shown,[3–10] at low concentrations, it significantly increased caspase activity. In line with previous results[33,34] 2%, DMSO treatment activated more caspase activity than HNE, with a maximum in the first 24 h of incubation. Caspases are well known for their role in cell apoptosis, but recent studies indicate that they also play a role in cell proliferation and differentiation, cleaving important substrates, in a variety of cells.[40–42] Higher concentrations of HNE had a significant inhibitory effect on protease activity. The most likely mechanism for this inhibition is a direct effect of HNE on caspase themselves, in agreement with observations that an excessive ROS production can block apoptosis causing cell death.[18,43] However, further investigations are required to better understand these complex biochemical pathways.

In contrast with results obtained on Jurkat T cells with H_2O_2,[44] in the present study as in our previous one,[25] we have noted that the enzymatic activity of SOD, GSH-Px and GSH-Rd increase at high HNE (and ozone) concentrations in a dose-dependent manner. An upregulation of antioxidant enzymes has been also detected in human nasal mucosa after ozone exposure.[45] The activity of G6PDH, the rate-limiting enzyme of the pentose phosphate pathway and the principal source of NADPH, was weekly potentiated at low HNE concentrations, but was strikingly inhibited at 50 and 100 μM. Several studies have shown that, under oxidative stress, G6PDH

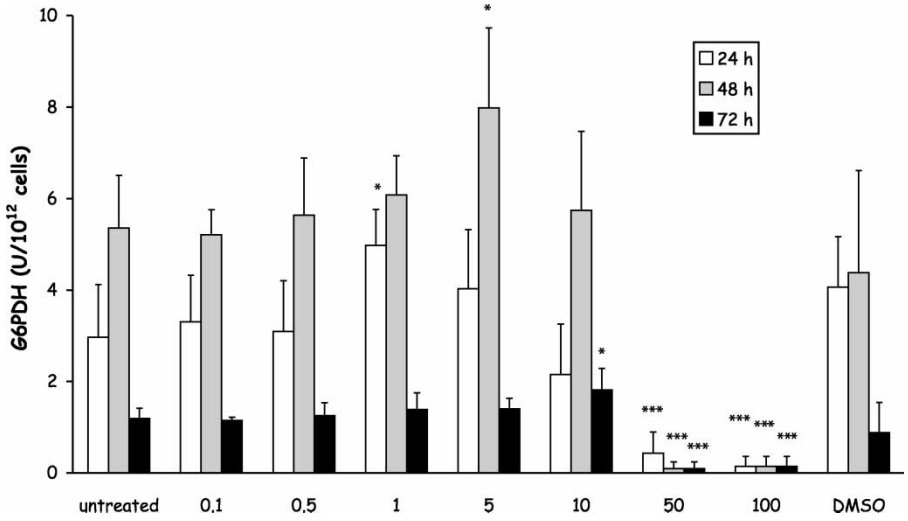

FIGURE 7 The diagram shows that Jurkat T cells displayed an increased G6PDH activity at low HNE concentrations (1 μM at 24 h, 5 μM at 48 h and 10 μM at 72 h). High concentrations of HNE significantly inhibit G6PDH activity. Symbols are the same of Fig. 1. Values are means and SD of six different experiments.

levels increase and that it plays a critical role in cell death by affecting the redox potential.[32,46,47] We can only speculate that the up- or down-regulation of these enzymes depends on different parameters such as levels of intracellular antioxidants and transcription factors that are likely to fluctuate against increasing HNE concentrations. The increased activity of antioxidant enzymes, as an adaptative protective response, may counteract lipid peroxidation. Antioxidant enzymes are critical in ROS defence, directly scavenging ROS and maintaining intracellular antioxidant pool. Depletion of intracellular GSH and caspase 3 activation has been detected in HNE treated Jurkat T cells[11] suggesting a cellular redox state-linked pathway. Further analysis, in particular Western Blot data, will be necessary to clarify whether changes on enzymes activity are due to an increase of enzymes synthesis.

We emphasize that our experiments have been performed in tissue culture medium with 20% FCS while most of previous studies[11,17–18,26,31–32] have employed 5 or 10% FCS. This difference seems important and may explain why, at variance with previous results,[11,13–17] we found a block of cell proliferation and partial cell death only when HNE concentrations were 50 and 100 μM. It is now well proven that serum or plasma is highly protective[48–51] and even a 10% difference in FCS concentration may partially inhibit the HNE toxicity. In line with this comment, Halliwell[52] has just critically reviewed to the often unphysiological conditions used for cell culture studies, likely leading to erroneous conclusions.

In conclusion, the present results indicate a dual effect of HNE and the adaptive responses elicited on intracellular antioxidant activity. This is interesting because ROS are implicated in the pathogenesis of different human diseases, including cancer, atherosclerosis, diabetes and neurodegenerative disorders.

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