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Aldehyde Reductase Gene Expression by Lipid Peroxidation End Products, MDA and HNE

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Membrane lipid peroxidation results in the production of a variety of aldehydic compounds that play a significant role in aging, drug toxicity and the pathogenesis of a number of human diseases, such as atherosclerosis and cancer. Increased lipid peroxidation and reduced antioxidant status may also contribute to the development of diabetic complications. This study reports that lipid peroxidation end products such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) induce aldehyde reductase (ALR) gene expression. MDA and HNE induce an increase in intracellular peroxide levels; *N*-Acetyl-L-cysteine (NAC) suppressed MDA- and HNE-induced ALR gene expression. These results indicate that increased levels of intracellular peroxides by MDA and HNE might be involved in the upregulation of ALR.

Keywords: redox regulation; peroxide; aldehyde reductase; lipid peroxidation

Abbreviations: RASMC, rat aortic smooth muscle cell(s); ALR, aldehyde reductase; MG, methylglyoxal; MDA, malondialdehyde; HNE, 4-hydroxynonenal; ROS, reactive oxygen species; BAPTA-AM, 1,2-bis(2-aminophenoxy)-ethane-N-N-N′-N′-tetra-acetic acid tetrakis(acetoxymethylester)

INTRODUCTION

It is generally recognized that oxidative stress is a major factor in the aging process [1–2]. In this context, mitochondrial oxidative alteration has been proposed as a possible cause of cell damage and tissue degeneration which, in turn, is associated with aging [3]. The generation of lipoperoxidative-derived aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) is considered to be involved in these processes.

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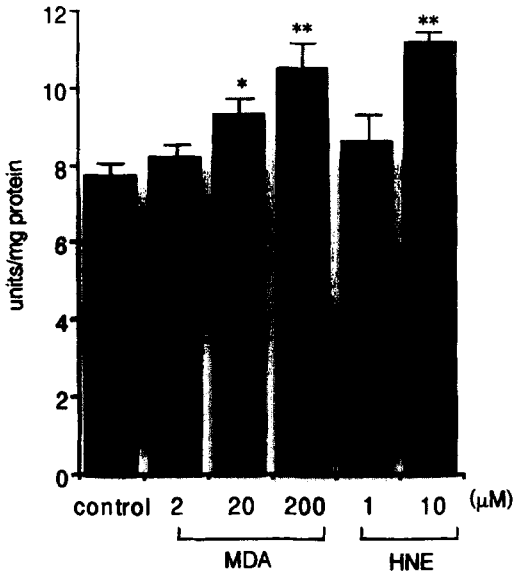

FIGURE 1 Effects of MDA and HNE on the ALR activity. RASMC were treated with 2–200 μM MDA and 1–10 μM HNE for 2 h. Enzyme activity of ALR was measured in cytosol fractions by the rate of decrease in the absorbance at 340 nm using methylglyoxal as a substrate. * denote $p<0.05$. ** denote $p<0.01$

It has been noted that streptozotocin-induced diabetic rats, which have a diminished ability to detoxify food peroxides in the gut, are reported to absorb peroxides from dietary oxidized fats to a greater extent than controls [4]. Diabetes mellitus is associated with increased levels of lipid peroxidation, that may contribute to vascular complications. One potential mechanism for the increased lipid peroxidation in diabetes involves lipid-linked advanced glycation. The increased production of lipoperoxidative aldehydes during the *in vitro* peroxidation of diabetic subcellular fractions occurrs in diabetic rats, irrespectively of the levels of α-tocopherol, compared with the levels of non diabetic rats [5]. Therefore, this suggests that, in the diabetic liver, a prooxidant stimulus which overcomes normal antioxidant defense, would be able to cause more severe damage than in the non diabetic liver.

Aldehyde reductase (ALR) is expressed in wide variety of organs and serves to reduce aldehyde carbonyl groups using NADPH as the hydrogen donor [6,7]. Recently, we and other investigators identified aldehyde reductase as the major enzyme in rat liver that detoxifies 3-deoxyglucosone [8,9]. Even more recently, we found that the overexpression of ALR protects PC12 cells from the cytotoxicity of MG or 3-DG [10]. These observations suggest that the increased level of lipid peroxidation in diabetes leads to cell death and that ALR plays a protective role in this process. However, the mechanism of ALR induction is not well understood.

In this study, we show that the treatment of rat aortic smooth muscle cells (RASMC) with MDA or HNE at physiological concentrations during oxidative injury leads to a transcriptional activation of the rat ALR gene. We demonstrate that MDA or HNE induce ALR in RASMC by increasing the level of intracellular peroxides. ALR expression by MDA and HNE may exert a protective effect against the directly or indirectly cytotoxicity of MDA and HNE.

MATERIALS AND METHODS

Materials

The HNE was provided by Prof. Hermann Esterbauer (Department of Biochemistry, University of Graz, Graz, Austria). MDA was synthesized according to a previously described method [11]. N-Acetylcysteine (NAC), L-buthionine-S,R-sulfoximine (BSO), BAPTA-AM were obtained from Sigma (St. Louis, MO) and 1,1,3,3-tetraethoxypropane (TEP) was purchased from the Wako Pure Chemical Industry (Osaka, Japan). 2′,7′-Dichlorofluorescin diacetate (H_2DCF-DA) was obtained from Molecular Probes, Inc. and the anti-phosphotyrosine antibody was purchased from Transduction Laboratories (Lexington, KY). Other chemicals were of the highest grade available.

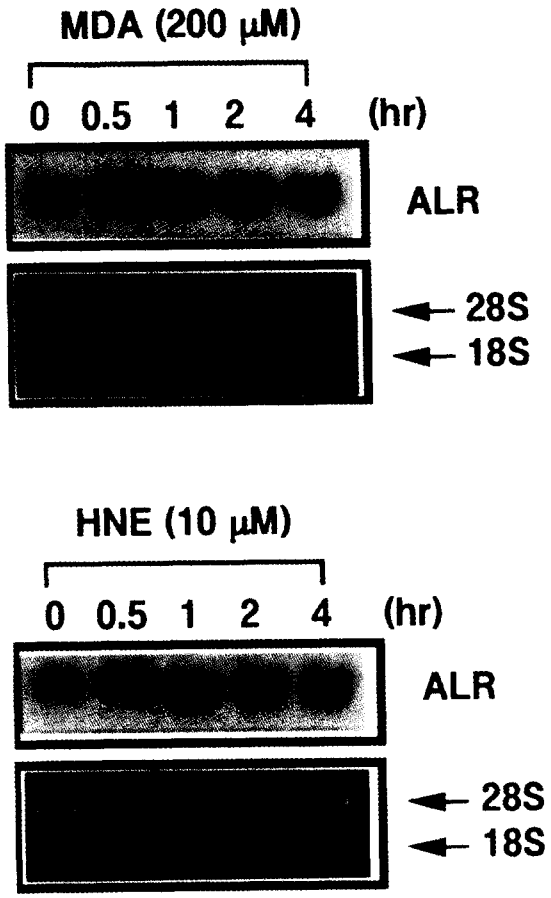

FIGURE 2 ALR mRNA induction by MDA and HNE. RASMC were treated with 200 μM MDA and 10 μM HNE for various periods of time. Cells were washed in phosphate-buffered saline twice. Total RNA was extracted, and 20 μg of the RNA were analyzed by Northern blotting with a ^{32}P-labeled rat ALR probe. 28S and 18S rRNA were used as controls

Cell cultures

Rat aortic smooth muscle cells (RASMC) were isolated from the thoracic aorta of a Wistar rat (body weight 200 g) as described previously [12] and was cultured in Dulbecco's modified Eagle's medium (DMEM; Nikken Bio Med Lab) containing 10 % fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5 % CO_2 at 37°C. The cells were passaged every 4–7 days. RASMC were cultured to about 80 % confluence and then further incubated with fresh medium containing the above reagents. Throughout these experiments, the cells were used within passages of 6–10.

RNA isolation and northern blot experiments

Total RNA was extracted with acid guanidium thiocyanate-phenol-chloroform as reported previously [13]. Twenty μg of the total RNA were run on a 1 % agarose gel containing 2.2 mol/l formaldehyde. The size-fractionated RNAs were transferred to Zeta-Probe membranes (Bio-Rad) overnight by capillary action. Rat ALR cDNA was labeled with [α-^{32}P] dCTP (DuPont-New England Nuclear) using random hexanucleotide primers (Multiprime DNA labeling system; Pharmacia). After hybridization with the labeled probes at 42°C in the presence of 50% formamide, the membrane was washed twice with 2 × sodium chloride-sodium citrate (SSC; 1 × SSC, 15 mM sodium citrate 150 mM NaCl, pH 7.5) which contained 0.1% sodium dodecyl sulfate (SDS) at 50°C for 60 min, and then washed with 0.2 × SSC with 0.1 % SDS at 50°C for 30 min. Kodak X-AR films were exposed for 1–2 days to an intensifying screen at −80°C. The intensities of the bands on X-ray films were quantitated with a CS-9000 gel scanner (Shimadzu, Japan).

Intracellular peroxide production

To assess the levels of intracellular peroxides, flow cytometric analyses were carried out using an oxidation-sensitive fluorescent probe, H_2DCF-DA as described previously [14]. The RASMC, which had been treated with MDA or HNE, were incubated with 5 μM H_2DCF-DA for 30 min. After the cells were harvested and washed them twice with ice-cold phosphate-buffered saline, the intracellular peroxide levels were measured using a FACScan instrument (Becton Dickinson, Mountain View, CA). For image analysis, cells were analyzed for fluorescence intensity using a lysis cell analysis system [15].

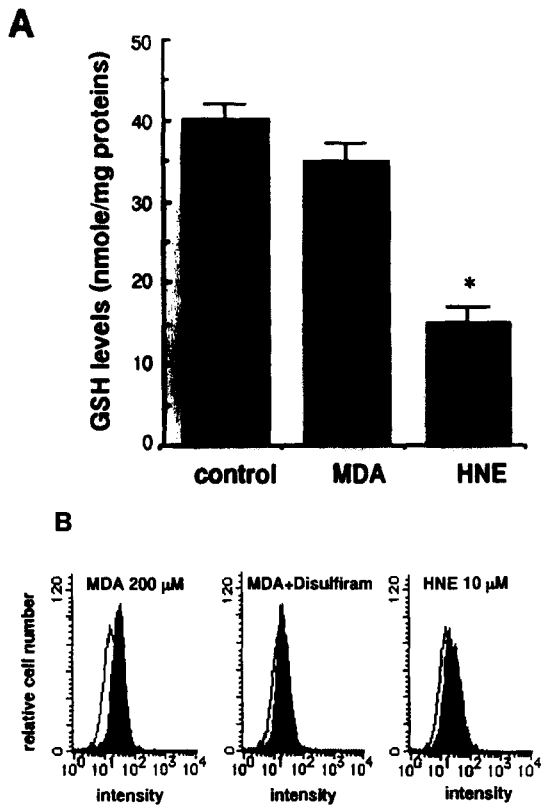

FIGURE 3 Intracellular peroxide production and redox alteration by MDA and HNE. (A) effect of MDA or HNE treatment on intracellular GSH levels. Cells were treated with 10 μM HNE or 200 μM MDA for 1 h. intracellular GSH levels were measured as described under "Materials and Methods". * denote $p<0.001$. (B) Cells were incubated with (black area) or without (white area) MDA (200 μM) or HNE (10 μM) for 30 min, and treated with a peroxide-sensative dye, H_2DCF-DA (5 μM) during the final 30 min of each treatment. The relative peroxide concentrations in the cells were then quantitated by flow cytometry. After preincubation with 50 μM disulfiram for 30 min, the cells were treated with (black area) or without (white area) 200 μM MDA, and subjected to flow cytometric analysis

Measurement of GSH

The GSH contents were determined by the method described by Anderson [16].

Enzyme assays

The enzyme activity of ALR was measured by the rate of decrease in the absorbance at 340 nm using methylglyoxal as a substrate. The standard assay mixture contained 100 mM Na-phosphate, ph 7.0/0.1 mM NADPH/10 mM methylglyoxal.

Statistical analysis

Data were analyzed by the Student's t-test, and the results were expressed as means ± S.D.

RESULTS

Induction of aldehyde reductase by MDA and HNE

The ALR activity in RASMC increased after treatment of 20, 200 μM MDA or 10 μM HNE (Fig. 1). Next, the effects of MDA and HNE on the ALR gene expression in RASMC were examined. An increase in the ALR mRNA expression level was observed 30 min after HNE or MDA treatment. ALR gene expression reached a maximal level at 1 h after MDA treatment and then remained constant for up to 4 h. In the case of HNE, ALR gene expression was increased moderately (Fig. 2).

The change of GSH by MDA and HNE

We examined the effect of MDA and HNE treatment on changes in cellular redox status. As shown in Figure 3A, a significant decrease in GSH levels was observed after 1 h of HNE treatment. The amount of GSH recovered to the basal level at 18 h (data not shown), indicates that the cell responded to the depletion of GSH. However, a significant decrease in the GSH level was not found when cells were treated with MDA.

Involvement of reactive oxygen species in MDA and HNE-induced gene expression

It has been reported that HNE produces reactive oxygen species (ROS) [17], which may contribute

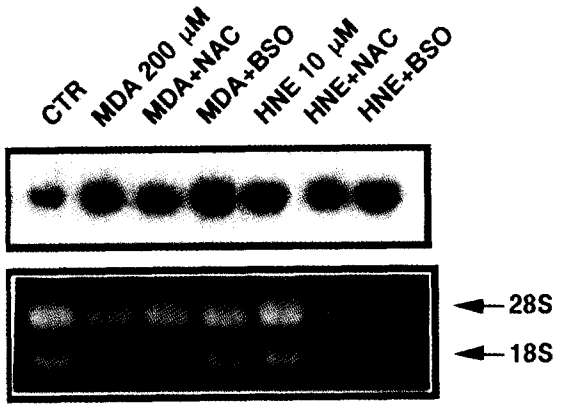

FIGURE 4 Effects of NAC and BSO on the induction of ALR mRNA by MDA and HNE. Following preincubation with 10 mM NAC or 10 μM BSO for 24 h, the chells were treated with or without MDA (200 μM) or HNE (10 μM), and Northern blotting was then carried out using a rat ALR cDNA probe

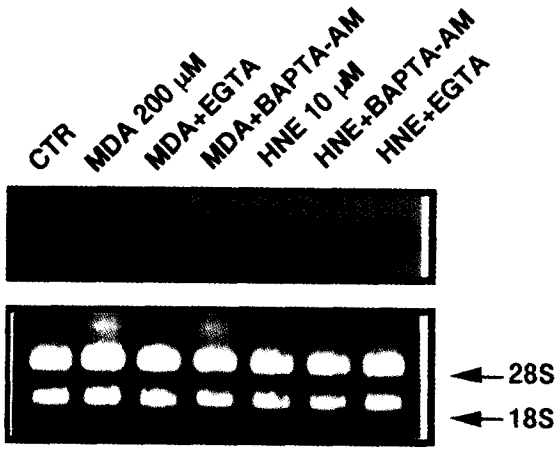

FIGURE 5 Effects of a calcium inhibitor on MDA and HNE-induced ALR gene expression. RASMC were treated with 200 μM MDA or 10 μM HNE for 1 h with EGTA (3 mM) or BAPTA-AM (10 μM). Northern blotting was then carried out using a rat ALR cDNA probe

to the induction of ALR in RASMC. We investigated the effect of MDA and HNE on intracellular ROS formation in RASMC by means of flow cytometric analysis using a peroxide-sensitive fluorescence probe H_2DCF-DA. MDA or HNE caused a significant increase in intracellular peroxide levels as early as 30 min (Figure 3B). An increase in intracellular peroxide levels, as the result of MDA treatment was blocked by disulfiram, a membrane permeable thiol-blocking agent. However, the increase in peroxide levels by HNE was not blocked by disulfiram (data not shown).

To investigate the role of ROS during the induction of ALR by MDA and HNE, the effect of N-acetylcysteine (NAC), a reducing agent known to alter the redox state of the cell, on MDA or HNE-induced ALR gene expression in RASMC was examined. Also, the effect of BSO, a reagent that depletes intracellular GSH levels, was examined. The cells were incubated with 10 mM NAC or 10 mM BSO for 24 h, and the cells were then treated with MDA or HNE, respectively, for an additional 1 h. The NAC pretreatment abolished both MDA and HNE-induced gene expression. In addition, the BSO pretreatment increased MDA and HNE-induced ALR gene expression (Figure 4). These results indicated that the upregulation of ALR is dependent on the ROS production by MDA and HNE.

Effects of Ca^{2+} on MDA and HNE-induced ALR gene expression

We tested the effect of Ca^{2+} flux by MDA on ALR gene expression. Agents that induce membrane lipid peroxidation (for example, Fe^{2+} and β-amyloid peptide) have been shown to impair the function of the plasma membrane Na^+/K^+- and Ca^{2+}-ATPase, the endoplasmic reticulum Ca^{2+}-ATPase and the glucose transporter [18,19]. Ten μM BAPTA-AM partially blocked 10 μM HNE-induced ALR gene expression (Figure 5). However, EGTA and BAPTA-AM had no effect on ALR mRNA induction by 200 μM MDA.

Effects of tyrosine phosphorylation on MDA and HNE-induced ALR gene expression

The promotion of tyrosine phosphorylation of cellular proteins may be the result of the activation of either protein tyrosine kinases or the inactivation of protein tyrosine phosphatases. Suc et

al. reported that oxLDL and HNE elicit tyrosine phosphorylation of the epithelial growth factor receptor (EGFR) and, as a result, the activation of its signaling pathway [20]. Therefore, we investigated the issue of whether tyrosine phosphorylation is involved in the MDA and the HNE-induced ALR mRNA expression in RASMC. Phosphoproteins from MDA-and HNE-treated RASMC were analyzed by western blotting using an antiphosphotyrosine antibody. As shown in Figure 6A, HNE and MDA caused an increase in the phosphorylation of tyrosine residues in a few cellular proteins. Next, the effect of inhibitors of tyrosine kinase on the MDA- and the HNE-induced ALR gene expression was studied. Cells were treated with 200 μM of MDA and 10 μM of HNE following pretreatment with 50 μM genistein (a non-selective tyrosine kinase inhibitor) or 250 nM AG1478 (an EGF receptor-specific tyrosine kinase inhibitor). ALR gene expression, induced by MDA or HNE, was not blocked by genistein and AG1478 (Figure 6B). This finding suggests that the promotion of tyrosine phosphorylation of cellular proteins is not required for ALR gene expression, as induced by HNE and MDA.

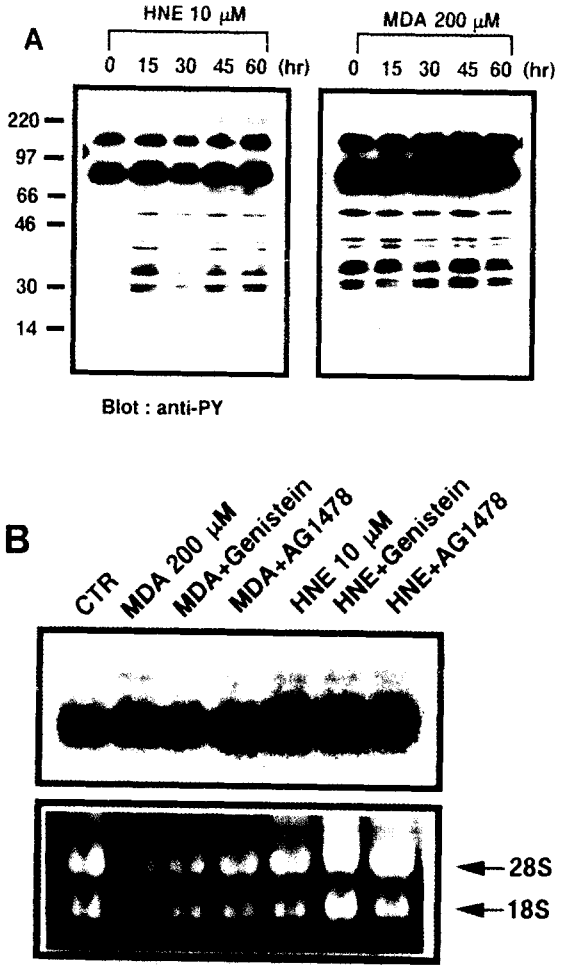

FIGURE 6 Effects of tyrosine phosphorylation on MDA and HNE-induced ALR gene expression. **(A)** Western blot analysis for tyrosine phosphorylation by HNE (10 μM) or MDA (200 μM) using an anti-phospho-tyrosine antibody. **(B)** Cells were treated with MDA (200 μM) or HNE (10 μM) for 1 h with or without pretreatment with 250 nM AG1478 or 50 μM genistein for 1 h. Northem blotting was then performed using a rat ALR cDNA probe

DISCUSSION

We report here that the exposure of rat aortic smooth muscle cells to lipid peroxidation products resulted in constitutive increases in the levels of ALR mRNA and its related activity. Enzymes which metabolize aldehydes produced during lipid peroxidation (aldehyde dehydrogenase, aldehyde reductase, alcohol dehydrogenase and glutathione-S-transferase) serve to maintain the steady-state concentration of aldehydes inside cells [21]. Generally in tumor tissues a marked increase in aldehyde dehydrogenase and aldehyde reductase activities is observed [22,23] in direct correlation with the degree of deviation. Recently, it was noted that HNE induces the transcription and expression of aldose reductase as an adaptive response [24]. However, the mechanism by which aldose reductase is expressed is not known. In this report, we demonstrate that lipid peroxidation products, such as HNE or MDA, induce aldehyde reductase in RASMC via ROS production. NAC inhibited MDA or HNE-induced ALR gene expression. These results suggest that ALR gene

expression is dependent on an increase of ROS by reactive aldehydes.

The HNE elicit tyrosine phosphorylation of the epithelial growth factor receptor (EGFR) and, as a result, the activation of its signaling pathway [20]. However, ALR upregulation by HNE or MDA is not blocked by genistein or AG1478. These results indicate that ALR upregulation by HNE is not dependent on the tyrosine phosphorylation of signal molecules but, rather, is dependent on oxidative stress via an increase peroxide levels.

In response to oxidative insults HNE accumulates in membranes at concentrations of 10 μM to 1 mM, and these levels of HNE are cytotoxic to hepatocytes, fibroblasts, and endothelial cells [25,26]. HNE itself may be a potential source of intracellular pro-oxidant. By interfering with the intracellular redox state, however, HNE triggers the inactivation of glutathione peroxidase (GPx), thereby leading to an increase in intracellular peroxide levels. The mechanism by which MDA causes an increase in ROS is unknown. The observation that disulfiram, a thiol-blocking agent, hindered ROS production by MDA not HNE suggests that MDA affects ROS production in a manner different from HNE. The mechanisms by which HNE and MDA induce ALR mRNA were shown to be different (Figure 5). Further study will be necessary, in order to elucidate the role of Ca^{2+} in the induction of ALR by HNE.

Increased peroxide levels, as the result of MDA or HNE may regulate the ROS-dependent antioxidative enzyme. In this cellular adaptive response, ALR, acts as an antioxidant enzyme, as would be expected. It has been suggested that ALR is present to detoxify 3-DG, a potent toxic dicarbonyl compound formed via the Maillard reaction *in vivo* [8,9]. Also, we noted that in ALR gene-transfected cells, resistance against the cytotoxicity of methylglyoxal and 3-DG, involved in ROS production, was increased [10]. These observations suggest that ALR upregulated by ROS may act as an antioxidative enzyme.

In conclusion, our data indicate that a mechanism for the upregulation of ALR, as the result of lipid peroxidation, is due to an increase in peroxide levels.

Acknowledgements

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