Cells Preconditioned with Mild, Transient UVA Irradiation Acquire Resistance to Oxidative Stress and UVA-induced Apoptosis

ROLE OF 4-HYDROXYNONENAL IN UVA-MEDIATED SIGNALING FOR APOPTOSIS*

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Because 4-hydroxynonenal (4-HNE) has been suggested to be involved in oxidative stress-mediated apoptosis (Cheng, J. Z., Sharma, R., Yang, Y., Singhal, S. S., Sharma, A., Saini, M. K., Singh, S. V., Zimniak, P., Awasthi, S., and Awasthi, Y. C. (2001) J. Biol. Chem. 276, 41213–41223) and UVA irradiation also causes lipid peroxidation, we have examined the role of 4-HNE in UVA-mediated apoptosis. K562 cells irradiated with UVA (3.0 milliwatts/cm²) for 5, 15, and 30 min showed a time-dependent increase in 4-HNE levels. As judged by the activation of caspases, apoptosis was observed only in cells irradiated for 30 min. Within 2 h of recovery in normal medium, 4-HNE levels in 5 and 15 min UVA-irradiated cells returned to the basal or even lower levels but in cells irradiated for 30 min, 4-HNE levels remained consistently higher. The cells irradiated with UVA for 5 min and allowed to recover for 2 h in normal medium (UVA-preconditioned cells) showed a remarkable induction of hGST5.8, which catalyzes conjugation of 4-HNE to glutathione (GSH), and RILIP76 (Ral BP-1), which mediates the transport of the conjugate, GS-HNE. In cells irradiated with UVA for 30 min the induction of RILIP76 or hGST5.8 was not observed. The preconditioned cells transported GS-HNE into the medium at a rate about 2-fold higher than the controls and the transport was inhibited (65%) by coating the cells with anti-RILIP76 IgG. Upon treatment with xanthine/xanthine oxidase (XA/XO), 4-HNE, or prolonged UVA exposure, the control cells showed a sustained activation of c-Jun N-terminal kinase (JNK) and apoptosis. However, in the UVA-preconditioned cells, apoptosis was not observed, and JNK activation was inhibited. This resistance of preconditioned cells to XA/XO, 4-HNE, or UVA-induced apoptosis could be abrogated when these cells were coated with anti-RILIP76 IgG to block the efflux of GS-HNE. These studies strongly suggest a role of 4-HNE in UVA-mediated apoptosis.

UVA irradiation affects cellular signaling mechanisms and is known to cause the activation of transcription factors such as NF-κB and the stress-related kinases including extracellularly regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 in different cell lines (1, 2). UVA causes oxidative stress in cells via the formation of reactive oxygen species (ROS) including singlet oxygen and hydrogen peroxide, which besides damaging other cellular constituents including DNA, can initiate lipid peroxidation in membranes (3–5). UVA irradiation causes the release of iron through degradation of ferritin, which can potentiate ROS-induced oxidative damage and lipid peroxidation (6). In addition, the impairment of the activities of cellular antioxidant enzymes (7) may also accelerate ROS induced lipid peroxidation leading to the formation of a variety of highly reactive and toxic compounds, including pentane and ethane radicals and α,β-unsaturated aldehydes. In particular, 4-hydroxynonenal (4-HNE), a relatively stable α,β-unsaturated aldehyde, which is the major degradation product of α,ω-unsaturated aldehydes, can be generated in relatively large amounts in cells exposed to UVA and other conditions of oxidative stress (8).

Exposure of cells to 4-HNE causes a wide range of biological outcomes such as necrosis, apoptosis, differentiation, and proliferation in a concentration-dependent manner (9–11). The intracellular concentrations of 4-HNE are regulated by a coordinated action of specific glutathione S-transferase isoforms that catalyze the Michael addition of 4-HNE to GSH to form the conjugate GS-HNE, and transporters, including RILIP76 (RalBP1), which catalyze the ATP-dependent efflux of GS-HNE from cells (12–16). In humans, two GST isoforms (GSTA4-4 and hGST5.8) with substrate preference to 4-HNE have been reported (17, 18). Transfection of HL-60 and K562 cells with murine enzyme mGSTA4-4 with substrate preference to 4-HNE has been shown to protect cells from apoptosis caused by oxidative stress as well as 4-HNE (9, 19). Also, a concomitant induction of hGST5.8 and RILIP76 has been shown to provide protection to several human cell lines in culture against oxidative stress-mediated apoptosis (12). Previous studies with a number of cell lines of human origin have dem-
ongestrated that cells preconditioned with mild, transient oxidative stress or heat shock acquire resistance to apoptosis caused by oxidative stress through an accelerated metabolism and exclusion of 4-HNE due to the induction of hGST5.8 and RLIP76. A coordinated action of these proteins causes an increased efflux of the GS-HNE across the cell membranes, lowers the intracellular concentration of 4-HNE, and protects against oxidative stress-induced apoptosis (12). These studies strongly indicate a role of 4-HNE in oxidative stress-mediated apoptosis.

Although UVA exposure causes lipid peroxidation (3–5) and also leads to apoptosis (20, 21), it is not known if lipid peroxidation products, particularly 4-HNE, are involved in UVA-mediated signaling for apoptosis. Therefore, present studies were designed to examine a possible role of 4-HNE in UVA-induced apoptosis. K562 cells were selected for these studies because we have previously characterized the enzyme systems, which regulate the intracellular concentrations of 4-HNE (12, 22) in these cells. It was hypothesized that if 4-HNE was involved in UVA-mediated apoptosis, then upon UVA exposure, 4-HNE levels should rise. This should be accompanied by a defense response of the cells against 4-HNE toxicity resulting in the induction of hGST5.8 and RLIP76. If this response occurs at non-toxic/non-lethal doses of UVA irradiation, cells preconditioned with mild and transient UVA exposure should become more resistant to apoptosis caused by prolonged UVA exposures. Furthermore, if UVA preconditioned cells also acquire resistance to oxidative stress-induced apoptosis, 4-HNE could be considered to be a common mediator of apoptosis in a variety of stress situations. To address the above questions, we have studied the effect of UVA exposure on the formation of 4-HNE, induction of hGST5.8 and RLIP76, and the efflux of GS-HNE from K562 cells. The effect of mild, transient UVA exposure on major antioxidant enzymes, which metabolize ROS has also been studied. Furthermore, we have examined whether cells exposed to mild UVA exposure acquire resistance to apoptosis caused by xanthine/xanthine oxidase (oxidative stress) and prolonged UVA exposure and if so, whether this protection can be abrogated by inhibiting the efflux of GS-HNE.

Results of these studies show that preconditioning with mild UVA exposure imparts to cells a partial resistance against oxidative stress- and UVA-induced apoptosis by preventing the activation of JNK and caspase, and that this resistance can be abolished by inhibiting the efflux of GS-HNE. These results strongly suggest an involvement of 4-HNE in UVA-induced signaling for apoptosis, and point out similarities in the mechanisms of apoptosis induced by oxidative stress and UVA.

EXPERIMENTAL PROCEDURES

Materials—1-chloro-2,4-dinitrobenzene (CDNB), glutathione (GSH), xanthine (2,6-dihydroxyprine) (XA), and xanthine oxidase (XO) were from Sigma. RPMI 1640 medium, fetal bovine serum, phosphate-buffered saline (PBS), and penicillin/streptomycin were purchased from Invitrogen. 4-HNE was purchased from Cayman Chemical Co. (Ann Arbor, MI) and hydrogen peroxide was obtained from Fisher Scientific (Fair Lawn, NJ). 4-[(1H)lll]HNE was synthesized as described previously (12). All reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western transfer were purchased from Invitrogen.

Antibodies—Polyclonal antibodies raised in rabbits against the Alp, Mu, and Pi classes of human GSTs were the same as those used in our previous studies (23). Polyclonal antibodies against recombinant mGSTA4-4, the mouse ortholog of hGST5.8, were raised in rabbits. In earlier studies, these antibodies have been shown to be specific to hGST5.8 among the human GSTs (24). Polyclonal antibodies raised in rabbit against the bacterially expressed recombinant RLIP76 were the same as those used in our previous studies (13). Purified IgG obtained by sequentially passing these antibodies over DEAE-50 and protein A-Sepharose columns were used in all the experiments. Polyclonal antibodies against recombinant hGSTA4-4 were raised in chicken as described by us previously (24). Monoclonal antibody against phospho-rylated JNK (G-7) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against β-actin was acquired from Sigma.

Cell Lines and Cultures—The human erythroleukemia K562 cells obtained from the American Type Culture Collection were grown as suspension cultures in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum and 1% penicillin/streptomycin, and maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

UV Irradiation—K562 (5 × 10⁶) cells were collected by centrifugation, washed with PBS, and resuspended in 5 ml of PBS. The cells were then transferred into a Petri dish (60 mm diameter) and irradiated under a 365-nm UV lamp (Model UVL-56, UVP Inc., San Gabriel, CA) in dark at a rate of 3 mW/cm² determined by the chemical actinometry method of Hatchard and Parker (25).

For the UVA exposure, fixed time periods, the cells were pelleted, and PBS was replaced by normal culture medium. The cells were allowed a 2 h recovery time at 37°C. In parallel, sham irradiated cells were subjected to an identical experimental protocol except that UV irradiation was omitted.

Preparation of Cell Extracts and Western Blot Analyses—The treated cells were pelleted, washed, and resuspended in RIPA buffer containing 1× PBS, pH 7.4, 1% Nonidet P-40 or Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 2 µg/ml pepstatin. The extract was passed through a 23-gauge needle to shear DNA, and was kept on ice for 30–60 min. The cell lysate was then centrifuged at 15,000 × g for 20 min at 4°C, and the resulting supernatant was used for Western blot analysis. For detection of phosphorylated JNK, cell extracts were subjected to 20× Tris-HCl, pH 7.4 containing 150 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, and freshly added protease and phosphatase inhibitor cocktails (Sigma), and were lysed by sonication (3 times, 5 s each at 40 watts).

Cell lysates containing 25–100 µg of protein were subjected to SDS-PAGE according to the method of Laemmli (26). For the detection of the expression of hGST5.8 in K562 cells, 250 µg of protein was loaded. For detection of phosphorylated JNK, 50 µg of protein was loaded. Western blot analysis was performed essentially according to the method of Towbin et al. (27). The chemiluminescent reagents from Pierce were used to develop the immunoblots by following the manufacturer’s instructions.

Determination of Intracellular 4-HNE Levels—Biotech LPO-586 228 kit (Oxis International, Portland, OR) was used to measure concentrations of MDA and MDA plus 4-HNE in K562 cells before recovery and after 2 h of recovery. For each determination, 1 × 10⁶ cells were collected by centrifugation at 500 × g for 10 min and washed twice with PBS. The pellet was resuspended in 0.2 ml of 20 mM Tris-HCl, pH 7.4, containing 5 mM BHT and frozen at −70°C until assayed. To each sample, 650 µl of N-methyl-2-phenylindole and 150 µl of either 12 mM HCl (for MDA determination) or 15.4 mM methanesulfonic acid (for 4-HNE plus MDA determination) were added. The reaction mixture was mixed by vortexing and incubated at 45°C for 60 min. After centrifugation at 15,000 × g for 10 min, the absorbance of the supernatant was determined at 586 nm. Standards of MDA and 4-HNE were prepared by the hydrolysis of 1,1,3,3-tetramethoxypropane in HCl and 4-HNE diethylester in methanesulfonic acid, respectively. Extinction coefficients for MDA and 4-HNE (1.1 × 10⁶ M⁻¹ cm⁻¹ and 1.3 × 10⁶ M⁻¹ cm⁻¹, respectively) determined from the standard curves were used, and the values were expressed as pmol of 4-HNE/mg of protein.

Enzyme Assays—Control or UVA-irradiated K562 cells were harvested by centrifugation and washed with PBS. The cells were resuspended in 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. Cells were sonicated in 0.1% mercaptoethanol buffer A. After lysis by sonication, the lysates were centrifuged for 45 min at 28,000 × g at 4°C. The supernatants were then assayed for their activities against different substrates. GST activity toward CDNB was determined by the method of Habig et al. (28) and that toward 4-HNE was determined according to the procedure described by Alin et al. (29). Catalase and superoxide dismutase (SOD) activities were determined by methods described in the same as those used for the measurement of 4-HNE by the method of Larsson (30), and Paolelli and Moceri (31), respectively. 2-Mercaptoethanol was excluded from the buffer A in preparation of the supernatants used for the determination of SOD activity.

Measurement of GS-HNE Efflux from the Cells—In order to investigate the effect of GS-HNE efflux on the mechanisms of UVA, oxidative stress, or 4-HNE-induced apoptosis, UVA-irradiated K562 cells were allowed to recover for an hour, treated with preimmune IgG or anti-RLIP76 IgG at a final concentration of 20 µg/ml, and incubated for an additional 1 h. In some experiments, as noted in the text, the antibody coating step was omitted. Instead, the cells were allowed to...
rest for 2 h, so that the total recovery period was 2 h in each treatment. After washing with PBS to remove IgG, the cells were then loaded with 4-[3H]HNE by incubating with 20 μM 4-[3H]HNE (specific activity 3,800 cpm/nmol) in culture medium for 30 min. The cells loaded with 4-[3H]HNE were harvested by centrifugation at 500 × g, washed with PBS (2 × 2 ml), resuspended in 2 ml of PBS, and incubated for 10 min at 37 °C for measuring the efflux of 4-[3H]HNE from the cells. After the incubation period, cells were harvested, the medium was quantitatively separated, and radioactivity was measured in the medium and in cells. For the identification of GS-HNE conjugate, the medium was lyophilized, extracted with 200 μl of 70% ethanol, and the conjugate was isolated and characterized by HPLC analysis and mass spectrometry as described previously (12). Determinations were made in triplicate in parallel experiments using equal number of cells under identical conditions.

Caspase Assay for in Situ Apoptosis—Control or 5 min of UVA-irradiated cells were allowed to recover for 2 h in complete medium. Apoptosis in separate experiments was induced by one of the following treatments (i) 20 μM 4-HNE for 2 h at 37 °C, (ii) UVA irradiation (3.0 mW/cm²) for 30 min, and (iii) 30 μM xanthine (XA) for 1 h prior to the addition of xanthine oxidase (XO, 20 milliunits) and incubation for additional 15 h. Where mentioned, after 5 min of UVA irradiation, cells were incubated for 1 h in complete medium followed by preincubation with preimmune IgG or anti-RLIP76 IgG at a final concentration of 20 μg/ml for an additional 1 h and, after washing with PBS, were treated with 4-HNE, XAO, or UVA irradiation as described above. Apoptotic cells were detected by staining with 10 μM CaspACE FITC-VAD-FMK (Promega) in situ marker for 30 min in the dark. The slides were rinsed with PBS twice, fixed with 4% paraformaldehyde for 1 h, mounted in a medium containing DAPI (1.5 μg/ml), and observed under fluorescent microscopy (Olympus, Japan).

RESULTS

Effects of Short Time UVA Irradiation on 4-HNE Levels and Apoptosis—In order to investigate a possible role of 4-HNE in UVA-mediated signaling for apoptosis, K562 human erythroleukemia cells were subjected to UVA (365 nm) irradiation (3.0 mW/cm²) up to a period of 30 min, and the intracellular levels of 4-HNE were examined at different time intervals. The results of these experiments presented in Fig. 1 showed that 5, 15, and 30 min of UVA irradiation caused 1.4-fold, 2-fold, and 2.6-fold increase, respectively, in the 4-HNE levels over that observed in the control cells. After UVA exposure, the cells were allowed to recover in complete medium at 37 °C for 2 h, and 4-HNE concentrations were re-examined after the recovery period. Results of these experiments (Fig. 1) showed that after 2 h of recovery, 4-HNE levels in the cells irradiated for relatively short time were remarkably reduced. For example, in cells irradiated for 5 min and rested for 2 h, the 4-HNE level was only about 40% of that observed immediately after UVA exposure. In fact, the 4-HNE level in these rested cells was even lower than the basal level observed without UVA irradiation. Likewise, in cells subjected to 15 min of UVA irradiation, the 4-HNE level was remarkably reduced after recovery period and was comparable to the basal 4-HNE level before irradiation. On the contrary, the 4-HNE level in cells irradiated for 30 min remained persistently high even after the recovery period (Fig. 1). These results indicated that UVA exposure caused an enhanced formation of 4-HNE and suggested that cells exposed to UVA for a short time acquired the capability to dispose of 4-HNE at an accelerated rate but lose this capability if irradiated for a prolonged period with UVA.

Effect of UVA on Apoptosis—UVA-irradiated cells after 2 h of resting were examined for apoptosis using the CaspACE FITC-VAD-FMK marker which specifically detects caspase activation in situ (Fig. 2). Results of these studies showed that after 2 h recovery, cells exposed to 5 and 15 min of UVA showed no significant activation of caspases indicating lack of significant apoptosis. However, a major fraction of the cells exposed to 30 min of UVA showed caspase activation, suggesting onset of apoptosis, which was also indicated by the characteristic nuclear condensation observed in DAPI-stained cells. These results are consistent with earlier reports that UVA causes apoptosis (20, 21) and suggest that the intracellular 4-HNE levels may correlate with the extent of UVA-mediated apoptosis.

Effects of UVA on Expression of Enzymes Regulating Intracellular Concentrations of 4-HNE—Since our results indicated that 4-HNE concentrations in cells exposed to 5 and 15 min of UVA were decreased after a 2-h recovery, we examined the expression of 4-HNE-metabolizing enzymes in cells rested after UVA exposure. Previous studies have shown that the majority of cellular 4-HNE is metabolized through its conjugation to GSH, catalyzed by GSTs (32). In humans, there are two GST isozymes, hGSTA4-4 (18), and hGST5.8 (17), which display high catalytic efficiency toward 4-HNE (33). Results presented in Fig. 3, A and B showed that hGST5.8 expression was strongly induced upon 5 min (20-fold) and 15 min (7-fold) of UVA irradiation but returned to barely detectable basal level in cells subjected to 30 min of UVA irradiation. These results are consistent with the results on 4-HNE levels presented in Fig. 1 and show that the 4-HNE-metabolizing GST isozyme hGST5.8 is remarkably induced upon initial exposure to UVA but this transient induction is abolished upon a prolonged exposure to UVA. The other 4-HNE-metabolizing GST isozyme, hGSTA4-4, was undetectable in K562 cells under physiological conditions as well as after UVA irradiation (data not presented). UVA exposure also did not affect the Pi and Mu class GSTs (data not presented), which comprise the bulk of constitutive GST protein in K562 cells (22). Accumulation of GS-HNE generated by GST-catalyzed conjugation of 4-HNE to GSH is inhibitory to GSTs. Therefore, GS-HNE should be pumped out of the cells to sustain the continuing conjugation of 4-HNE. Previous studies have shown that RLIP76 mediates the ATP-dependent trans-
port of GSH conjugates, and that it accounts for ~70% of the total transport activity toward GS-HNE in various human cells (14, 15). We therefore examined the expression of RLIP76 in cells immediately after UVA irradiation and subsequent recovery after 2 h. Results of Western blot analyses shown in Fig. 4 indicated that RLIP76 was induced in cells irradiated for 5 min (3-fold) and 15 min (3-fold) but not in cells irradiated for 30 min. These results were consistent with previous studies (13) showing that in SDS gels, RLIP76 appears as multiple bands due to its proteolytic cleavage. All these bands are recognized by anti-RLIP76 IgG as shown in Fig. 4 and are induced proportionally after UVA exposure. Together, these results showed that there was a concomitant induction of hGST5.8 and RLIP76 upon a mild UVA irradiation.

**Effect of UVA Irradiation on GST Activity toward 4-HNE**—Results presented in Table I showed that the GST activity toward 4-HNE in 5 and 15 min of UVA-irradiated cells was about 7- and 5-fold higher, respectively, than the activity in

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**FIG. 2.** **Time course of UVA-induced apoptosis.** 5 × 10⁶ K562 cells suspended in 5 ml of PBS in a 60-mm Petri dish were irradiated with UVA for indicated times as shown in the figure. The cells were pelleted, resuspended in full culture medium, and were allowed to rest for 2 h. After resting, an aliquot of cells (1 × 10⁶) were incubating with 10 μM CaspACE FITC-VAD-FM in situ marker (Promega) for 30 min in the dark and then cytophosphed to poly-l-lysine-coated slides. The slides were fixed with 4% paraformaldehyde, mounted with mounting medium (Vectorshield) containing DAPI (1.5 μg/ml), and observed under fluorescent microscopy (Olympus, Japan). All the pictures were taken at ×400 magnifications. In each treatment, *panel A* represents the slide observed through the filter with excitation/emission wavelength at 360/457 nm to visualize DAPI-stained nuclei. *Panel B* in each treatment represents the same slide observed through the filter with excitation/emission wavelength at 490/528 nm to visualize the activation of caspases bound to CaspACE FITC-VAD-FM. The results are representative of two independent experiments.

**FIG. 3.** **Effect of UVA irradiation on expression of GSTs.** K562 cells were subjected to UVA irradiation as detailed in the legend of Fig. 2 for indicated times and allowed for 2 h rest in full culture medium. The cells were then lysed with RIPA buffer as detailed under “**Experimental Procedures.**” *Panel A*, cell extracts used to detect hGST5.8 by Western blot analysis using antibodies against recombinant mgSTA4-4, which are highly specific to hGST5.8. β-Actin expression was used as internal control. The fold of hGST5.8 induction in UVA-irradiated cells compared with that in control cells was determined by densitometry (*panel B*) and normalized with the intensity of β-actin bands.

**FIG. 4.** **Effect of UVA irradiation on expression of RLIP76.** 5 × 10⁶ K562 cells were exposed to UVA irradiation for the indicated times as detailed in the legend of Fig. 2. After 2 h recovery, the cells extracts were subjected to Western blot analysis using antibodies to RLIP76 (*A*). Multiple bands in *panel A* are due to the proteolytic cleavage of RLIP76 as described previously (13). β-Actin expression was used as internal control. *Panel B*, the fold induction of RLIP76 in UVA-irradiated cells was determined by densitometry and normalized with the intensity of β-actin bands.
control cells. In contrast, the GST activity toward 4-HNE in cells irradiated with UVA for 30 min was lower than control. These results were consistent with those of Western blot analysis (Fig. 3A) and indicated that the induced hGST5.8 protein was enzymatically active. In contrast, UVA irradiation failed to induce either the Alpha class GSTs hGSTA1-1 and hGSTA2-2, or the Mu and Pi class GSTs (data not shown). The latter enzymes are quantitatively predominant and thus responsible for the majority of GST activity toward CDNB, which is generally regarded as the common substrate for most of the GSTs. The selective induction of hGST5.8 by UVA irradiation therefore explains the observed increase in cellular GST activity for 4-HNE but not for CDNB (Table I). Previous studies have shown that extended UVA irradiation affects the activity of antioxidant enzymes (7). Results presented in Table I showed that under the mild UVA irradiation conditions used in the present study, there was no significant change in SOD or CAT activity in the cells. A small but noticeable decrease in CAT activity was, however, seen in cells irradiated for 30 min. The Alpha class GSTs, hGSTA1-1 and hGSTA2-2, displayed GPx activity toward lipid hydroperoxides and thus may decrease intracellular 4-HNE concentrations by interrupting autocatalytic chain reactions of lipid peroxidation (23, 33). As already mentioned, results of the Western blot analysis using antibodies specific to hGSTA1-1 and hGSTA2-2 demonstrated that, in K562 cells, there was neither constitutive nor UVA-inducible expression of hGSTA1-1/A2-2. Taken together, these results showed that a mild, short time (5 and 15 min) UVA irradiation caused a transient induction of GST activity toward 4-HNE, which was abolished upon prolonged UVA exposure. The activity of the primary antioxidant enzymes was not affected under these conditions.

Effect of UVA Irradiation on the Efflux of GS-HNE—In order to evaluate the functional consequences of the induction of RLIP76 by UVA, we compared the efflux of GST in control and UVA-irradiated cells. In these experiments, cells were loaded with 4-[3H]HNE, which was intracellularly converted to its GSH conjugate GS-[3H]HNE. The efflux of GS-[3H]HNE from the medium was measured in the UVA-irradiated and rested cells versus the control cells without UVA exposure using the experimental protocol described by us previously (12). The results of these experiments presented in Fig. 5 showed that 5 min of UVA irradiation caused about 2-fold increase in the rate of GS-HNE efflux compared with that of cells given sham irradiation. In order to assess the contribution of RLIP76 in the transport of GS-HNE, the cells were incubated with 20 μg/ml preimmune IgG or RLIP76 IgG during 2 h of recovery after UVA irradiation, and the efflux of GS-[3H]HNE in the medium was measured. The results of these experiments (Fig. 5) indicated that anti-RLIP76 IgG inhibited ~65% of GS-HNE efflux in 5 min UVA-irradiated cells, which was consistent with our previous studies (12). On the other hand, coating cells with preimmune IgG did not have any significant effect on GS-HNE transport. In order to confirm that the radioactivity of the medium was, in fact, due to GS-HNE, HPLC analysis of the medium collected from control cells and cells exposed to 5 min UVA was performed. The results of HPLC indicated the presence of major peak of radioactivity at retention time: 28.5 min., which coincided with that of standard sample of GS-HNE (data not presented). The identity of this peak with GS-HNE was further confirmed by mass spectral analysis, which showed a molecular ion peak at m/z 462.3(M+1) with additional peaks at m/z 444 (M-OH) and m/z 306 (glutathionyl moiety). As previously shown, these peaks correspond to the hemiacetal of 3-(4-hydroxynonanoyl) glutathione (12). Taken together, the results showed that RLIP76-mediated transport of GS-HNE was accelerated in cells preconditioned with a mild UVA exposure, and that the accelerated efflux of the conjugate correlated with the induction of hGST5.8 and RLIP76 proteins.

Table I

| Specific activity | UVA irradiation time |
|------------------|----------------------|
|                  | 0   | 5   | 15  | 30  |
| GST activity towards 4-HNE (nmol/min/mg protein) | 23.5 ± 3.5 | 151.6 ± 6.2* | 114.2 ± 8.2* | 17.8 ± 0.6 |
| GST activity towards CDNB (nmol/min/mg protein) | 131.2 ± 2.0 | 151.6 ± 10.3 | 116.7 ± 9.1 | 126 ± 3.2 |
| SOD activity (μmol/min/mg protein) | 19.98 ± 0.76 | 21.35 ± 2.11 | 21.90 ± 1.26 | 19.6 ± 4.21 |
| CAT activity (μmol/min/mg protein) | 20.12 ± 1.48 | 21.88 ± 0.72 | 18.21 ± 0.10 | 16.74 ± 0.81* |

Fig. 5. Efflux of GS-HNE in control and UVA-pretreated K562 cells. 5 × 10⁶ K562 cells were UVA preconditioned by irradiation with UVA in PBS for 5 min and allowed to recover for 2 h in full culture medium as described under “Experimental Procedures.” The cells were then incubated with 20 μg/ml 4-[3H]HNE (specific activity 8,000 cpm/nmol) in 2 ml of medium for 30 min at 37°C. After washing with PBS three times to remove extraneous 4-[3H]HNE, cells were incubated for additional 10 min at 37°C. The cells were centrifuged, and the medium containing transported GS-HNE was lyophilized, resuspended in 200 μl of 70% ethanol, and radioactivity was determined. To determine the effect of anti-RLIP76 IgG on the transport of 4-[3H]HNE, the efflux rate in these IgG–coated cells was determined as described above.

Role of 4-Hydroxynonenal in UVA-mediated Signaling for Apoptosis—Since our results showed that cells subjected to a transient mild UVA irradiation acquired an increased capacity to conjugate 4-HNE and to transport the resulting GS-HNE, we...
reasoned that UVA-preconditioned cells should be less susceptible to apoptosis caused by 4-HNE than control cells. Since 4-HNE may be involved in the mechanisms of apoptosis caused by UVA exposure (present work) as well as by oxidative stress (8, 34), UVA-preconditioned cells should also be resistant to apoptosis caused by both these factors. These possibilities were explored in the following experiments. First, the effect of UVA-preconditioning on 4-HNE induced apoptosis was examined. The results presented in Fig. 6A showed that incubation of control cells with 20 μM 4-HNE for 2 h caused a prominent activation of caspases in most cells, but the preconditioning of cells with UVA exposure for 5 min blocked 4-HNE induced activation of caspases (Fig. 6B), indicating inhibition of apoptosis. We then examined whether UVA-preconditioned cells also acquire resistance to apoptosis caused by oxidative stress. XA/XO exists ubiquitously in mammalian cells, and is involved in generation of O$_2^−$, an initial reactive oxygen species, under physiological conditions. Therefore, we added XA (30 μM) and 20 milliunits of XO to the medium of control and UVA-preconditioned cells and incubated for 16 h. The results presented in Fig. 7A showed that XA/XO caused a remarkable activation of caspases in control cells indicating apoptosis, which was almost completely inhibited in the UVA-preconditioned cells (Fig. 7B).

Lastly, we demonstrated that UVA-preconditioned cells also acquired resistance to apoptosis caused by a prolonged UVA exposure (Fig. 8B). Taken together, these results indicate a similarity in the signaling mechanisms for apoptosis by oxidative stress and UVA exposure, and suggest a role of 4-HNE during apoptosis caused by both these factors.

Resistence of Preconditioned Cells to Apoptosis Is Abrogated by Blocking the Efflux of GS-HNE—Consistent with earlier studies (13, 30), the data presented in Fig. 5 indicated that coating cells with RLIP76 IgG blocked about 65% of the GS-HNE efflux, suggesting that RLIP76 was the major transporter of GS-HNE in K562 cells. We reasoned that if the preconditioned cells acquire resistance to UVA and oxidative stress-mediated apoptosis by disposing of 4-HNE at a faster rate, then the resistance of these cells to apoptosis should be compromised by blocking GS-HNE efflux. To explore this possibility, we coated control and UVA-preconditioned cells with preimmune IgG or with anti-RLIP IgG, and treated the cells with 20 μg of 4-HNE for 2 h. Results presented in Fig. 6C showed that coating the preconditioned cells with preimmune IgG did not cause any significant apoptosis and the resistance of these cells to 4-HNE mediated apoptosis was maintained. In contrast, coating the preconditioned cells with anti-RLIP76 IgG abrogated this resistance, and a significant fraction of cells underwent apoptosis (Fig. 6D).

Likewise, blocking GS-HNE efflux from the preconditioned cells with RLIP76 IgG also abrogated their resistance to UVA and oxidative stress induced apoptosis and sensitized these cells to apoptosis caused by XA/XO (Fig. 7D) and UVA (Fig. 8D). Together, these results indicated that 4-HNE was involved in the mechanism of apoptosis caused by oxidative stress as well as UVA exposure, and that upon preconditioning with mild UVA exposure, the cells acquire resistance to oxidative stress and UVA induced apoptosis by acquiring the capacity to dispose of 4-HNE at a faster pace through the induction of hGST5.8 and RLIP76.

UVA Preconditioning Inhibited JNK Activation Caused by 4-HNE, UVA, and XA/XO—JNK/SAPK is activated by various stresses such as heat shock, oxidative stress, deprivation of growth factor, cytokines, etc. (35, 36). It has also been suggested that persistent activation of JNK is an early signal for apoptosis (37, 38). Therefore, we treated control and UVA-preconditioned cells with 4-HNE for different time periods up to 2 h, and determined JNK activation in these cells by monoclonal antibody specific to phosphorylated JNK1. Results shown in Fig. 9 indicated that treating control cells with 4-HNE caused an early and persistent activation of JNK which supported the idea that 4-HNE was involved in the signaling for apoptosis in these cells. In contrast to control cells, the activation of JNK was remarkably inhibited in preconditioned cells subjected to further UVA irradiation (Fig. 9B). Our results (see Fig. 2) showed that a 30 min UVA irradiation caused apoptosis in control cells but not in cells preconditioned with UVA. We therefore compared the activation of JNK in the control and UVA-preconditioned cells irradiated with UVA for a period up to 30 min. UVA irradiation of control cells resulted in an early and persistent activation of JNK. However, the activation of JNK was markedly inhibited in preconditioned cells subjected to further UVA irradiation (Fig. 9B). Similar results were obtained when the activation of JNK was compared in control and UVA-preconditioned cells upon treatment with XA/XO (data not presented).

Collectively, these results indicated that the JNK activation
caused by 4-HNE, UVA, or oxidative stress was suppressed in cells preconditioned with mild UVA exposure and that it may be attributed to the capacity of the preconditioned cells to exclude 4-HNE at a faster rate.

**DISCUSSION**

Cells exposed to UVA undergo apoptosis. The mechanisms of apoptosis caused by stress factors including UV, oxidants, heat, or inflammatory cytokines are not completely understood but activation of SAPK/JNK by stress factors has been extensively studied, and it is believed that a sustained activation of JNK may be required for the signaling for apoptosis (37, 38). The events upstream of the activation of JNK caused by UV or other stress factors and the nature of chemical species initiating the cascade of signaling for stress-mediated apoptosis are not fully understood. However, some studies suggest that the lipid peroxidation product, 4-HNE may be involved in the upstream events leading to apoptosis at least in some cell types exposed to H₂O₂ (12, 39). Results presented in this communication strongly suggest that 4-HNE is involved in the initial events leading to UVA-mediated apoptosis of K562 cells.

Present studies show that even under the conditions of mild UVA exposure which cause no apparent deleterious effects on cells, lipid peroxidation is initiated as indicated by an increase in 4-HNE levels. The cells respond to the increase in 4-HNE levels by rapidly inducing the enzymes responsible for metabolizing 4-HNE, in an attempt to maintain the intracellular levels of 4-HNE. Our results show that mild UVA pre-exposed and rested cells referred to as preconditioned cells in this communication transport GS-HNE at a much faster rate as compared with the untreated controls in order to exclude the excess of 4-HNE from the intracellular environment. This is consistent with the observed induction of hGST5.8 and RLIP76, which catalyze the conjugation of 4-HNE to GSH (17) and the subsequent efflux of GS-HNE (13), respectively. A coordinated action of these enzymes brings down the levels of 4-HNE in UVA preconditioned cells. In cells exposed to UVA for longer time, the induction of hGST5.8 and RLIP76 is not observed, which is consistent with high levels of 4-HNE observed in cells exposed to UVA for 30 min even after 2 h resting. These results suggest that after a certain threshold of stress, the initial adaptive response to upregulate the mechanisms to detoxify 4-HNE is abrogated.
pho-JNK antibody. (no preconditioning) was detected by Western blot analysis using phos-JNK activation in these UVA-preconditioned cells and the control cells.

Fig. 9. UVA preconditioning inhibits 4-HNE and UVA-induced activation of JNK. 5 × 10⁵ K562 cells were exposed to UVA for 5 min and rested for 2 h. After which, the cells were incubated with 20 μg of 4-HNE (panel A) or UVA irradiation for the indicated times (panel B). JNK activation in these UVA-preconditioned cells and the control cells (no preconditioning) was detected by Western blot analysis using phospho-JNK antibody. β-Actin expression was shown to confirm equal amount of protein (50 μg) was loaded in each sample.

Results of our studies with UVA preconditioned cells strongly suggest a role of 4-HNE in UVA-mediated apoptosis. We demonstrate that the resistance of the UVA-preconditioned cells to 4-HNE mediated activation of JNK, caspases, and eventual apoptosis correlates with their ability to exclude 4-HNE from the intracellular environment at a faster rate as compared with control cells. When 4-HNE is included in the medium, control cells show a persistent activation of JNK and a significant increase in apoptosis is observed within 2 h. On the other hand, the preconditioned cells resist 4-HNE-mediated activation of JNK and caspases, and do not undergo apoptosis under the identical conditions of 4-HNE treatment. Since the preconditioned cells express SOD, CAT, and Gpx at levels comparable to those of control cells, the resistance of preconditioned cells to apoptosis cannot be attributed to their capability to resist the primary oxidative stress. Barring a possible role of unknown factors, the ability of preconditioned cells to exclude 4-HNE by transporting GS-HNE at a faster rate can perhaps best explain the mechanism of their resistance to UVA- and 4-HNE-induced apoptosis.

Our results showing that the resistance of preconditioned cells to 4-HNE as well as UVA-induced apoptosis can be compromised by blocking the efflux of GS-HNE from these cells are consistent with the idea that the resistance of the preconditioned cells is due to the accelerated metabolism of 4-HNE and efflux of GS-HNE from these cells. We have previously shown (12, 15) that RLIP76-mediated transport of GS-HNE can be blocked by anti-RLIP76 IgG. Consistent with these observations, the results of present studies show that when the preconditioned cells are coated with anti-RLIP76 IgG, the transport of GS-HNE is substantially inhibited, and that the cells become sensitive to the apoptotic effects of both 4-HNE and UVA. Oxidative stress is known to cause apoptosis in a variety of cells in culture (9, 10, 12, 39). As expected, our results show that the inclusion of XA/XO in the growth medium leads to apoptosis of the control K562 cells. However, the preconditioned cells show resistance to XA/XO-induced JNK activation, caspase activation, and apoptosis. The resistance of the preconditioned cells to XA/XO induced apoptosis can also be compromised by blocking the efflux of GS-HNE by anti-RLIP76 IgG.

Together, these results indicate similarities in the signaling mechanisms for apoptosis caused by UVA and oxidative stress. More importantly, these results suggest that 4-HNE is involved in the events upstream of JNK and caspase activation in signaling for apoptosis caused by UVA and/or oxidative stress.

The overexpression of antioxidant enzymes which specifically protect against lipid peroxidation by reducing lipid hydroperoxides has been shown to block activation of JNK, caspase, and eventual apoptosis (20), suggesting a role of lipid peroxidation products in oxidative stress induced apoptosis. Likewise, overexpression of the 4-HNE-metabolizing murine isozyme mGSTA4-4 has been shown to protect against apoptosis caused by H₂O₂ (19), which is consistent with the idea that 4-HNE is involved in oxidative stress induced signaling for apoptosis. The results of present studies support these previous observations and demonstrate that lipid peroxidation leading to the accumulation of 4-HNE may be a factor in signaling for apoptosis by UVA as well as by oxidative stress. Previous studies have shown that the cells preconditioned with mild, transient exposure to H₂O₂ acquire resistance to toxicity by further H₂O₂ challenge without any noticeable induction of the classical antioxidant enzymes (40). It is possible that UVA- and H₂O₂-preconditioned cells acquire resistance to oxidative stress through similar mechanisms.

4-HNE formed during lipid peroxidation initiated by UVA and oxidative stress is toxic and can also cause apoptosis (9, 10). Thus, it should not be surprising that as an initial adaptive response to mild UVA exposure, the cells upregulate the mechanisms that maintain 4-HNE homeostasis. In fact, previous studies have shown that when cells are subjected to a mild heat shock, 4-HNE levels rise quickly and there is a rapid but transient induction of hGST5.8 and RLIP76. Cells preconditioned with mild heat exclude 4-HNE from intracellular space at a much higher rate than the control cells, and acquire resistance to H₂O₂-mediated apoptosis by blocking the activation of JNK and caspases. These earlier findings (12), together with the result of present studies, strongly indicate a key role of 4-HNE in signaling for apoptosis triggered by stress factors, which cause membrane lipid peroxidation.

It is possible that hGST5.8 and RLIP76 induction during UVA preconditioning may also affect the cascade for apoptosis signaling through other mechanisms. No functions other than protection against lipid peroxidation are known for hGST5.8. However, RLIP76 is a previously described GTPase-activating protein (GAP) that is believed to bridge the Rac and Rho pathways (41). It is a Rac effector protein with GTPase-activating activity for Cdc42/Rac proteins. Recent studies suggest that RLIP76 (RalBP1) is multifunctional and besides catalyzing the transport of GSH conjugates including GS-HNE and leukotrienes (15), it can also regulate the expression of HSP70 through its interaction with heat shock factor (HSF1) (42). While we demonstrate a correlation of RLIP76 induction with increased 4-HNE levels and enhanced efflux of GS-HNE, our present studies are focused only on the transport function of RLIP76 and its role in maintaining the intracellular levels of 4-HNE. Since the stress-mediated activation of JNK/SAPK involves GAP, Rac, and Cdc42 (43–45), the interaction of RLIP76 with Cdc42/Rac may also potentially affect the stress-mediated signaling for apoptosis. Therefore, further studies to define the significance of RLIP76 induction as an early adaptive response to UVA are needed to elucidate the mechanisms of UVA-mediated apoptosis.
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