Accelerated Metabolism and Exclusion of 4-Hydroxynonenal through Induction of RLIP76 and hGST5.8 Is an Early Adaptive Response of Cells to Heat and Oxidative Stress*

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From the ‡Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77555-1067, §Department of Chemistry and Biochemistry, University of Texas, Arlington, Texas 76019-0065, ¶Department of Pharmacology and University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213, and ||Department of Internal Medicine and Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences and Central Arkansas Veterans Healthcare System, Little Rock, Arkansas 72205

To explore the role of lipid peroxidation (LPO) products in the initial phase of stress mediated signaling, we studied the effect of mild, transient oxidative or heat stress on parameters that regulate the cellular concentration of 4-hydroxynonenal (4-HNE). When K562 cells were exposed to mild heat shock (42 °C, 30 min) or oxidative stress (50 μM H₂O₂, 20 min) and allowed to recover for 2 h, there was a severalfold induction of hGST5.8, which catalyzes the formation of glutathione-4-HNE conjugate (GS-HNE), and RLIP76, which mediates the transport of GS-HNE from cells (Awasthi, S., Cheng, J., Singhal, S. S., Saini, M. K., Pandya, U., Pikula, S., Bandorowicz-Pikula, J., Singh, S. V., Zimniak, P., and Awasthi, Y. C. (2000) Biochemistry 39, 9327–9334). Enhanced LPO was observed in stressed cells, but the major antioxidant enzymes and HSP70 remained unaffected. The stressed cells showed higher GS-HNE-conjugating activity and increased efflux of GS-HNE. Stress-pre-conditioned cells with induced hGST5.8 and RLIP76 acquired resistance to 4-HNE and H₂O₂-mediated apoptosis by suppressing a sustained activation of c-Jun N-terminal kinase and caspase 3. The protective effect of stress pre-conditioning against apoptosis was abrogated by coating the cells with anti-RLIP76 IgG, which inhibited the efflux of GS-HNE from cells, indicating that the cells acquired resistance to apoptosis by metabolizing and excluding 4-HNE at a higher rate. Induction of hGST5.8 and RLIP76 by mild, transient stress and the resulting resistance of stress-pre-conditioned cells to apoptosis appears to be a general phenomenon since it was not limited to K562 cells but was also evident in lung cancer cells, H-68, H-226, human leukemia cells, HL-60, and human retinal pigmented epithelial cells. These results strongly suggest a role of LPO products, particularly 4-HNE, in the initial phase of stress mediated signaling.

4-Hydroxy-t-2,3-nonenal (4-HNE),¹ a highly reactive but relatively stable end product of lipid peroxidation (LPO), has drawn a great deal of attention in recent years because of its possible involvement in signaling mechanisms. 4-HNE has been shown to cause apoptosis (1–5), differentiation (6–9), and induction of enzymes including c-Jun kinase (JNK)/stress-activated protein kinase (1, 10), protein kinase C (11), adenylate cyclase (12), and phospholipase C (13). Available evidence suggests that depending upon its intracellular concentration, 4-HNE may differentially affect the cell cycle regulation. For example, it has been shown that at low concentrations, 4-HNE causes proliferation of aortic smooth muscle cells (14) and K562 cells (6), but at relatively higher concentrations, it causes differentiation and apoptosis in these cells (6). Thus, the intracellular concentrations of 4-HNE must be stringently controlled. Because the formation of 4-HNE results from LPO, an uncontrolled process depending on the levels of cellular redox status, the intracellular levels of 4-HNE must be controlled through its metabolism and elimination of the metabolites from cells. Glutathione S-transferase (GST) mediated conjugation of 4-HNE to glutathione (GSH), resulting in the formation of the GST-conjugate (GS-HNE), is the major pathway for its metabolism (15). GSH conjugates are known to be transported out of cells through an ATP-dependent primary active efflux mechanism (16–19). In humans, GST isozymes designated as hGST5.8 (20–22) and hGSTA4-4 (23, 24) preferentially conjugate 4-HNE to GSH, and GS-HNE thus formed is transported across the membrane by transport proteins, including RLIP76, a Ran binding GTPase-activating protein (25), which has been shown to account for the ATP-dependent transport of GS-HNE in K562 cells and human erythrocytes (26–29). Thus, the levels of expression and activities of hGST5.8 or/and hGSTA4-4 and RLIP76 may be the major determinants of the intracellular concentrations of 4-HNE.

¹ The abbreviations used are: 4-HNE, 4-Hydroxy-t-2,3-nonenal; JNK, c-Jun kinase; GST, glutathione S-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione; GS-HNE, glutathione-4-HNE conjugates; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RPE, retinal pigmented epithelial; HPLC, high performance liquid chromatography; ECL, chemiluminescence; PARP, poly(ADP-ribose) polymerase; HSP, heat shock protein; MDA, malonaldehyde; GPx, glutathione peroxidase; SOD, superoxide dismutase; TUNEL, Tdt-mediated dUTP nick-end labeling; RLIP, Ran-interacting protein; LPO, lipid peroxidation; PBS, phosphate-buffered saline.

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In aerobic organisms, reactive oxygen species such as O₂⁻, H₂O₂, OH⁻ are continually generated, and their overproduction during stress (oxidative, chemical, heat) conditions causes adverse effects. Under most of these stress conditions LPO is increased with an expected increase in the formation of 4-HNE. Pro-apoptotic agents such as H₂O₂ or a variety of xenobiotics (e.g. doxorubicin) also lead to the generation of reactive oxygen species and induce LPO, suggesting a possible role of LPO products in apoptotic signaling. This idea is consistent with the results of our recent studies showing that attenuation of LPO in K562 by transfection with hGSTA2-3, which specifically reduces lipid hydroperoxides, blocks H₂O₂-induced apoptosis in these cells (30). A clear link between LPO and stress-mediated apoptotic signaling is, however, not established. We reasoned that if 4-HNE derived from LPO is involved in stress-mediated signaling for apoptosis, then one of the early responses of the cells may be to eliminate 4-HNE through its enhanced conjugation to GSH catalyzed by hGST5.8 and the subsequent transport of the conjugate, GS-HNE, by RLIP76. Therefore, during the present studies we have examined the effect of relatively low levels of heat (30 min, 42 °C) and oxidative (50 μM H₂O₂, 20 min exposure) stress on the expression and functions of hGST5.8 and RLIP76. K562 cells were chosen for these experiments because these cells constitutively express hGST5.8 (31) as well as RLIP76 and, when transfected with RLIP76, show enhanced efflux of GS-HNE (27). Results of these studies demonstrate that the induction of RLIP76 and hGST5.8 is an early adaptive response of cells exposed to mild heat shock (42 °C, 30 min) or low levels of transient oxidative stress (50 μM H₂O₂, 20 min), and the cells pre-exposed to these stress conditions suppress H₂O₂- and 4-HNE-mediated activation of JNK and caspase 3 and acquire resistance to apoptosis. These findings suggest that hGST5.8 and RLIP76 play regulatory roles in mechanism in stress-mediated apoptosis.

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 medium, phosphate-buffered saline (PBS), penicillin/streptomycin, and 100-base pair DNA markers were purchased from Life Technologies, Inc. (Grand Island, NY). DNA blood mini kit for genome DNA preparation was purchased from Qiagen Inc (Valencia, CA). (Glycine-2-³H)GSH (specific activity 43.8 Ci/mmol) was purchased from PerkinElmer Life Sciences.

Antibodies—Polyclonal antibodies raised in rabbits against the Alpha, Mu, and Pi classes of human GSTs were the same as used in our previous studies (30). Polyclonal antibodies against recombinant hGSTA4-4 were raised in chickens, and the specificity of the antibodies was stringently established (22). Polyclonal antibodies against recombinant mGSTA4-4, the mouse ortholog of hGST5.8, were raised in rabbits, and their specificity only to hGST5.8 among human GSTs has been established (20–22, 32). Polyclonal antibodies raised in rabbit against recombinant RLIP76 were the same as these used in our previous studies (27, 28). IgG fractions from all these antibodies purified through DE-52 and protein A-agarose columns were used. Anti-heat shock protein 70 (HSP70) goat polyclonal antibodies (K20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phospho-c-Jun (Ser-63) antibodies and GST-c-Jun(1–89) fusion protein were obtained from New England Biolabs, Inc. Antibodies against poly(ADP-ribose) polymerase (PARP) (H-250) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and those against caspase 3 were obtained from Cayman, San Diego, CA. Antibodies against β-actin were purchased from Sigma.

Cell Lines and Cultures—Human leukemia K562 and HL-60, human small cell lung cancer cell line H-69, and human non-small cell lung cancer cell line H-226 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. K562, HL-60, and H-69 cells were grown as suspension cultures in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 1% penicillin/streptomycin. Monolayer cultures of H-226 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1% (v/v) penicillin/streptomycin solution, 2 mM l-glutamine, 4.5 μg/ml amphotericin B, 0.1 mg/ml HEPES, 1 mM 4-phenylmorpholine, 4.5 μg/ml glucose, and 1.5 g/liter sodium bicarbonate. Cultures of simian virus 40-transformed fetal male pigmented epithelial (RPE) cells (33) were the same as those used in our previous studies (34) and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% (v/v) penicillin/streptomycin, 10 mM HEPES, pH 7.4. The cells were transferred and passaged every 5 days.

Heat Shock and H₂O₂ Treatment—For heat shock the cells were exposed to 42 °C for different time periods, brought back to 37 °C, and allowed to recover for 2 h at 37 °C before use in further experiments. For the transient exposure to H₂O₂, the cells were treated with 50 μM H₂O₂ in the medium for 20 min, after which cells were pelleted, washed with H₂O₂-free medium, resuspended in medium, and allowed to recover for 2 h at 37 °C.

Lipid Peroxidation—LPO was measured by determining the thiobarbituric acid-reactive substance as described by Wagner et al. (38). For each determination, 1 × 10⁵ cells collected by centrifugation at 500 × g for 10 min and washed twice with PBS were used. Cells were resuspended in 1 ml of 10 mM potassium phosphate buffer, pH 7.0, containing 0.4 mM butylated hydroxytoluene, vortexed vigorously, and immediately used for thiobarbituric acid-reactive substance assay.

Determination of Intracellular Malonaldehyde and 4-HNE Levels—Malonaldehyde (MDA) and 4-HNE levels were determined using Bio-tech LPO-586™ kit (Oxis International, Portland, OR) according to the manufacturer’s instructions. For each determination, 5 × 10⁶ cells were collected by centrifugation at 500 × g for 10 min and washed twice with PBS. The harvested cells were resuspended in 0.2 ml of 10 mM potassium phosphate buffer, pH 7.0, containing 4 mM butylated hydroxytoluene and 0.1 mM 1-chloro-2,4-dinitrobenzene (CDNB), vortexed vigorously, and immediately used for thiobarbituric acid-reactive substance assay.

Enzyme Assays—Catalase, glutathione peroxidase (GPx), and glutathione reductase activities were determined by the methods described by Wagner et al. (38). GPx activity toward cumene hydroperoxide, pH 7.0, containing 0.4 mM butylated hydroxytoluene, was measured by the methods described by Beers and Sizer (42). The extinction coefficient of 1.1 × 10⁵ M⁻¹ cm⁻¹ was determined from the standard curves of MDA and 4-HNE, was used, and the values were expressed as pmol of MDA or 4-HNE/mg of protein.

GST Purification—K562 cells (50 × 10⁶) treated with heat shock or H₂O₂ as described above were harvested by centrifugation and washed with PBS. The cells were resuspended in 10 mM potassium phosphate buffer, pH 7.0 (buffer A), containing 1.4 mM 2-mercaptoethanol, lyzed by sonication, and centrifuged for 45 min at 28,000 × g at 4 °C. Total GSTs were purified from the 28,000 × g supernatants using GSH affinity chromatography as described in our previous studies (30).

Synthesis of GS-HNE—Unlabeled and 4-[³H]-GSH were synthesized enzymatically using purified human GSTs. Briefly, 1 μmol of GSH was synthesized by the method of Faucette et al. (45) and the synthesis was verified by the methods described by Beers and Sizer (42) and Paolini and Mocari (43), respectively. Glutathione reductase activity was determined by the method of Carlberg and Mannervik (44), and GSH was measured as non-protein thiols according to the method of Beutler et al. (45).
was incubated with 10 μmol of 4-[3H]HNE in 50 mM potassium phosphate buffer, pH 7.2, and two units GST at 37 °C for 30 min. GS-HNE conjugate was purified by thin layer chromatography over silica gel G plates as described in Xiao et al. (46). The authenticity of GS-HNE was confirmed through high performance liquid chromatography (HPLC) and mass spectra as described previously (15). The specific activity of [3H]GS-HNE was determined by measuring radioactivity in a liquid scintillation counter (Beckman LS-6800), and the concentration of GS-HNE was determined by the previously described colorometric assay (47).

**GS-HNE Transport**—In these experiments, 50 × 10^6 cells were incubated at 37 °C for 10 min in 2 ml of PBS buffer containing 4-[3H]HNE (specific activity 3,800 cpm/nmol). The cells loaded with 4-[3H]HNE were harvested by centrifugation at 500 g, resuspended in 1 ml of PBS, and incubated for 2 h at 37 °C for measuring the transport. After incubation, the cells were harvested, the media was quantitatively separated, and the radioactivity associated with the media and that retained within the cells was determined. Determinations were made in triplicate for the controls (untreated), heat shock-treated and H2O2-treated, and antibody-coated cells in parallel experiments under identical conditions using equal number of cells. For isolation and characterization of GS-HNE, the medium was lyophilized and extracted with 200 μl of 70% ethanol, and the extract was used for HPLC analysis to isolate and characterize [3H]GS-HNE as described above for synthetic GS-HNE. The specific activity of 4-HNE for 2 h at 37 °C was determined by measuring radioactivity in a liquid scintillation counter (Beckman LS-6800), and the concentration of GS-HNE was determined by the previously described colorometric assay (47).

**TUNEL Assay for Apoptosis**—The cells (5 × 10^6) were allowed to recover for 2 h after a 30-min heat shock and then treated with 20 μM 4-HNE for 2 h. Subsequently, the cells were washed twice with PBS and resuspended in 5 ml of PBS at a density of 1 × 10^6 cells/ml. Cell aliquots (100 μl containing 1 × 10^5 cells) were layered onto poly-L-lysine-coated slides using Cytospin (500 × g for 5 min) and fixed by treating with 4% paraformaldehyde for 20 min at 4 °C, and a TUNEL assay was performed using the fluorescein apoptosis detection system (Promega) according to the protocol provided by the manufacturer. In the experiments to determine the effect of RIP1L76 antibodies on heat shock protection against 4-HNE-induced apoptosis, pre-immune IgG or anti-RIP1L76 IgG was added in the media (final concentration 20 μg/ml) after 1 h of the recovery period. The cells were allowed to recover for an additional 1 h and incubated with 20 μM 4-HNE for 2 h, and apoptosis was detected by the TUNEL assay.

**DNA Laddering Assay**—To detect apoptosis by DNA-laddering assay, 5 × 10^6 cells (control or stress-pre-conditioned) were incubated with 20 μM 4-HNE for 2 h at 37 °C. Cells were pelleted, washed twice with PBS, and resuspended in 200 μl of PBS, and the genomic DNA was isolated using QIAamp DNA blood mini kit (Qiagen) according to the manufacturer’s instructions. The DNA-laddering assay was performed as described by us previously (30).

**JNK Assay, Caspase 3 Activation, and PARP Cleavage**—The JNK assay was performed essentially according to the method of Uchida et al. (1), with slight modifications as described by us previously (30). Caspase 3 activation and PARP cleavage were determined by Western blot analysis. Briefly, total cell lysates containing 25–50 μg of protein were separated by SDS-polyacrylamide gel electrophoresis (12% gels) and transferred onto nitrocellulose membranes (Bio-Rad). Immunoblot were developed with the ECL according to the manufacturer’s instructions using the antibodies against caspase 3 and PARP (H-250).

**Cytotoxicity Assay**—The sensitivity of the cells to 4-HNE was measured using the MTT assay (48) as described by us previously (30).

**RESULTS**

**Effect of Heat Shock on LPO—**K562 cells exposed to 42 °C for 30 min or 50 μM H2O2 for 20 min followed by a 2-h recovery did not show any apparent effects on their gross morphology or vitality as measured by an MTT assay. Comparison of the extent of LPO in the control and heat shock-treated cells by the conventional thiobarbituric acid-reactive substance assay showed no detectable expression of hGSTA4-4 in these cells (Fig. 1, C). Cells exposed to longer periods of heat shock (Fig. 2, A and E) showed no noticeable effect of heat shock on the expression of the cationic Alpha-class isozymes hGSTA1–1, hGSTA2–2, hGSTA3–3, the Mu-class isozymes, and the Pi-class isozyme, GSTP1–1 (data not presented). The GST activity of heat shock-treated cells toward CDNB (Table I) was not significantly changed as compared with the control, which was consistent with the results showing no effect of heat shock on expression of the Alpha-, Mu-, or Pi-class GSTs, which account for the bulk of GST activity of K562 cells toward CDNB (31). On the other hand, heat shock caused a transient induction (about 3-fold) of the 4-HNE-metabolizing GST isozyme, hGST5.8, in cells exposed to a 30-min heat shock that declined gradually in cells exposed to longer periods of heat shock (Fig. 2, A and E). Consistent with the induction of hGST5.8 protein, an increase in the GST activity toward 4-HNE was also observed in heat shock-treated cells (Table I). In human tissues two immunologically distinct GST isozymes, hGSTA4-4 (23, 24) and hGST5.8 (20–22), with high catalytic efficiency for 4-HNE have been reported. Western blot analysis of the control or heat shock-treated K562 cells using specific hGSTA4-4 antibodies showed no detectable expression of hGSTA4-4 in these cells (Fig. 2B). In heat shock-treated cells, the activities of glutathione peroxidase toward H2O2 or cumene hydroperoxide, catalase, superoxide dismutase, and glutathione reductase remained unaltered (Table II). However, GSH levels in heat shock-treated cells (78.6 ± 0.85 ng/mg of protein in control versus 151.1 ± 3.0 ng/mg of protein in heat shock-treated cells, n = 3) were increased significantly (p < 0.01).

**Effect of Heat Shock and Oxidative Stress on RIP1L76 and HSP70—**GSH conjugates (e.g. GS-HNE) of electrophilic compounds inhibit GSTs, and therefore, these conjugates must be eliminated from the cells to sustain GST conjugation reactions. It has been recently shown that RIP1L76 mediates the transport of GS-HNE in K562 cells (27) and erythrocytes (29). We therefore studied the effect of heat shock on the expression of RIP1L76 and the transport of GS-HNE. Western blot analysis (Fig. 2C) showed that RIP1L76 was induced by about 3.7-fold in cells subjected to a 30-min of heat shock (Fig. 2E). Similar to hGST5.8, the levels of induction of RIP1L76 declined in cells exposed to longer periods of heat shock (Fig. 2, C and E). RIP1L76 is known to show a band at 95 kDa in SDS gels even recover at 37 °C for 2 h showed no noticeable effect of heat shock on the expression of the cationic Alpha-class isozymes hGSTA1–1, hGSTA2–2, hGSTA3–3, the Mu-class isozymes, and the Pi-class isozyme, GSTP1–1 (data not presented). The GST activity of heat shock-treated cells toward CDNB (Table I) was not significantly changed as compared with the control, which was consistent with the results showing no effect of heat shock on expression of the Alpha-, Mu-, or Pi-class GSTs, which account for the bulk of GST activity of K562 cells toward CDNB (31). On the other hand, heat shock caused a transient induction (about 3-fold) of the 4-HNE-metabolizing GST isozyme, hGST5.8, in cells exposed to a 30-min heat shock that declined gradually in cells exposed to longer periods of heat shock (Fig. 2, A and E). Consistent with the induction of hGST5.8 protein, an increase in the GST activity toward 4-HNE was also observed in heat shock-treated cells (Table I). In human tissues two immunologically distinct GST isozymes, hGSTA4-4 (23, 24) and hGST5.8 (20–22), with high catalytic efficiency for 4-HNE have been reported. Western blot analysis of the control or heat shock-treated K562 cells using specific hGSTA4-4 antibodies showed no detectable expression of hGSTA4-4 in these cells (Fig. 2B). In heat shock-treated cells, the activities of glutathione peroxidase toward H2O2 or cumene hydroperoxide, catalase, superoxide dismutase, and glutathione reductase remained unaltered (Table II). However, GSH levels in heat shock-treated cells (78.6 ± 0.85 ng/mg of protein in control versus 151.1 ± 3.0 ng/mg of protein in heat shock-treated cells, n = 3) were increased significantly (p < 0.01).

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TABLE I  
Effect of heat shock and H$_2$O$_2$ on the GST activity in K562 cells

| Treatment | CDNB units/mg | 4-HNE units/mg  |
|-----------|--------------|----------------|
| None      | 15.45 ± 0.18 | 1.10 ± 0.43    |
| 42 °C, 30 min | 15.82 ± 1.38 | 9.13 ± 0.93$^a$ |
| 42 °C, 60 min | 13.21 ± 1.55 | 7.58 ± 0.73$^a$ |
| 42 °C, 120 min | 13.45 ± 0.25 | 5.98 ± 0.49$^a$ |
| 42 °C, 300 min | 14.37 ± 0.36 | 1.69 ± 0.56    |
| 50 µM H$_2$O$_2$, 20 min | 14.00 ± 0.57 | 9.32 ± 2.16$^a$ |

$^a$ Statistically significant differences between treated and control cells evaluated with Student's $t$ test ($P < 0.05$).

Although its calculated molecular mass is 76 kDa (25), previous studies show that RLIP76 readily undergoes proteolytic degradation to yield fragments of varying molecular masses (27–29). Results presented in Fig. 2C were consistent with these studies and a proportional increase in the intensities of the 95 kDa, and the lower molecular mass peptides were observed in cells exposed to 30 min of heat shock. We also compared the expression of HSP70, RLIP76, and hGST5.8 in cells exposed to heat shock for increasing time periods (0.5–5 h). Western blot analysis (Fig. 2D) showed no significant effect on the expression of HSP70 in cells exposed to 42 °C for a period up to 1 h. However, a gradual increase in the expression of HSP70 was observed in cells exposed to heat shock for more than 1 h. This was in contrast to the effect of heat shock on hGST5.8 and RLIP76, where maximal induction was observed in cells subjected only to a 30-min heat shock that gradually declined with longer periods of heat shock (Fig. 2, A, C, and D). Our results showing a robust and sustained activation of HSP70 on prolonged heat shock are consistent with previous studies demonstrating the protective role of HSP70 against stress (49–54). Results presented in Fig. 3 showed that the effect of mild transient oxidative stress (50 µM H$_2$O$_2$, 20 min) on the levels of hGST5.8, RLIP76, and HSP70 was similar to that of heat shock, indicating that oxidative stress or heat shock affected the expression of these proteins in a similar manner.

Effect of Heat Shock on Transport of GS-HNE.—To investigate the functional consequences of increased overexpression of RLIP76 in heat shock-treated cells, we compared the transport of GS-HNE in the control and heat shock-treated cells. In these experiments, the control and heat shock or H$_2$O$_2$-treated cells (after a 2-h recovery period) were loaded with 4-[$^3$H]HNE by incubating cells with 20 µM 4-[$^3$H]HNE in PBS for 10 min at 37 °C. We have previously shown that 4-HNE is readily taken up by the cells and is quickly converted to GS-HNE (27). The cells were pelleted and washed 3 times with PBS to remove extracellular 4-HNE. After loading, cells were resuspended in media and incubated at 37 °C for 2 h, after which 4-HNE was quantitated in the medium and the cells. The results presented in Table III showed that the amount of GS-HNE transported from cells subjected to heat shock for 30 min was about 3-fold higher compared with untreated controls. These results are consistent with previous studies showing increased efflux of GSH-conjugate from cells after heat shock (55). A gradual decline in the efflux of GS-HNE was observed in the cells subjected to increasing periods of heat shock (data not presented), which was consistent with the decreasing RLIP76 expression in cells upon prolonged heat shock (Fig. 2, C and E). In cells coated with anti-RLIP76 IgG, the transport of GS-HNE was inhibited (Table III) by about 65%, whereas the pre-immune serum did not have any noticeable effect on transport. These results demonstrated that in these cells, RLIP76 was the major transport protein for the efflux of GS-HNE, which was consistent with our previous studies showing that RLIP76 ac-
counted for more than two-thirds of the transport of the electrophile-GSH conjugates from human erythrocytes (29).

Identification of GS-HNE in the Medium—Analysis of the authentic GS-HNE on a reverse phase HPLC column showed four major peaks at retention times of 22.0, 24.2, 28.7, and 31.7 min (Fig. 4A). This was consistent with previous studies showing multiple peaks for GS-HNE due to its diastereoisomers (26). The conjugation of 4-HNE to GSH results in two chiral carbon atoms, and the subsequent ring closure of GS-HNE yields another chiral carbon, giving rise to the possibility of up to a maximum of eight diastereoisomers of GS-HNE (26). To determine whether or not the radioactivity in the medium represented [3H]GS-HNE, the components of the medium were analyzed. The media collected from the control and heat-treated cells were separately lyophilized and extracted with 70% ethanol, and the extracts were subjected to HPLC. The result of MTT assays to assess the rate of its transport in heat shock-treated cells expressing hGST5.8 and RLIP76 Induction Suppresses JNK Activation

Table II

| Treatment                        | GSH (ng/mg of protein) | Catalase (µmol/min/mg of protein) | SOD (µmol/min/mg of protein) | GPx toward H₂O₂ (µmol/min/mg of protein) | GPx toward CUOOH (µmol/min/mg of protein) |
|----------------------------------|------------------------|----------------------------------|-------------------------------|------------------------------------------|------------------------------------------|
| Control                          | 78.65 ± 0.85 (3)       | 28.02 ± 1.12 (4)                 | 14.27 ± 1.46 (3)              | 2.52 ± 0.26 (4)                          | 0.053 ± 0.081 (3)                        |
| Heat shock                       | 151.11 ± 3.06 (3)      | 31.26 ± 2.54 (4)                 | 14.53 ± 0.32 (3)              | 2.68 ± 0.18 (4)                          | 0.0467 ± 0.075 (3)                       |

* Statistically significant differences evaluated by Student’s t test (P < 0.01).

Fig. 3. Effect of transient H₂O₂ exposure on expression of hGST5.8, RLIP76, and HSP70 in K562 cells. K562 cells in complete growth medium were treated with 50 µM H₂O₂ for 20 min, pelleted, washed with PBS (3 × 5 ml) to remove H₂O₂, resuspended in complete growth medium, and incubated for 2 h at 37 °C for recovery. The cells were then collected and lysed in lysis buffer by sonication (30 s, 50 W) to remove H₂O₂, resuspended in complete growth medium, and incubated for 2 h at 37 °C for recovery. The cells were then collected and lysed in lysis buffer by sonication (30 s, 50 W), and aliquots of the supernatants were used for analysis. Values are the means ± S.D. of determinations, given in parentheses. CUOOH, cumene hydroperoxide.

Table III

| Treatment                        | [3H] GS-HNE (nanomoles) |
|----------------------------------|-------------------------|
| Only H₂O₂ treatment              | 1.87 ± 0.11             |
| No H₂O₂ treatment control       | 4.98 ± 0.64             |
| Only heat shock                  | 1.68 ± 0.17             |
| No H₂O₂, 20 min                  | 4.65 ± 0.14             |
| Heat shock and antibodies        | 5.22 ± 0.35             |
| 42 °C, 30 min + pre-immune IgG   | 1.78 ± 0.22             |
| 42 °C, 30 min + anti-RLIP76 IgG  | 2.68 ± 0.18             |

* K562 cells (5 × 10⁶ cells) were exposed to 42 °C for 30 min and allowed to recover for 2 h in medium at 37 °C. The cells were centrifuged, and [3H]GS-HNE transport was measured as described above. Values are the means ± S.D. (n = 3 separate experiments).

**The Effect of Heat Shock on 4-HNE and H₂O₂ Induced Cyto-toxicity and Apoptosis—**The result of MTT assays to assess the...
The cytotoxicity of H$_2$O$_2$ and 4-HNE showed that the cells pre-exposed to heat shock were significantly more resistant to the cytotoxic effects of H$_2$O$_2$ (about 1.7-fold) and 4-HNE (about 2-fold) compared with untreated control cells. Results presented in Fig. 6 demonstrated that stress-pre-conditioned cells acquired resistance against 4-HNE-induced apoptosis. Although a 2-h treatment with 20 $\mu$M 4-HNE caused apoptosis in the control cells (Fig. 6A, lane 3), no apoptosis was observed in stress-pre-conditioned cells (Fig. 6A, lanes 5 and 7). The resistance of the stress-pre-conditioned cells to 4-HNE-induced apoptosis was confirmed by the results of experiments showing that PARP (a substrate of caspase 3) cleavage was observed only in the control cells treated with 4-HNE (Fig. 6B, lane 2) but not in stress-pre-conditioned cells (Fig. 6B, lanes 4 and 6). Stress-pre-conditioned cells were also resistant to H$_2$O$_2$-induced apoptosis, as indicated by results showing that treatment with 100 $\mu$M H$_2$O$_2$ for 2 h caused activation of caspase 3 and apoptosis only in the control cells and not in the stress-pre-conditioned cells (data not presented). These results demonstrated that stress-pre-conditioned cells, with higher rates of

**Fig. 4.** A, HPLC profile of authentic GS-HNE conjugate. The conjugate was synthesized as described under “Experimental Procedures” and subjected to HPLC analysis on a RPC$_18$ column (Waters, Symmetry 3.9 $\times$ 150 mm, 5 $\mu$m) using a 60-min linear gradient of 0 to 100% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The elution of GS-HNE conjugate was monitored by a dual wavelength UV-visible detector at 224 and 254 nm. The peak c, corresponding to 28.7, was identified with the hemiacetal form of GS-HNE (26). B, identification of 4-[3H]HNE, transported by K562 cells. Control and heat shock-treated K562 cells ($1 \times 10^6$) were separately incubated with 20 $\mu$M 4-[3H]HNE (specific activity 3800 cpm/nmol) in 2 ml of PBS at 37 °C for 10 min. The cells were pelleted, washed with PBS (3 $\times$ 5 ml), and incubated with 2 ml of PBS for 2 h at 37 °C. The supernatants were separated by centrifugation, lyophilized, and extracted with 200 $\mu$l of 70% ethanol, and 20-$\mu$l aliquots of the extracts were subjected to HPLC as described for panel A. The radioactivity profiles of 1-ml fractions from the control ($\triangle$) and heat-treated cells, (■) are superimposed on the actual HPLC profile (solid line). Rt, retention time.

**Fig. 5.** Negative ion mass spectrum of the GS-HNE conjugate. The major peak fraction (retention time 28.7 min) obtained during the HPLC analysis (Fig. 4B) of the cell supernatant was subjected to mass spectral analysis on a Micromass Q-TOF 2 electrospray mass spectrometer. The sample was introduced in water:methanol (50:50) at a flow rate of 5 $\mu$l/min. The cone and capillary voltages were kept at 20 and 3000 V, respectively.

hGST5.8 and RLIP76 Induction Suppresses JNK Activation
4-HNE metabolism and exclusion of its GSH conjugate, acquired resistance to apoptosis caused by H$_2$O$_2$ or by 4-HNE through blockage of caspase 3 activation.

**Anti-RLIP76 Antibodies Abolish the Protective Effect of Heat Shock Pre-conditioning against 4-HNE-induced Apoptosis**—We have previously demonstrated that the majority of the ATP-dependent transport activity of erythrocyte membrane toward GS-HNE and other GS conjugates is inhibited (65%) by the polyclonal antibodies against recombinant RLIP76 (29). We reasoned that if the protection to heat-pre-conditioned cells from 4-HNE-induced apoptosis was due to induction of RLIP76 and increased efflux of GS-HNE from the cells, it should be abolished by inhibiting GS-HNE efflux by RLIP76 antibodies. Therefore, heat shock-treated cells were incubated with anti-RLIP76 IgG after a 1-h recovery period, allowed to recover for an additional 1 h, and then tested for 4-HNE-induced apoptosis using TUNEL assay. Results presented in Fig. 7 showed that heat shock alone did not cause apoptosis (Fig. 7A). Treatment with 4-HNE caused apoptosis in control cells (Fig. 7B), whereas heat shock-pre-conditioned cells were resistant to 4-HNE-induced apoptosis (Fig. 7C). In contrast, in heat shock-pre-conditioned cells coated with anti-RLIP76 IgG, 4-HNE-induced apoptosis was observed (Fig. 7D), indicating that these antibodies abrogated the protective effect of heat shock pre-conditioning. In cells coated with the preimmune IgG, the protective effect of heat shock pre-conditioning against 4-HNE-induced apoptosis was retained (data not presented). These results taken together with inhibition of GS-HNE transport by anti-RLIP76 antibodies (Table III) strongly suggested that the protection provided to the heat-pre-conditioned cells was directly linked to the induction of RLIP76 and its associated transport function.

**Effect of Heat Shock and Transient Oxidative Stress on JNK Activation**—A number of cellular stress conditions including heat shock and oxidative stress activate the JNK cascade, and increased efflux of GS-HNE from the cells was electrophoresed on 2% agarose gels containing 10 μg/ml ethidium bromide. B, Western blot analysis of cell extracts containing 25 μg of protein using anti-PARP (H-250) antibodies. Lanes representing different treatments in both panels are marked in the figure.

**Effect of anti-RLIP76 IgG on 4-HNE-mediated apoptosis in heat shock-pre-conditioned cells.** Aliquots (50–100 μl) containing 1–2 × 10$^6$ cells were fixed onto poly-l-lysine-coated slides by Cytofix at 500 × g for 5 min, and the TUNEL apoptosis assay was performed as described under "Experimental Procedures." The slides were analyzed by fluorescence microscope (Nikon Eclipse 600, Japan) using a standard fluorescein filter (EX 450–490, DM 505, BA 520, B–2A). Photomicrographs at 400× magnification are presented. Apoptotic cells showed characteristic green fluorescence. A, control K562 cells pre-treated with heat shock (42 °C, 30 min) and allowed to recover for 2 h at 37 °C. B, control cells without heat shock pre-treatment, incubated with 20 μM 4-HNE for 2 h. C, cells pre-treated with heat shock, allowed to recover for 2 h at 37 °C, followed by incubation in medium containing 20 μM 4-HNE for 2 h at 37 °C. D, heat shock-pre-treated cells, allowed to recover for 1 h at 37 °C, after which anti-RLIP76 IgG was added to medium (20 μg/ml final concentration) and incubated for an additional 1 h. Cells were then incubated for 2 h at 37 °C in medium containing 20 μM 4-HNE. Stress-pre-conditioned cells in our studies acquired significant resistance to 4-HNE- and H$_2$O$_2$-induced apoptosis compared with control cells. Therefore, we compared 4-HNE-mediated activation of JNK in control and stress-pre-condi-
untreated, and H₂O₂ pre-

conditioned, cells, 4-HNE- and H₂O₂-mediated activation of JNK that was prominent at 0 h during the recovery period (Fig. 9, A and B, lanes 2) and not in stress-pre-conditioned cells (Fig. 10, lanes 2). Likewise, 20 μM 4-HNE caused apoptosis in control RPE and H₂O₂ cells (Fig. 10, lanes 2) but not in stress-pre-

conditioned cells. Collectively these results suggested that an early induction of hGST5.8 and RLIP76 in response to heat shock appears to be a generalized phenomenon and that the cells pre-conditioned with a mild transient heat shock acquire resistance to apoptosis caused by the LPO product, 4-HNE.

**DISCUSSION**

The activation of JNK/stress-activated protein kinase cascade upon exposure to heat, UV radiation, or inflammatory cytokines and its involvement in apoptotic signaling has been extensively studied. Available evidence suggests that activation of these kinases in response to stress is mediated by GT-Pase-activating proteins, in particular Rac and Cdc42 (60–62). It has been suggested that activated Cdc42 binds to and activates PAK65, which activates stress-activated protein kinase enzyme/JNK kinase (SEK/JNKK) by phosphorylating JNKK at the serine residue 219 (63). In turn, activated stress-activated protein kinase enzyme/JNK kinase (SEK/ JNKK) causes activation of JNK/stress-activated protein kinase, which binds and phosphorylates c-Jun at specific residues. The initial events leading to the activation of JNK/ stress-activated protein kinase and the nature of chemical species initiating this cascade in response to stress are, however, poorly defined.

The results of the present studies shed light on the mechanisms of the response of the cells to stress in the initial phase and demonstrate a role of lipid peroxidation products, particularly 4-HNE, in stress-mediated signaling. We demonstrate that in cells subjected to a mild, transient stress (heat or oxidative), lipid peroxidation ensues, resulting in an increase in the cellular levels of 4-HNE. The cells respond to this increase in 4-HNE levels by acquiring the capability to exclude 4-HNE from their environment at a faster rate. This response occurs earlier than the induction of heat shock proteins or the antioxidant enzymes, which are known to be induced by stress. Within 30 min of exposure to mild stress, cells showed about a 50% increase in the 4-HNE levels that was accompanied by a concomitant activation of JNK, whose sustained activation has been suggested to be a prerequisite for activation of caspase 3 and subsequent apoptosis (64, 65). However, within a 2-h resting period after the transient stress, the cells were able to exclude 4-HNE by transporting its GSH conjugate (GS-HNE) at a severalfold higher rate as compared with the control cells. The cells acquire this capability through a rapid induction of...

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**Fig. 8. Suppression of 4-HNE-mediated sustained JNK activation in K562 cells pre-conditioned with heat shock or H₂O₂.** JNK activity was determined in cell extracts by solid-phase kinase assay (1) using purified GST-c-Jun-(1–89) as substrate, and the phosphorylated activity was determined in cell extracts by solid-phase kinase assay (1) using purified GST-c-Jun-(1–89) as substrate, and the phosphorylated activity was determined in cell extracts by solid-phase kinase assay (1) using purified GST-c-Jun-(1–89) as substrate, and the phosphorylated...
hGST5.8 and RLIP76, which, respectively, catalyze the conjugation of 4-HNE to GSH and subsequent efflux of GS-HNE. This is indicated by our results showing increased expression of hGST5.8 and RLIP76 proteins, increased GST activity toward 4-HNE, and an accelerated transport of GS-HNE in the cells exposed to a mild, transient stress. As a result of the accelerated exclusion of 4-HNE-GSH conjugate, within a 2-h resting period after stress, 4-HNE levels were restored to normal physiological or basal levels, and the JNK activity was restored to constitutive levels. These results strongly suggest a role of 4-HNE in the activation of JNK either through directly acting on JNK or through interaction(s) with the kinases upstream to JNK. This postulate needs to be substantiated by further studies.

Our results showing acquisition of a limited resistance to H$_2$O$_2$ and 4-HNE-mediated apoptosis in stress-pre-conditioned cells overexpressing hGST5.8 and RLIP76 further suggest the role of 4-HNE in stress-mediated signaling mechanisms. Our studies show that when 4-HNE is included in the medium for 2 h, the control cells undergo apoptosis that is accompanied by a sustained activation of JNK and caspase 3, whereas apoptosis or a sustained activation of JNK and caspase 3 is not observed in stress-pre-conditioned cells. Stress pre-conditioning either by heat shock or H$_2$O$_2$ by itself caused transient activation of JNK, which returned to normal levels within 2 h when constitutive levels of 4-HNE were restored in these cells, probably due to the induction of hGST5.8 and RLIP76 and accelerated 4-HNE metabolism and transport of GS-HNE. Stress-pre-conditioned cells also acquire resistance to apoptosis by H$_2$O$_2$. It is to be noted that the stressed-pre-conditioned cells should have similar capabilities to decompose H$_2$O$_2$ because the levels of the antioxidant enzymes, catalase, GPx, and SOD in the controls and conditioned cells are similar. Therefore, the resistance of pre-conditioned cells to H$_2$O$_2$-induced apoptosis may also be attributed to their ability to metabolize and transport 4-HNE at a faster rate. This contention is supported by our results showing that the resistance of stress-pre-conditioned cells to 4-HNE-induced apoptosis can be abrogated by coating the cells with anti-RLIP76 IgG, which inhibit the transport of GS-HNE. Collectively, these results demonstrate that the intracellular concentration of 4-HNE or its conjugate GS-HNE may be one of the major determinants for the signaling mechanisms leading to the activation of JNK, caspase 3, and subsequent apoptosis.

Activation of JNK is involved in stress-mediated apoptosis. Our results show that only a 50% increase in 4-HNE levels over its constitutive physiologic levels appears to initiate JNK activation. It has been reported that relatively low concentrations of 4-HNE promote proliferation of aortic smooth muscle cells (14) and that K562 cells transfected with mGSTA4-4, which have lower levels of 4-HNE, grow at a faster rate than the vector-transfected controls (6). These findings imply that at least in some cell types, low concentrations of 4-HNE may promote proliferation, whereas relatively higher concentrations of 4-HNE may promote apoptosis. Therefore, the mechanisms that determine the intracellular concentrations of 4-HNE should be crucial in the early phases of signaling. Our observations showing an early induction of hGST5.8 and RLIP76 in response to stress support this idea. Furthermore, our results demonstrate that tumor cells of diverse origins respond to stress in a similar manner, and an early induction of hGST5.8 and RLIP76 is observed in all the cell lines tested in the present work. This suggests that in the initial phase, the cells in general respond to stress (which causes lipid peroxidation) by up-regulating the mechanisms for metabolism and exclusion of 4-HNE to avoid apoptosis. The results of our studies, showing that only a small increase in the concentrations of 4-HNE leads to the activation of JNK, which is quickly suppressed to basal levels when 4-HNE levels are restored to normal, suggest that there is a narrow range of constitutive levels of 4-HNE, above which the apoptotic cascade is initiated. It is possible that suppression of the 4-HNE levels below the
constitutive levels may promote proliferation, such as that observed in K562 cells transfected with mGSTA4-4 having sub-basal levels of 4-HNE (6). Further studies are needed to substantiate these possibilities.

GSTs account for the metabolism of a major fraction of cellular 4-HNE (15). Recent studies show that in human tissues, two immunologically distinct GST isozymes with high catalytic efficiency ($k_{cat}/K_m$ of about 2500–3000 s$^{-1}$·mM$^{-1}$) for 4-HNE are expressed in relatively low abundance in a tissue-specific manner (22). One of these isozymes, hGSTA4-4, has been cloned, but the gene encoding hGST5.8 has not yet been cloned.

In tumor cells used in the present studies, detectable expression of hGSTA4-4 was not observed either in control cells or in the stressed cells. Our results showed that low levels of hGST5.8 were constitutively present in all these cells, and a mild stress caused an early and transient induction of this isozyme. These results suggest that hGST5.8 is induced to interrupt 4-HNE-mediated signaling for apoptosis, and it is rapidly degraded after performing this function, as indicated by our results showing that within 5 h after stress, hGST5.8 expression returns to the usual low constitutive levels. This may explain the difficulty in cloning of hGST 5.8 from human libraries, because even though partial primary structure of hGST 5.8 has been established (20), attempts to clone its protein with GTPase-activating protein activity for Cdc42/Rac has not been successful, perhaps due to its low abundance in cDNA libraries. Recent studies (66) identifying a pseudogene corresponding to the peptide sequences of hGST5.8 may be helpful in its cloning.

RLIP76 is a GTPase-activating protein that is believed to bridge the Ral and Rho pathways (25). It is a Ral effector protein with GTPase-activating protein activity for Cdc42/Rac, and it has recently been demonstrated that RLIP76 induction as an early response to stress.

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Accelerated Metabolism and Exclusion of 4-Hydroxynonenal through Induction of RLIP76 and hGST5.8 Is an Early Adaptive Response of Cells to Heat and Oxidative Stress

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