Published in final edited form as:

Free Radic Biol Med. 2005 February 15; 38(4): 463–471. doi:10.1016/j.freeradbiomed.2004.10.031.

4-Hydroxynonenal increases γ -glutamyl transpeptidase gene expression through mitogen-activated protein kinase pathways

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Abstract

γ -Glutamyl transpeptidase (GGT) plays key roles in the metabolism of glutathione. Previous studies have shown that GGT expression was increased by oxidants, but the mechanism remains unclear. In the present study, the effects of 4-hydroxy-2-nonenal (HNE), an electrophilic end product of lipid peroxidation, on GGT expression were investigated in rat lung epithelial type II (L2) cells. We demonstrated that HNE increased GGT activity and mRNA content in both time- and dose-dependent manners. Actinomycin D, an RNA transcription inhibitor, blocked HNE-stimulated increase in GGT mRNA, suggesting transcriptional regulation of GGT mRNA by HNE. Of the seven GGT mRNA transcripts known to be produced from the single rat GGT gene, we found that types I, II, and V-2 were constitutively expressed in L2 cells, but only types I and V-2 were increased by HNE. PD98059 and SB203580, relatively specific inhibitors of the ERK and the p38MAPK kinase pathway, respectively, significantly attenuated HNE induction of both GGT activity and mRNA content. In contrast, studies with JNK inhibitor I, a cell-permeable peptide, indicated that JNK was not involved in the GGT induction by HNE. We also found that GGT induction by HNE could be completely blocked by a cocktail of PD98059 and SB203580, suggesting a combined effect of ERK and p38MAPK pathways in HNE-mediated GGT induction. In conclusion, our results demonstrate that HNE increased GGT expression in rat alveolar type II cells and that the induction of GGT by HNE was mediated through activation of the ERK and p38MAPK pathways.

Keywords

Glutathione; GGT; HNE; MAPK; Oxidative stress; Free radicals

γ -Glutamyl transpeptidase (γ -glutamyl transferase; GGT), a glycosylated enzyme located on the outer surface of the plasma membrane, catalyzes the transfer of the γ -glutamyl moiety from peptide donors such as glutathione (GSH) to a variety of acceptors, including amino acids, dipeptides, and H₂O [1]. The breakdown of extracellular GSH provides cysteine, the rate-limiting amino acid for glutathione synthesis. As such, GGT plays a key role in GSH and cysteine homeostasis [2–5]. GGT also catalyzes the metabolism of natural biomolecules such as leukotriene C₄, xenobiotics, and carcinogens and chemotherapeutic drugs after their conjugation with GSH [6]. GSH conjugates are cleaved by GGT as the initial step of their

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conversion to mercapturic acids, a process that is usually considered a detoxification pathway [7,8].

GGT is mainly expressed in epithelial cells with absorptive or secretive functions, e.g., renal proximal tubule cells and bile duct epithelial cells [9]. In the lung, GGT is mainly expressed in Clara cells and type II epithelial cells, whereas other alveolar space cells, including alveolar macrophages, have undetectable GGT mRNA and protein although the macrophage can adsorb the enzyme from the extracellular fluid [10,11]. The ELF GSH content has been reported to be as high as 400 μ M in humans [12] and 2 mM in rats [13], which is about 100-fold higher concentration than in the plasma. This alveolar GSH pool provides not only an extracellular antioxidant defense, but also cysteine for alveolar space cells after the cleavage of GSH by GGT followed by cysteinylglycine cleavage by dipeptidases found on cell surfaces. GGT deficiency alters the GSH/GSSG ratio and decreases the content of cysteine and GSH in alveolar space cells, which show signs of oxidative stress even under normal oxygen concentration [5,14]. GGT-deficient mice are more susceptible to pulmonary oxygen toxicity than wild-type mice, demonstrating the important roles of GGT in maintaining antioxidant function in the lung [4,5,14–16].

Many physiological and pathological factors regulate GGT expression. GGT expression is increased in whole lung after birth [10], but is decreased in adult liver compared to fetal liver [17]. GGT activity is increased in certain liver diseases, after stroke, in tumor tissues, and in other pathologies [18–23]. Many substances, especially those that generate reactive oxygen/nitrogen species (ROS/RNS) and/or perturb the redox balance, such as aflatoxin B1, NO₂, redox cycling quinones, and alcohol, can increase GGT expression in various cells and tissues [24–30]. As an essential component of the cellular antioxidant defense system, upregulation of GGT upon oxidative stress facilitates de novo GSH synthesis and the metabolism of GSH S-conjugates, which renders cells more resistant to further oxidative challenge. Despite these important findings, the mechanism of GGT regulation in response to oxidative stress remains unclear.

4-Hydroxy-2-nonenal (HNE), an end product of lipid peroxidation, is both a toxic electrophile that forms adducts with various molecules at high concentrations and a powerful signaling molecule at subtoxic concentrations [31–33]. HNE activates various signaling pathways including MAP kinase pathways [34–40] and is involved in cellular functions such as proliferation [41–44], differentiation [45–48], and apoptosis [49–52]. A variety of genes can be regulated by HNE, especially genes involved in stress adaptation and detoxification, such as glutamate cysteine ligase [53–56] and aldose reductase [57]. In the present study, we investigated the regulatory effect of HNE on GGT expression and the possible signaling pathways involved in rat lung epithelial type II cells (L2 cell). GGT gene structure is complex. In the rat, GGT is a single copy gene whose expression is controlled by five tandem-arranged promoters from which seven transcripts are generated through alternative splicing [58]. These transcripts share a common coding sequence but have different 5' untranslated regions. The mechanism regulating the expression of different types of GGT mRNA is not clear. In this study, we also examined the response of these different types of GGT mRNA to HNE.

Materials and methods

Chemicals and reagents

Unless otherwise noted, all chemicals were from Sigma (St. Louis, MO, USA). HNE was purchased from Cayman Chemical (Ann Arbor, MI, USA). Glycylglycine was from ICN Biochemical (Aurora, OH, USA). L- γ -Glutamyl 7-amino-4-methylcoumarin (γ -Glu AMC) was purchased from Bachem Bioscience (King of Prussia, PA, USA). PD 98059, SB 203580, and JNK inhibitor I were purchased from Calbiochem (La Jolla, CA, USA). Antibodies for

Western blotting were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). TRIzol reagent was from Life Technologies (Grand Island, NY, USA). DNA-free was from Ambion (Austin, TX, USA). TaqMan reverse transcription reagent and SYBR Green PCR Master Mix were from Applied Biosystems (Foster City, CA, USA). All chemicals used were at least analytical grade.

Cell culture and treatments

L2 cells (from the American Type Culture Collection) were cultured in F-12K medium (Life Technologies) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified incubator containing 5% CO₂ at 37°C.

HNE was dissolved in ethanol. PD98059 and SB203580 were dissolved in dimethyl sulfoxide (DMSO), and JNK inhibitor I (JNKi) was dissolved in PBS. The final concentration of ethanol and DMSO in the medium was 0.05 and 0.1%, respectively. L2 cells were treated at about 90% confluence with different compounds as indicated under Results. Cells were rinsed with cold PBS before being harvested using rubber policemen.

GGT activity assay

GGT activity was measured according to the method described by Forman et al. [59], with slight modifications for use on a fluorescence microplate reader. Specificity of GGT activity was confirmed by acivicin, a specific GGT inhibitor. One unit of GGT activity was expressed as 1 pmol AMC produced per milligram protein per minute.

GGT mRNA assay

Total RNA was extracted using TRIzol reagent and treated with DNA-free reagent according to the manufacturers' protocols. DNA-free RNA samples were reverse transcribed using the TaqMan reverse transcription system (Applied Biosystems) and real-time PCRs were run with a Cepheid 1.2 real-time PCR machine. Briefly, 5 µl of reverse transcription reaction product was added to reaction tubes containing 12.5 µl SYBR Green PCR Master Mix and primer pair specific for total or types of GGT mRNA; the total PCR sample was 25 µl. GAPDH was used as internal control (25 µl PCR: 2.5 µl RT reaction, 12.5 µl SYBR Green PCR Master Mix, primers, and water). Table 1 shows the specific primer pairs for GAPDH and total and type-specific GGT mRNA. Specificity of PCR products was confirmed by DNA sequencing.

Western blotting assay

Briefly, protein was extracted with M-PER extraction buffer (Pierce) and 35 µg of total protein was heated for 15 min at 95°C in a 2× loading buffer containing sodium dodecyl sulfate (Tris base, pH 6.5, glycerol, DTT, and pyronin Y), electrophoresed under denaturing conditions on a 10% Tris-glycine acrylamide gel (Invitrogen, Carlsbad, CA, USA), and then electroblotted onto a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA, USA). Membranes were blocked with 5% fat-free milk at room temperature for 1 h and then incubated overnight at 4°C with appropriate primary antibody in 5% milk in Tris-buffered saline (TBS). After being washed with TBS containing 0.05% Tween 20 (TTBS), the membrane was incubated with appropriate secondary antibody at room temperature for 2 h. After TTBS washing, the membrane was treated with an enhanced chemiluminescence (ECL Plus; Amersham, Arlington Heights, IL, USA) reagent mixture for 5 min. Film exposure was done with Hyperfilm ECL film (Amersham).

Statistical analysis

We used the comparative $\Delta\Delta C_T$ method for the relative mRNA quantitation. The relative quantification of target, normalized to an internal control (GAPDH), and an untreated sample

is given by relative quantitation = $2^{-\Delta\Delta C_T}$, $\Delta\Delta C_T$ being defined as the difference of mean ΔC_T (HNE treated sample) and mean ΔC_T (untreated sample) and ΔC_T as the difference in mean C_T (GGT) and C_T (GAPDH) as the internal control. Threshold cycles (C_T) were selected in the line in which all samples were in logarithmic phase.

All data were expressed as the mean $\pm$ standard error. Sigma Stat software was used for statistical analysis and statistical significance was accepted when $p < 0.05$. The Student t test was used to analyze GGT activity data, and the Tukey test was used for comparison of mRNA level.

Results

HNE exposure increased both GGT activity and total GGT mRNA content in L2 cells

HNE is present in the free form at 0.3–0.7 μM in human plasma in controls and can increase 10 times or more during oxidative stress in vivo [60–63]. The HNE concentrations used in this study (5–20 μM) did not inhibit cell growth or cause any morphological changes. Exposure of L2 cells to 5–20 μM HNE increased GGT activity significantly (Fig. 1A). No significant increase in GGT activity was observed at 6 h after HNE treatment. However, 10 and 20 μM HNE significantly increased GGT activity by 12 h and all three concentrations used increased GGT activity by 24 h.

The change in GGT mRNA content was measured using real-time PCR. GGT mRNA was increased significantly after a 6-h treatment at all HNE concentrations used. Maximal induction of GGT mRNA was reached 12 h after HNE treatment. By 24 h, the mRNA content decreased compared to that at 12 h but was still significantly higher than control (Fig. 1B). Thus, exposure to HNE increased both GGT activity and total mRNA content in time- and dose- dependent manners in L2 cells.

HNE increased GGT mRNA at the transcription level

To elucidate whether HNE increased GGT mRNA at the transcriptional or the mRNA stability level, L2 cells were preincubated with actinomycin D, an RNA transcription inhibitor, for 1 h and then treated with HNE. In the presence of actinomycin D, HNE caused neither an increase in GGT mRNA nor a change in the rate of GGT mRNA degradation, suggesting transcriptional regulation of GGT mRNA by HNE (Fig. 1C). These results suggest that HNE increases GGT mRNA by stimulating gene transcription rather than by increasing mRNA stability.

HNE activated ERK, p38 MAPK, and JNK kinase pathways in L2 cells

HNE has been shown to initiate signaling cascades within the cell, resulting in gene expression, and this has often been identified as being dependent on mitogen-activated protein kinase (MAPK). Using immunoblotting to the phosphorylated forms of these kinases, we demonstrated that HNE activated all three major MAP kinases in L2 cells (Fig. 2). Phosphorylated ERK was significantly increased after 2 h of HNE treatment and remained activated for at least 8 h (Fig. 2A). Phosphorylation of p38MAPK became significant 30 min after HNE treatment. Activation of JNK was observed as early as 15 min and remained active for several hours after HNE exposure. These data indicate that HNE provokes a complex of signaling pathways, each with unique kinetics.

Both PD98059 and SB203580 attenuated the HNE-mediated increase in GGT activity and GGT mRNA

To investigate whether any the three major MAPK pathways were involved in the HNE-mediated induction of GGT in L2 cells, chemical inhibitors with relatively high specificity for each of the three major MAPK pathways were used over a range of concentrations. As shown

in Fig. 3A, pretreatment of L2 cells for 1 h with increasing concentrations of PD98059, the ERK pathway inhibitor, significantly attenuated HNE-mediated induction in GGT activity. Over the same concentration range, PD98059 also produced increasing inhibition of the phosphorylation of ERK1/2 (Fig. 3C). Nonetheless, complete inhibition of the ERK pathway resulted in only partial inhibition of HNE induction of GGT (maximum inhibition was $49.1 \pm 4.9\%$). At concentrations as low as $5 \mu\text{M}$, the p38MAPK inhibitor SB203580 also significantly reduced the increase in GGT activity by HNE. As doubling the concentration of SB203580 produced no greater effect on HNE induction of GGT, it seems that p38MAPK also is only partially responsible for GGT induction by HNE (maximum inhibition was $66.8 \pm 6.0\%$). In contrast, JNKi had no effect on GGT activity induction by HNE at a concentration that completely inhibited the phosphorylation of c-Jun by JNK1/2 (Fig. 3D). The effects of the major three MAPK inhibitors on GGT mRNA induction by HNE are consistent with their effects on GGT activity induction (Fig. 3B). Therefore, our results suggest that at least one of the two p38MAPK isoforms, α and β , that SB203580 inhibits, and at least one of the ERK isoforms, ERK1 and ERK2, that PD 98059 inhibits, were partially responsible for HNE-mediated GGT induction. On the other hand, the data indicate that although JNK1/2 was activated by HNE, it is not involved in GGT induction by HNE in L2 cells.

As the maximum percentage inhibition of the elevation in GGT expression by either PD98059 or SB203580 alone was incomplete, we determined whether inhibiting ERK and p38MAPK simultaneously would produce complete inhibition. L2 cells were pretreated with PD98059 ($50 \mu\text{M}$) and SB203580 ($5 \mu\text{M}$) together before cells were exposed to HNE. We found that the presence of both inhibitors blocked the HNE-induced GGT expression (both activity and mRNA, Fig. 4) completely, indicating that the two inhibitors act on separate pathways rather than on different steps along one pathway.

HNE exposure increased GGT mRNA types I and V-2 expression in L2 cells

Rat GGT is a single-copy gene from which seven transcripts are produced that share the same coding sequence but differ in their 5' untranslated regions. To investigate which type of GGT mRNAs are induced by HNE we used specific primers for each rat GGT transcript and real-time PCR to measure the expression pattern of specific GGT mRNA types after HNE exposure. We found that L2 cells, which are derived from Lewis rat alveolar epithelial type II cells, constitutively express mRNA types I, II, and V-2. HNE exposure increased types I and V-2, but not type II (Fig. 5). The data suggest that the expression of different types of GGT mRNA is regulated by different mechanisms. Thus, HNE induces specific GGT mRNA types in L2 cells, demonstrating differential regulation through the various promoters.

Discussion

We and others have shown the increase in GGT expression in response to oxidants. Here our results showed that HNE, an end product of lipid peroxidation, increased GGT expression at the enzymatic activity and mRNA levels. GGT is a glycosylated protein and Western blots therefore show smears. GGT is not made as a proenzyme and therefore activity should correlate with protein. Indeed, using an antibody to a GGT peptide domain, we have previously demonstrated that GGT protein correlated with activity in both GGT induction in L2 cells by menadione [64] and transfection of human GGT into rat L2 cells [65].

Although the JNK, ERK, and p38MAPK pathways were all activated, only the ERK and p38MAPK pathways were involved in GGT induction by HNE. In addition, although L2 cells express types I, II, and V-2 GGT transcripts, only type I and type V-2 GGT mRNA were induced. This indicates the presence of HNE-responsive elements in the promoter regions for specific transcripts that will be pursued in the future.

It is well established that despite the toxicity of HNE, this strong electrophile is able to signal for the synthesis of enzymes such as aldose reductase [57], glutamate cysteine ligase (the rate-limiting enzyme for de novo GSH synthesis) [56,66], and glutathione *S*-transferase [67–69], which are involved in its detoxification as well as that of other endogenous and xenobiotic electrophiles. To this list of HNE-inducible enzymes, we now add GGT, which is involved in supplying cysteine for GSH synthesis. The increase in GSH synthesis in response to HNE exposure can maintain GSH level and allow cells to adapt to oxidative stress and toxicity of electrophiles.

Many extracellular stimuli elicit specific biological responses through the activation of MAPK cascades. Previously, Sinha et al. found that rat liver epithelial cells became GGT positive after transformation with Ras [70,71], which could activate ERK via the Ras–Raf–ERK pathway. All three MAPKs were activated by HNE in L2 cells. Nonetheless, very little is known about the possible involvement of the MAPK signaling pathways in increased expression of GGT, particularly by oxidative stress. Therefore to begin an investigation of the mechanism underlying increased expression of GGT by HNE in L2 cells, we investigated the involvement of the three major MAPK pathways (ERK, p38MAPK, and JNK) by using MAPK inhibitors. Considering that chemical inhibitors for a signaling pathway may have nonspecific effects on molecules other than their targets at higher concentration, we used inhibitors at concentrations that are specific [72–74]. PD98059 and SB203580, specific inhibitors for the ERK and p38MAPK signaling pathways, respectively, attenuated the HNE-mediated increase in GGT activity and mRNA content, but the maximal inhibitory effect of either alone was not complete. In combination, however, PD98059 and SB203580 blocked HNE-induced GGT expression completely, suggesting that the ERK1/2 and p38MAPK signaling pathways were separate in their effects upon the HNE-mediated increase in GGT expression. Interestingly the maximal inhibition by the inhibitors added to nearly 100%. For the increase in GGT activity by HNE, maximal PD98059 inhibition was $49.1 \pm 4.9\%$, whereas SB203580 maximally inhibited $66.8 \pm 6.0\%$. For the increase in GGT mRNA by HNE, PD98059 inhibited $38.7 \pm 3.2\%$, whereas SB203580 inhibited $57.2 \pm 4.0\%$. These results suggest that the ERK and p38MAPK pathways acted independently to produce part of the increase in HNE-induced expression of GGT but together accounted for the whole induction.

Our experience with several lung epithelial cell lines has shown that transient transfection rates are too low (<10%) for successful dominant negative protein expression using a variety of commercial liposomal delivery systems. Therefore, for PD98059, which had its maximal effect at the relatively high concentration of 50 μM , and JNKi, which did not inhibit, it was important to show that they actually inhibited their targets at the concentrations used. This was demonstrated here for PD98059 by the proportional increase in the inhibition of ERK phosphorylation and GGT expression. Indeed, such concentration dependence of the small-molecular-weight inhibitors has an advantage over the use of dominant negative proteins that must be expressed at high enough concentrations to outcompete their endogenous counterpart, running the risk of nonspecific protein–protein interactions.

JNK, also called stress-activated protein kinase, can be activated by various stimuli, such as irradiation, heat shock, hyperosmotic shock, inflammatory cytokines, and oxidative stress [75]. JNK activation can positively regulate the expression of genes involved in cellular response to stresses. As GGT is a necessary component of cellular adaptation to oxidative stress and HNE activated JNK in L2 cells, we hypothesized that JNK could play a positive role in HNE-induced expression of GGT. However, the JNK inhibitor has no effect on HNE-mediated induction of GGT activity although it inhibited c-Jun phosphorylation at the concentration used. These data suggest that the JNK1/2 pathway is not involved in GGT induction by HNE.

In the rat, GGT is a single-copy gene whose expression is regulated by five tandemly arranged promoters; alternative splicing from two of these generates seven transcripts. The expression of these seven GGT mRNAs varies with tissue and development phase [58]. It was found that in Sprague–Dawley rats, fetal rat lung mainly expressed types I and II GGT mRNAs, which were replaced by type III after birth [76]. Our results showed, however, that types I, II, and V-2 of GGT mRNA were the major types of GGT mRNA expressed in L2 cells, a cell line derived from lung epithelial type II cells of the Lewis rat. We did not find expression of type III GGT mRNA in L2 cells. Whether this indicates a difference in cell type distribution or rat strains is unclear but as the lung is composed of 40 cell types, a difference between whole tissue and one derived cell line is not surprising.

Increased GGT expression in response to oxidative stress was first shown in rat L2 cells in this laboratory [29]. Subsequently, others have shown that GGT expression in the lung can be increased during oxidative stress caused by various agents, including the quinones (menadione, DMNQ, and TBHQ), NO₂, and hyperoxia [25,26,30,77]. Takahashi et al. [25] then showed that oxidative stress specifically induced type I GGT mRNA in Sprague–Dawley rat lungs. Here, we report that the expression of both types I and V-2 but not type II GGT mRNA was increased after HNE exposure. Previously, type V mRNA (both V-1 and V-2) was found to be expressed in undifferentiated rat H5 hepatoma cells [78], and types II, III, and IV were shown to be inducible by aflatoxin B1, hyperoxia, and xanthine/xanthine oxidase in a tissue-specific manner [24,76,79]. Thus, GGT expression is redox-regulated, although each promoter is regulated by both tissue-specific factors and redox-sensitive factors. Although the physiological significance of having multiple transcripts coding for the same protein remains unclear, perhaps the multiple promoters provide a fail-safe redundancy mechanism allowing cells an increased probability of increasing GGT in response to a variety of stimuli. Yet, little is known about the regulation of any of these mRNA types beyond the observation that oxidants and electrophiles can cause an increase in the various transcripts.

The lung is frequently exposed to ROS/RNS from air pollutants (NO₂, ozone, particulates, or smoke) or released by neutrophils recruited in lung inflammation (i.e., chronic obstructive pulmonary disease and cystic fibrosis). In response to oxidative stress, cells increase the expression of genes involved in antioxidant and detoxification systems as an adaptive response. GGT, which breaks down circulating glutathione and provides cells with cysteine for de novo GSH synthesis, plays an essential role in defense against oxidants and electrophiles, including xenobiotics in the lung, as demonstrated in GGT knockout mice [4,5,16]. In the present study, we provided evidence that 4-hydroxy-2-nonenal, an end product of lipid peroxidation during oxidative stress, increased both GGT activity and mRNA content in L2 cells. We also showed that HNE increased GGT expression via activation of ERK and p38MAPK signaling pathways.

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Fig. 1.

GGT activity and mRNA in HNE-treated L2 cells. (A) HNE increased GGT activity in a dose- and time-dependent manner. Ethanol was used as vehicle control. Data are means $\pm$ SEM; $n = 3$. * $p < 0.05$; ** $p < 0.01$. (B) GGT mRNA content was increased by HNE in a dose- and time-dependent manner. Cells were treated and GGT mRNA was determined using the real-time PCR assay. The identity of the PCR product was confirmed by DNA sequencing. * $p < 0.05$; ** $p < 0.01$, $n = 5$. (C) GGT mRNA decay curve. Cells were pretreated with 1 μ g/ml actinomycin D for 4 h before being treated with/without HNE, and the total GGT mRNA was determined at different time points. $n = 3$.

Fig. 2.

HNE activated the major three MAP kinase pathways in L2 cells. (A) Phospho-ERK1/2 (pERK1/2) and phospho-p38 kinase (pp38) and (B) phospho-JNK1/2 (pJNK1/2). Actin was used as a loading control.

Fig. 3.

Effects of MAP kinase inhibitors on HNE-mediated induction of GGT expression. L2 cells were pretreated with MAPK inhibitors as shown for 1 h before HNE treatment: 24 h for activity assay, 12 h for mRNA assay, and 1 h for Western blots. (A) Effects of MAP kinase inhibitors on GGT activity. (B) Effects of MAP kinase inhibitors on total GGT mRNA. Final concentration of MAP kinase inhibitors in the medium: PD98059, 50 μM; SB203580, 5 μM; and JNKi, 10 μM. (C) Effects of different concentrations of PD98059 on phosphorylation of ERK1/2. (D) JNKi-blocked phosphorylation of c-Jun increased by HNE. * $p < 0.01$ in comparison with vehicle control; # $p < 0.05$, ## $p < 0.01$ in comparison with HNE treatment, $n = 3$.

Fig. 4.

Combined effects of PD98059 and SB203580 on GGT induction. L2 cells were pretreated with the cocktail of PD98059 (50 μM) and SB203580 (5 μM) for 1 h before HNE treatment. 24 or 12 h after HNE exposure, cells were collected for activity or mRNA assay, respectively. (A) Effects on GGT activity. (B) Effects on GGT mRNA. * $p < 0.01$, comparison with vehicle control, $n = 3$.

Fig. 5. HNE increased types (A) I and (B) V-2 GGT mRNA. Specific primer pairs against the 5' untranslated region of each type of GGT mRNA were used in the real-time PCR assay, and the PCR products were confirmed with DNA sequencing. * $p < 0.05$, ** $p < 0.01$, $n = 5$.

Table 1

Primer pairs for GGT mRNA real-time PCR assay

GGT mRNA type	Primers
All GGTs	Forward 5'-ACCACTCACCCAACCGCCTAC-3' Reverse 5'-ATCCGAACCTTGCCGTCCTT-3'
I	Forward 5'-GCTCATCACATCAGGCACCC-3' Reverse 5'-AAACCCACAGCCAATCTTCC-3'
II	Forward 5'-CCACCAGTGTGACCATCCTC-3' Reverse 5'-AAACCCACAGCCAATCTTCC-3'
III	Forward 5'-ATCCCAAGCCCTCCTCACC-3' Reverse 5'-AAACCCACAGCCAATCTTCC-3'
IV	Forward 5'-GCTTGTTGACCTTGGGCATCTG-3' Reverse 5'-AAACCCACAGCCAATCTTCC-3'
V	Forward 5'-GCTTGTTGACCTTGGGCATCTG-3' Reverse 5'-AAACCCACAGCCAATCTTCC-3'
GAPDH	Forward 5'-ACCCCAATGTATCCGTTGT-3' Reverse 5'-TACTCCTTGGAGGCCATGT-3'

Primer pairs for types IV and V each generate two products, due to alternative splicing. Only mRNA types I, II, and V-2 were detectable in L2 cells. PCR products were confirmed by DNA sequencing after gel purification.