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Effects of 2(*RS*)-*n*-propylthiazolidine-4(*R*)-carboxylic acid on 4-hydroxy-2-nonenal-induced apoptotic T cell death

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Abstract

4-Hydroxy-2-nonenal (HNE), the aldehydic product of lipid peroxidation, is associated with multiple immune dysfunctions, such as HIV and hepatitis C virus infection. HNE-induced immunosuppression could be due to a decrease in $CD4^+$ T lymphocyte activation or proliferation. Glutathione (GSH) is the most abundant endogenous antioxidant in cells, and an adduct between HNE and GSH has been suggested to be a marker of oxidative stress. Our earlier studies showed that HNE induced cytotoxicity and Akt inactivation, which led to the enhancement of FasL expression and concomitantly decreased cellular FLICE-like inhibitory protein (c-$FLIP_S$) levels. In this study, we found that HNE caused intracellular GSH depletion in Jurkat T cells, and we further investigated the role of 2(*RS*)-*n*-propylthiazolidine-4(*R*)-carboxylic acid (PTCA), a GSH prodrug, in attenuating HNE-induced cytotoxicity in $CD4^+$ T lymphocytes. The results show that PTCA protected against HNE-induced apoptosis and depletion of intracellular GSH. PTCA also suppressed FasL expression through increasing levels of Akt kinase as well as antiapoptotic c-$FLIP_S$ and decreasing the activation of type 2 protein serine/threonine phosphatase. Taken together, these data demonstrate a novel correlation between GSH levels and Akt activation in T lymphocyte survival, which involves FasL down-regulation and c-$FLIP_S$ expression through increasing intracellular GSH levels. This suggests that PTCA could potentially be used in the treatment of oxidative stress-induced immunosuppressive diseases.

Keywords: Hydroxynonenal; Glutathione; PTCA; Apoptosis; Fas ligand; $CD4^+$ T lymphocytes

1. Introduction

There is an increasing body of evidence showing that reactive aldehydic molecules generated as a consequence of lipid peroxidation are causally linked in most of the pathophysiological effects associated with oxidative stress in cells and tissues [1]. 4-Hydroxy-2-alkenals represent the most prominent lipid peroxidation-specific aldehydes [2]. Among them, 4-hydroxy-2-nonenal (HNE) is believed to be largely responsible for the cytopathological effects observed during oxidative stress in vivo [1]. Clinical observations show that HNE is associated with multiple pathophysiological conditions involving immune dysfunction, such as HIV and hepatitis C virus infection [3–5]. HNE plays a central physiological role in the down-regulation of cell proliferation and in the modulation of T cell differentiation [6,7]. In T lymphocytes, thiols play an important role in regulating cell proliferation and programmed cell death. The restoration of cellular glutathione (GSH) levels is known to modulate the effect of inflammatory cytokines and increase the $CD4^+$ cell count of HIV-infected patients [8–10]. Several studies demonstrated an increase in human $CD4^+$ T lymphocyte count after supplementation with antioxidants such as N-acetyl cysteine (NAC), oxothiazolidine carboxylic acid and vitamin E among patients with immune diseases, indicating that oxidative stress can affect human $CD4^+$ T lymphocyte survival [11,12]. NAC, a prodrug of GSH, has been used in HIV infection to prevent the activation of nuclear factor kappa-B and the replication of HIV [10]. Taken together, these results suggest an important role of GSH in oxidative stress-induced T cell toxicity and dysfunction.

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2(*RS*)-*n*-Propylthiazolidine-4(*R*)-carboxylic acid (PTCA) is a sulfhydryl-protected compound containing a cyclized form of L-cysteine (Cys) that can be nonenzymatically converted into Cys at physiological pH and room temperature levels [13]. Like NAC, PTCA has been shown to attenuate acetaminophen- and para-aminophenol-induced hepatic GSH depletion in mice [14] and hamsters [15]. Whether PTCA can protect against HNE-induced cytotoxicity and depletion of GSH has not been investigated.

Depletion of GSH by oxidants or by depleting agents such as diethyl maleate and buthionine sulfoximine (BSO) is known to increase cell vulnerability to oxidative stress and disturb the redox balance [16]. Recently, studies demonstrated that GSH depletion is probably an early signaling event in apoptotic cell death, which is characterized by the activation of protein kinase C-δ [17]. Others showed that cell viability and activation of Mek1 and Akt decreased when *ETV6-NTRK3*-transformed fibroblastic cells were incubated with BSO, suggesting that GSH plays a critical role in Akt activation in the transformed cells [18]. However, there is no direct link between GSH content and the protein kinase B/Akt survival signaling pathway.

The role of Akt kinase in T lymphocyte survival is well documented [19–21]. Activation of Akt plays an important role in the promotion of cell survival and prevention of proapoptosis in the intracellular signaling pathway in T lymphocytes [22]. In contrast to the activation of Akt by PDK1, the mechanisms of the inactivation of Akt have not been clearly delineated. The cellular FLICE-like inhibitory protein (c-FLIP$_S$) is an antiapoptotic cytoplasmic protein with sequence homology to caspase 8 and hence functions as a dominant-negative inhibitor of caspase 8, thereby preventing Fas-induced apoptosis [23]. The data obtained from our previous studies showed that HNE inhibited Akt activation and decreased c-FLIP$_S$ expression with a correspondent increase in caspase-8 activity that led to Fas-mediated apoptosis [22]. Although several studies have identified the antiapoptotic functions of Akt and c-FLIP$_S$ in T lymphocytes, the potential role of GSH in regulating Akt activation and c-FLIP$_S$ expression resulting in Fas-mediated apoptotic signaling has not been examined.

We hypothesized that GSH directly correlates with Akt expression and survival of CD4$^+$ T lymphocytes, as well as Fas-mediated death signaling. Therefore, our aim in this study was to test this hypothesis by modulating GSH status with PTCA and BSO in the HNE-induced cytotoxicity of Jurkat cells. We also explored whether PTCA can attenuate HNE-induced cytotoxicity by blocking the Fas-mediated apoptotic signaling pathway through Akt activation.

2. Materials and methods

2.1. Cell culture

Jurkat (clone E6-1) cells were obtained from ATCC (Rockville, MD, USA) and grown in RPMI-1640 supplemented with 10% fetal bovine serum, 10 U/ml of penicillin and 10 μg/ml of streptomycin in a 5% CO_2 incubator at 37°C.

2.2. Chemicals

HNE and malondialdehyde (MDA) were kindly provided by Dr. Sanjay Srivastava (Department of Internal Medicine). The antioxidant, PTCA, was synthesized and kindly provided by Dr. Herbert T. Nagasawa (VA Medical Center, Minneapolis, MN, USA). BSO, Nonidet P40, sodium orthovanadate and benzamidine were purchased from Sigma (St. Louis, MO, USA). FasL (C-178) and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FLIP$_{\gamma/\delta}$ (191–209) antibody was purchased from Calbiochem (La Jolla, CA, USA). *p*-Akt (Ser 473) and total Akt antibodies were purchased from Cell Signaling (Beverly, MA, USA). Goat antirabbit, antimouse and antirat antibodies conjugated with horseradish peroxidase were purchased from Bioscience International (Rockville, MD, USA). Phenylmethylsulfonyl fluoride was obtained from Boehringer Mannheim (Indianapolis, IN, USA). β-Glycerophosphate was purchased from TCI America (Portland, OR, USA). Fetal bovine serum, penicillin/streptomycin and trypan blue were purchased from Invitrogen (Grand Island, NY, USA).

2.3. Analysis of GSH and other thiols (SH) and disulfides (SS)

Cell pellets were dispersed with 0.25 ml of 4% metaphosphoric acid and centrifuged at 10,000×*g* for 2 min. The supernatants were collected for GSH analysis. Reduced GSH, oxidized GSH (GSSG), cysteine and cystine were simultaneously quantified by high-performance liquid chromatography with dual electrochemical detection according to our previous method [24]. In brief, 20-μl samples were injected onto a 250×4.6-mm and 5-μm C-18 column (Val-U-Pak HP, fully end-capped ODS, ChromTech, Apple Valley, MN, USA). Samples (20 μl) were injected onto the column and eluted isocratically with a mobile phase consisting of 0.1 M of monochloroacetic acid, 2 mM of heptanesulfonic acid and 2% acetonitrile at pH 2.8 and then delivered at a flow rate of 1 ml/min. The compounds were detected in the eluant with a Bioanalytical Systems model LC4B dual electrochemical detector using two Au–Hg electrodes in series with potentials of −1.2 and 0.15 V for the upstream and downstream electrodes, respectively. Current (in nanoamperes) was measured at the downstream electrode. Analytes were quantified from peak area measurements using authentic external standards.

2.4. Trypan blue dye exclusion

Cells were stained with trypan blue dye, counted by light microscopy with a minimum of 100 total cells counted per slide and then scored as cells that were able to exclude the dye (alive) or unable to exclude the dye (dead) to measure their viability [25].

2.5. DNA fragmentation ELISA assay

The treated Jurkat cells were lysed after 6 h to measure cell apoptosis. DNA fragmentation was quantified using a Cell Death ELISA kit (Roche, Indianapolis, IN, USA) by a method described elsewhere [22].

2.6. Akt (pS473) ELISA assay

Treated Jurkat T cells were lysed at 6 h to determine Akt. The concentration of phosphorylated Akt was determined by ELISA using an Akt (pS473) kit (BioSource International, Camarillo, CA, USA) according to the manufacturer's instructions.

2.7. Western blot analysis

Jurkat T cells (15×10^6) were pretreated with PTCA for 1 h and left untreated or treated with HNE for 6 h. Following treatment, cells were immunoprecipitated and immunoblotted as described previously [22]. Protein mass was assessed by quantifying the intensity of protein bands using Quantity One software (Bio-Rad, CA, USA).

2.8. Type 2 protein serine/threonine phosphatase enzymatic activity assay

Treated Jurkat T cells were lysed for 30 min to determine type 2 protein serine/threonine phosphatase (PP2A) activity. PP2A enzymatic activity was assessed following PP2A immunoprecipitation using a malachite green-based phosphatase assay (PP2A Immunoprecipitation Phosphatase Assay Kit, Upstate) according to the manufacturer's instructions. Phosphate concentrations were calculated from a standard curve created by using serial dilutions of a standard phosphate solution (0–2,000 pmol). Data were normalized to the protein concentration, and PP2A activity was expressed as picomoles of phosphate per microgram of protein per 10 min.

2.9. RNA isolation and real time polymerase chain reaction analysis

Reverse transcriptase polymerase chain reaction (RT-PCR) assays were used to assess FasL messenger RNA (mRNA) levels in Jurkat cells and performed as described elsewhere [22]. The following primers were used in real time PCR:

hGAPDH-RT-FP1: 5′ TGGGCTACACTGAGCACCAG 3′
hGAPDH-RT-RP1: 5′ GGGTGTCGCTGTTGAAGTCA 3′
hFasL-RT-FP: 5′ GGCCTGTGTCTCCTTGTGAT 3′
hFasL-RT-RP: 5′ TGCCAGCTCCTTCTGTAGGT 3′

The parameter C_t (threshold cycle) was defined as the fraction cycle number at which fluorescence passed the threshold. The relative gene expression of FasL was analyzed using the $2^{-\Delta\Delta C_t}$ method [26] by normalizing it with GAPDH gene expression in all the experiments.

2.10. Statistical analysis

Statistical analysis was performed with the use of SPSS 11.5 (SPSS, Chicago, IL, USA) for Windows XP. Differences between the means of two groups were evaluated by a two-tailed t test for independent samples. Differences between three or four groups of means were evaluated by single-factor analysis of variance and further assessed by Tukey tests. Differences were considered to be statistically significant at the $P<.05$ level.

3. Results

3.1. Effects of PTCA on HNE cytotoxicity and GSH concentrations

HNE produced a time- and dose-dependent depletion of intracellular GSH concentrations. The basal GSH concentration was 37.1±8.6 nmol/mg protein. It was depleted by 70% 15 min after HNE (2.5 μM) treatment and reached a maximal depletion of 90% at 1 h and then remained low throughout 3 h (Fig. 1A). The dose effect of HNE on GSH concentration at 3 h is shown in Fig. 1B. At the lowest HNE concentration (2.5 μM), GSH was depleted by 80%. At higher HNE concentrations (5–20 μM), GSH was completely depleted. GSH concentrations in cells pretreated with PTCA (250 μM) were maintained at about 80% of the

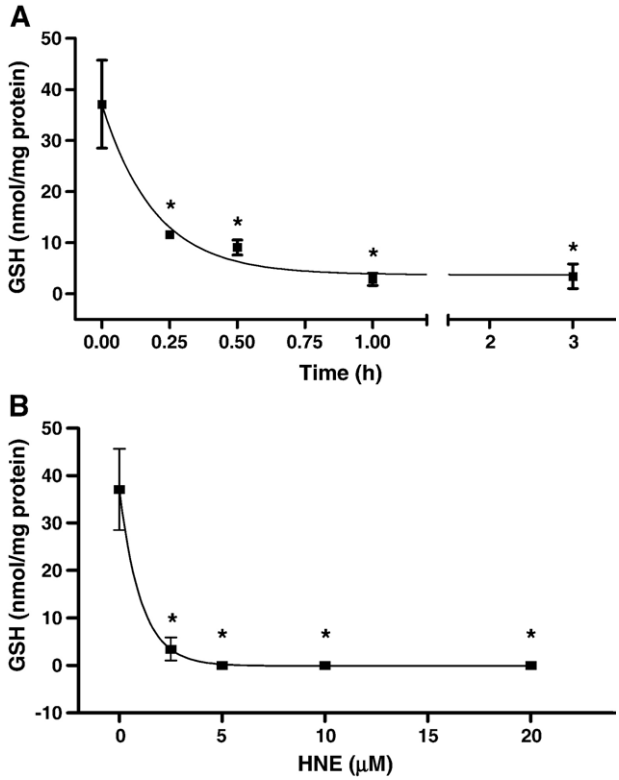

Fig. 1. Time and dose effects of HNE on intracellular GSH concentrations. (A) Cells were untreated (UT) or treated with HNE (2.5 μM). Cells were harvested at time points from 15 min to 3 h, and intracellular GSH concentrations were measured as described in Materials and methods. (B) Cells were UT or treated with various concentrations of HNE (2.5–20 μM). Cells were harvested at 3 h, and intracellular GSH concentrations were measured. Data were normalized to the protein concentration (nmol/mg protein) and are presented as the mean±S.D. of three experiments. *$P<.05$ compared with UT.

untreated value, as compared with merely 8% with HNE alone (2.5 μM) (Fig. 2). In addition, when Jurkat T cells were pretreated with PTCA, cell viabilities increased to about 80% of the untreated value, as compared with 25% survival with HNE alone (2.5 and 5 μM) (Fig. 3A). Similarly, PTCA pretreatment decreased DNA fragmentation by approximately 20% as compared with HNE alone (Fig. 3B). Thus, pretreatment of Jurkat T cells with PTCA markedly attenuated the HNE-induced decrease in survival and in intracellular GSH concentrations.

Based on the cited results, the relationship between cytotoxicity and GSH concentrations was plotted. As shown in Fig. 4, a close correlation (R^2=0.9908) between GSH concentrations and cytotoxicity was observed; it appears that when GSH concentrations were depleted by about 80–90% of the control (threshold) levels, cytotoxicity ensued.

3.2. Effects of PTCA on Akt activation

The serine/threonine kinase (protein kinase B/Akt) has been shown to be critical for T lymphocyte survival (e.g., inhibition of Akt leads to apoptotic T cell death) [27]. In untreated Jurkat T cells, Akt is constitutively phosphorylated at Ser 473, and the basal concentration of the *p*-Akt was 28.6±1.0 U/mg protein. *p*-Akt levels decreased by 73% and 11% of the untreated value when cells were exposed to HNE at 2.5 and 5 μM, respectively. When Jurkat T cells were pretreated with PTCA followed by HNE administration, *p*-Akt concentrations increased to about 115% (2.5 μM) and 70% (5 μM) of the untreated value (Fig. 5A). PTCA alone had no effect on the *p*-Akt levels.

We confirmed the effect of HNE on *p*-Akt concentrations by immunoblotting analysis on *p*-Akt and Akt expressions. As shown in Fig. 5B, treatment of Jurkat T cells with HNE (2.5 and 5 μM) for 6 h decreased *p*-Akt expression but PTCA pretreatment protected against this decrease. Similarly, total Akt degradation of HNE at 5 μM was protected by PTCA. These results suggest that the protection by PTCA against HNE-induced cytotoxicity may be mediated through the activation of Akt.

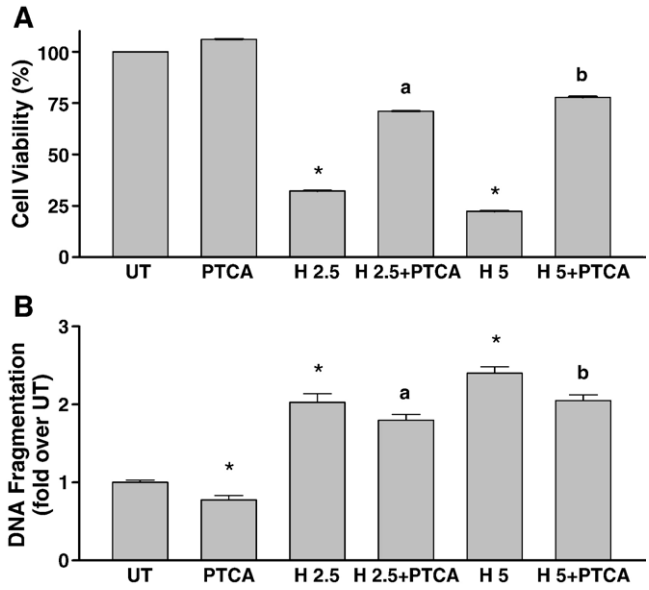

Fig. 3. PTCA protects against HNE-induced cytotoxicity. Sample preparation was as that described in Fig. 2. Cell extracts were collected at 6 and 24 h. (A) Cell survival was quantified by trypan blue exclusion at 24 h. Data were normalized to the UT control, set to 100% and presented as the mean±S.D. of five experiments. (B) Cytoplasmic extracts were prepared and analyzed at 6 h for DNA fragmentation by a Cell Death ELISA kit as described in Materials and methods. Data were normalized to the UT control, set to 1 and presented as the mean±S.D. of four experiments. In each experiment, duplicate wells were assayed separately for each treatment. *P<.05 compared with UT. [a]P<.05 compared with HNE 2.5 μM. [b]P<.05 compared with HNE 5 μM.

Since HNE significantly decreased T cell survival and GSH concentrations, we examined the effect of another lipid peroxidation product (i.e., MDA) on Akt expression and cell survival. MDA or BSO alone had no effect on *p*-Akt expression; however, the combined treatment significantly decreased *p*-Akt and total Akt expressions (Fig. 6A). Furthermore, MDA alone had no effect on cell viability, but pretreatment with BSO, an inhibitor of de novo GSH biosynthesis, produced profound cell death (Fig. 6B). These results demonstrate that BSO sensitizes Jurkat T cells to

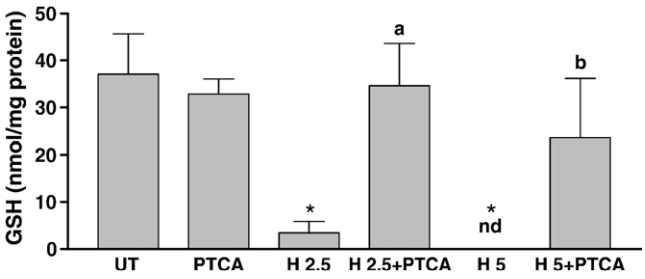

Fig. 2. PTCA attenuates HNE-induced GSH depletion and cytotoxicity. Jurkat T cells were pretreated with PTCA for 1 h before the addition of HNE. Cells were UT or treated with PTCA (250 μM), HNE (2.5 or 5 μM) or PTCA plus HNE. Cells were harvested at 3 h, and intracellular GSH concentrations were measured. *nd* indicates below detection limit. *P<.05 compared with UT. [a]P<.05 compared with HNE 2.5 μM. [b]P<.05 compared with HNE 5 μM.

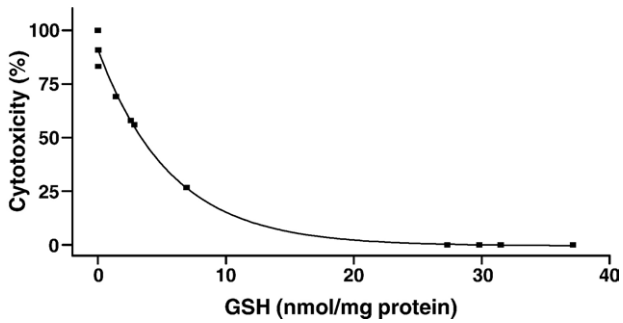

Fig. 4. Relationship between GSH concentration and Jurkat T cell death. GSH concentration and cell viability were determined at 3 and 24 h, respectively, after HNE treatment. Each point represents the value obtained with a given treatment. The plot includes data from all experiments in cells in which both cytotoxicity and GSH were determined.

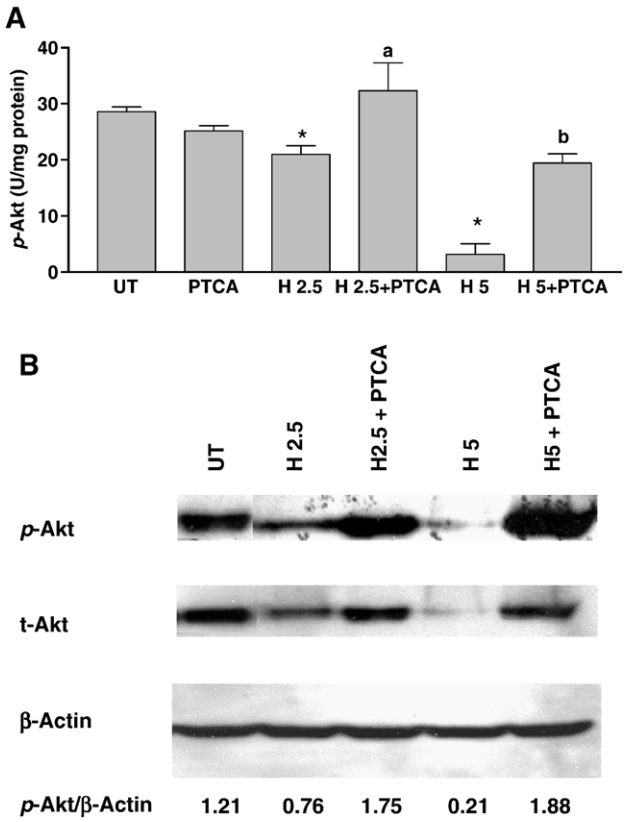

Fig. 5. PTCA protects against HNE-induced down-regulation of Akt kinase expression. Sample preparation was as that described in Fig. 2. Cell extracts were harvested at 6 h after HNE treatment. (A) Data were normalized to the protein concentration, and *p*-Akt concentration was expressed as U/mg protein. Data are presented as the mean±S.D. of three experiments. $^{*}P<.05$ compared with UT. $^{a}P<.05$ compared with HNE 2.5 μM. $^{b}P<.05$ compared with HNE 5 μM. (B) Akt and *p*-Akt were detected by immunoblotting using anti-Akt and *p*-Akt antibodies at 6 h.

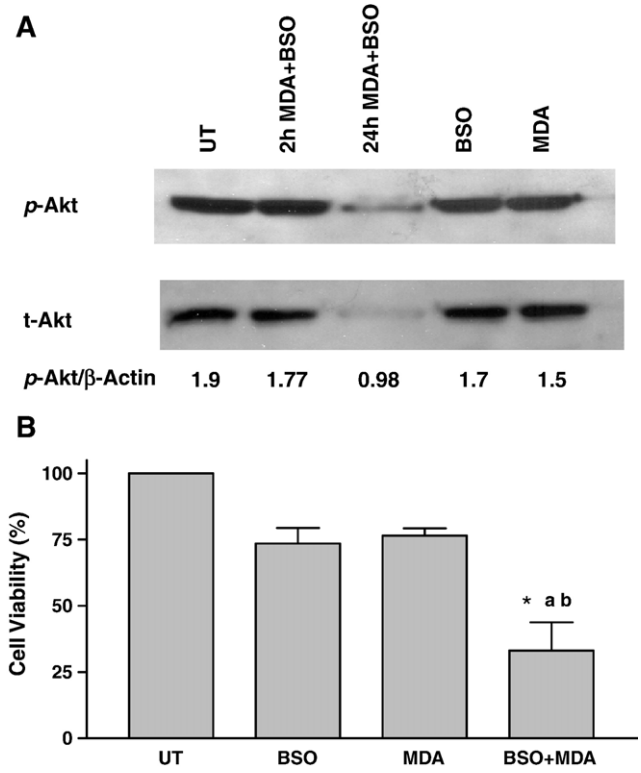

Fig. 6. Role of GSH in MDA-induced Akt activation and cell viability. Jurkat T cells were pretreated with BSO for 24 h before the addition of MDA at 2 or 24 h. Cells were UT or treated with BSO (5 mM), MDA (10 μM) or BSO plus MDA. (A) Akt and *p*-Akt were detected by immunoblotting using anti-Akt and *p*-Akt antibodies at 2 and 24 h. (B) Cell survival was quantified by trypan blue exclusion at 24 h. Data were normalized to the UT control, set to 100% and presented as the mean±S.D. of five experiments. $^{*}P<.001$ compared with UT. $^{a}P<.01$ compared with BSO. $^{b}P<.01$ compared with MDA.

lipid peroxide-induced Akt inactivation and cytotoxicity, suggesting that GSH plays an important role in T cell survival.

In addition, a close correlation ($R^2=0.9668$) between GSH concentrations and cell survival, as indexed by *p*-Akt concentrations, was observed (Fig. 7). Thus, it appears that the inactivation of Akt occurs when 80–90% of cellular GSH was depleted.

3.3. Effects of PTCA on PP2A activation

It is well known that the activation/inactivation of Akt depends on the phosphorylation/dephosphorylation of the active site of Akt [27]. PP2A has been shown to dephosphorylate Akt in vitro, which is highly associated with survival [28]. We therefore examined the effect of HNE on the overall catalytic activity of PP2A. As shown in Fig. 8, PP2A activities were significantly increased (210% of the untreated value) in cells exposed to 5 μM but not 2.5 μM of HNE for 30 min. When Jurkat T cells were pretreated with PTCA for 1 h before the addition of HNE, PP2A activities decreased by 121% (2.5 μM) and 104% (5 μM) of the untreated value. PTCA alone had no statistically significant effect on PP2A activity. Our results demonstrate that HNE induced increases in PP2A activity, whereas pretreatment with PTCA blocked these effects.

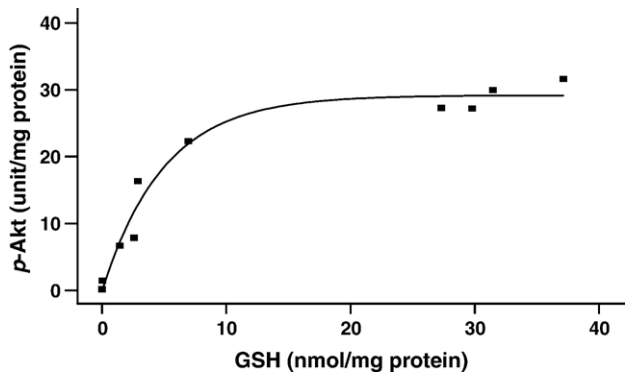

Fig. 7. Relationship between GSH concentration and *p*-Akt concentration in Jurkat T cells. GSH concentration and *p*-Akt concentration were determined at 3 and 6 h, respectively, after HNE treatment. Each point represents the value obtained with a given treatment. The plot includes data from all experiments in cells in which both GSH and *p*-Akt concentrations were determined.

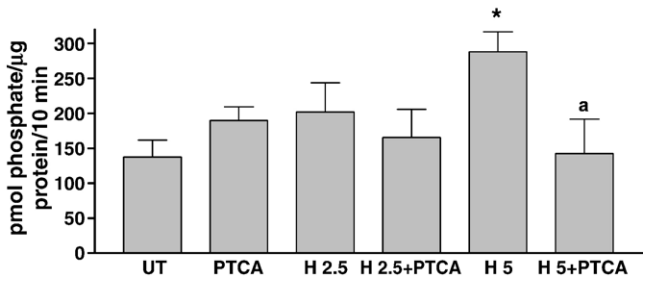

Fig. 8. PTCA inhibits HNE-induced PP2A activation. Sample preparation was as that described in Fig. 2. Cell extracts were harvested at 30 min after HNE treatment. PP2A activity was measured using a PP2A immunoprecipitation assay kit as described in Materials and methods. Data were normalized to the protein concentration, and PP2A activity was expressed as pmol phosphate/μg protein/10 min. Data are presented as the mean±S.D. of three experiments. $^{*}P<.05$ compared with UT. $^{a}P<.06$ compared with HNE 5 μM.

3.4. Effects of PTCA on Fas-mediated signaling

We previously demonstrated that HNE-induced apoptosis was through FasL expression and FADD recruitment to the death-initiating signaling complex [29]. In the present study, we further examined the effect of PTCA on FasL expression. The results show that HNE at 5 μM increased FasL expression but that PTCA pretreatment attenuated this effect (Fig. 9A). We then analyzed FasL mRNA levels by real time PCR. As shown in Fig. 9B, HNE at 2.5 μM had no effect on FasL mRNA, but at 5 μM, the FasL mRNA increased fourfold relative to control. The data show that in correspondence with FasL protein expression, FasL mRNA was significantly reduced (approximately to the untreated level) after 1 h of pretreatment with PTCA.

c-FLIP$_S$ is an antiapoptotic protein and has been shown to inhibit Fas-mediated apoptosis [30]. Since PTCA protects against HNE-induced apoptosis and up-regulates FasL expression, we examined the effects of PTCA on c-FLIP$_S$ expression. As shown in Fig. 10, c-FLIP$_S$ levels were decreased after HNE treatment, whereas PTCA pretreatment led to significant increases in c-FLIP$_S$ levels. Thus, PTCA circumvented HNE-induced c-FLIP$_S$ down-regulation.

4. Discussion

Our earlier studies demonstrated that HNE induced Fas-mediated apoptosis and cytotoxicity in Jurkat T cells. However, the underlying mechanism was not clear. Numerous studies suggest that intracellular events such as GSH depletion, caspase activation, DNA fragmentation and chromatin condensation may be involved in the apoptotic pathway initiated by HNE [1]. The present study focused on the role of GSH in HNE-induced toxicity and the possibility for cytoprotection by the GSH precursor PTCA.

GSH is the most abundant antioxidant in the body. In many cultured cell lines, including Jurkat T lymphocytes, GSH plays an important role in cell cycle, function, proliferation and survival [31]. HNE decreased intracellular GSH in a dose- and time-dependent manner, which correlated with the induction of DNA fragmentation (Figs. 1 and 3). The HNE-induced decreases in intracellular GSH concentrations could be due to the sequestration of GSH by HNE to form HNE–GSH adducts since we did not find any increase in GSSG after HNE treatment (data not shown). Moreover, GSH rebound effects for increased GSH resynthesis, which is controlled by GSH concentration-dependent feedback, were not observed.

PTCA functions similarly to NAC by supplying Cys in a prodrug form for GSH biosynthesis; however, PTCA, unlike NAC, releases Cys spontaneously and is independent of any enzymatic action [13]. NAC has been shown to block HNE-induced DNA fragmentation in Jurkat cells [6] and in HIV-infected patients; its administration resulted in a relative

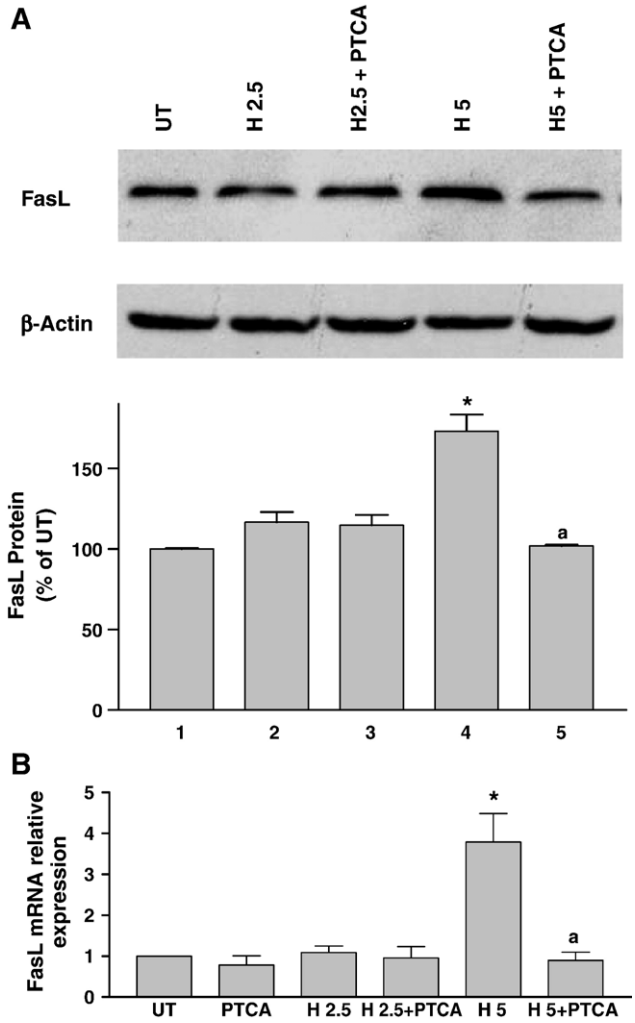

Fig. 9. PTCA protects against HNE-induced up-regulation of FasL. Sample preparation was as that described in Fig. 2. (A) FasL was detected by immunoblotting using an anti-FasL-specific antibody at 6 h. Densitometry results are represented as the mean±S.D. of three experiments. $^{*}P<.05$ compared with UT. $^{a}P<.05$ compared with HNE (5 μM). (B) Total RNA was isolated from cells at 6 h after treatment. FasL mRNA levels were determined by real time PCR. Data are presented as the mean±S.D. of five experiments. $^{*}P<.001$ compared with UT. $^{a}P<.001$ compared with HNE (5 μM).

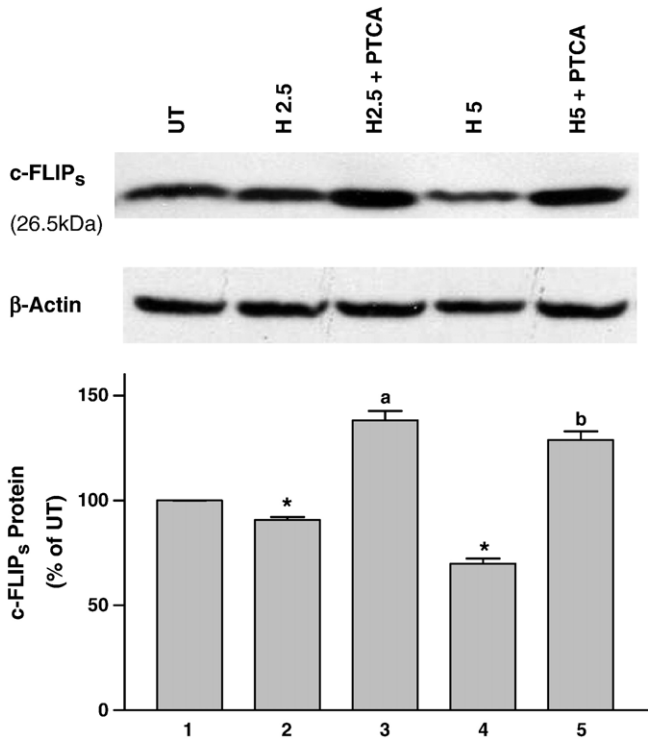

Fig. 10. PTCA protects against HNE-induced down-regulation of c-FLIP$_S$. Sample preparation was as that described in Fig. 2. c-FLIP$_S$ was detected by immunoblotting using an anti-c-FLIP$_{\delta/\gamma}$ antibody at 6 h. Densitometry results are presented as the mean±S.D. of three experiments. *P<.05 compared with UT. [a]P<.05 compared with HNE 2.5 μM. [b]P<.05 compared with HNE 5 μM.

increase in the CD4/CD8 ratio [8] and a slower decline of the CD4$^+$ T cell count [9]. In the present study, we demonstrated for the first time that PTCA protected against HNE-induced cytotoxicity, possibly mediated by GSH restoration, thereby confirming the important role of GSH in HNE-induced cytotoxicity. As in one in vivo study [15], a GSH threshold phenomenon in HNE-induced cytotoxicity in CD4$^+$ T lymphocytes was also observed here. Thus, intracellular GSH appears to play an important role in cell viability since cytotoxicity occurred only when GSH was depleted by 80–90%, suggesting the likely depletion of mitochondrial GSH.

The serine/threonine kinase Akt is critical for survival and protection against apoptosis in various cells [19,21]. It is well known that down-regulation of Akt is associated with its dephosphorylation by PP2A and is important for cytotoxicity in vitro [7]. HNE inhibited Akt phosphorylation as well as activation and concomitantly increased PP2A activity, whereas PTCA attenuated HNE-induced Akt down-regulation (Fig. 5). NAC, another antioxidant, has been shown to activate Akt and decrease apoptosis in Jurkat T cells by blocking adaphostin-mediated reactive oxygen species (ROS) generation [32]. It is likely that NAC, as well as PTCA, reversed ROS-induced Akt dephosphorylation. Our studies demonstrated that PTCA pretreatment also attenuated HNE-induced increases in PP2A activation in Jurkat T cells (Fig. 8). Others have shown that NAC completely reversed the N-ethylmaleimide-induced inhibition of Akt phosphorylation and apoptosis mediated by the inactivation of PP2A in vascular smooth muscle cells [33]. Interestingly, Liu et al. [7] showed that pretreatment of the Jurkat T cells with Cys before HNE restored the active Akt almost to the untreated level. They further demonstrated that HNE-induced Akt dephosphorylation was prevented by okadaic acid, a PP2A inhibitor [7]. Taken together, these data demonstrate an important role of PP2A in HNE-induced apoptosis via phosphorylation and dephosphorylation of target proteins in the survival pathway. In addition, a novel finding here is that PTCA protects against HNE-induced inactivation of Akt, possibly mediated by restoring the phosphorylation of Akt kinase through down-regulation and association of PP2A.

Unlike the profound effect of HNE on T cell loss and GSH depletion, our results show that MDA, another lipid peroxidation product that is less reactive than HNE, had no effect on Akt activation and cell viability. However, pretreatment with BSO (a GSH depletor) before the addition of MDA caused a significant decrease in T cell viability (Fig. 6). Spitz et al. [34] demonstrated that pretreatment of Chinese hamster fibroblasts and clonogenic cells with BSO potentiated HNE-induced cytotoxicity. Others have shown that depletion of GSH by BSO inhibited Akt phosphorylation in *ETV6-NTRK3*-transformed murine fibroblast cells [18]. Interestingly, we also found that by pretreatment of cells with BSO before MDA addition, *p*-Akt and total Akt were markedly decreased (Fig. 6). Thus, BSO, by inhibition of GSH biosynthesis, can sensitize cells to peroxide-induced inhibition of Akt and cell viability.

A plot of *p*-Akt levels versus GSH concentrations under different experimental conditions in Fig. 7 shows a GSH threshold phenomenon similar to that of HNE-induced loss of survival in CD4$^+$ T cells (Fig. 4). Cells are viable as long as the intracellular GSH concentration is maintained at about 20% of the basal level. This study demonstrated for the first time the close relationship between GSH, cytotoxicity and cell survival. The results also suggest that active Akt is tightly regulated by intracellular GSH concentrations.

Since PTCA had an effect on the Akt survival pathway, we further examined its effect on the FasL-dependent death pathway. We showed that PTCA prevented HNE-induced FasL expression at the protein and transcriptional levels (Fig. 9), possibly through restoration of intracellular GSH. Others have found that NAC and GSH protected against *tert*-butylhydroperoxide-induced FasL expression and apoptosis in human retinal pigment epithelial cells [35]. In addition to its effect on Akt activation, we demonstrated that PTCA also prevented HNE-induced c-FLIP$_S$ down-regulation. To our knowledge, this is the first report to show that GSH plays a role in HNE-induced Fas-mediated apoptosis by preventing the loss of Akt phosphorylation/activation and c-FLIP$_S$ expression in human T lymphocytes. Others have shown that pretreatment of multiple myeloma cells with NAC blocked oleanane triterpenoid-induced apoptosis and c-FLIP$_S$ down-

regulation [36]. Our results clearly indicate that restoration of GSH by PTCA protects against HNE-induced Fas-mediated apoptotic cell death, which is mediated through redox-sensitive Akt and c-FLIP$_S$ signaling molecules.

Overall, our study demonstrates that, in addition to its known antiapoptotic functions, GSH regulates Akt kinase in T lymphocytes. This involves the regulation of Fas-mediated upstream apoptotic cell death as well as FasL and c-FLIP$_S$ expression. Importantly, PTCA protects against HNE-induced toxicity and could potentially be used in the treatment of oxidative stress-induced immunosuppressive diseases.

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