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Thioproline Serves as an Efficient Antioxidant Protecting Human Cells from Oxidative Stress and Improves Cell Viability

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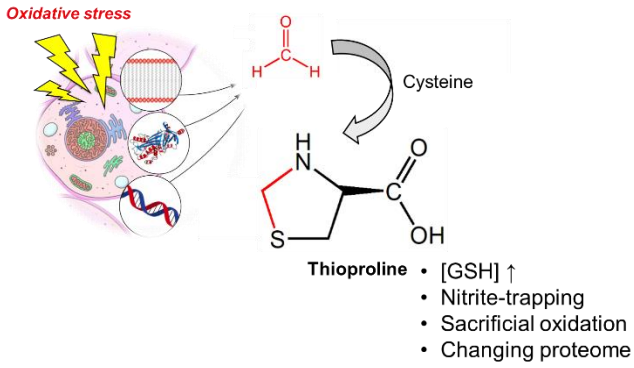
Oxidative stress
Cysteine
Thioproline
• [GSH] ↑
• Nitrite-trapping
• Sacrificial oxidation
• Changing proteome

Abstract

Oxidative stress is associated with the pathophysiology of many degenerative human diseases, including Alzheimer's disease, atherosclerosis, Parkinson's disease, and cancers. We discovered in our previous study that thioproline (SPro), a proline analogue, is generated in oxidant-exposed cells. With the prior observation that SPro served as an efficient nitrile trapping agent, we tested in this study the hypothesis that this oxidative stress generated cysteine-formaldehyde adduct, SPro, may serve as an antioxidant protecting cells from oxidative stress. Interestingly, results showed that HeLa cells cultured in SPro-supplemented culture media are more tolerant of oxidative stress, indicated by a dosage-dependent increase in cell viability. Investigation of the molecular mechanism of the observed increase in cell tolerance to oxidative stress revealed SPro acting as an effective antioxidant by sacrificial oxidation. Results also showed that SPro had been incorporated into cellular proteins and induced changes in protein expression profiles of treated cells. Despite it is yet to determine the participation of individual factors to the observed increase cell tolerance to oxidative stress, this study sheds light on the potential use of SPro as a dietary supplement protecting human from oxidative stress-associated degenerative human diseases.

INTRODUCTION

Thiazolidine are sulfur-containing compounds that are produced when cysteine condenses with reactive carbonyls, such as formaldehyde (**Figure 1**)[1-3]. These compounds exhibit a wide variety of pharmaceutical properties,[4-10] and thiazolidine-4-carboxylic acid (thioproline, SPro) in particular, has attracted the most notable interest for its hepatoprotective,[6] anti-cancer,[7] and anti-aging properties.[8] Most remarkably, SPro was demonstrated to be an effective endogenous nitric oxide-trapping agent that could reduce the risk of cancer.[11-13] Currently, SPro is being commercialized as a dietary supplement.[14]

We recently demonstrated that formaldehyde-producing oxidizing agents induced a dosage-dependent formation of SPro in *E. coli* cells,[15] which eventually became incorporated into cellular proteins.[16,17] Given that SPro is generally considered to be a protective agent against degenerative human diseases,[4-10] we hypothesized that the generation of this formaldehyde adduct of cysteine, SPro, when cells are challenged by oxidative stress, could have been an anti-oxidative survival mechanism of cells to resist oxidative stress-induced toxicity.

To test this hypothesis, we initiated the study by measuring the viability of SPro-treated HeLa cells in the presence of oxidative stress-inducers (Fe^{2+}-EDTA, NaOCl or H_2O_2). The results showed that SPro-treated HeLa cells can indeed better tolerate conditions of oxidative stress, and the mortality rate of these cells dropped with increasing dosage of SPro. To the best of our

knowledge, this is the first report demonstrating SPro's ability to improve cell survival in an oxidative environment.

The study was then extended to investigate the molecular mechanism behind the increased tolerance to oxidative stress. To this end, we developed liquid chromatography-tandem mass spectrometry coupled with stable isotope-dilution methods to quantitate the dosage-dependent incorporation of SPro into cellular proteins, the relative concentration of oxidized SPro (*N*-formyl-L-cysteine, **Figure 1**),[18] and the ratio of reduced to oxidized glutathione (GSH/GSSG ratio) in the intracellular fluid of the oxidative stress-exposed HeLa cells. These results confirmed that SPro indeed acted as an effective antioxidant shielding cells from oxidation-induced damages and that human cells appear to utilize this modification as a scavenging system for formaldehyde-producing oxidants.

Experimental Methods Section

Chemicals and Reagents. All chemicals and reagents were of the highest purity available and were used without further purification unless otherwise stated. L-Thioproline, L-cysteine, *N*-acetyl-L-cysteine, [^{13}C,^{2}H$_2$]formaldehyde, glutathione-([^{13}C$_2$,^{15}N]glycine), protease from bovine pancreas, oxidized and reduced glutathione were purchased from Sigma-Aldrich (St. Louis, MO). Heavy isotope-labeled *L*-cysteine, i.e. [^{13}C$_3$]-L-cysteine, was obtained from Toronto Research Chemicals Inc. (Ontario, Canada). Isotope-labeled L-thioproline ([^{13}C,^{2}H$_2$]-L-thioproline) was synthesized by reacting [^{13}C,^{2}H$_2$]-formaldehyde with L-cysteine and purified by reversed-phase HPLC, as described previously.[19] Using a similar approach, [^{13}C$_3$]-L-thioproline was also synthesized by reacting [^{13}C$_3$]-L-cysteine with formaldehyde. Isotope-labeled oxidized glutathione was synthesized by incubating glutathione-([^{13}C$_2$,^{15}N]glycine) in 0.5 M H_2O_2 at 37 °C for 3 h and purified by reversed-phase HPLC. Reference standard of *N*-formyl-L-cysteine (oxidized SPro),[18] was produced by incubating SPro in 0.5 M H_2O_2 at 37 °C for 3 h, and the reaction product characterized by high resolution MS and MS/MS analyses (**Figures S1** and **S2**). HPLC grade acetonitrile were purchased from Tedia (Fairfield, OH). Ultrapure water was produced with a Cascada laboratory water purification system (PALL; Port Washington, NY) and used in all experiments.

Instrumental Analyses. HPLC purification of isotope-labeled internal standards were performed on an Agilent 1100 HPLC system equipped with a photodiode array detector (Waltham, MA). High-resolution mass spectrometry (MS) and collision-induced dissociation MS/MS experiments

were performed on a Xevo G2 Q-Tof mass spectrometer equipped with a standard ESI interface coupled with an Acquity UPLC system (Waters Corporation, Milford, MA). LC−MS/MS analyses were performed on a Waters Acquity UPLC (Milford, MA) coupled with an API 4000 QTRAP (AB Sciex; Foster City, CA) or a Waters TQ-XS (Milford, MA) tandem mass spectrometer. The mass spectrometers were equipped with electrospray ionization ion sources and operated in the multiple-reaction monitoring (MRM) mode. Protein concentration was measured by UV absorbance at 280 nm using a Jenway UV-visible spectrophotometers (Staffordshire, UK). Shotgun proteomics analysis was conducted on a orbitrap fusion lumos nano-LC−MS/MS system (Thermo Scientific, Bremen, German).

Cell Culture and Treatment with Oxidants. HeLa cells were obtained from the American Type Culture Collection (Camden, NJ). The cells were cultured in Dulbecco's Modified Eagle's Media supplemented with 10% fetal bovine serum, 1% penicillin (Thermo-Fisher; Waltham, MA), and added with different concentrations of SPro (0, 0.1, 0.25, 0.5, and 1 mM) (or *N*-acetyl-cysteine for comparative analysis of antioxidative capacity; or 0, 0.25 mM, 0.5 mM [$^{13}C_3$]-L-thioproline for demonstrating the incorporation of [$^{13}C_3$]-L-thioproline during protein synthesis). After washing twice with PBS, the SPro-treated cells were used to investigate thioproline's dosage-dependent protection to cells from oxidative insults and the underlying molecular mechanisms.

We first investigated the effect that SPro-treatment had on cells in their tolerance to oxidative stress. To this end, we incubated the SPro-treated cells with Fe^{2+}-EDTA (1 mM, 18 h), NaOCl (100 μM, 6 h) or H_2O_2 (100 μM, 6 h) at 37 °C. These conditions were chosen because they were

tested to produced ~50 % mortality in preliminary experiments. Cells were then washed twice using PBS and detached from the plate by trypsin-EDTA. Cell viability was then determined using the trypan blue staining assay.

Quantitation of Proteinic Thioproline. To quantitate the proteinic SPro concentration in SPro-treated cells, detached cells were re-suspended in lysis buffer containing 8 M urea and lysed at 4 °C with the assistance of ultrasonic cell disruptor. The lysed cells were centrifuged at 15,000 g for 2 min at 4 °C, the supernatant was collected, and added with 4 volumes of cold (−20 °C) acetone to precipitate the proteins. They were then collected by centrifugation (15,000 g, 15 min at 4 °C), washed with cold 70% (v/v) acetone, and dried under ambient environment. Prior to analysis, the precipitated protein samples were re-solubilized in a buffer solution (4 M urea and 25 mM ammonium bicarbonate), quantitated using UV absorption spectrophotometry (A_{280}), added with [2-^{13}C,$^{2}H_2$]-L-thioproline, and hydrolyzed using protease (2 μg/100 μg protein, incubated at 37 °C for ≥16 h), as described previously.[16] SPro concentration in the hydrolysates was analyzed using the LC−MS/MS method described below. Using a similar approach, the concentration of [$^{13}C_3$]-SPro in cellular proteins of [$^{13}C_3$]-SPro-treated cells was also quantitated.

Proteomics analysis. Cellular proteins isolated from control and SPro-exposed cells (1 mM) were also analyzed using the shotgun proteomics approach. Solution contains 40 μg of extracted proteins was incubated with 10 mM dithiothreitol (DTT) at 37 °C for 1 h to reduce the disulfide bonds. Iodoacetamide was added to a final concentration of 20 mM and the treated samples were incubated at room temperature in the dark for 30 mins. The reduced and alkylated proteins were

digested with sequencing grade trypsin (0.8 μg) at 37 °C for 10 h. The digestion was terminated by addition of 1% formic acid. The insoluble materials were removed by centrifugation at 15, 000 g for 10 mins and the supernatant was desalted and purified by a ZipTipC18 (C18-resin packed pipette tip). The purified samples were dried with SpeedVac, and stored at −80 °C before LC-MS/MS analysis.

Analysis of GSHs and Oxidized Thioproline in Cells. To quantitate GSH, GSSG, and *N*-formyl-L-cysteine in the sPro-pretreated, and oxidant-exposed cells, 1 x 10^6 cells were lysed in 200 μL of water at 4°C, added with 20 μL of internal standard (mixture of 10 μM [$^{13}C_2$,^{15}N]GSH and 10 μM [$^{13}C_4$,$^{15}N_2$]GSSG) and then passed through a PALL 10K nanosep membrane to remove cellular proteins. The filtrates were then analyzed for GSH, GSSG, and *N*-formyl-cysteine using the isotope dilution LC−MS/MS methods described below.

LC−MS/MS Analyses. LC separation of SPro, GSH and GSSG were performed on a Phenomenex RP 18 column (100 × 2 mm inner diameter, 5 μm; Phenomenex, Torrance, CA). Five microliters of the protein hydrolysate or sample extracts were injected into the column, and eluted at a flow rate of 0.3 mL/min using 0.2% acetic acid in water (A) and acetonitrile (B) as the mobile phase. The gradient elution was programmed to start at 1% B (v/v) for 2 min, increased to 30% B in 4 min, then ramped to 90% B in 0.1 min and held for 3 min, before being re-equilibrated to initial mobile phase conditions. The LC eluate was directed to an API 4000 QTRAP LC−MS/MS system operated in positive electrospray ionization (ESI) mode for the analyses.

For enhanced chromatographic performance, separation of *N*-formyl-cysteine were performed on a Phenomenex HILIC column of the same column parameters, and with the same mobile phase components as stated above. The LC gradient elution started from 100% B (v/v) and held for 2 min, decreased to 40% B in 7 min, held for 2 min, before being re-equilibrated to initial conditions. The LC eluate was diverted to a TQ-XS for analysis.

The LC−MS/MS systems were both operated at multiple reaction monitoring (MRM) mode using the optimized instrumental parameters. The *m/z* values for the qualifying and quantifying MRM transition of the target analytes and internal standard are listed in **Table 1**, with the dwell time for each transition set to 50 ms. Authentic standard of *N*-formylcysteine, the oxidation product of SPro, is not commercially available, cysteine was used as a surrogate to establish a calibration curve for its quantitation because of their similar ESI-MS response (**Figure S3**).

Proteomics Analysis. *LC−MS/MS Analysis.* A nano-LC coupled with an ultra-high resolution MS was used for the proteomics study. A 15 cm ×100 μm inner diameter, 3μm, C18 capillary column (Thermo fisher Scientific, San Jose, CA) was used for LC separation for the shotgun proteomics analysis, and gradient elution was performed using an Ultimate 3000 nanoLC system (Thermo fisher Scientific, San Jose, CA) with a flow rate of 350 nL/min. The mobile phase A was 0.1% (v/v) formic acid in water and mobile phase B was 0.1% (v/v) formic acid in acetonitrile. The gradient elution was programmed to start at 2% B (v/v), increased to 10% B in 4 min, then increase to 35% B in 60 min and ramped up to 60% in 6 mins, followed by a

washing and equilibration step where solvent B increased to 95% in 5 min, was held for 10 mins, before being re-equilibrated to initial mobile phase conditions.

The LC eluate was diverted to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, San Jose, CA) operated in positive ion mode was used for analysis. MS/MS data was recorded in parallel in a data-dependent mode, whereby the top 10 most intense multiply-charged precursor ions were fragmented by collision-induced dissociation using 30% collision energy. Following a full scan in the mass range of *m/z* 350 to 1600, and MS/MS was performed with top speed mode.

Data Analysis. Progenesis Qi automatically aligned the MS raw data by selecting one of the LC-MS files as reference. Following the peak-picking step only features within 0−90 min retention time, mass to charge ratios of 350−1600 and charge states of +2, + 3, or +4 were considered for peptide statistics, analysis of variance (ANOVA) and principal component analysis (PCA). MS/MS spectra exported directly from Progenesis Qi in an mgf format were subsequently searched against a decoy database of proteome. The *Homo sapiens* proteome database was downloaded from Uniprot and Mascot 2.4.0 (Matrix Science) search engines were applied. The search criteria were set as follows: (1) trypsin specificity and up to 2 missed cleavage sites; (2) Cysteine carboxamidomethylation (+57.0215 Da) was set as a fixed modification, and methionine oxidation (+15.9949 Da), proline modification (+17.9564 Da) were set as a variable modification; (3) Precursor mass tolerance was set to 10 ppm within the calculated average mass, and fragment ion mass tolerance was set to 10 mmu of their monoisotopic mass. Peptide matches

that pass the filter associated with FDR < 0.01 were used for analysis. The results from the database search were imported back into Progenesis Qi for relative protein quantitation. For each protein, the average of the normalized abundances (obtained from Progenesis) from the triplicate analyses was calculated to determine the fold change between the treated and control samples. STRING was employed to predict the protein-protein interaction network of the proteins that were significantly changed with high confidence (Minimum required interaction score was set to 0.7).[20] DAVID was used for pathway enrichment analysis, with the EASE score set to 0.01.[21,22]

RESULTS AND DISCUSSION

Effect of Thioproline-Treatment on Cell Viability in Oxidizing Environment. With prior observation that SPro scavenges oxidative stress-generated nitric oxides[11-13] and our recent finding that SPro was generated in cells by cysteine reacting with oxidative stress-generated formaldehyde,[15-16] we hypothesized that the oxidative stress-induced generation of SPro may have protected cells from oxidative damages and improved cell viability. To test this hypothesis, we investigated whether a dosage-dependent protective effect of SPro to HeLa cells exist under oxidative environments.

To this end, we cultured HeLa cells with media containing different concentrations of SPro (0.1 to 1.0 mM). This concentration range was chosen because it does not affect cell proliferation and viability, but yet observed to provide protection again oxidative insults, as discussed below. After growing to 90% confluence, the SPro-containing media were removed and cells were incubated in PBS containing 1 mM Fe^{2+}-EDTA for 18 hours, or freshly prepared 100 μM NaOCl or 100 μM H_2O_2 for 6 h. Control groups were prepared using cells cultured in the same manner without adding SPro and separately treated with identical oxidizing agents and conditions. Cell viability was then determined using the trypan blue staining assay.

The decrease in mortality rate for cells grown in media supplemented with SPro compared to control groups that did not receive SPro, clearly indicated that SPro-pretreated cells can better tolerate oxidative stress induced by all three oxidants (**Figure 2**). This protective effect was also

found to be proportional to the dosage of SPro that the cells received. To the best of our knowledge, this is the first report on a cellular survival system that utilized cysteine to capture formaldehyde into a stable adduct that protected against formaldehyde-producing oxidants.

Quantitation of Thioproline in Cellular Proteins of Thioproline-Treated HeLa Cells. With the observation that SPro pretreatment increased the tolerant capacity of oxidative stress, we set to determine the molecular mechanism underlying. Since we had demonstrated in our previous study that SPro was produced and incorporated into cellular protein in formaldehyde and oxidants-exposed *E. coli* cells,[16] we first determined the exposure-duration-related and dosage-dependent incorporation of SPro into cellular proteins of SPro pretreated HeLa cells using our previously developed LC−MS/MS coupled with stable isotope dilution method,[15] as descried above. Depicted in **Figure 3A** is a typical chromatogram obtained from LC−MS/MS analysis of SPro in HeLa cells that were pretreated with 0.5 mM of SPro for 6 h.

As shown in **Figure 3B**, the analysis detected an increase in proteinic SPro concentration that was dependent on both the duration of exposure and the dosage of SPro. Evidence from our previous studies could not confirm whether these detected proteinic SPro in oxidative stress- and formaldehyde-exposed cells were a result of post-translational modification of cysteine residues by condensation with oxidative stress-generated formaldehyde, or a result of incorporating oxidative stress-generated SPro directly during protein synthesis.[16,17] In order to better distinguish the origin of proteinic SPro, we exposed HeLa cells to different concentrations of synthetic $^{13}C_3$-labelled SPro and observed a concentration-dependent incorporation of [$^{13}C_3$]-

SPro into cellular proteins (**Figure 3C**), while the concentrations of non-labelled SPro showed no significant difference between the [$^{13}C_3$]-SPro-treated and control cells. The detection of proteinic ^{13}C-labelled SPro in these results provided direct evidence that SPro were incorporated into cellular proteins during protein synthesis in the translation process. Despite that the underlying mechanisms have yet to be determined, the protein incorporation of SPro may have increased cell tolerance to oxidants.

Determination of GSHs in Thioproline Pretreated, Oxidants-Exposed HeLa Cells. In a parallel study, we investigated the potential of SPro to act as an antioxidant protecting cells from oxidative insult. In this regard, we employed the LC−MS/MS coupled with stable isotope-dilution method stated above, to determine the concentration ratios of the reduced (GSH) to oxidized (GSSG) forms of glutathione in the SPro-pretreated, oxidants-exposed HeLa cells (**Figure S3**).

As expected, analysis of the H_2O_2, NaOCl, and Fe(II)EDTA exposed cells with no SPro pretreatment detected a tremendous, and oxidant concentration-dependent decrease in GSH/GSSG ratio (**Figure 4**). Furthermore, results suggested that SPro pretreatment would offer a dosage-dependent anti-oxidative protection to oxidant-exposed cells, as revealed by the increasing GSH/GSSG ratios as the cells received higher concentrations of SPro, though the ratios are still below that of the control. After the SPro pretreatment step, SPro-enriched media was removed from the cells and SPro-free media was added in for oxidants exposure. Given that SPro treatment alone did not alter the GSH/GSSG ratio, as demonstrated in the control

experiment, it is possible that the cells received antioxidative protection from hydroxyl radicals from the sacrificial oxidation of SPro, or collectively by altering the activity of certain SPro-incorporated proteins and changing in protein expression, as will be discussed in the following section.

Interestingly, when the SPro-pretreated cells were exposed to hydroxyl radical generator, Fe(II)EDTA, the GSH/GSSG ratio increased significantly, which is higher than that of the control. This is probably attributed to the Ferric-chelate reductase, which convert $NADP^+$ to NADPH that facilitates the conversion of GSSG to GSH, as NADPH is the essential reducing agent in the conversion.[23] A similar phenomenon of an accelerating GSH/GSSG ratio was also observed when cells were exposed to Fe^{2+} ions, indicating that Fe^{2+} contributed to the dramatic elevation of GSH/GSSG ratio in the Fe(II)EDTA-exposed cells (**Figure S4**).

Quantitation of Oxidized Thioproline in Thioproline-Treated and Oxidants-Exposed HeLa Cells. Being an analogue of proline, thioproline is also a substrate for L-proline dehydrogenase and can be oxidized into *N*-formylcysteine *in vivo* (**Figure 1**).[18,24,25] With the observation that SPro increased the oxidizing tolerance of cells (**Figure 2**), we also determined the concentrations of *N*-formylcysteine (oxidized SPro) in toxicant-exposed cells. Result showed an oxidant concentration-dependent formation of *N*-formyl-cysteine in NaOCl-exposed cells (**Figure 5**), thus it is probable that SPro increased cell tolerance to oxidative stress also by being sacrificially oxidised to *N*-formyl-cysteine, as reported previously.[18,24,25]

It is worth-mentioning that a relatively high background concentration of *N*-formyl-cysteine was detected in the control cells that were not treated with SPro, at ~1 x 10^7 molecules per cell. This result showed that cells might have produced SPro by reacting endogenously generated formaldehyde with cysteine and had used it as a natural antioxidant protecting from oxidation stress. Furthermore, SPro-pretreatment produced a SPro concentration-dependent formation of *N*-formyl-cysteine in the NaOCl-exposed cells (**Figure 5**), which further corroborating the anti-oxidative effect of SPro to oxidant-exposed cells.

Comparative Proteomics Analysis. To differentiate potential proteome changes following SPro exposure, non-targeted proteomics study was conducted by coupling nano-LC to an orbitrap mass spectrometer. The acquired data were analyzed using a two-tailed Student's t-test to assess the significance of protein fold-changes. Proteins that are common in the replicate runs, having a fold-change > 2 and peptide count > 3 were considered to be significantly affected by the exposure. The analysis revealed a total of 117 protein's expressions were significantly altered, of which 107 proteins were upregulated in the SPro-treated group compared to the control group, and the remainders were downregulated (**Table S1**).

The differentially expressed proteins were then subjected to protein interaction network (**Figure 6A**) and functional enrichments (**Figure 6B**) analyses, as stated in the *Experimental Methods* section. Pathway enrichment analysis of the differentially expressed proteins revealed that pathways relating to cell-cell adhesion, translational initiation, rRNA processing, mRNA

splicing, regulation of apoptosis and protein folding were altered. In particular, cell-cell adhesion and protein folding functions were found to enhanced in the SPro-treated group.

Results showed SPro-exposure appear to have enhanced the expression of pyruvate kinase PKM, basigin, and calpastatin, three proteins which are involved in cell adhesion to the extracellular matrix. Because cell adhesion plays an important role in regulating cell survival and proliferation, the increased level of these proteins could be contributing to the improved cell survival in S-Pro treated cells.

The analysis also found an increased expression of proteins involved in the protein folding process, which may be necessitated since polypeptide chains with S-Pro are less likely to fold correctly due to thioproline's reduced conformational rigidity when compared with proline. [27,28] Proline family amino acid metabolic process proteins are also upregulated, which is reasonable as SPro could act as a surrogate for Pro in cells and stimulate related metabolic processes. Expression of aminoacyl-tRNA synthetases are also upregulated, which may be caused by the need to replace tRNAs misincorporated with S-Pro. [29,30] Moreover, it is reasonable to presume that SPro-incorporation would lead to many cellular proteins losing their biological activities, triggering the cell to compensate for them by producing more copies of these proteins. This may explain the observed increase in the expression of proteins involved in protein synthesis.[31-33]

Comparative Analysis of Antioxidant Capacity. After completed investigating the effectiveness of SPro treatment on increasing human cell's tolerance to oxidative stress-inducers and the underlying molecular mechanisms, we compared the antioxidant capacity of SPro *vs N*-acetyl-L-cysteine, one of the commonly used health supplement. Results show both SPro and *N*-acetyl-L-cysteine pre-treatments offered similar levels of protection to oxidative stress, as indicated by the similar GSH/GSSG ratios in toxicants-exposed HeLa cells (**Figure S5**). The combined effects of increasing cell tolerance to oxidative stress, together with the previously observed hepatoprotective, anti-cancer, and anti-aging properties,[6-8] revealed SPro as a potentially beneficial health supplement.

CONCLUSION

In summary, we discovered in this study for the first time that the oxidative stress-derived SPro, formed by reacting oxidative stress-generated formaldehyde with cysteine, protected cells from oxidative insults. Investigation on the molecular mechanism of the observed increase in tolerance to oxidative stress revealed SPro acted as a highly efficient antioxidant by sacrificial oxidation and by inducing changes in protein composition and expression. It is possible that the use of SPro as dietary supplement may protect human against oxidative stress-associated human diseases, such as Alzheimer's disease, atherosclerosis, cancers, and Parkinson's disease.

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Notes

The authors declare no competing financial interest.

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Supporting Information Available

Typical chromatogram obtained from the HPLC analysis of thioproline after incubating thioproline in H_2O_2 at 37 °C, together with the spectra from high resolution MS and MS/MS analyses of the oxidation product of thioproline; Chromatogram obtained from LC−MS/MS analysis of GSH and GSSG in HeLa cells. GSH/GSSG ratio in thioproline pre-treated Hela cells that were exposed to Fe^{2+}. Comparative analysis of the antioxidative capacity of thioproline and *N*-acetyl-cysteine. This material is available free of charge via the Internet at http://pubs.acs.org.

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Table 1 Multiple-Reaction Monitoring Mass Transitions used for the Quantitative and Qualitative Analyses of Proteinic Thioproline, $^{13}C_3$-L-thioproline, Oxidized Thioproline, and Cellular Glutathione in Cells.

	MRM transition		
	Quantitative	Qualitative	Internal standard
Thioproline	134→88	134→59	137→91
$^{13}C_3$-L-thioproline	137→90	137→61	N/A
Oxidized thioproline [a]	150→132	150→105	125→108 [b]
Glutathione, reduced	308→179	308→76	311→182
Glutathione, oxidized	613→484	613→355	619→490

[a] *N*-formyl-cysteine, analysis performed on a HILIC column and quantitated using cysteine as surrogate; [b] $^{13}C_3$-cysteine was used as internal standard.

FIGURE LEGEND

Figure 1. Summary scheme of how oxidative stress-derived thioproline by reacting formaldehye with cysteine protects cells from oxidative insult by acting as an antioxidant.

Figure 2. Mortality rate of thioproline treated, oxidants exposed HeLa cells. Thioproline pre-treated Hela cells were incubated at 37°C with (A) 100 µM H_2O_2, (B) 100 µM NaOCl, and (C) 1 mM Fe^{2+}-EDTA, cell viability determined by trypan blue staining assay. The data represent mean ± standard deviation of results obtained from three independent experiments. Student's t-test at 95% confidence interval was conducted to determine the mortality rate of at increasing thioproline concentration. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 3. (A) Typical chromatogram obtained from the LC−MS/MS analysis of thioproline in cellular protein isolated from HeLa cells cultured in thioproline supplied culture media. Shown in the insets are the MS/MS spectrum of the $[M+H]^+$ ion of the thioproline and the cleavage reactions for the formation of major fragment ions found in MS/MS analysis. (B) Thioproline concentration- (0, 0.5 mM, and 1 mM) and thioproline exposure duration- (2, 4, 6, and 9 days) dependent incorporation of thioproline in cellular protein in HeLa cells cultured in thioproline supplied culture media. Together with the concentrations of thioproline and $^{13}C_3$-thioproline in cellular protein of HeLa cells cultured in $^{13}C_3$-thioproline supplied culture media (C). The data represent mean ± standard deviation of results obtained from three independent experiments.

Figure 4. GSH/GSSG ratio in thioproline pre-treated Hela cells incubated with different concentrations of (A) H_2O_2, (B) NaOCl, and (C) Fe^{2+}-EDTA as determined by LC−MS/MS analysis of intracellular fluid of the toxicants-exposed cells. ■ control; ● 10 µM; and ▲ 20 µM.

The data represent mean ± standard deviation of results obtained from three independent experiments.

Figure 5. Concentration of *N*-formyl-L-cysteine in thioproline pre-treated Hela cells incubated with different concentrations of NaOCl, as determined by LC−MS/MS analysis of intracellular fluid of the toxicants-exposed cells. ■ control; ● 10 μM; and ▲ 20 μM. The data represent mean ± standard deviation of results obtained from three independent experiments.

Figure 6. Differentially protein expression in the thioproline-treated human cell, as depicted in their interaction networks by STRING (A). Each circle represents one protein found to be differentially expressed in the presence of thioproline. And classification of the altered proteins based on their cell functions (B).

Figure 1

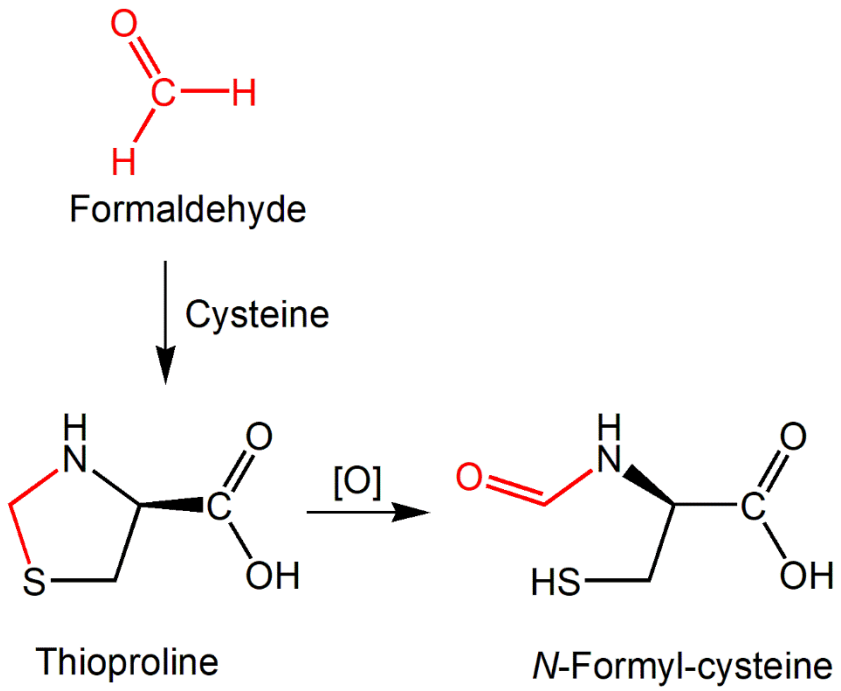

O
C
H
H
Formaldehyde
Cysteine
H
N
O
C
S
OH
[O]
O
H
N
O
C
HS
OH
Thioproline
N-Formyl-cysteine

Figure 2

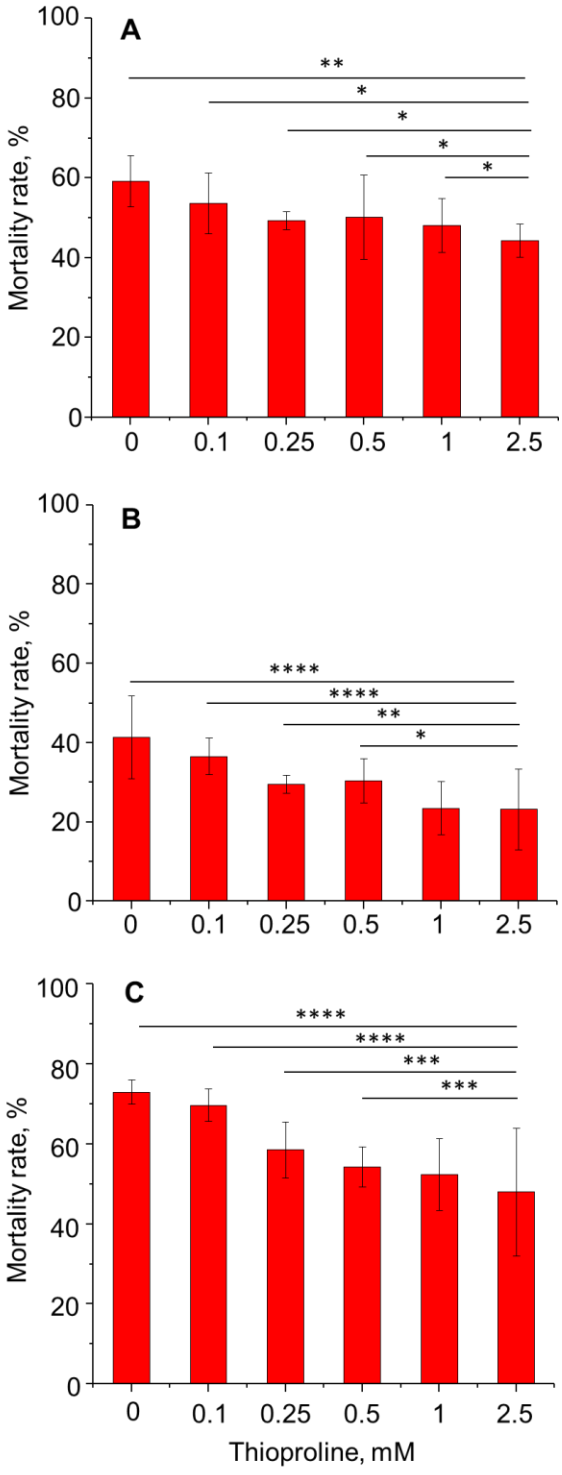

A
B
C
Mortality rate, %
Thioproline, mM
0
0.1
0.25
0.5
1
2.5
20
40
60
80
100
**
*
****
***

Figure 3

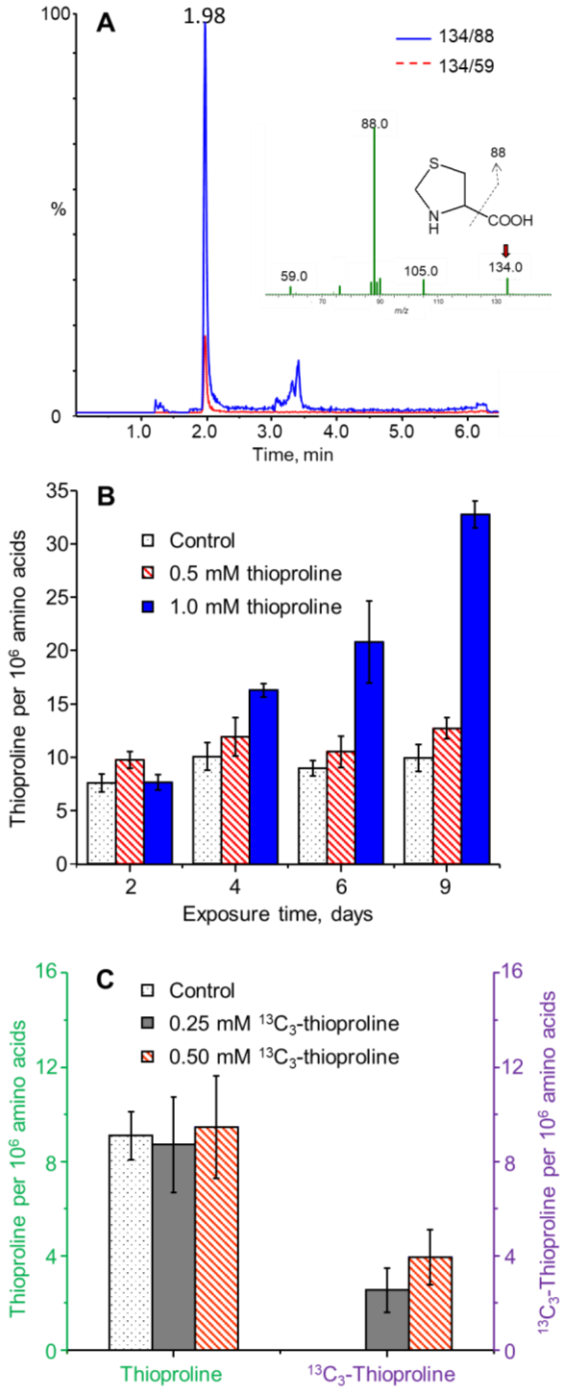

A
1.98
134/88
134/59
88.0
88
COOH
59.0
105.0
134.0
100
%
0
1.0
2.0
3.0
4.0
5.0
6.0
Time, min
B
Control
0.5 mM thioproline
1.0 mM thioproline
Thioproline per 10^6 amino acids
35
30
25
20
15
10
5
0
2
4
6
9
Exposure time, days
C
Control
0.25 mM 13C3-thioproline
0.50 mM 13C3-thioproline
Thioproline per 10^6 amino acids
13C3-Thioproline per 10^6 amino acids
16
12
8
4
0
Thioproline
13C3-Thioproline

Figure 4

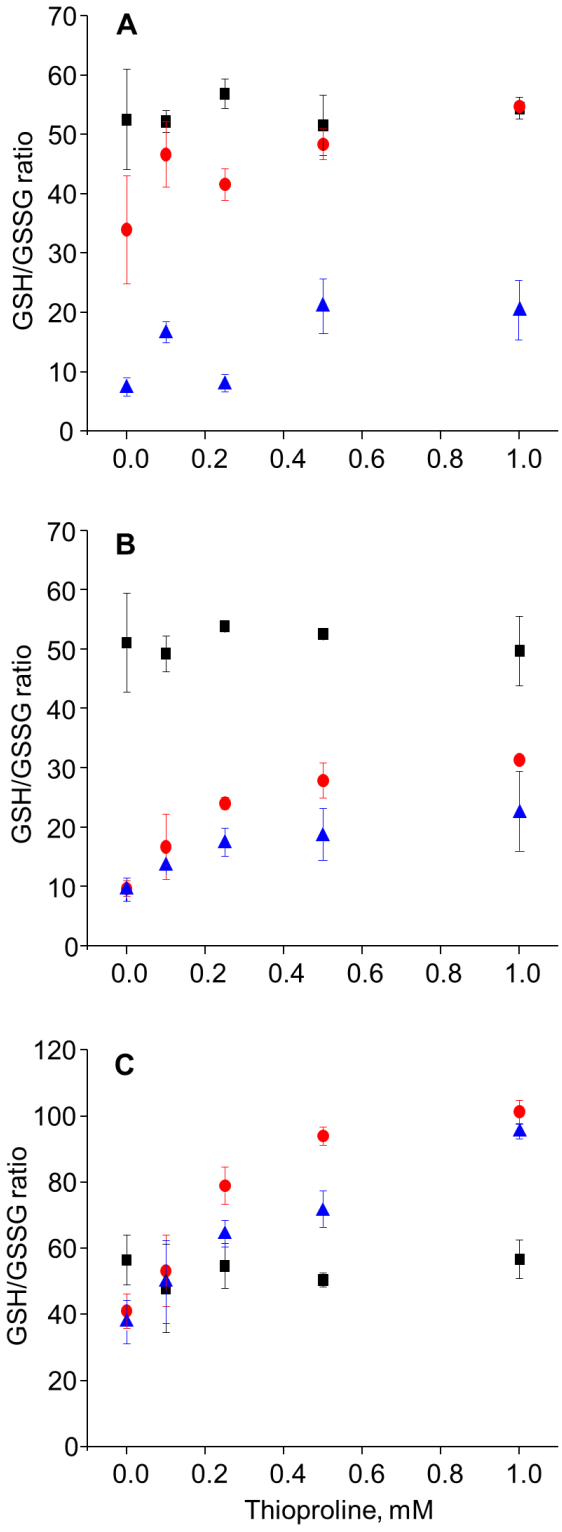

A
B
C
GSH/GSSG ratio
GSH/GSSG ratio
GSH/GSSG ratio
0
10
20
30
40
50
60
70
80
100
120
0.0
0.2
0.4
0.6
0.8
1.0
Thioproline, mM

Figure 5

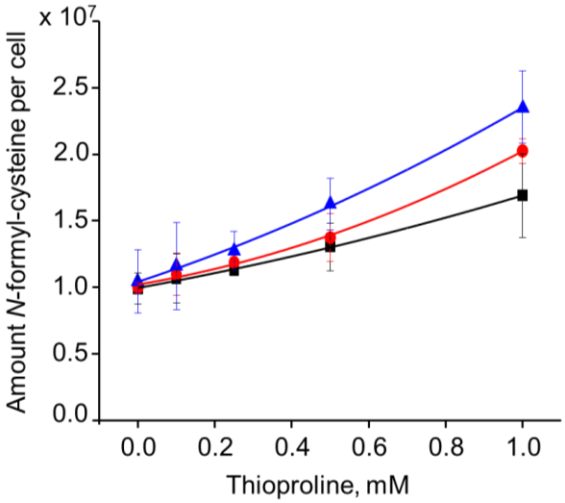
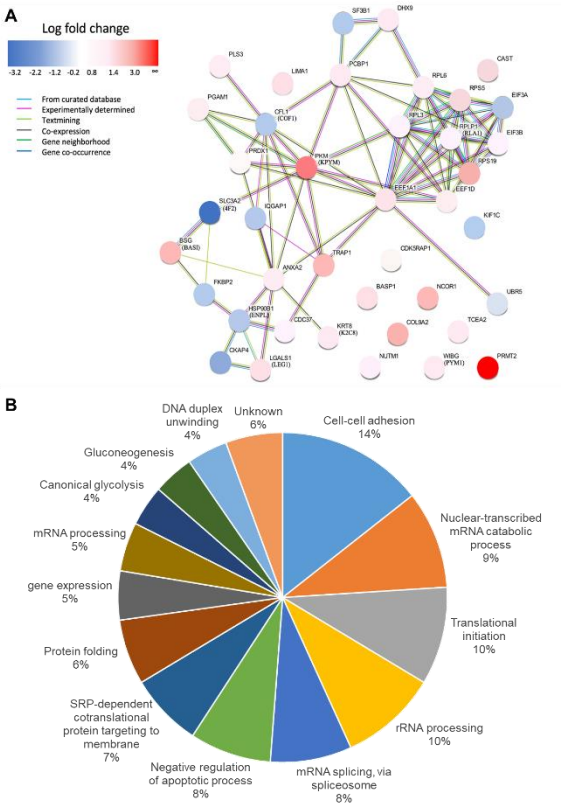

x 10^7
Amount N-formyl-cysteine per cell
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.2
0.4
0.6
0.8
1.0
Thioproline, mM

Figure 6


A
Log fold change
-3.2 -2.2 -1.2 -0.2 0.8 1.4 3.0
From curated database
Experimentally determined
Textmining
Co-expression
Gene neighborhood
Gene co-occurrence
B
DNA duplex unwinding 4%
Unknown 6%
Cell-cell adhesion 14%
Gluconeogenesis 4%
Canonical glycolysis 4%
Nuclear-transcribed mRNA catabolic process 9%
mRNA processing 5%
gene expression 5%
Translational initiation 10%
Protein folding 6%
rRNA processing 10%
SRP-dependent cotranslational protein targeting to membrane 7%
mRNA splicing, via spliceosome 8%
Negative regulation of apoptotic process 8%