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Proline modulates the intracellular redox environment and protects mammalian cells against oxidative stress

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Abstract

The potential of proline to suppress reactive oxygen species (ROS) and apoptosis in mammalian cells was tested by manipulating intracellular proline levels exogenously and endogenously by overexpression of proline metabolic enzymes. Proline was observed to protect cells against H_2O_2, *tert*-butyl hydroperoxide and a carcinogenic oxidative stress inducer but was not effective against superoxide generators such as menadione. Oxidative stress protection by proline requires the secondary amine of the pyrrolidine ring and involves preservation of the glutathione redox environment. Overexpression of proline dehydrogenase (PRODH), a mitochondrial flavoenzyme that oxidizes proline, resulted in 6-fold lower intracellular proline content and decreased cell survival relative to control cells. Cells overexpressing PRODH were rescued by pipecolate, an analog that mimics the antioxidant properties of proline, and by tetrahydro-2-furoic acid, a specific inhibitor of PRODH. In contrast, overexpression of the proline biosynthetic enzymes Δ^1-pyrroline-5-carboxylate (P5C) synthetase (P5CS) and P5C reductase (P5CR) resulted in 2-fold higher proline content, significantly lower ROS levels and increased cell survival relative to control cells. In different mammalian cell lines exposed to physiological H_2O_2 levels, increased endogenous P5CS and P5CR expression was observed indicating upregulation of proline biosynthesis is an oxidative stress response.

Keywords

proline; proline oxidation; proline biosynthesis; reactive oxygen species (ROS); oxidative stress protection

Introduction

A hypothesis emerging from proline metabolic studies on plants, fungi, and mammals, is that proline may have opposing effects on the intracellular redox environment. In eukaryotes, proline is oxidized to glutamate by two mitochondrial enzymes, proline dehydrogenase (PRODH) (also known as proline oxidase) and Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH). The PRODH flavoenzyme catalyzes the rate-limiting two-electron oxidation of proline to Δ^1-pyrroline-5-carboxylate (P5C) and the subsequent transfer of reducing

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equivalents from the reduced flavin cofactor to the mitochondrial electron transport chain [1]. P5C is then converted to glutamate in a NAD^+-dependent reaction catalyzed by P5CDH [2]. Phang and others have shown that in mammalian cells PRODH expression is activated by p53 with proline oxidation leading to increases in intracellular reactive oxygen species (ROS) and induction of cellular apoptosis in various cancer cell lines [3–9]. Thus, proline serves as a pro-oxidant in apoptotic signaling via PRODH activity which may be an important mechanism for reducing carcinogenesis [6].

Proline also has an established role in defending against various abiotic and biotic stresses with its properties as a compatible solute benefiting a broad-range of organisms [10–20]. Proline has been shown to diminish aggregation of the polyQ region of the huntington protein suggesting a preventive role of proline in neurodegenerative diseases [19]. Besides its natural osmolyte properties, proline appears well suited to combat ROS, which is frequently elevated in stressful environments. In plants, proline accumulation has been shown to protect against free radicals and UV stress [21,22]. We have shown that proline acts as an antioxidant in the fungal pathogen *Colletotrichum trifolli* and in *Sacchromyces cereviseae* [23,24]. The ability of proline to protect skin cells against photooxidative damage from UVA generated singlet oxygen ($^1O_2$) has also been reported [25]. Thus, proline may be a broad-based antioxidant, a property which has been relatively unexplored in mammalian systems.

Here we examine the effects that proline metabolism has on intracellular ROS levels and the ability of proline to protect mammalian cells against oxidative stress and prevent apoptosis. Endogenous proline levels were differentially modulated in HEK 293 cells by upregulating PRODH and the proline biosynthetic enzymes, P5C synthetase (P5CS) and P5C reductase (P5CR, encoded by the *P5CR2* gene). The mitochondrion enzyme P5CS converts glutamate to P5C which is then reduced to proline in the cytosol by the NADPH-dependent enzyme P5CR [26,27]. Our results suggest that the intracellular accumulation of proline is an adaptive stress response that affords oxidative stress protection in certain mammalian cells.

Experimental procedures

Materials and constructs

Unless stated otherwise, all chemicals, enzymes and buffers were purchased from Fisher Scientific and Sigma-Aldrich, Inc. HEK 293 cells (human embryonic kidney cell line transformed with SV40), HeLa cells (cervical cancer cell line), HepG2 cells (hepatocellular liver carcinoma cell line), and Jurkat cells (T lymphocytes) were obtained from the American Tissue Type Collection. BJAB cells (Burkitt's lymphoma cell line) were provided by Prof. Charles Wood at the University of Nebraska-Lincoln. The cDNA clones of the *PRODH* and *P5CS* (short isoform) genes were generously provided by Prof. Bert Vogelstein at John Hopkins Oncology Center and Prof. Andy Hu at the University of New Mexico Medical School, respectively. Human brain cDNA library for cloning the *P5CR2* gene was purchased from Clontech. Sequence-specific synthetic oligonucleotides were purchased from Integrated DNA Technologies. Vectors pcDNA3.1, pFlag-CMV3 and pIRES2-EGFP were from Novagen. The *PRODH*, *P5CS*, and *P5CR2* genes were inserted into pcDNA3.1 (pcDNA3.1-PRODH, pcDNA3.1-P5CS, and pcDNA3.1-P5CR) for overexpression in HEK 293 cells. The *PRODH*, *P5CS*, and *P5CR2* genes were also subcloned into vectors pFlag-CMV3 and pIRES2-EGFP for Western blot analysis and for evaluation of transfection efficiencies by flow cytometry, respectively. Further details about the cloning procedures and the primers used are provided in the Supplementary Materials.

Enzyme overexpression, PRODH purification and assays

HEK 293, HeLa, and HepG2 cell lines were propagated in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Invitrogen) at 37 °C in 10 cm plates. Jurkat and BJAB cells were cultured using Roswell Park Memorial Institute medium with 10% fetal calf serum in 25 cm^2 culture flasks at 37 °C. For transfection of HEK 293 cells, a 10 cm plate of confluent HEK 293 cells was incubated with 2 μg of vector DNA (empty or containing PRODH, P5CS, or P5CR) and 3 μl of FuGENE 6 Transfection Reagent (Roche Applied Bioscience) in growth medium for 24 h. Transfection experiments were performed with PRODH, P5CS, and P5CR enzymes expressed as non-tagged proteins using the pcDNA3.1 vector unless stated otherwise. Overexpression of PRODH, P5CS, and P5CR in HEK 293 was confirmed by Western Blot analysis. For Western blot analysis, PRODH, P5CS, and P5CR were expressed as C-terminal flag-fusion proteins in HEK 293 using the pFlag-CMV3 vector. After transfection, cells were lysed by vortexing gently for one minute in ice-cold buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, and 0.2% Triton X-100), unless otherwise indicated, and the lysates were centrifuged for 10 min at 16,000 × *g* at 4 °C. Total protein concentration in the soluble portion of the cell lysates was determined using bicinchoninic acid (Pierce) and bovine serum albumin as a standard. Western blot analysis of cell extracts (10 μg) was performed as previously described except that the immunoreactive bands were detected using a monoclonal anti-flag fluorescein isothiocyanate (FITC) conjugate (Sigma-Aldrich) and were visualized using a LI-COR Odyssey Imager [24].

Transfection efficiencies were determined by flow cytometry using the pIRES2-EGFP vector in which GFP is coexpressed with PRODH, P5CS, or P5CR. Transfection efficiencies averaged between 70–80% for all of the proline metabolic gene constructs. In addition, expression of the pIRES2-EGFP-PRODH, pIRES2-EGFP-P5CS and pIRES2-EGFP-P5CR constructs in HEK 293 cells resulted in cell survival rates that were similar to those observed using the pcDNA3.1 vector without GFP (see Supplementary Fig. 1S and Fig. 4B).

Recombinant human full-length PRODH was expressed with an N-terminal 6xHis tag in *E. coli* strain BL21(DE3) using a pET14b-PRODH construct in the presence of 0.5 mM IPTG at 20 °C for 10 h. Cells were resuspended in potassium phosphate buffer (pH 7.4) containing 50 mM KCl, 20% glycerol, 20% sucrose, and 4 mM imidazole and broken by sonication as previously described [28]. PRODH was then partially purified by Ni-NTA chromatography using previously described methods and stored at 4 °C in potassium phosphate buffer containing 100 mM KCl, 10% glycerol, and 10% sucrose [28]. PRODH activity was detected using the proline:dichloroindophenol (DCPIP) oxidoreductase assay as previously described [29]. Proline:O_2 activity was determined by following the reduction of cytochrome c at 550 nm and the production of P5C in air-saturated 20 mM MOPS (pH 7.5) buffer as previously described [30,31]. P5C was detected by adding *o*-aminobenzaldehyde (*o*-AB) (4 mM final concentration) to the proline:O_2 assay mixture and following the formation of the *o*-AB-P5C yellow complex at 443 nm (ε = 2,900 M^{-1} cm^{-1}) [30,32].

Stress treatment and ROS measurements

For stress treatments, mammalian cells were grown to about 80% confluence. Cells were then treated for 3 h with 0.5–2.5 mM H_2O_2 in the absence and presence of 5 mM proline. H_2O_2 concentrations were chosen so that control cells exhibited 50% cell survival after stress treatment. For testing the protective ability of other amino acids and proline analogs, HEK 293 cells were treated for 3 h with 0.5 mM H_2O_2 in the absence and presence of each reagent (5 mM). HEK 293 cells were also stressed with *tert*-butyl hydroperoxide (tBH) (0–10 mM), and 50 μM each of menadione, methyl viologen and fumonisin for 3 h in the absence and presence of proline (5 mM). HEK 293 cells overexpressing PRODH, P5CS, and P5CR were incubated with and without H_2O_2 (0.5 mM) for 3 h at 37 °C after 24 h of transfection. Percent cell survival

after stress treatment was estimated by counting live and dead cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the recommendations of the manufacturer (Promega). In initial stress studies with HEK 293 cells, cell death was also observed by staining dead cells with Trypan blue after the H_2O_2-treated cells were washed with phosphate-buffered saline (PBS) solution (pH 7.4). To evaluate potential stress responses of *P5CR*, *P5CS* and *OAT* gene expression HEK 293 cells were exposed to a continual 20 μM H_2O_2 concentration for 0–24 h at 37 °C. Increased concentrations of H_2O_2 (20–100 μM) were also imposed on HEK 293 cell cultures for evaluating changes in P5CR and P5CS expression. Changes in *P5CR*, *P5CS*, and *OAT* gene expression were also evaluated in HeLa, HepG2, BJAB, and Jurkat cell lines by exposing cell cultures to H_2O_2 (100 μM) for 0–24 h at 37 °C. H_2O_2 concentrations were maintained by measuring H_2O_2 levels as previously described and supplementing cultures using a 0.5 M H_2O_2 stock concentration [28].

Intracellular ROS levels were evaluated by fluorescence microscopy (see Supplementary Material) and flow cytometry. After oxidative stress treatment as described above, cells (5×10^5) were washed with PBS and incubated with dichlorodihydrofluorescein diacetate (DCHF-DA) (100 μM) for 15 min in the dark at room temperature. Cells were then washed twice with PBS to remove excess DCHF-DA and resuspended in PBS containing 10 mM EDTA. The cells were then analyzed using FACScan flow cytometry (Becton-Dickinson, San Jose, CA). The mean fluorescence channel of each sample was calculated using FACS-equipped CELLQUEST^R software and is reported as the percent fluorescence observed in the live cell population. In each assay, matching cells not treated with DCHF-DA served as a control. The M1 line was set at 0.5% fluorescence using the control cells (untreated) so that any signals < 0.5% were considered as background fluorescence. Signals to the right of the M1 line were thus considered to be due to DCF fluorescence. The number of cells counted in the flow cytometry experiments ranged from 5300–7400 cells.

DNA fragmentation

HEK 293 cells were treated with H_2O_2 (1 mM) in the absence and presence of 5 mM proline and were collected by centrifugation and washed twice with cold PBS. The cell pellets were resuspended in 0.4 ml of 100 mM Tris-HCl (pH 8.5) containing 0.2% SDS, 0.2 mg/ml proteinase K and 5 mM EDTA and incubated at 37 °C for 16 h. The nuclear debris was then removed by centrifugation and the nucleic acids were precipitated using established protocols. DNA samples were analyzed by non-denaturing agarose gel electrophoresis (1.5%) and visualized by ethidium bromide staining.

Proline, P5C, GSH, and GSSG measurements

For measuring intracellular proline and P5C content, HEK 293 cells were washed twice with PBS, pelleted by centrifugation, and resuspended in 0.5 ml of PBS with 0.1% Triton X-100. Cells were then lysed by vortexing and the cellular debris was removed by centrifugation. Proline levels in HEK 293 cell lysates were determined as previously described using the acid-ninhydrin method and 200 μl of the resulting supernatant [24,33]. Proline concentrations were estimated using a proline standard curve of 0 – 3 mM proline. The reported proline levels are the concentration of proline in the final toluene extract and are an average value of three independent determinations. P5C levels were estimated in the lysed cell supernatants by detecting the yellow dihydroquinazolinium complex with *o*-AB at 443 nm [32]. For glutathione measurements, ~ 10^6 cells were resuspended in PBS and lysed with metaphosphoric acid. Intracellular glutathione (GSH) and glutathione disulfide (GSSG) levels were measured by HPLC (Bondapak amine column) using a previously described method for quantitating free thiols in cell extracts [34,35].

RNA isolation and quantitative reverse transcription PCR (RT-PCR)

Mammalian cells were incubated with and without H_2O_2 for 24 h at 37 °C as described above. For HEK 293, HeLa, and HepG2 cell cultures, about 10^6 cells were grown as a monolayer on 10 cm plates and directly lysed at different time points (0, 3, 6, 9, 12, and 24 h) during the incubation. BJAB and Jurkat cells (~ 10^6 cells) were collected by centrifugation (3000 × *g*) prior to being lysed. Total RNA was then isolated according to the recommendation of the manufacturer (Invitrogen) and used to generate a cDNA pool by RT-PCR. The resulting cDNA served as a template for PCR amplification of the *P5CS*, *P5CR2* and *OAT* cDNA products. Relative changes in gene expression were estimated using Quantity One 4.6.1 software and by normalizing P5CR, P5CS, OAT products to control GAPDH levels. More information about the experimental conditions and PCR primers can be found in the Supplementary Material.

Results

Proline protects against H_2O_2-induced cell death

The protective role of proline was first investigated by examining whether proline supplementation could suppress H_2O_2 induced cell death and apoptosis in mammalian cell cultures. Treating HEK 293 cells with H_2O_2 (1 mM, 3 –6 h) causes significant cell death and DNA fragmentation which is a characteristic feature of apoptosis (Fig. 1 and see Supplementary Fig. 2S) [36]. Fig. 1 shows that when HEK 293 cells are treated with H_2O_2 for 3 and 6 h, the genomic DNA is cleaved resulting in the formation of a DNA ladder. When cell cultures are supplemented with proline, however, HEK 293 cells are protected from H_2O_2 induced cell death and no DNA fragmentation is observed (Fig. 1). TUNEL assays also provided evidence that proline prevents apoptosis as H_2O_2 treated HEK 293 cells exhibited a high population of TUNEL positive cells indicative of DNA fragmentation while in the presence of proline no TUNEL positives were detected (see Supplementary Fig. 3S). Thus, proline suppresses H_2O_2 induced cell death and apoptosis. Survival of various cell lines after H_2O_2 stress treatment was then estimated by MTT assays. In HEK 293 cells, cell survival after exposure to H_2O_2 (0.5 mM, 3 h) was 39% in the absence of proline and 77% in the presence of 5 mM proline. If the H_2O_2 and proline were removed after the 3 h stress treatment and cells were returned to normal medium for 24 h, cell survival rates increased to ~ 57% and ~ 82% in untreated and proline treated cells, respectively, indicating HEK 293 cells are able to recover after acute H_2O_2 stress. In HepG2 and HeLa cells exposed to H_2O_2 (1 mM, 3 h) the survival rate increased from 55% in control cells to around 77% in cell cultures supplemented with proline (5 mM). Protection was also observed in BJAB and Jurkat cells (2–2.5 mM H_2O_2, 3 h) with control cells exhibiting about 50% survival rates while cells supplemented with proline had survival rates of 67% (BJAB) and 76% (Jurkat cells). We also evaluated a longer stress period with HEK 293 cells using a lower H_2O_2 concentration (0.3 mM) for 24 h in the absence and presence of proline. Cell survival rates were 39 ± 4% and 57± 2% in the absence and presence of proline, respectively. The 1.5-fold protection provided by proline was slightly lower than the 2-fold protection observed with the 3 h H_2O_2 (0.5 mM) stress treatment.

Fig. 2 shows the concentration dependence of proline protection against H_2O_2 stress in HEK 293 cells. Protection of HEK 293 cells was significantly dependent on proline concentration from 0 – 1.0 mM proline. At 0.5 mM proline in the external medium proline, survival of HEK 293 cells increased to 65%. The concentration of proline in human plasma is normally around 0.2 – 0.3 mM and in type I hyperprolinaemia plasma proline levels have been reported up to 2.2 mM [37]. Therefore, proline concentrations in the medium at which protection against H_2O_2 is observed are near physiological levels.

The ability of proline to protect against oxidative stress was then compared with other amino acids. Survival of HEK 293 cells after H_2O_2 treatment (0.5 mM, 3 h) in the absence and

presence of each amino acid was quantitated by MTT assays (see Table 1). Cysteine and tryptophan supplementation were observed to increase cell survival (66–68%) to nearly the same level as proline (77%). Other amino acids affording HEK 293 cells protection against H_2O_2 exposure included threonine, histidine, and methionine but the protection (57–59% cell survival) is less than that observed with proline (Table 1). Thus, amino acids proline, cysteine, and tryptophan appear to be the most potent at combating H_2O_2 stress in HEK 293 cell culture.

Proline was also tested for its protective ability against other oxidative stress agents (see Supplementary Table 1S). HEK 293 cells were treated with increasing concentrations of the organic peroxide, tBH. 50% cell death was observed at 0.9 mM and 3.3 mM tBH in the absence and presence of proline, respectively (data not shown). Thus, proline afforded nearly a 4-fold increase in resistance to tBH induced oxidative stress. Proline was also observed to protect HEK 293 cells against fumonisin treatment (50 μM, 3h), a carcinogenic mycotoxin that induces intracellular oxidative stress in mammalian cells, with cell survival rates of 51% and 88% in the absence and presence of proline, respectively [38]. Proline, however, did not protect HEK 293 cells against methyl viologen or menadione which generates intracellular superoxide radicals. Tiron, a superoxide scavenger, did protect HEK 293 cells against menadione (50 μM, 3 h).

The increased cell survival and suppression of apoptosis elicited by proline correlates with lower intracellular ROS levels. Flow cytometry analysis of H_2O_2-treated cells in Fig. 3 show that in the absence of proline, cells exhibit 55% fluorescent positives while in cells supplemented with proline the fluorescence levels are 10%. Control cell populations without H_2O_2 treatment exhibited ~ 6% fluorescent positives.

Proline analogs and protection of GSH levels

Various proline analogs were tested for the ability to protect HEK 293 cells against H_2O_2 stress. Table 2 shows the survival rates of HEK 293 cells supplemented with different proline analogs. Except for L-tetrahydro-2-furoic acid (L-THFA), each of the analogs share the secondary amine of proline which is thought to be a key feature for its reactivity with •OH species [39]. The ionization potentials of the secondary amine in each analog are expected to be close to that of proline except for azetidine-2-carboxylic acid which has a smaller heterocyclic ring structure and therefore a slightly higher ionization potential than proline [40]. Pipecolate and thiazolidine-4-carboxylate both confer significant protection similar to L-proline (80–83% survival rates). These findings agree with a previous study that reported thiazolidine-4-carboxylate can function as an antioxidant [41]. D-proline, L-azetidine-2-carboxylic acid, and 4-hydroxyproline provided only a modest increase in survival rates (53–59%) relative to cells without any supplement (45%). The lower protective ability of D-proline relative to L-proline is likely due to the stereoselectivity of the mammalian IMINO transporter, indicating that the scavenging of ROS by proline is largely an intracellular process [42]. L-THFA offered no oxidative stress protection which confirms the necessity of the secondary amine of proline in ROS scavenging.

We next tested how glutathione levels are impacted by proline during H_2O_2 stress treatment. Intracellular GSH and GSSG levels were measured in HEK 293 cells after treatment with 0.5 mM H_2O_2 for 3 h (37 °C) in the absence and presence of 5 mM proline. Glutathione was also measured in control cells incubated for 3 h without H_2O_2 treatment. The intracellular GSH levels decreased from 61 ± 2 nmol/mg of protein in control cells to 35 ± 2 nmol/mg of protein in cells exposed to H_2O_2. The intracellular GSH/GSSG ratio correspondingly decreased from 46 ± 8 in control cells to 12 ± 2 in cells under oxidative stress. Adding 5 mM proline to cells under H_2O_2 stress maintained the GSH/GSSG ratio at 47 ± 11 with GSH at 60 ± 4 nmol/mg of protein. Thus, proline protects the intracellular GSH pool and the GSH/GSSG redox status of the cell under oxidative stress.

Down-regulation of proline levels

Endogenous proline levels in HEK 293 cells were decreased by overexpressing PRODH, the rate limiting enzyme of proline catabolism. Overexpression of PRODH in HEK 293 cells was confirmed by Western blot analysis (Fig. 4A). Overexpression of PRODH in different cancer cell lines was previously shown to generate ROS and reduce cell viability [8]. HEK 293 cells expressing PRODH also exhibit diminished cell viability with ~ 68% survival compared to control cells (98%) transfected with the empty pcDNA3.1 vector (see Fig. 4B). PRODH transfected cells were more sensitive to oxidative stress with a cell survival rate of about 10% after H_2O_2 exposure relative to controls cells which exhibited a 53% survival rate after stress treatment (Fig. 4B). HEK 293 cells overexpressing PRODH were characterized by 6-fold lower intracellular proline content (0.03 ± 0.002 mM) than control cells (0.18 mM proline) (Fig. 4C). Correspondingly, intracellular levels of P5C increased nearly 6-fold in PRODH transfected cells (56 ± 2 μM) relative to control cells (< 10 μM). In addition, fluorescence microscopy showed ROS levels were elevated in HEK 293 cells expressing PRODH compared to control cells (see Supplementary Fig. 4S). Fig. 5 shows that after H_2O_2 exposure, a significant population of the PRODH transfected cells are fluorescent positive (90%) consistent with the lower stress tolerance observed in Fig. 4B.

Superoxide formation by PRODH and rescue of PRODH toxicity

PRODH overexpression may have a dual effect on the viability of HEK 293 cells due to depletion of proline and intrinsic ROS generation. Previously, Phang's group showed that overexpression of PRODH in mammalian cells leads to toxic levels of superoxide anion radicals and that coexpression of mitochondrial superoxide dismutase (SOD) diminishes this effect [43]. During proline oxidation, superoxide may be formed directly by PRODH during catalytic turnover or indirectly by downstream events in the mitochondrial electron pathway. To distinguish these two possibilities, we tested whether superoxide is generated during PRODH catalytic turnover with proline using a preparation of recombinant human PRODH expressed from *E. coli.* SDS-PAGE analysis of recombinant PRODH shows a major protein band of about 68-kDa consistent with the calculated molecular weight of full-length PRODH (see Fig. 6A). Mass spectral analysis also confirmed the identity of the band as PRODH. A specific activity of 0.1 U/mg was determined for recombinant PRODH using the proline:DCPIP oxidoreductase assay with a K_m value for proline of 15 ± 2 mM. The ability of PRODH to react with molecular oxygen and potentially generate superoxide anion during catalytic turnover was next assessed. Catalytic turnover with proline using oxygen as an electron acceptor was observed with PRODH by monitoring the formation of P5C and by following the reduction of cytochrome c at 550 nm. Proline:O_2 activity of PRODH was similar to the proline:DCPIP oxidoreductase activity with a specific activity of around 0.09 U/mg. Formation of superoxide anion during PRODH catalytic turnover with proline and molecular oxygen was determined by including SOD in the cytochrome c reduction assay. As shown in Fig. 6B, PRODH activity leads to the reduction of cytochrome c at 550 nm while the addition of SOD blocks cytochrome c reduction demonstrating the production of superoxide. L-THFA, a competitive inhibitor of the PRODH homolog from *Escherichia coli*, PutA, also prevents cyctochrome c reduction indicating that L-THFA is an inhibitor of human PRODH and that superoxide is formed at the active site (Fig. 6B, inset) [44]. These results are consistent with a previous study of PRODH from *Thermus thermophilus* which was also shown to generate superoxide during catalytic turnover and is thought to be an important model for understanding human PRODH [45]. To our knowledge, this is the first characterization of purified human PRODH and the in vitro assays demonstrate that superoxide is generated directly during catalytic turnover with proline. Thus, PRODH activity can cause immediate oxidative changes in the cellular environment by simultaneously depleting intracellular proline and producing superoxide.

We then attempted to rescue cells from PRODH toxicity by supplementing cell cultures with 5 mM L-THFA. Fig. 7 shows that L-THFA dramatically increases the survival of cells overexpressing PRODH during H_2O_2 stress to ~ 76% relative to cells without L-THFA (~16% survival). Because L-THFA does not afford H_2O_2 stress protection to HEK 293 cells (see Table 2), the protective effect of L-THFA is due to the inhibition of proline-dependent superoxide generation by PRODH as shown in Fig. 6B. We next tested whether PRODH expressing cells could be rescued with a proline analog that exhibits the same ROS scavenging properties of proline. DL-pipecolate, which is neither a substrate nor an inhibitor of PRODH, was found to protect HEK 293 cells against H_2O_2 stress similarly to proline (see Table 2). Fig. 7 shows that pipecolate increases the H_2O_2 stress survival of HEK 293 cells overexpressing PRODH by > 2-fold (~ 38%) relative to cells without pipecolate. Thus, providing proline-depleted cells (Fig. 4C) with an antioxidant analog of proline partially rescues HEK 293 cells from PRODH induced toxicity. These results indicate that PRODH exerts a dual impact on the intracellular redox environment by simultaneously generating superoxide radicals and reducing proline levels.

Up-regulation of proline levels

The cytoprotective benefits of increasing intracellular proline levels were explored by overexpression of P5CS and P5CR in HEK 293 cells. Overexpression of P5CS and P5CR was confirmed by Western blot analysis (Fig. 4A). Unlike the results with PRODH, HEK 293 cells were more tolerant to overexpression of P5CS and P5CR with survival rates of 85–90% (Fig. 4B). Proline content in P5CS overexpressing HEK 293 cells was found to be similar to control cells while P5CR overexpression generated a noticeable increase (0.34 ± 0.02 mM) (Fig. 4C). P5C levels in P5CS overexpressing HEK 293 cells were significantly elevated (70 ± 5 μM) relative to control cells (< 10 μM) and slightly higher than cells expressing PRODH. Although P5C has been reported to elicit apoptotic events and have detrimental effects in yeast and mammalian cells, apparently, P5C does not accumulate to a toxic threshold in our experiments [46,47].

HEK 293 cells transfected with P5CS or P5CR were then exposed to H_2O_2 stress. Cell survival rates after oxidative stress ranged from 45 to 50% for the P5CS and P5CR overexpressing cells, respectively, similar to control cells (Fig. 4B). Co-transfection of HEK 293 cells with P5CS and P5CR (P5CS/P5CR), however, lead to a significant increase in resistance to oxidative stress with ~ 80% survival (Fig. 4B). Endogenous proline content was > 2-fold higher in the P5CS/P5CR cells (0.44 ± 0.03 mM) relative to control cells (Fig. 4C). Accordingly, P5CS/P5CR cells showed the lowest ROS content (Fig. 5). Flow cytometry analysis of P5CS and P5CR transfected cells after H_2O_2 stress estimated 40% and 20% fluorescent positive cells, respectively (Fig. 5). P5CS/P5CR overexpressing cells showed the lowest population of fluorescent positives (5%) with levels similar to that of control cells not exposed to H_2O_2 stress (5%). Thus, endogenous proline content inversely correlates with ROS levels consistent with proline protecting against H_2O_2 stress.

Stress response of proline biosynthesis

To address whether proline accumulation is an adaptive stress response, we exposed HEK 293 cells to low concentrations of H_2O_2 (20 μM) over 24 h and monitored expression levels of *P5CR2* and *P5CS*. In three independent experiments, P5CS and P5CR transcript levels were observed to increase ~ 3-fold during H_2O_2 stress. Fig. 8 shows a representative time course of changes in *P5CR2* and *P5CS* expression during H_2O_2 exposure. In control cell cultures without H_2O_2 exposure, no upregulation of P5CS and P5CR expression was observed over the same time period (Fig. 8). These results demonstrate that proline biosynthesis is upregulated in cells exposed to mild H_2O_2 stress. Consistent with higher expression levels of P5CS and P5CR, proline content increased by about 2-fold in H_2O_2-stressed HEK 293 cells (24 h) while non-

stressed HEK 293 cells exhibit no significant change in proline content (see Supplementary Table 2S). Increased expression of *P5CR2* (2-fold) and *P5CS* (3-fold) were also observed over 24 h in HEK 293 cells exposed to higher but still physiological H_2O_2 concentrations (~ 100 μM) (see Supplementary Fig. 5S). In addition to proline, P5C can be converted into ornithine by ornithine δ-aminotransferase (OAT) linking proline metabolism with the urea cycle. This alternative fate for P5C could potentially diminish proline accumulation during stress. We have observed, however, that OAT expression is significantly down regulated in HEK 293 cells during H_2O_2 stress suggesting a coordinated effort to direct P5C toward proline biosynthesis (Fig. 8). Thus, proline accumulation appears to be an adaptive response under H_2O_2 stress conditions. We also attempted to see whether PRODH expression was down regulated during H_2O_2 stress but we were unable to detect a PRODH transcript under our cell growth conditions.

Expression levels of the proline biosynthetic genes in other cell lines exposed to H_2O_2 stress were also explored. Similar to the results with HEK 293, upregulation of P5CS and P5CR and down regulation of OAT was observed in HeLa, HepG2, and BJAB cell lines exposed to 100 μM H_2O_2 (0–24 h) (Fig. 8). In cells without H_2O_2 treatment, P5CS and P5CR expression appeared constant while a decrease in OAT expression was evident in HeLa and BJAB cells. In Jurkat cells, however, changes in the expression of P5CR, P5CS, and OAT were less noticeable and were not significantly different than that observed in the control cells without H_2O_2 stress indicating that proline biosynthesis is not a H_2O_2 stress response in this particular cell line.

Discussion

Previously, we reported that proline protects filamentous fungi and yeast against oxidative stress [23,24]. Here we extend our study to mammalian cells suggesting proline functions as a universal antioxidant. Besides proline, cysteine and tryptophan also provide HEK 293 cells significant protection against H_2O_2 stress. These findings are consistent with previous work ranking these amino acids as potent hydroxyl free radical (•OH) scavengers [48–50].

In polypeptides, exposure of prolyl residues to hydroxyl free radicals generates 4-hydroxyprolyl and glutamic semialdehyde [51]. For the free imino acid, in vitro studies have demonstrated that incubation of proline with a Fe (II)-H_2O_2 hydroxyl radical generating system leads to the formation of nitroxide radicals and 3- and 4-hydroxyproline products [48,52]. In our cell culture studies the toxicity of H_2O_2 is most likely mediated by •OH arising from the reaction of H_2O_2 with transition metals. Interestingly, we found that proline was ineffective at protecting cells against superoxide generators such as menadione. Recently, it was reported that proline does not interact with superoxide in vitro [53]. Our cellular studies concur with this finding and suggest that proline exhibits specificity toward H_2O_2-induced stress. In addition to reacting with •OH, proline is an efficient quencher of $^1O_2$ and has been shown to protect keratinocytes against photodamage [25,54]. $^1O_2$ is generated mainly by UV light but is also formed by peroxidases causing tremendous oxidative damage to proteins, lipids, and DNA [55]. The quenching of $^1O_2$ by secondary amines is well documented and enables polyamines to protect DNA from oxidative damage [39,54,56,57].

Protection of cells by proline against oxidative stress appears to rely on its unique chemical properties even though proline has a modest second order rate constant for reacting with •OH [58,59]. The low ionization potential and the secondary amine of the pyrrolidine ring are key factors in the chemical and physical quenching activity of proline with •OH and $^1O_2$, respectively [39]. The ability of pipecolate to protect against oxidative stress and rescue cells from PRODH toxicity argues that proline scavenging of intracellular ROS occurs via a chemical pathway. Pipecolate, which has a larger heterocyclic ring size (six-member), is anticipated to have a lower ionization potential than proline and protect cells against oxidative

stress as we observed. Both L-proline and L-pipecolate are transported into cells by the mammalian IMINO transporter system suggesting similar bioavailability [42]. Whether the lack of protection observed with other proline analogs such as L-azetidine-2-carboxylic acid is due to differences in chemical properties is not clear as cell permeability and transport issues may be the determining factor. We also observed that proline preserves the intracellular glutathione pool, which is the major redox buffer of the cell. Proline protection of GSH was previously observed in *Chlamydomonas reinhardtii* exposed to toxic Cd levels [60]. Thus, proline accumulation appears to be an effective means for protecting the glutathione redox state of the cell under stress conditions perhaps by directly scavenging ROS. Alternatively, increased levels of proline may activate GSH synthesis or stabilize antioxidant enzymes during oxidative stress and invoke signaling pathways that upregulate cellular antioxidant defenses. In our previous work with an oncogenic Ras mutant of *C. trifolli*, proline treatment increased endogenous catalase activity by nearly 4-fold [23]. In addition, proline was observed to protect against paraquat (a superoxide generator) in filamentous fungi and yeast, thus, in these organisms a cellular pathway may mediate proline cytoprotection against superoxide [23,24]. Uncovering potential mechanisms by which proline protects cells against oxidative assaults will require exploring these possibilities.

Another intriguing question is how proline metabolism is regulated in response to stress. Our results provide evidence that upregulation of proline biosynthesis occurs during H_2O_2 exposure and that proline accumulation is a stress response mechanism in certain human cell lines such as HEK 293, Hela, and HepG2. We also found that OAT expression is down regulated during H_2O_2 stress indicating that the P5C-ornithine pathway is suppressed in order to funnel the P5C intermediate to proline synthesis. Of the proline metabolic genes used in this study, most is known about the regulation of *PRODH* gene expression in cellular apoptosis. Different studies have established that *PRODH* is activated by p53 and the peroxisome proliferator-activated receptor gamma (PPARγ) regulator with increased PRODH expression leading to oxidative stress [3–8]. Hepatocyte nuclear factors HNF4α and HNF1α have also been found to regulate proline degradation [61]. In yeast, DNA microarray analysis revealed expression of the PRODH gene (PUT1) increased with aging suggesting proline oxidation may contribute to accumulation of ROS with age and associated oxidative damage [62]. Identifying factors that regulate proline metabolism during different physiological stresses in mammalian cells will be an important future pursuit.

Proline metabolism has generally been considered to generate a more oxidative environment via mitochondrial PRODH activity [3–6,47,63,64]. Here, we have shown that PRODH generates superoxide directly during turnover with proline. We also provide evidence of a proline-dependent mechanism that opposes intracellular ROS formation. These findings suggest that proline metabolism is more pivotal in maintaining redox homeostasis than previously thought with proline exhibiting dual functions as a pro-oxidant via PRODH and as a ROS scavenger.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

GAPDH, glyceraldehdye-3-phosphate dehydrogenase
PRODH, proline dehydrogenase
P5CDH, Δ^1-pyrroline-5-carboxylate dehydrogenase
P5CS, Δ^1-pyrroline-5-carboxylate synthetase
P5CR, Δ^1-pyrroline-5-carboxylate reductase
OAT, ornithine δ-aminotransferase
TUNEL, Terminal deoxynucleotide-mediated dUTP nick end labeling
DCHF-DA, dichlorodihydrofluorescein diacetate
DAPI, 4′-6-diamidino-2-phenylindole
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
HEK, human embryonic kidney
ROS, reactive oxygen species
SOD, superoxide dismutase
o-AB, *o*-aminobenzaldehyde
tBH, *tert*-butyl hydroperoxide
GSH, glutathione
GSSG, glutathione disulfide

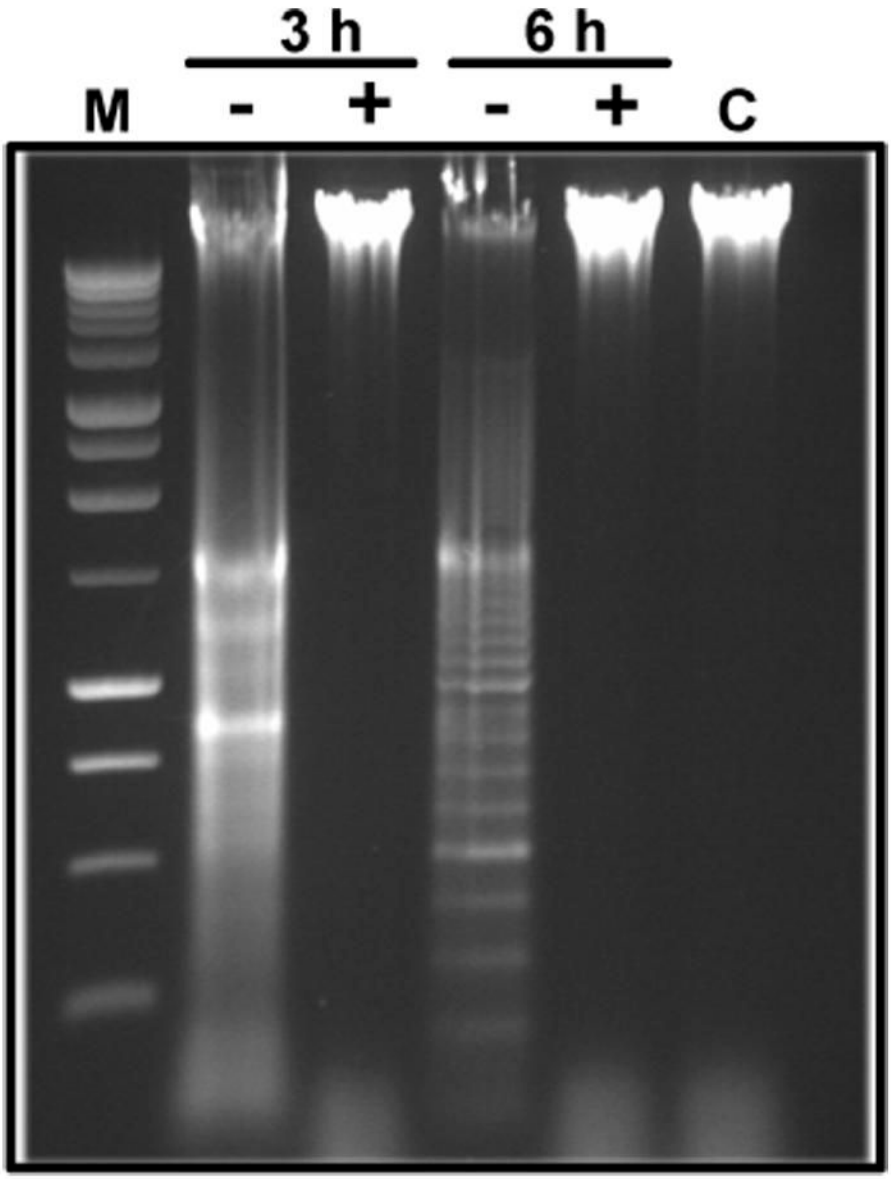

Fig. 1.

Proline prevents DNA laddering induced by H_2O_2. Agarose (1.5%) gel electrophoresis of DNA extract from HEK 293 cells treated with H_2O_2 (1 mM) for 3 and 6 h in the absence (−) and presence (+) of 5 mM proline. Far left-hand lane shows a 1.0 kB DNA ladder (M) and far right-hand lane shows DNA extract from untreated HEK 293 control cells (C).

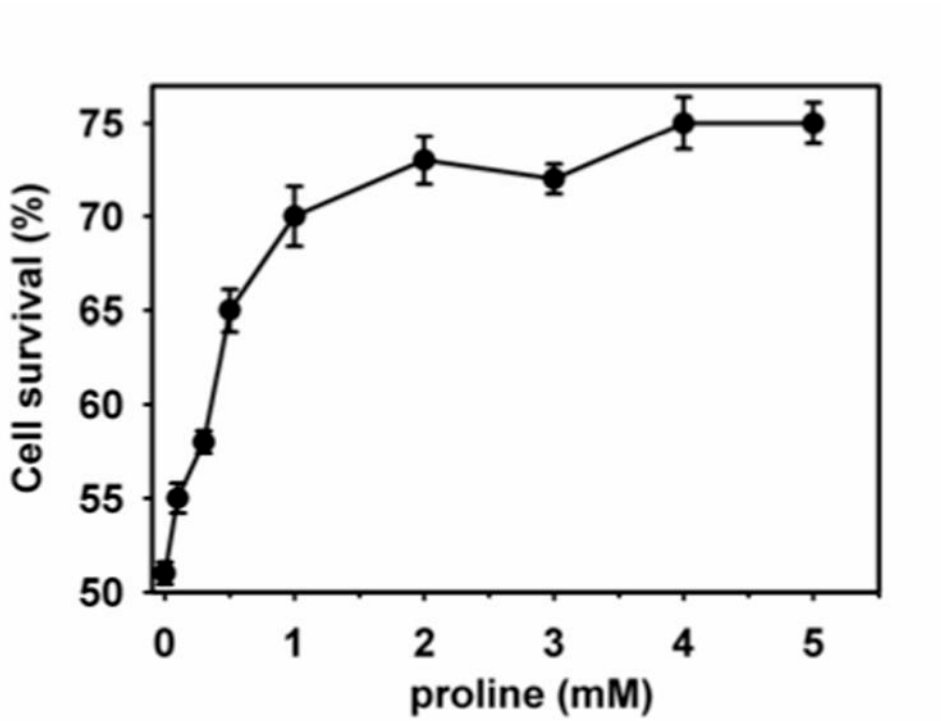

Fig. 2.

Concentration dependence of proline protection against oxidative stress. HEK 293 cells were incubated with 0.5 mM H_2O_2 (3 h) in the presence of increasing amounts of proline (0–5 mM) supplemented in the medium. The proline concentration in the medium alone is ~ 10 μM.

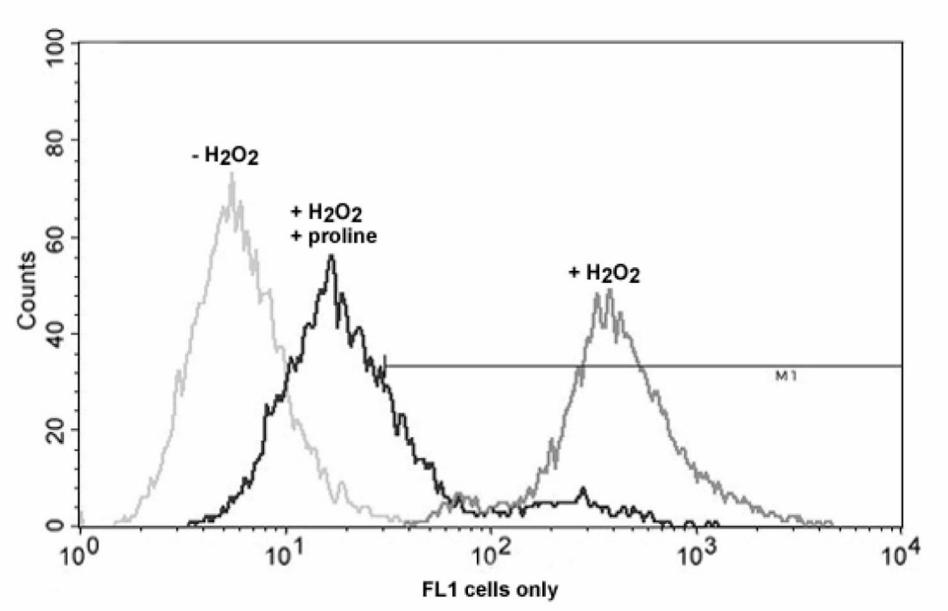

Fig. 3.

Flow cytometry analysis of ROS levels in HEK 293 cells. Fluorescent positives from HEK 293 cells without H_2O_2 stress treatment ($-H_2O_2$) and HEK 293 cells incubated with H_2O_2 (0.5 mM) for 3 h at 37 °C in the absence ($+ H_2O_2$) and presence of 5 mM proline ($+ H_2O_2$ + proline). Cells were treated with DCHF-DA prior to analysis.

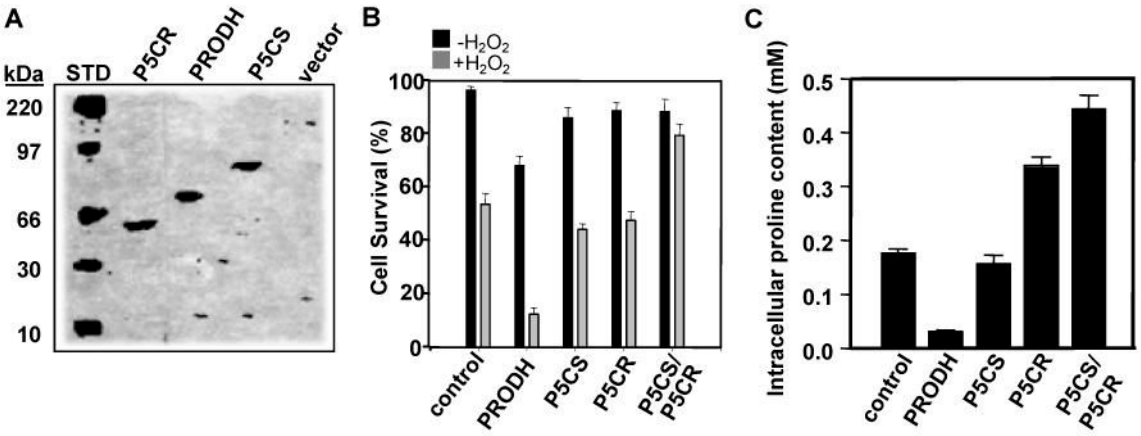

Fig. 4.

Proline accumulation correlates with increased cell survival. (A) Western analysis of HEK 293 cells transfected with P5CR-pFlag-CMV3, PRODH-pFlag-CMV3, and P5CS-pFlag-CMV3 and vector alone for 24 h at 37 °C. Protein extracts were separated by SDS-PAGE and immunoblotted with an anti-flag FITC conjugate antibody and visualized by fluorescence imaging. Protein standards visualized by the same detection system are shown in the left-hand lane. The anticipated molecular sizes of the pFlag fusion proteins are 34.7 kDa (P5CR), 69.15 kDa (PRODH), and 88.2 kDa (P5CS). (B) Survival rates of transfected HEK 293 cultured cells after 3 h incubation (37 °C) in the absence and presence of H_2O_2 (0.5 mM). HEK 293 cells were transfected with pcDNA3.1 vector alone (control) or with PRODH, P5CS, P5CR, and P5CS/P5CR for 24 h before treatment with H_2O_2. Survival rates were determined by the MTT assay. (C) Proline content in HEK 293 cells after 24 h transfection with pcDNA3.1 vector alone (control), PRODH, P5CS, P5CR, and P5CS/P5CR.

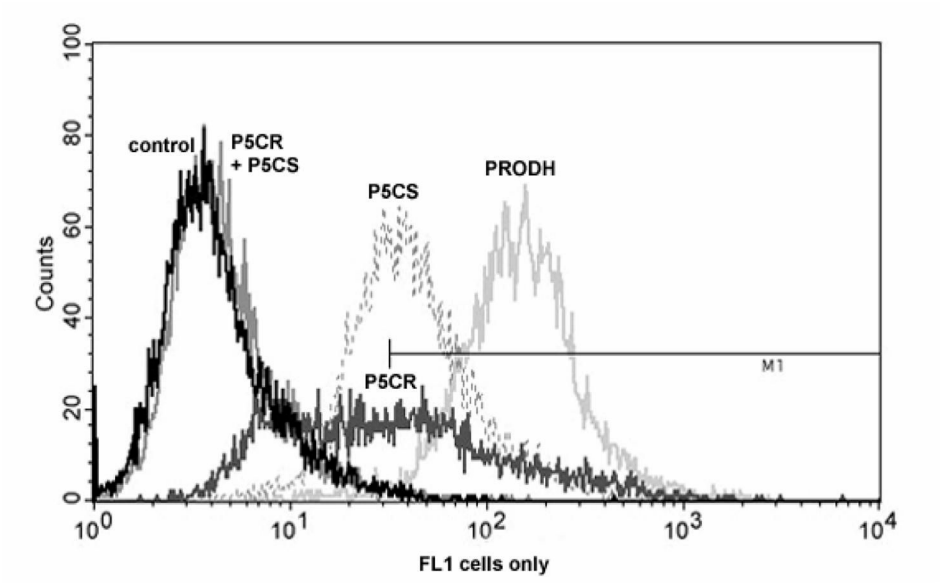

Fig. 5.

Flow cytometry analysis of intracellular ROS levels. Fluorescent positives from HEK 293 cells transfected with PRODH, P5CS, P5CR, and P5CS/P5CR and exposed to 0.5 mM H_2O_2 for 3 h at 37 °C. Fluorescent positives from transfected control cells (pcDNA3.1 alone) without H_2O_2 exposure are also shown. The oxidant-sensitive probe DCHF-DA was used to detect intracellular ROS levels.

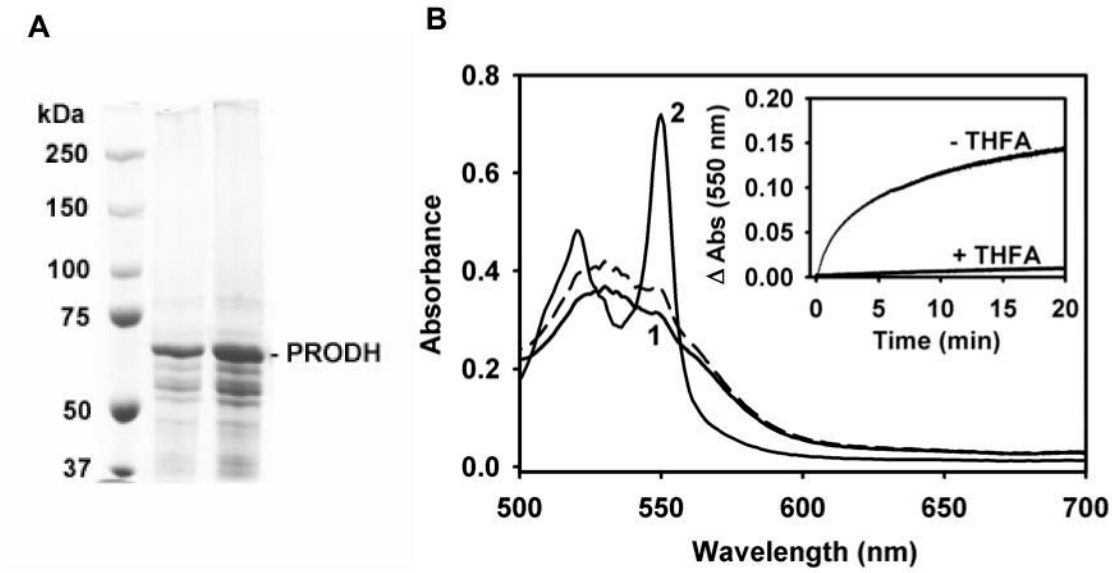

Fig. 6.

Superoxide formation by PRODH. (A) SDS-PAGE analysis of recombinant PRODH expressed in *E. coli*. Left, protein molecular weight standard; Middle, 3 μg of PRODH preparation; Right, 7 μg of PRODH preparation. The molecular weight of the PRODH band estimated by SDS-PAGE is 67.4 kDa. (B) Recombinant PRODH (20 μg) was incubated in proline:O_2 assay buffer (pH 7.5) with 150 mM proline and cytochrome c (40 μM) for 60 min at 23 °C in the absence and presence of SOD (36 U). Solid curves show the absorbance spectra of the assay mixture at 0 (curve 1) and 60 min (curve 2) without SOD. The dashed curve is the spectrum of the assay mixture supplemented with SOD and incubated for 60 min. The inset shows the rate of cytochrome c reduction in the absence (−THFA) and presence of 5 mM THFA (+ THFA) in the assay mixture.

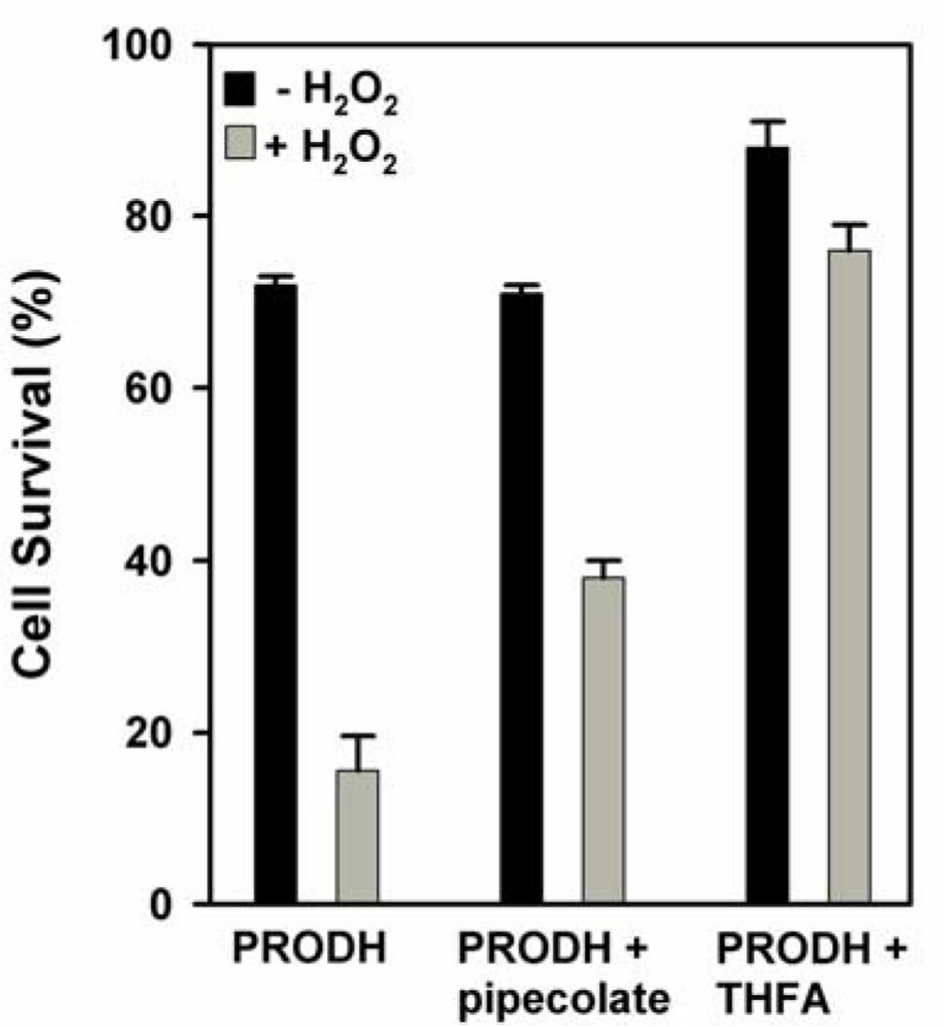

Fig. 7.

Rescue of PRODH toxicity in HEK 293 cells. HEK 293 cells were transfected with PRODH for 24 h in medium alone and medium supplemented with DL-pipecolate (5 mM) or L-THFA (5 mM) prior to treatment with H_2O_2. PRODH transfected HEK 293 cultured cells were then incubated for 3 h in the absence and presence of H_2O_2 (0.5 mM). Survival rates were determined by the MTT assay. HEK 293 cells transfected with pcDNA3.1 vector alone (control) exhibited 96% viability without H_2O_2 treatment.

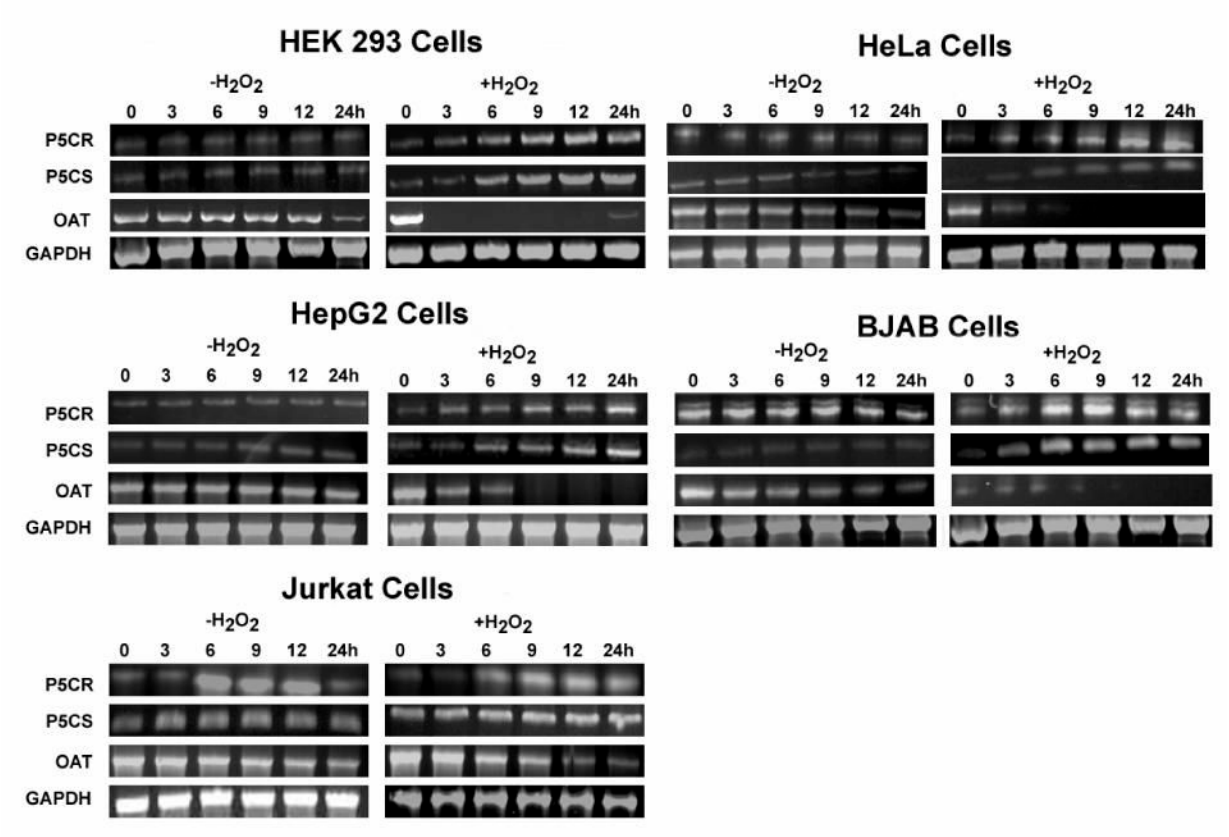

Fig. 8.

Expression profiling of P5CS, P5CR and OAT during H_2O_2 stress treatment. Total RNA was extracted at different time points from mammalian cells incubated at 37 °C from 0–24 h in the absence and presence of H_2O_2 maintained at 20 μM (HEK 293 cells) or 100 μM (HeLa, HepG2, BJAB, and Jurkat cells) in the cell culture medium. RT-PCR products for P5CR, P5CS, OAT and GAPDH transcripts are shown from non-stressed ($-H_2O_2$) and H_2O_2-stressed ($+H_2O_2$) cells at 0–24 h of incubation.

Table 1

Survival rates of HEK 293 cells exposed to H_2O_2 and supplemented with different amino acids[a]

Supplement	Cell Survival (%)
none	39
proline	77
cysteine	68
tryptophan	66
threonine	59
histidine	58
serine	58
methionine	57
lysine	54
phenylalanine	51
arginine	50
glutamine	49
alanine	48
asparagine	48
valine	44
leucine	43
isoleucine	42
tyrosine	41
glutamate	35
aspartate	35
glycine	33
control (no H_2O_2)	97

[a]Cell cultures were supplemented with individual amino acids (5 mM) and treated with 0.5 mM H_2O_2 for 3 h at 37 °C. Survival rates were determined by MTT assays and are an average of three independent experiments with standard errors < 10%.

Table 2

Survival rates of HEK 293 cells exposed to H_2O_2 and supplemented with different proline analogs[a]

Supplement	Cell survival (%)
none	45
DL-pipecolate	83
thiazolidine-4-carboxylic acid	81
L-proline	80
D-proline	59
L-azetidine-2-carboxylic acid	54
trans-4-hydroxy-L-proline	53
L-tetrahydro-2-furoic acid (THFA)	48
Control (no H_2O_2)	99

[a]Cell cultures were supplemented with proline analogs (5 mM) and treated with 0.5 mM H_2O_2 for 3 h at 37 °C. Survival rates were determined by MTT assays and are an average of three independent experiments with standard errors < 10%.