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Proline dehydrogenase is essential for proline protection against hydrogen peroxide induced cell death

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Abstract

Proline metabolism has an underlying role in apoptotic signaling that impacts tumorigenesis. Proline is oxidized to glutamate in the mitochondria with the rate limiting step catalyzed by proline dehydrogenase (PRODH). PRODH expression is inducible by p53 leading to increased proline oxidation, reactive oxygen species (ROS) formation, and induction of apoptosis. Paradoxical to its role in apoptosis, proline also protects cells against oxidative stress. Here we explore the mechanism of proline protection against hydrogen peroxide stress in melanoma WM35 cells. Treatment of WM35 cells with proline significantly increased cell viability, diminished oxidative damage of cellular lipids and proteins, and retained ATP and NADPH levels after exposure to hydrogen peroxide. Inhibition or siRNA-mediated knockdown of PRODH abolished proline protection against oxidative stress whereas knockdown of Δ^1-pyrroline-5-carboxylate reductase, a key enzyme in proline biosynthesis, had no impact on proline protection. Potential linkages between proline metabolism and signaling pathways were explored. The combined inhibition of the mammalian target of rapamycin complex 1 (mTORC1) and mTORC2 eliminated proline protection. A significant increase in Akt activation was observed in proline treated cells after hydrogen peroxide stress along with a corresponding increase in the phosphorylation of the fork head transcription factor class O3a (FoxO3a). The role of PRODH in proline mediated protection was validated in the prostate carcinoma cell line, PC3. Knockdown of PRODH in PC3 cells attenuated phosphorylated levels of Akt and FoxO3a and decreased cell survival during hydrogen peroxide stress. The results provide evidence that PRODH is essential in proline protection against hydrogen peroxide mediated cell death and that proline/PRODH helps activate Akt in cancer cells.

Keywords

Proline; proline dehydrogenase; oxidative stress; pyrroline-5-carboxylate reductase; Akt

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Introduction

Proline is a multi-functional imino acid with important roles in primary carbon and nitrogen metabolism, osmotic and oxidative stress protection, protein chaperoning, cell signaling, programmed cell death, and nutrient adaptation and survival [1–4]. Proline metabolism impacts cell survival and death outcomes by influencing the intracellular redox environment during oxidative and nutritional stress conditions [2, 5, 6]. The oxidation of proline to glutamate involves two enzymatic steps, which in eukaryotes occurs in the mitochondrion [7]. In the first step, proline is oxidized to P5C by the flavin-dependent enzyme proline dehydrogenase (PRODH) also known as proline oxidase (POX) (Fig. 1). The intermediate, P5C, is hydrolyzed nonenzymatically to γ-glutamate semialdehyde (GSA) which is then oxidized by P5C dehydrogenase (P5CDH) to glutamate [8]. Glutamate derived from proline can subsequently enter the TCA cycle via α-ketoglutarate. Conversion of glutamate back to proline involves three enzymatic steps with the initial two steps catalyzed by the bifunctional enzyme P5C synthetase (P5CS) to generate P5C [9]. P5C is subsequently reduced to proline by the NADPH-dependent P5C reductase (PYCR). Metabolic cycling of proline and P5C via PRODH and PYCR has been shown to increase flux through the pentose phosphate pathway by helping maintain $NADP^+$ levels in the cytosol [5, 7].

The gene encoding PRODH (*PRODH1*) is inducible by p53 [10, 11]. PRODH is associated with the inner mitochondrial membrane with activity likely coupled to reduction of ubiquinone in the electron transport chain [12, 13]. Upregulation of PRODH by p53 results in formation of proline-dependent reactive oxygen species (ROS) and induction of intrinsic and extrinsic apoptotic pathways [11, 14]. Co-expression of manganese superoxide dismutase blocks apoptosis induced by PRODH, suggesting the involvement of superoxide [15, 16]. Overexpression of PRODH was also shown to suppress tumorigenesis in xenografted nude mice [17]. Thus, proline oxidative catabolism has a well-established role in apoptosis and cancer prevention [11, 17].

Despite its pro-oxidant effects, proline has been shown to protect fungi, plants, and mammalian cells against oxidative stress [3, 6, 18, 19]. In mammalian cells proline was observed to decrease apoptosis induced by different oxidative stress agents such as H_2O_2, tert-butyl hydroperoxide and the carcinogen, fumonisin [6]. Though numerous studies have demonstrated the role of proline in stress protection, deciphering the mechanism of proline protection has been enigmatic. In different organisms proline protection has been postulated to involve protein chaperoning, direct scavenging of ROS, protection and upregulation of antioxidant enzymes such as catalase, and maintenance of key redox molecules such as glutathione and $NADPH/NADP^+$ that are critical for proper redox balance [3, 6, 18, 20, 21].

Here, we seek to provide new insights into the mechanism of proline protection against oxidative stress by exploring the role of proline metabolic enzymes and signaling pathways possibly influenced by proline. For this study, we used Wistar melanoma 35 (WM35) cells, a non-tumorigenic cell line derived from primary, radial growth phase melanoma [22], and subsequently generalized our findings in the prostate carcinoma line, PC3. WM35 has been shown to maintain wild type p53 and cell cycle responses similar to melanocytes [22]. Furthermore, a metabolic profiling study showed WM35 cells exhibit significant proline metabolic flux and TCA cycle activity [23]. We report evidence that PRODH activity is essential for proline mediated protection of WM35 cells against oxidative stress and identify signaling pathways that potentially lead to increased cell survival.

Experimental procedures

Materials

Unless stated otherwise, all chemicals, enzymes and buffers were purchased from Fisher Scientific and Sigma-Aldrich, Inc. WM35 cells were provided by Dr. Adam Richardson and Dr. Jeffrey Smith at the Sanford-Burnham Medical Research Institute, La Jolla, CA. RWPE-1 and PC3 cells were purchased from ATCC.

Antibodies

Antibodies recognizing VDAC/Porin and β-actin were from Sigma. Antibodies against phospho-Akt (T308), phospho-Akt (S473), total Akt, phospho-FoxO1 (T24), phospho-FoxO1 (S256), phospho-FoxO3a (T32), and total FoxO1 were from Cell Signaling Technology. Polyclonal antibodies against PRODH, PYCR1, PYCR2 were custom-made by Proteintech. Horse radish peroxidase (HRP)-labeled anti-mouse, anti-goat and anti-rabbit secondary antibodies were from GE Healthcare.

Stress tests and cell viability

WM35 cells were grown in Rosewell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS) (Phenix) and non-essential amino acids at 37 °C. For WM35 cell culture media, 0.25 μg/ml of antibiotic-antimycotic solution was used (Invitrogen). PC3 cells were grown in Improved MEM (IMEM) supplemented with 10% FBS and non-essential amino acids. RWPE-1 cells were grown in keratinocyte serum free medium (K-SFM) supplemented with 10% FBS, bovine pituitary extract and human recombinant epidermal growth factor (Invitrogen). For stress treatments, WM35, RWPE-1and PC3 cells were grown to 80% confluence. For the initial time study with WM35 cells, cells were pretreated with proline (5 mM) for various time points (1–24 h) in serum containing medium prior to H_2O_2 stress (0.5 mM, 3 h) in serum free medium. A 12 h pretreatment period with proline (5 mM) in serum containing medium was then chosen for all subsequent stress tests with WM35 and RWPE-1 cells. Stress tests were performed by pretreatment of cells with and without proline (5 mM) in serum containing medium for 12 h. The media was then changed and cells were incubated for 3 h in serum free medium with and without H_2O_2 (50 and 500 μM). During the 3 h H_2O_2 stress treatment, proline (5 mM) was supplemented in the serum free medium for the proline treated cells whereas control cells were not supplemented with proline. After stress treatment, cell viability was quantified using the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) according to the recommendation of the manufacturers (Promega). The CellTiter-Glo Luminescent assay (Promega) was also used as an alternative method according to the recommendation of the manufacturers. Percent cell survival was determined relative to the appropriate controls. Stress tests were also performed with WM35 cells using different inhibitor compounds. For inhibition of PRODH, WM35 cells were incubated with 0–5 mM L-tetrahydro-2-furoic acid (L-THFA) with and without proline (5 mM) for 12 h prior to 0.5 mM H_2O_2 stress (3 h). To inhibit the pentose phosphate pathway, WM35 cells were incubated with dehydroepiandrosterone (DHEA) (50 μM) and 6-aminonicotinamide (50 μM) for 24 h before adding proline (5 mM) to the cell culture. After a 12 h incubation with proline, WM35 cells were then treated with H_2O_2 as described above. Inhibition of the mTOR pathway was achieved by incubating WM35 cells with rapamycin (Calbiochem) at 10 nM and 100 nM concentrations and with KU-0063794 (Selleck) at 10 nM and 20 nM concentrations for 2 h prior to H_2O_2 stress. Stock solutions of the inhibitor compounds DHEA, 6-aminonicotinamide, rapamycin, and KU-0063794 were prepared in dimethyl sulfoxide (DMSO).

G6PDH assay

Glucose-6-phosphate dehydrogenase (G6PDH) activity was measured according to the recommendation of the manufacturer (BioVision). One unit of enzyme is defined as 1 μmol of product formed per min at 37 °C. Total protein concentration in the cell lysates was determined using the bicinchoninic acid (BCA) protein assay (Pierce) using bovine serum albumin as a standard.

Oxidative stress markers

Malondialdehyde was measured using the thiobarbituric acid method [24]. Protein carbonyl content formed was measured using 2,4-dinitro-phenyl hydrazine and calculated using an extinction coef3cient of 22 $mM^{-1}cm^{-1}$ [25]. Total thiol content was measured using dithio-bis-(2-nitro benzoic acid) and expressed as nanomoles per milligram protein as described [26].

Proline, ATP, and NADPH measurements

Intracellular proline levels were measured using the acid-ninhydrin method as described earlier [6, 27]. Briefly, WM35, RWPE-1, and PC3 cells were trypsinized, washed twice with PBS, pelleted by centrifugation, and resuspended in 0.5 ml of Mammalian protein extraction reagent (M-PER). Cell lysates were prepared according to the manufacturer's instruction with the addition of mammalian protease inhibitor cocktail (Sigma) and were centrifuged at 17,000 × g for 10 min to obtain a cell free supernatant for the assays. Proline concentrations were estimated using a standard curve of proline (0–10 mM). The reported proline levels are the concentration of proline in the final toluene extract and are an average value of four independent experiments. ATP (Promega) and NADPH/$NADP^+$ (Cell Technology) levels were estimated according to the manufacturer's recommendation.

Western blot analysis

Cells grown in 10 cm diameter dishes were rinsed once with ice cold PBS and cell lysates were prepared using M-PER as described above, along with the addition of mammalian protease inhibitor cocktail (Sigma) and phosphatase inhibitor (Pierce). Lysates containing 50–100 μg of protein were resolved by SDS-PAGE. Proteins were transferred to a PVDF membrane and visualized by immunoblotting. Band intensities from Western blots were analyzed using TotalLab Quant software. Phosphorylated Akt levels were reported with respect to total Akt and phosphorylation of FoxO3a was normalized with respect to β-actin.

Mitochondrial isolation

Cells were released with trypsin and washed twice via centrifugation with ice cold PBS and then cell pellets were resuspended in Tris buffer (5 mM, pH 7.4) containing 0.25 M sucrose and 1 mM EDTA. Cells were hand homogenized using a Dounce homogenizer for 1 min on ice and centrifuged at 17,000 × g for 15 min (4 °C). The supernatant containing the mitochondrial-free cytosolic fraction was removed and the pellet was resuspended in a solution containing 8 M urea, 2 M thiourea, 2% CHAPS and used for Western analysis. Total protein concentrations were quantified using the Bradford method (Bio-Rad).

siRNA transfection

Cells were transfected with On-Target plus smart pool siRNA of PRODH, PYCR1 and PYCR2 and On-Target plus Non-targeting Pool referred to as control siRNA (Dharmacon RNA technologies). siRNA transfection was allowed to proceed for 48–96 h before doing the stress test or whole cell extract collection. Knockdown was confirmed by Western analysis. Following transfection, stress tests were performed as described above.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using student's t-test. A P value of <0.05 was considered to be statistically significant.

Results

Proline protects WM35 cells

Proline was previously shown to protect several mammalian cell types against H_2O_2-induced cell death [6]. To first test whether proline would protect WM35 cells against oxidative stress, we pretreated WM35 cells with proline. Cells were pretreated with proline for various time points (1–24 h) prior to H_2O_2 stress treatment (0.5 mM, 3h). We found that 6 h of pretreatment with proline (5 mM) significantly increased survival of WM35 cells in the presence of H_2O_2 relative to control cells not given proline (Fig. 2A). Additional increased cell survival was observed at incubation times > 12 h. We confirmed the protective effect of proline addition by luminescence quantification of ATP generation, in which survival increased from 44% to 78% (Fig. 1B). In further support of engaged oxidative stress protective mechanisms, WM35 cells exposed to H_2O_2 exhibited considerably higher levels of malondialdehyde and protein carbonyl content relative to control cells indicative of oxidative damage (Fig. 2C and D). In contrast, malondialdehye was not elevated by H_2O_2 treatment in the presence of proline, and protein carbonyl levels were significantly lower after H_2O_2 stress compared to cells without proline. H_2O_2 stress decreased total thiol content in the absence of proline but not when proline cells were pretreated (Supplementary Fig. 1). Thus, consistent with improving cell survival, proline limits thiol depletion and oxidative modification of lipids and proteins.

Because proline has been postulated to directly scavenge ROS, we tested the possibility of proline reacting with H_2O_2 in the cell medium. The reactivity of proline with H_2O_2 was compared to that of pyruvate, a well-established scavenger of H_2O_2 [28]. Proline did not significantly diminish H_2O_2 levels whereas in medium supplemented with pyruvate H_2O_2 was quickly degraded (Supplementary Fig. 2). We also did not find any evidence for direct scavenging of hydroxyl radicals by proline using previously published hydroxyl-radical scavenger assays (data not shown) [29, 30]. Thus, it was concluded that proline protection against oxidative stress does not involve direct scavenging.

In addition, protection by proline was not mediated through the action of the antioxidant enzymes catalase, glutathione peroxidase, glutathione S-transferase and glutathione reductase (Supplementary Fig. 3), since these activities were not altered in the presence of proline.

Inhibition and siRNA mediated knockdown of PRODH

We next examined the effect of reducing endogenous PRODH activity and expression. WM35 cells were treated with different concentrations of L-THFA, an inhibitor of human PRODH [31], during proline supplementation. L-THFA decreased proline protection against H_2O_2-induced cell death in a dose-dependent manner (Fig. 3A). At 5 mM L-THFA, proline did not protect. L-THFA alone had no effect on cell survival. To test whether L-THFA addition to the culture medium affects proline uptake, we measured intracellular proline levels in cells treated with L-THFA and proline. Proline treatment increased intracellular proline by 3-fold relative to controls (Fig. 3B). Addition of L-THFA or H_2O_2 to the proline treated cells did not alter intracellular proline levels, suggesting proline transport is not affected by THFA nor significantly diminished after H_2O_2 stress (Fig. 3B). The impact of inhibiting PRODH activity was then tested using PRODH siRNA (siRPODH) transfection of WM35 cells. Western blot analysis of isolated mitochondria from WM35 cells showed a

significant loss of PRODH expression after 48 h of siPRODH treatment relative to cells treated with control siRNA (Fig. 3C). Proline protection against H_2O_2-induced cell death was eliminated in cells transfected with siPRODH compared to cells transfected with control scrambled siRNA (Fig. 3D). These results suggest proline protection is dependent on PRODH.

siRNA mediated knockdown of PYCR

Recently, fibroblasts isolated from patients with a mutation in the *PYCR1* gene were reported to have altered mitochondrial morphology, mitochondrial membrane potential and an increased apoptotic rate upon oxidative stress [32]. To test the impact of interrupting the proline-P5C cycle through PYCR, we performed siRNA knockdown of PYCR1 and PYCR2. Proline protection against oxidative stress-induced cell death was unaffected even with virtually complete knockdown of both PYCR isozymes (Fig. 4A-D). Thus, unlike PRODH, diminished PYCR1 and PYCR2 expression does not appear to impact proline protection against H_2O_2 stress, indicating that the proline-P5C cycle may not be critical for proline protection. Consistent with this result, high flux through the pentose phosphate pathway was also not required for proline protection (Supplementary Fig. 4).

Cellular ATP and NAPH/NADP$^+$

PRODH may help maintain cellular energy during stress by coupling the oxidation of proline to the mitochondrial electron transport chain [5]. To test this, we measured the effect of proline on intracellular ATP and NADPH/NADP$^+$ after 3 h H_2O_2 stress treatment. ATP levels and the NADPH/NADP$^+$ ratio were significantly decreased after 3 h of 0.5 mM H_2O_2 treatment relative to control cells. In cells treated with proline, ATP levels and the NADPH/NADP$^+$ ratio were near that of control cells (Fig. 5). Thus, proline helps maintain ATP levels and NADPH/NADP$^+$ in H_2O_2 stressed cells.

Inhibition of mammalian target of rapamycin complex (mTORC)

Since amino acids have been shown to activate the mammalian target of rapamycin complex 1 (mTORC1) [33, 34], we tested whether mTORC1 is a component of proline protection against H_2O_2. Incubation of WM35 cells with 10 nM rapamycin did not alter proline protection against H_2O_2 stress suggesting that mTORC1 is not involved in proline protection (Fig. 6A). However, treatment of cells with a higher concentration of rapamycin (100 nM) did prevent proline protection (Fig. 6A). Because the rictor-complex of mTOR (mTORC2) has been reported to be sensitive to rapamycin at high concentrations [35], we tested the effect of KU-0063794, a compound which inhibits both mTORC1 and mTORC2 [36]. Treatment of WM35 cells with KU-0063794 abolished proline protection against oxidative stress (Fig. 6B), indicating a role for mTORC2 in proline protection.

Activation of Akt and FoxO3a during H_2O_2 stress

Akt/PKB (Protein kinase B) is a downstream target of mTORC2 activated in response to oxidative stress, and a well-known promoter of tumor cell survival, proliferation and metastasis [37]. The activation of Akt was examined by immunoblot analysis of phosphorylation sites Thr308 and Ser473 after exposure of cells to H_2O_2 with or without proline treatment. At 0.5 h of H_2O_2 stress, 12-fold and 13-fold increases were observed for phospho-Akt at Thr308 and Ser473, respectively, relative to control cells (Fig. 7A–C). Phospho-Akt levels were found to be even higher in proline treated cells exposed to H_2O_2 stress with 30-fold and 44-fold increases observed for Thr308 and Ser473 sites, respectively, relative to control cells (Fig. 7A–C). Initial activation of Akt occurs via phosphorylation at Thr308 by phosphatidylinositol-3,4,5-triphosphate dependent kinase (PI3K) and full activation of Akt is catalyzed by mTORC2 at S473 [36]. After 3 h, the levels of phospho-

Akt at Thr308 remained significantly upregulated in cells exposed to H_2O_2 stress (Fig. 7E and F) whereas phospho-Akt (Ser473) was no longer upregulated. Proline treatment did not effect phospho-Akt levels at Thr308 (Fig. 7E) or Ser473 at 3h H_2O_2 stress.

Complete activation of Akt is known to phosphorylate several downstream targets including the fork head family of transcription factor class O (FoxO) [38]. We examined the phosphorylation status of FoxO in H_2O_2 stressed cells with or without proline treatment. No significant differences in the phosphorylation of FoxO1 (Ser256) and FoxO4 (Ser193) were observed after 0.5 and 3 h of H_2O_2 stress in cells with and without proline (Fig. 7A and E). In contrast, in cells exposed to H_2O_2 without proline, phospho-FoxO3a (Thr32) was 6-fold higher, and ≈16-fold higher when proline was also provided (Fig. 7A and D). Thus, phospho-FoxO3a was significantly upregulated in proline treated cells, consistent with significantly higher levels of phospho-Akt (Ser473) observed at 0.5 h of H_2O_2 stress in cells with proline (Fig. 7C). At 3h, phospho-FoxO3a (Thr32) had returned to the basal level in all conditions. Since upregulation of phospho-FoxO3a (Thr32) follows the transient profile observed for phospho-Akt (Ser473), increased cell survival in the presence of proline may involve inactivation of FoxO3a via Akt phosphorylation.

Proline protection at lower H_2O_2 concentration

Proline protection of WM35 cells was also tested using a lower concentration of H_2O_2 (50 μM) to more closely mimic pathophysiological conditions. Cell survival after H_2O_2 exposure (50 μM, 3h) increased from 68% to 88% in cells treated with proline (Fig 8A). Furthermore, phospho-Akt (Thr 308 and Ser 473) and phospho-FoxO3a (Thr 32) were found to be significantly higher in proline treated cells after 50 μM H_2O_2 exposure (0.5 h) relative to H_2O_2 alone treated cells (Fig. 8B–E). These results are similar to that observed with higher concentrations of H_2O_2 (500 μM, 3h) consistent with proilne protecting against pathophysiological levels of H_2O_2.

PRODH and Akt in prostate cancer cells

To validate and generalize the roles of PRODH and Akt in proline protection during oxidative stress injury, we repeated the experiments in a prostate cancer cell line. Proline biosynthesis is upregulated in clinical samples of prostate cancer suggesting that proline metabolism may have a role in prostate cancer development [39, 40]. We first measured intracellular proline levels in normal non-tumorigenic human prostate epithelial cells (RWPE-1) and in PC3, an androgen independent cancer cell line with constitutively active Akt [41]. The intracellular proline content was 3-fold higher in PC3 cells (28 ± 1 μg/mg protein) relative to RWPE-1 cells (9.5 ± 1 μg/mg protein), consistent with the elevated expression of proline biosynthesis genes previously reported [42]. Addition of proline to RWPE-1 cells increased survival in the presence of H_2O_2 (1 mM, 3 h) by about 26% relative to control cells without proline (Fig. 9A). Because the intracellular proline content of PC3 cells is already similar to that found in cells treated with proline, cells were not incubated with proline prior to H_2O_2 stress. Instead, the impact of PRODH in stress protection was tested by siRNA knockdown of PRODH in cells grown in medium without proline supplementation. PC3 cells were highly resistant to oxidative stress, with 50% cell survival still observed at 8 mM H_2O_2 in cells treated with control siRNA (Fig. 9B). At all H_2O_2 concentrations tested, survival of PC3 cells treated with siPRODH was decreased by 20–30% relative to scrambled siRNA treated cells, with 25% cell survival observed at 8 mM H_2O_2 (Fig. 9B). Knockdown of PRODH was confirmed by Western analysis (Fig. 9C). Levels of phospho-Akt (Thr308 and Ser473) were ≈80% lower in cells treated with siPRODH relative to control cells (Fig. 9D and E). Similarly, phospho-FoxO3a (Thr32) was also reduced by ≈80% in these cells. These results support a general mechanism implicating PRODH oxidative metabolism of proline, either from exogenous sources or endogenous

stores, in the downstream Akt signaling pathways that influence cell survival during oxidative stress.

Discussion

The mechanism by which proline protects cells against oxidative stress induced apoptosis and cell death has been relatively unexplored. Here, we show that PRODH activity is essential for proline protection as knockdown of PRODH activity abrogates proline protection in WM35 cells and significantly decreases survival of PC3 cells during oxidative stress. Potential linkages between proline metabolism and other pathways that could be beneficial for cell survival during oxidative stress were also examined. The proline-P5C cycle has been implicated as an important mechanism for transferring reducing equivalents of NADPH from the cytosol into the mitochondria and, helping maintain $NADP^+$ for the pentose phosphate pathway [5, 43, 44]. In this study, however, knockdown of PYCR1/2 and inhibition of pentose phosphate pathway enzymes did not block proline protection suggesting the proline-P5C cycle is not essential. However, ATP levels and the NAPDH/$NADP^+$ ratio were retained in proline treated cells during oxidative stress suggesting proline helps combat depletion of ATP and NADPH. A previous study also showed that PRODH helps maintain ATP levels in cells under nutrient deprivation, thus, proline metabolism appears to influence cellular ATP during oxidative and nutrient stress [5, 7].

A possible role for mTOR in proline protection was tested as amino acids have been shown to influence mTORC1 and mTORC2 signaling pathways [45]. Proline protection was lost only under conditions known to inhibit both mTORC1 and mTORC2 complexes, suggesting mTORC2 may help mediate cell survival. Upregulation of mTORC2 has previously been shown to augment Akt phosphorylation at Ser473 and inhibit FoxO-dependent transcription [38, 45]. Consistent with the involvement of mTORC2, the cytoprotective effects of proline appear to be mediated by the Akt survival pathway. The Akt pathway is inducible by H_2O_2 stress [46]. Unexpectedly, phospho-Akt levels were observed to be significantly higher in cells treated with proline. Concomitant with the transient increase in phospho-Akt (Ser473), significant induction of phosphorylated FoxO3a was observed in proline treated cells during stress (0.5 h) (Fig. 7D and 8E). Phosphorylated FoxO binds to the 14-3-3 binding protein resulting in translocation of FoxO from the nucleus to the cytosol. Removal of FoxO from the nucleus prevents activation of pro-apoptotic gene targets thereby promoting cell survival [45, 47]. Thus, increased phospho-FoxO3a is anticipated to inhibit FoxO3a induced cell death leading to increased survival of proline treated cells during oxidative stress.

The effect of proline and PRODH on the Akt pathway was tested further in prostate cells. Our motivation for examining PC3 cells was from previous studies showing a significant increase in proline metabolism in prostate cancer (PCa) metastatic samples suggesting a clinically relevant proline metabolic shift in PCa progression [42]. Proline levels were found to be significantly increased in metastatic PCa samples and the key enzyme in proline biosynthesis, PYCR, is highly expressed in metastatic tumors. Furthermore, it is already established that activation of phospho-Akt by mTORC2 leads to cell survival in prostate cancer cells [48] and that androgen independent prostate cancer cells such as PC3, exhibit higher resistance to H_2O_2 compared to normal prostate epithelial cells [49].

In agreement with previous studies on prostate cancer clinical samples [39], we found that PC3 cells have considerably higher levels of proline relative to normal prostate cells (RWPE-1). Knockdown of PRODH led to a significant decrease in resistance to H_2O_2 stress in PC3 cells (Fig. 9B). In addition, significantly decreased levels of phospho-Akt and phospho-FoxO3a were found in PC3 cells with PRODH knockdown. Importantly, the effect of PRODH knockdown on Akt phosphorylation in PC3 cells was observed under non-stress

conditions. Thus, PRODH appears to contribute to Akt activation in PC3 cells, the activation of which is constitutive due to the lack of functional PTEN in PC3 cells [41]. These results provide support for a generalized model in which Akt acts as a downstream effector of proline oxidative metabolism in cancer cells.

Figure 10 summarizes a general model for how proline provides a protective effect against H_2O_2 stress. Proline catabolism via PRODH is proposed to help maintain cellular energy, minimize oxidative damage, and increase activation of the Akt survival pathway. The exact mechanism by which PRODH influences Akt is not apparent but may involve mTORC2. Other factors, however, may be involved. In addition to mTORC2, Akt (Ser473) phosphorylation has been suggested to be catalyzed by several other kinases including MAP kinase-activated protein kinase 2, protein kinase C isoforms, integrin-linked kinase, and 3-phosphoinositide dependent protein kinase 1 [50–53]. Interestingly, mTORC2 mediated full activation of Akt has been shown to induce peroxisomes proliferators activated receptor (PPAR-γ) [54, 55], which has been shown to induce PRODH expression [56].

Finally, while preparing this manuscript a novel study was published reporting that proline catabolism extends lifespan in worms [57]. Knockdown of PRODH in a *C. elegans daf-2* mutant with impaired insulin and IGF1 signaling was shown to significantly decrease lifespan. Supplementation of wild-type worms with proline was also found to extend lifespan [57]. Proline catabolism was proposed to generate transient ROS signals that activate the worm homologues of p38 MAP kinase and Nrf2, leading to increased expression of antioxidant enzymes and lifespan [57]. So far, we have not found any evidence of transient proline-dependent ROS that could potentially impact the Akt signaling pathway (data not shown), and Nrf2 does not appear to be necessary for proline protection against oxidative stress induced cell death (Supplementary Figure 5). Future studies which aim to elucidate the mechanisms by which proline catabolism influences the Akt pathway should reveal new insights into mitochondrial dependent signaling in stress response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DHEA	dehydroepiandrosterone
ETC	electron transport chain
FoxO	fork head family of transcription factor class O
G6PDH	glucose-6-phosphate dehydrogenase
GSA	γ-glutamate semialdehyde
mTORC	mammalian target of rapamycin complex

MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]
PPAR	peroxisomes proliferators activated receptor
PI3K	phosphatidylinositol-3,4,5-triphosphate dependent kinase
PRODH	proline dehydrogenase
POX	proline oxidase
P5C	Δ^1-pyrroline-5-carboxylate
P5CDH	P5C dehydrogenase
PYCR	P5C reductase
P5CS	P5C synthetase
THFA	tetrahydro-2-furoic acid
VDAC	voltage-dependent anion channel

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:

Highlights

- The mechanism of proline protection against hydrogen peroxide stress was examined
- Inhibition/knockdown of proline dehydrogenase (PRODH) eliminated proline protection
- Proline retained ATP/NADPH levels and enhanced the Akt survival pathway
- Knockdown of PRODH in a prostate carcinoma cell line decreased cell survival
- A general model for proline protection is proposed in which PRODH is essential

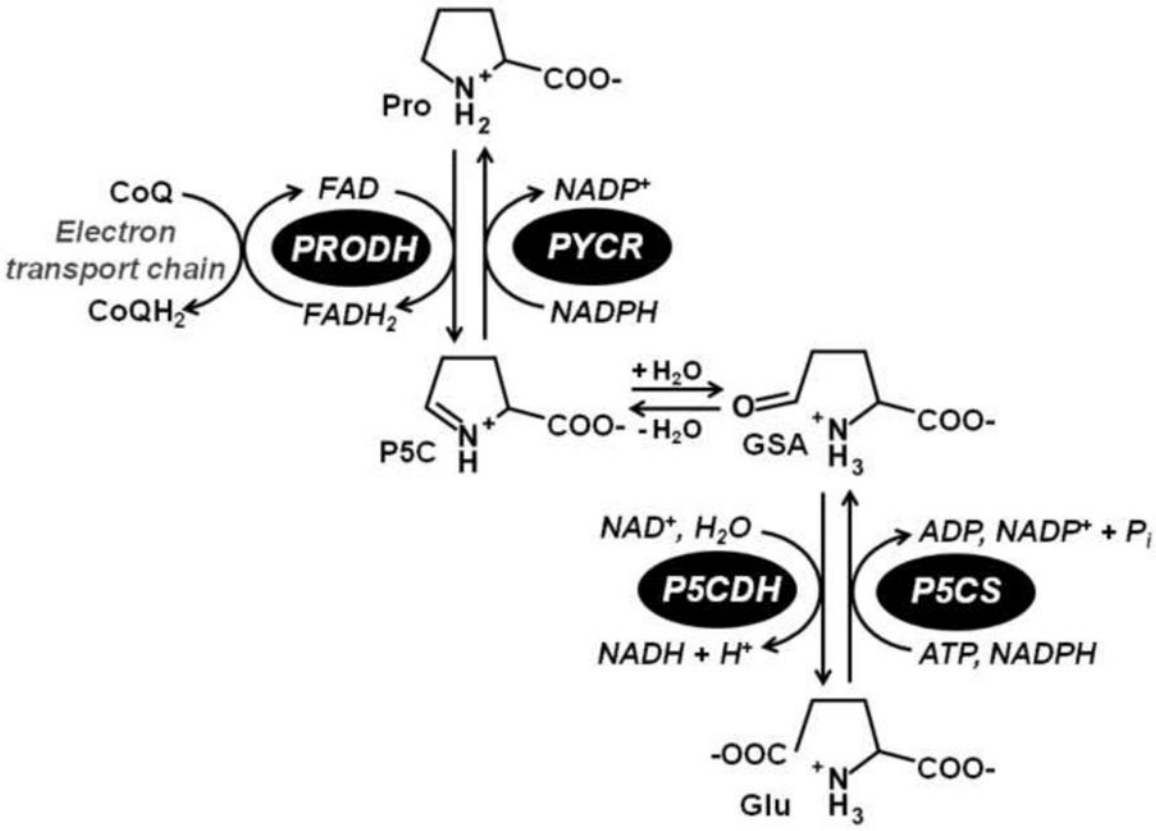
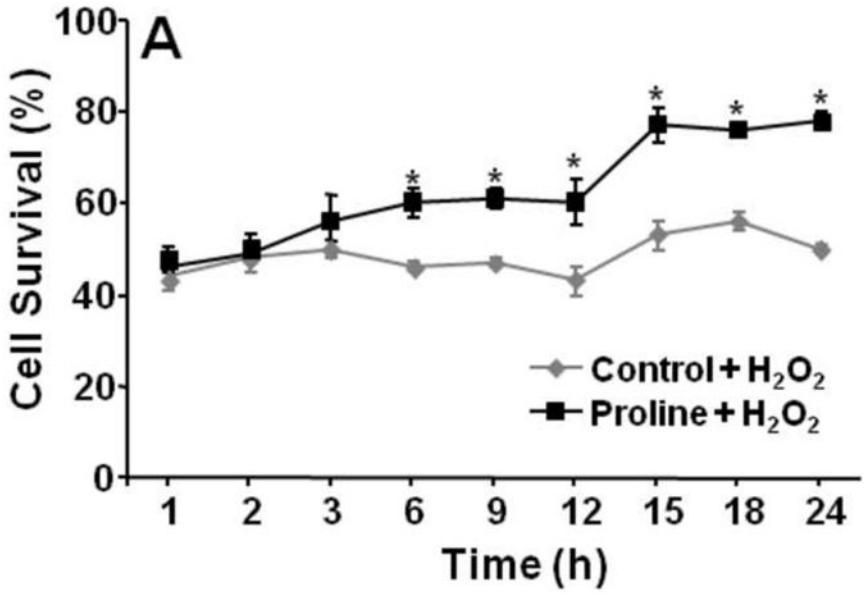
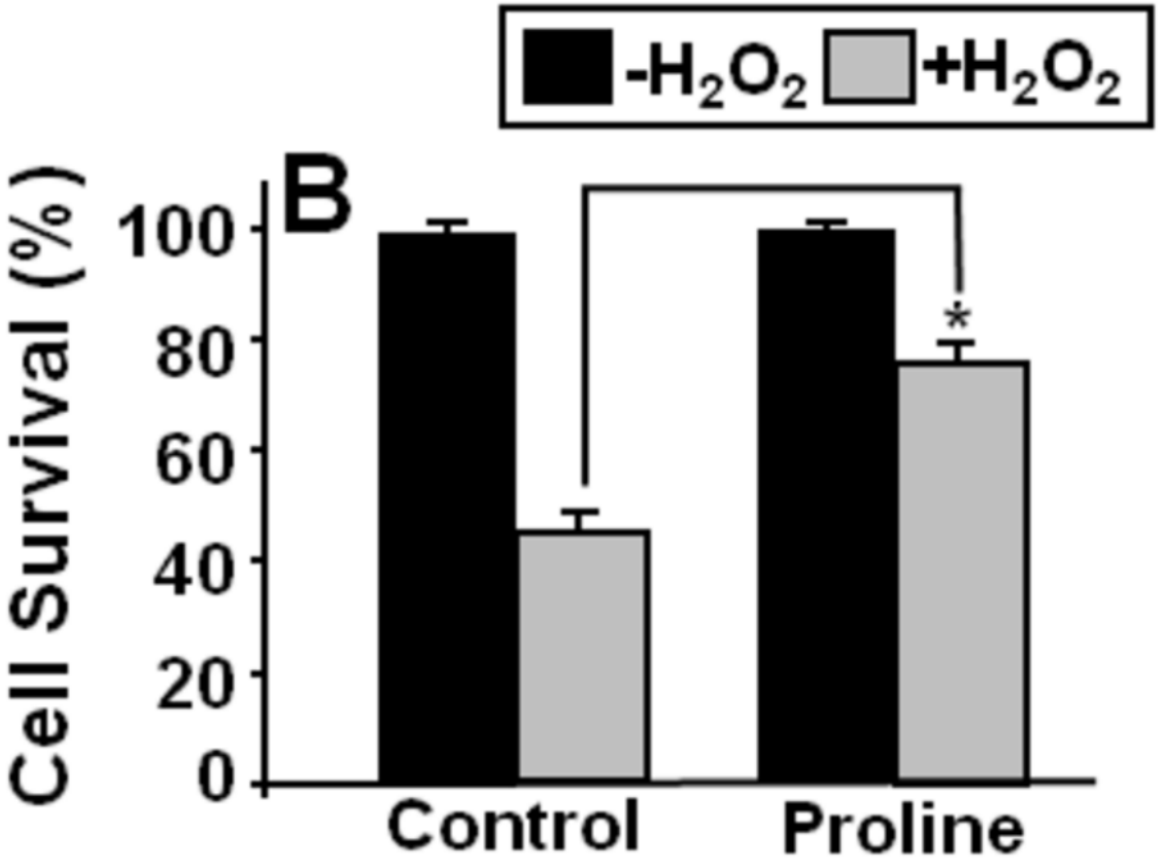


Fig. 1.

Proline metabolic pathway. Proline is oxidized to glutamate by proline dehydrogenase (PRODH) and P5C dehydrogenase (P5CDH) in the mitochondrion. PRODH couples proline oxidation to reduction of CoQ in the electron transport chain. The enzymes P5C synthetase (P5CS) and P5C reductase (PYCR) convert glutamate into proline.





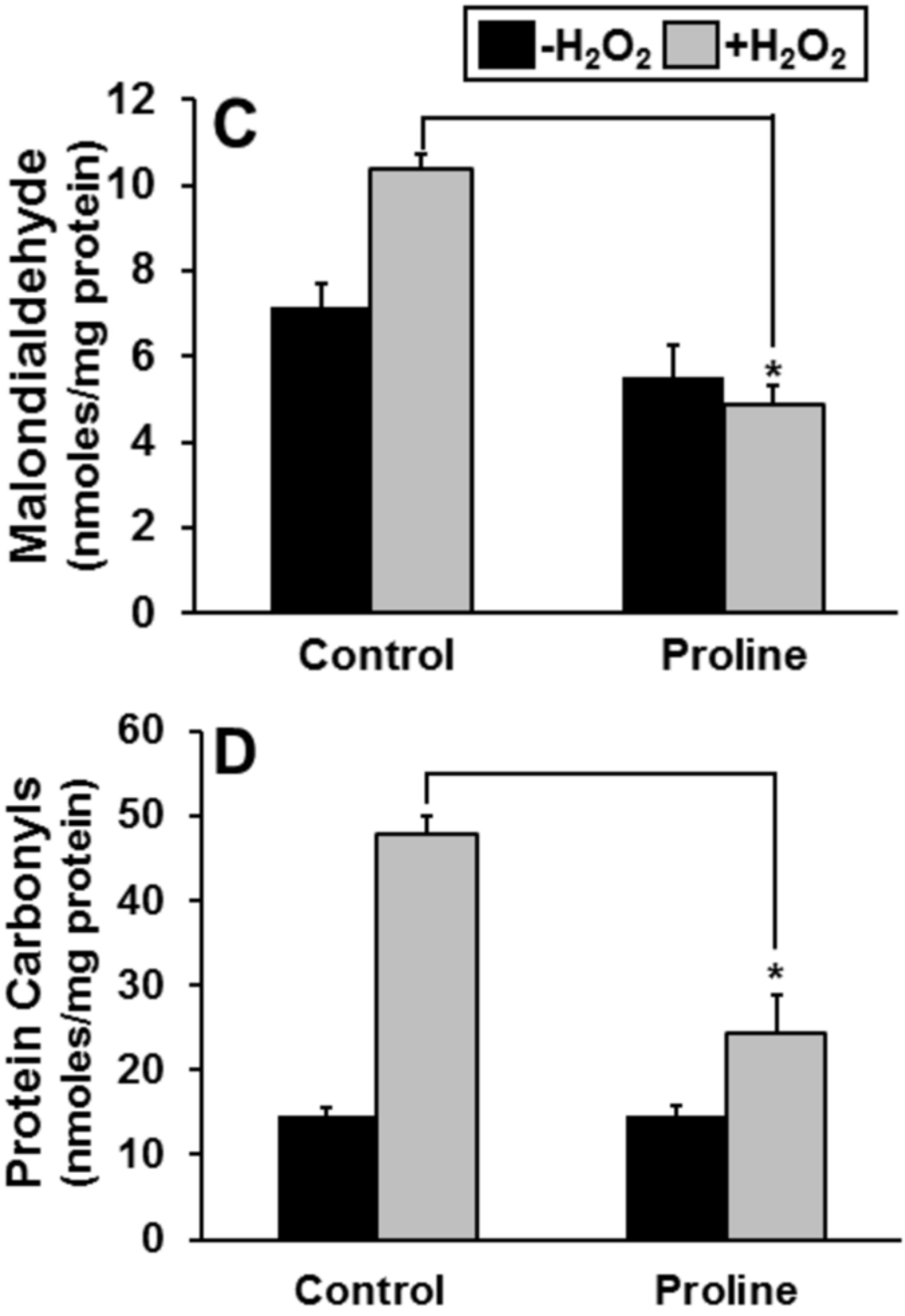
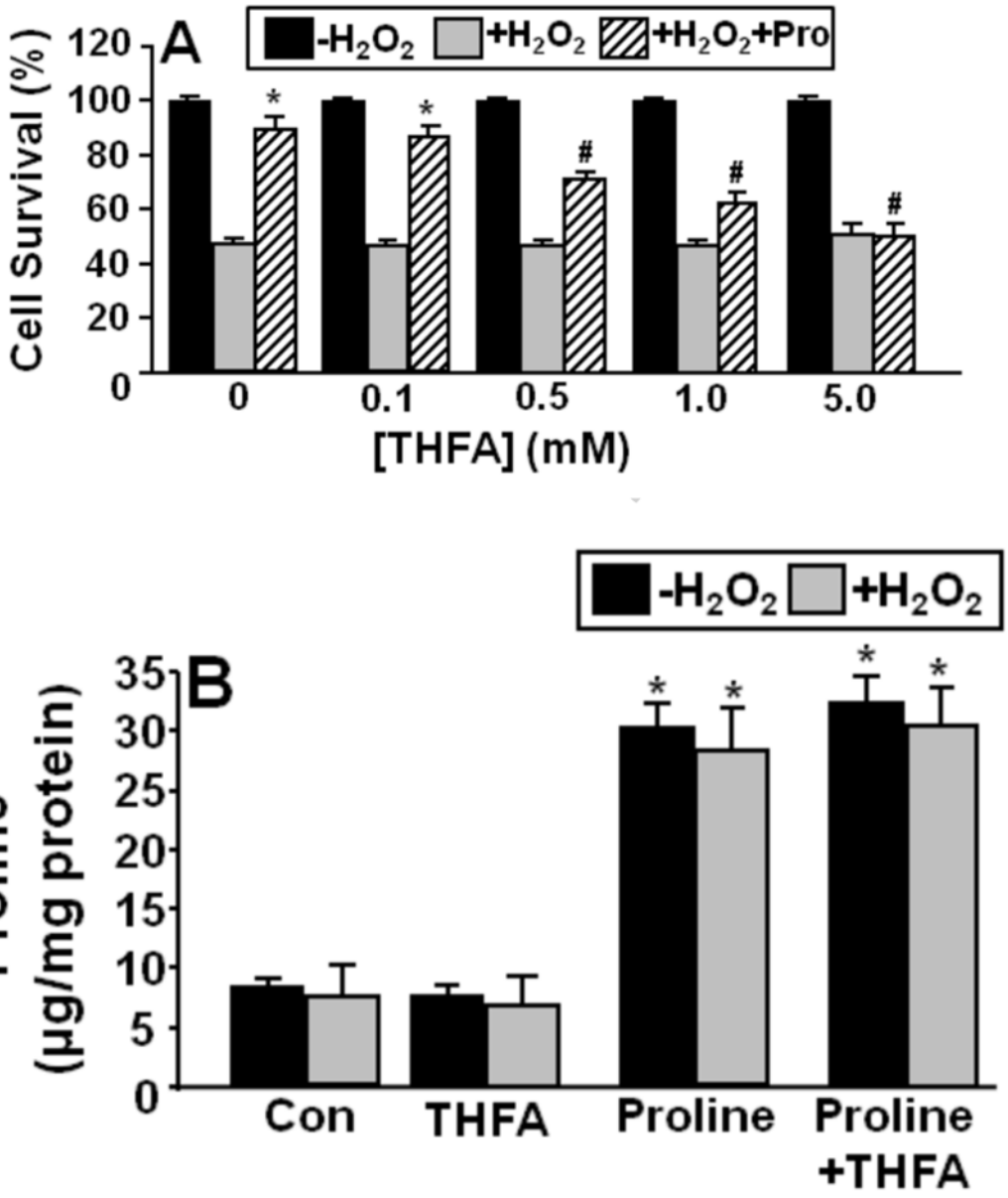


Fig. 2.

Proline protects WM35 cells against oxidative stress. (A) WM35 cells were treated with (black squares) and without (gray diamonds) proline (5 mM) for 1–24 h and then incubated with 0.5 mM H_2O_2 for 3 h in serum free medium. Percent cell survival was estimated using

the MTS cell viability assay. (B) WM35 cells were treated with (proline) and without (control) proline (5 mM) for 12 h and then incubated with and without 0.5 mM H_2O_2 (3 h) in serum free medium. Cell survival measurements were determined using the Cell Titer-Glo Luminescent assay. (C and D) WM35 cells were treated with (proline) and without (control) proline (5 mM) for 12 h and incubated with and without 0.5 mM H_2O_2 (3 h) in serum free medium. Oxidative stress markers (C) malondialdehyde and (D) protein carbonyls were then measured as described. Each value represents mean ± SD from five different experiments (*$P < 0.05$).



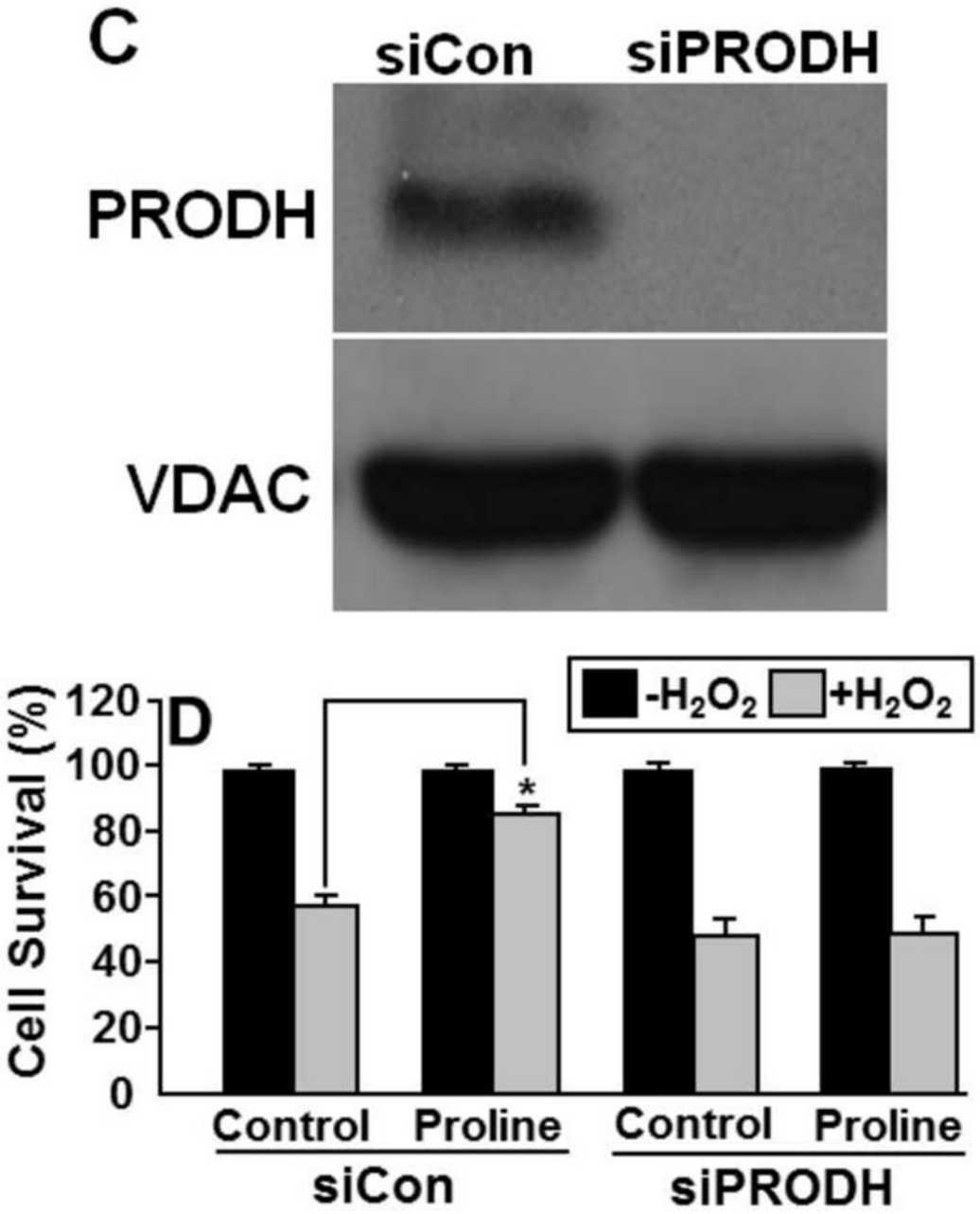
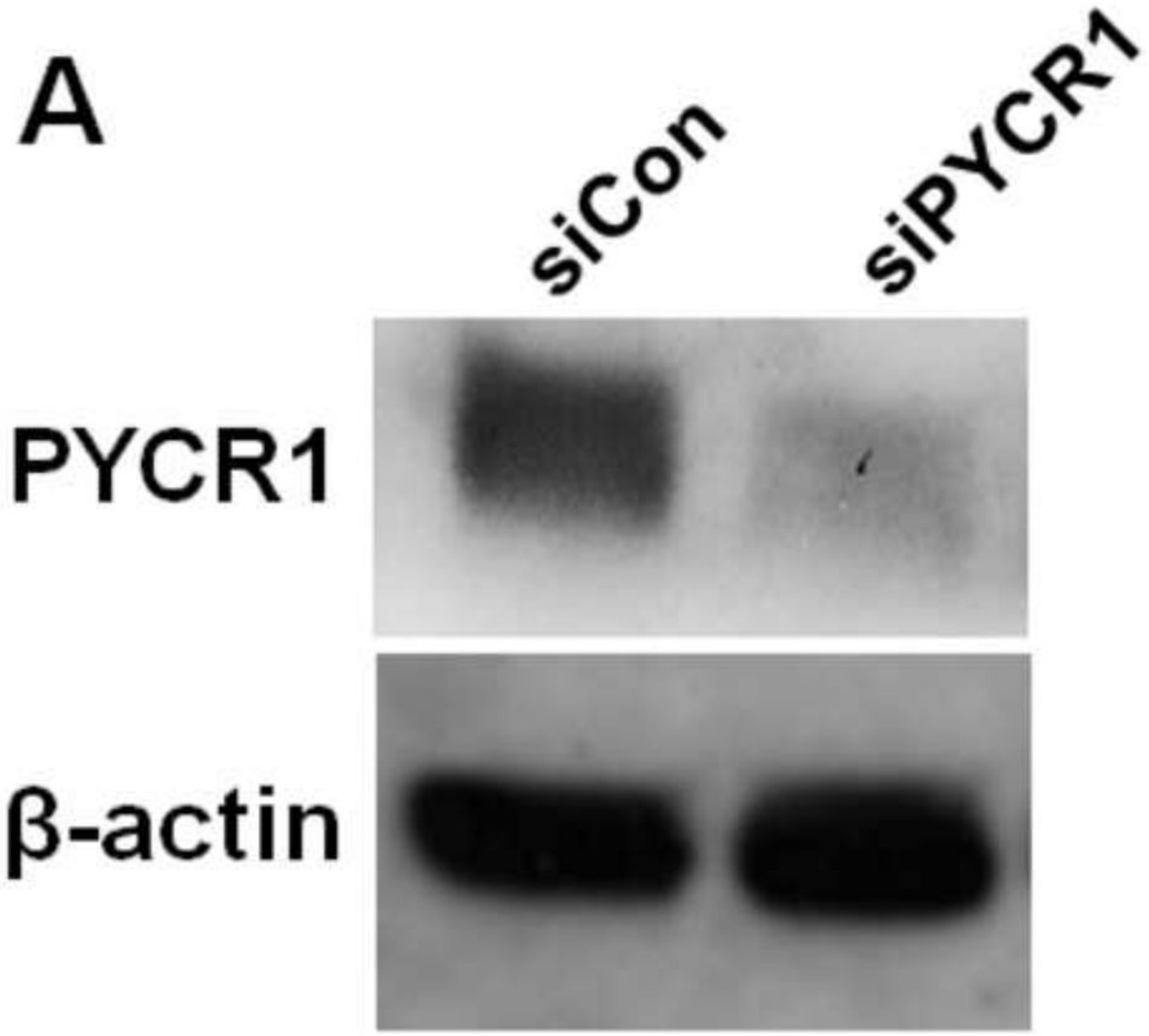
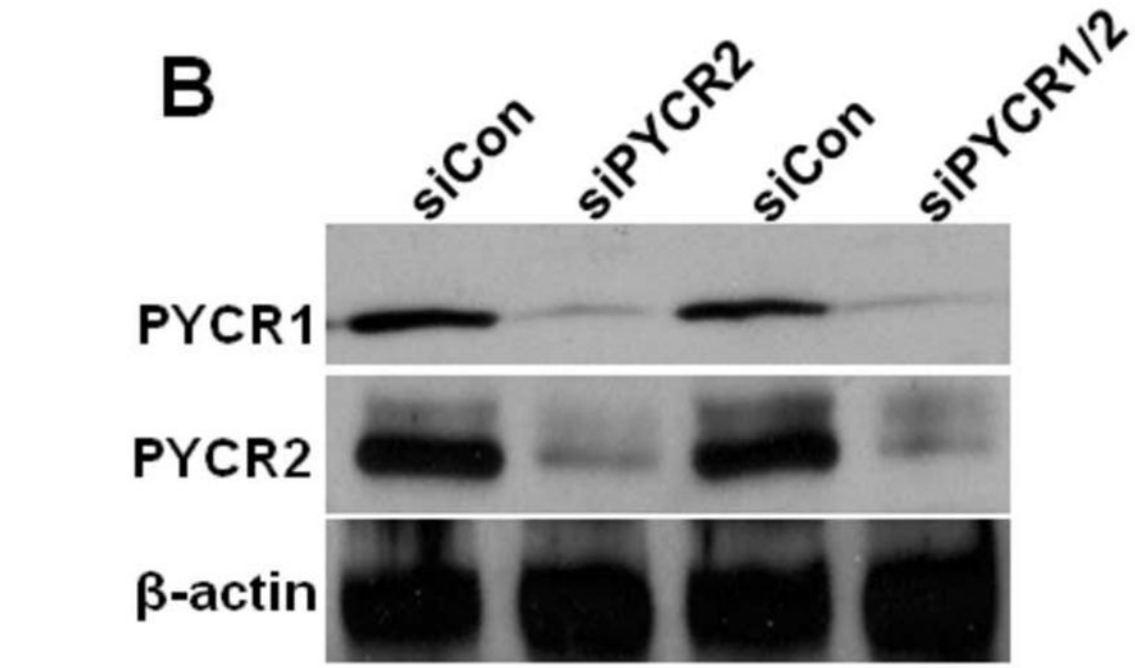


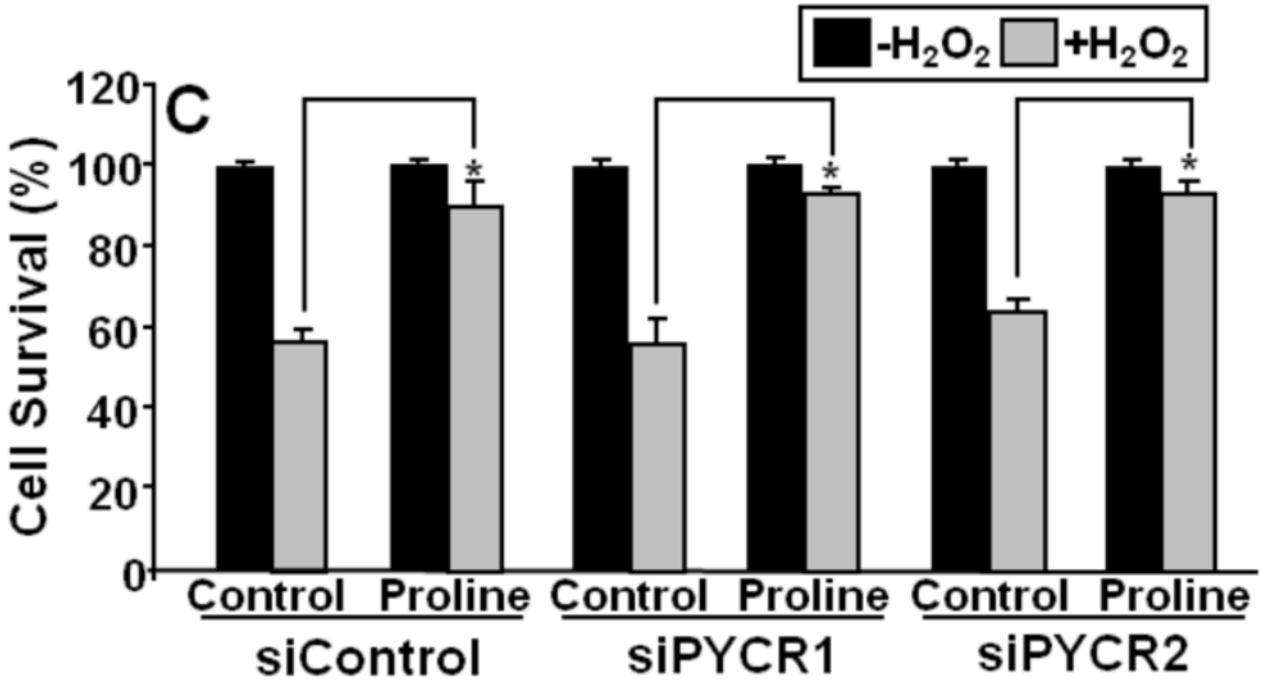
Fig. 3.

Knockdown of PRODH abolishes proline protection. (A) WM35 cells were treated (12 h) with and without proline (5 mM) in the presence of increasing concentrations of THFA (0.1–5 mM). Cells were then incubated with and without 0.5 mM H_2O_2 (3 h) in serum free medium. Percent cell survival was estimated using the MTS cell viability assay. Each value represents mean ± SD of separate experiments (n = 5). $^{*}P < 0.05$ when compared to H_2O_2 stress without proline, $^{\#}P < 0.05$ when compared to H_2O_2 stressed cells treated with proline and 0.1 mM THFA. (B) Intracellular proline levels in control cells, THFA (5 mM) treated cells, proline (5 mM) treated cells, and THFA + proline treated cells with and without H_2O_2 stress (0.5 mM, 3 h). Each value represents mean ± SD of separate experiments (n = 5) ($^{*}P < 0.05$). (C) Western analysis of PRODH in WM35 cells transfected with control siRNA

(siCon, 50 nM) and PRODH siRNA (siPRODH, 50 nM) for 48 h. VDAC is shown as a control. (D) Percent cell survival of control siRNA (siCon) or PRODH siRNA (siPRODH) treated WM35cells with and without H_2O_2 stress in the absence (control) and presence of proline (5 mM). Percent cell survival was estimated using the MTS cell viability assay. Each value represents mean ± SD of separate experiments (n = 4) (*$P < 0.05$).







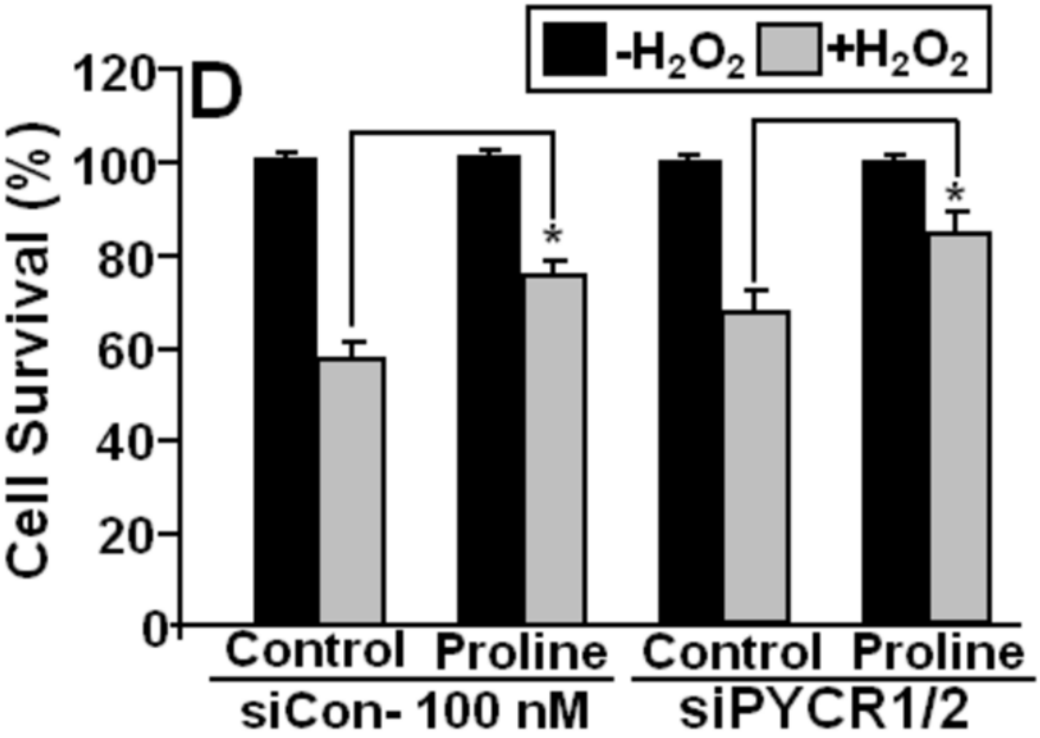


Fig. 4.

Knockdown of PYCR. (A) Western blot analysis of PYCR1 in WM35 cells transfected with control siRNA (siCon, 50 nM) and PYCR1 siRNA (siPYCR1, 50 nM) for 48 h. β-actin is shown as a control. (B) Western analysis of PYCR1 and PYCR2 in cells transfected with siCon (50 nM), siPYCR2 alone (50 nM), and siPYCR1 (50 nM) and siPYCR2 (50 nM) combined (siPYCR1/2, 100 nM total). For siPYCR1/2, 100 nM of siCon was used. β-actin is shown as a control. (C). Percent cell survival of siCon, siPYCR1, and siPYCR2 transfected cells with and without H_2O_2 stress in the absence (control) and presence (proline) of proline (5 mM). (D) Percent cell survival of WM35 cells transfected with siCon and siPYCR1/2 with and without H_2O_2 stress in the absence (control) and presence of proline (5 mM). Percent cell survival was estimated using the MTS cell viability assay. Each value represents mean ± SD of separate experiments (n = 4) (* $P < 0.05$).

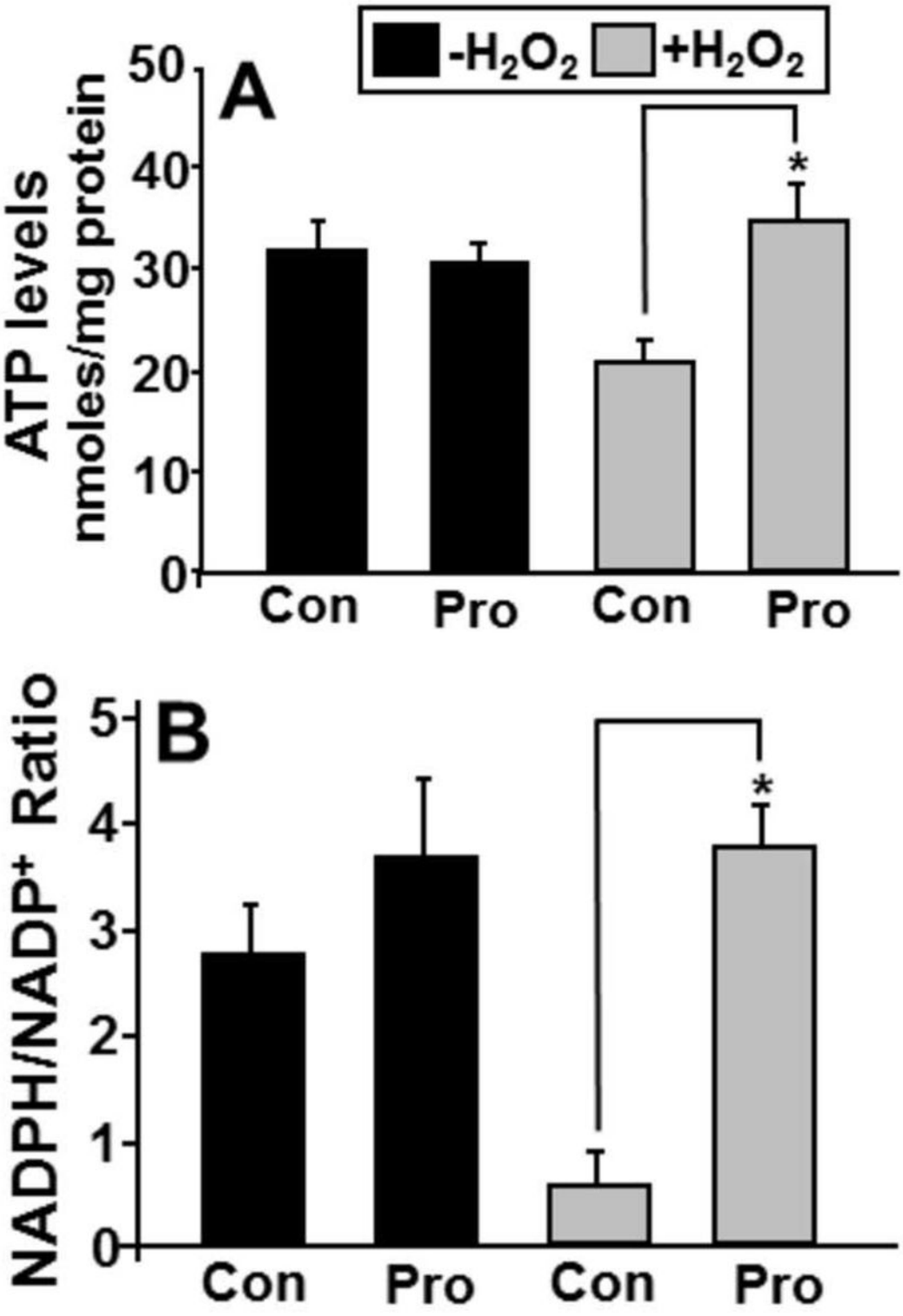


Fig. 5.

(A) ATP levels and (B) NADPH/$NADP^+$ ratio were measured in control and proline treated cells with or without 0.5 mM H_2O_2 stress (3 h). Each value represents mean ± SD of separate experiments (n = 4) (*$P < 0.05$).

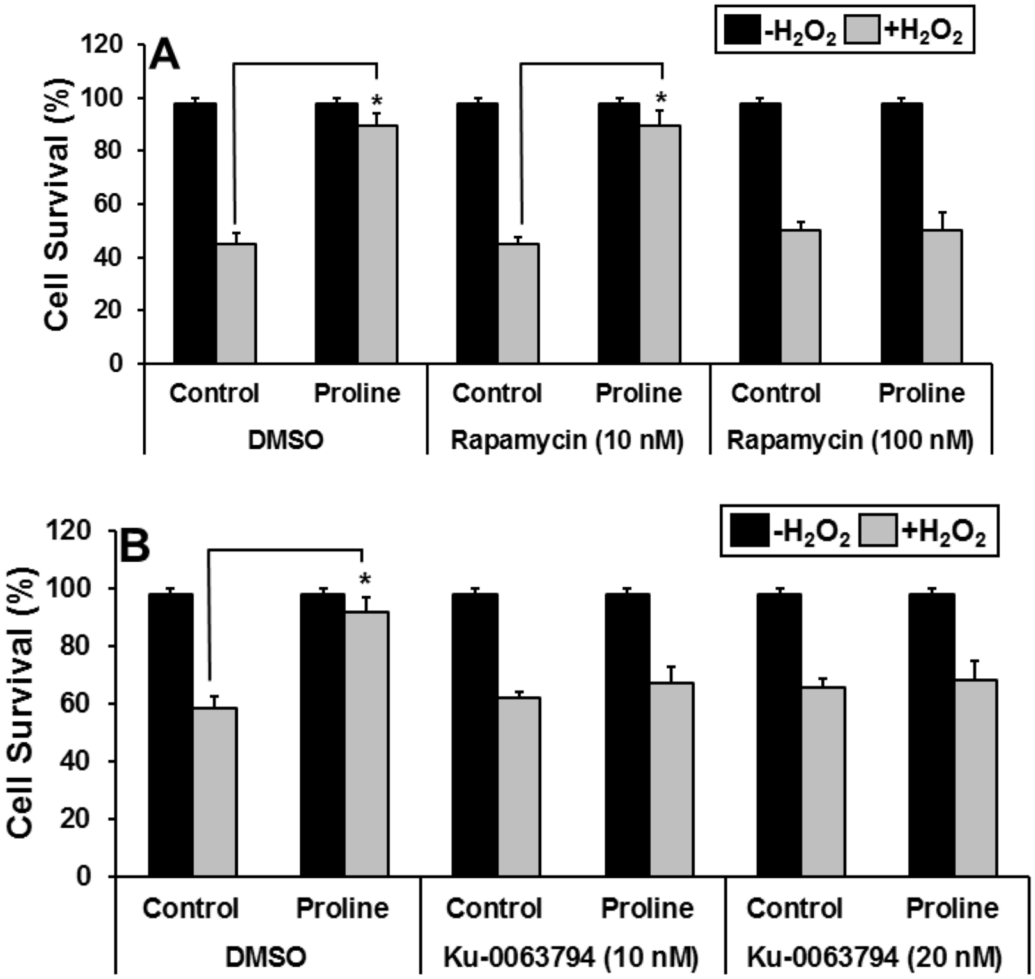
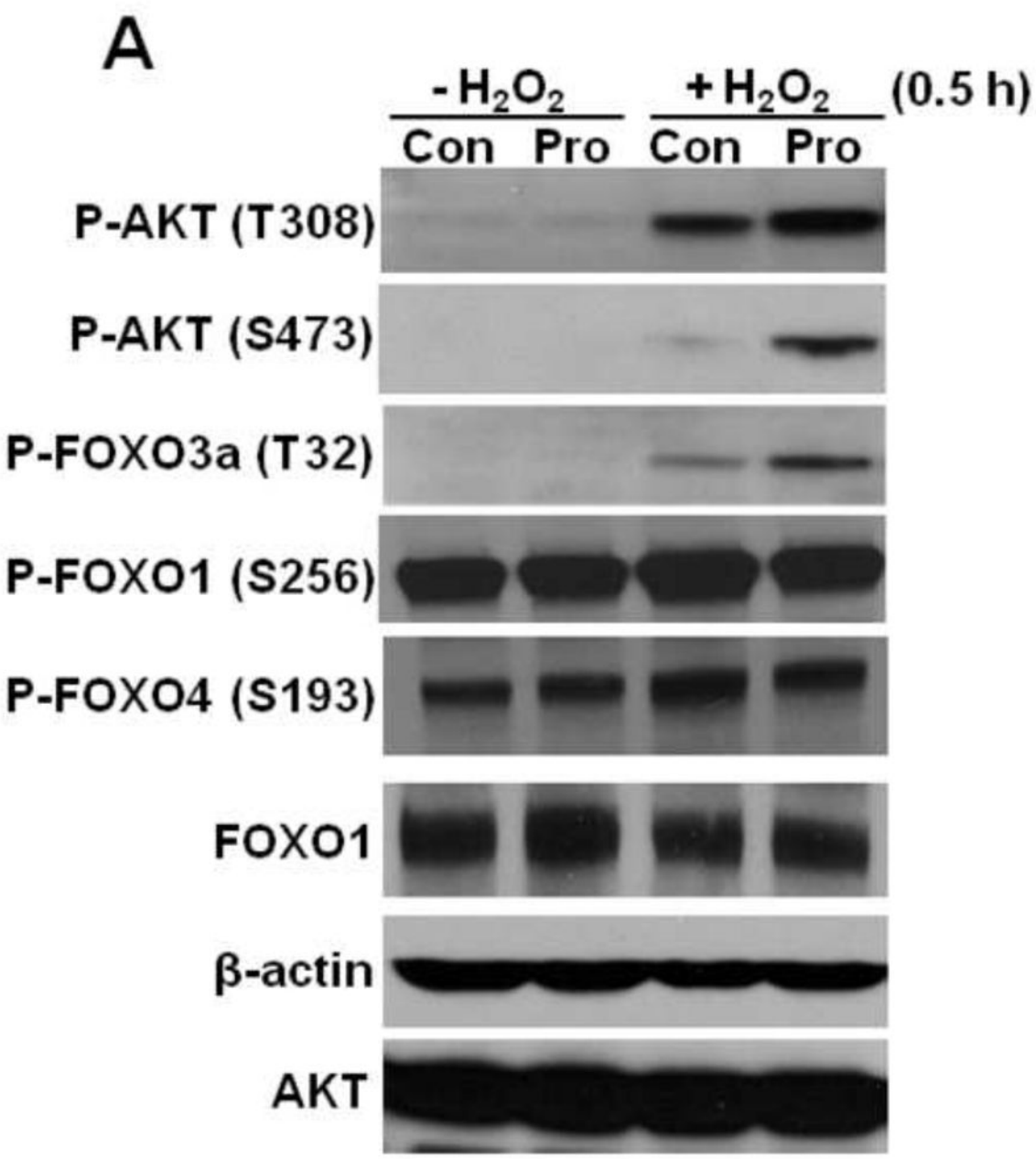
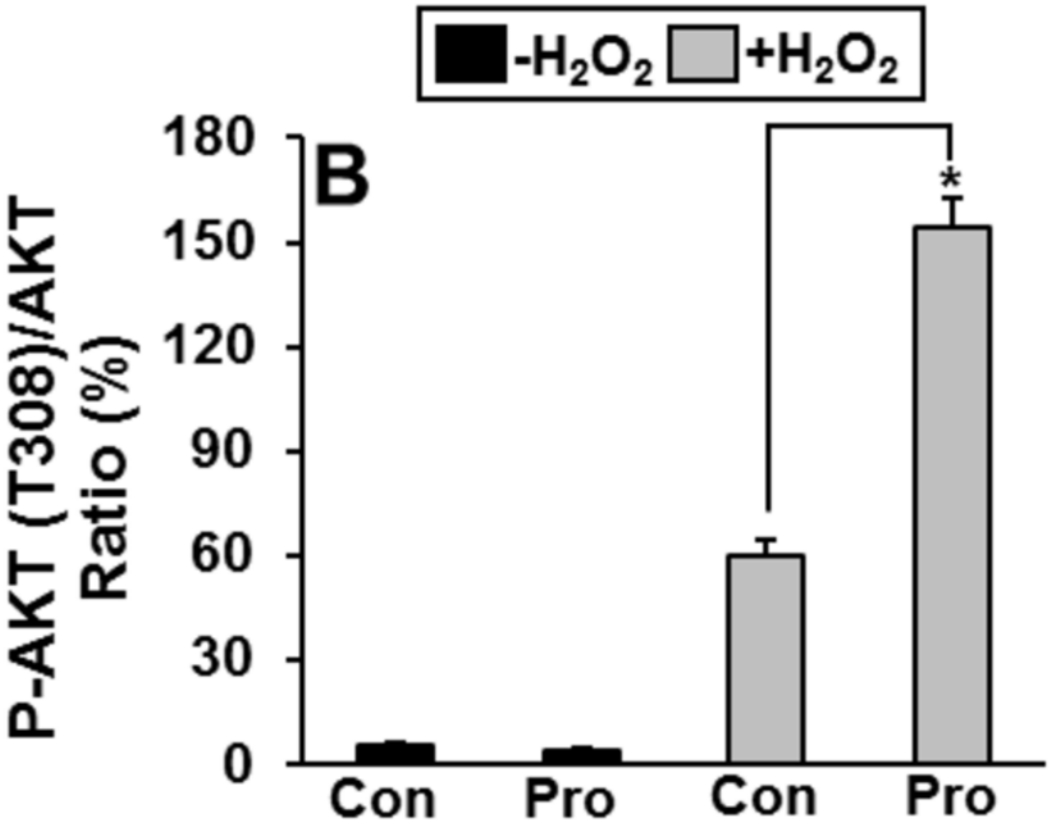
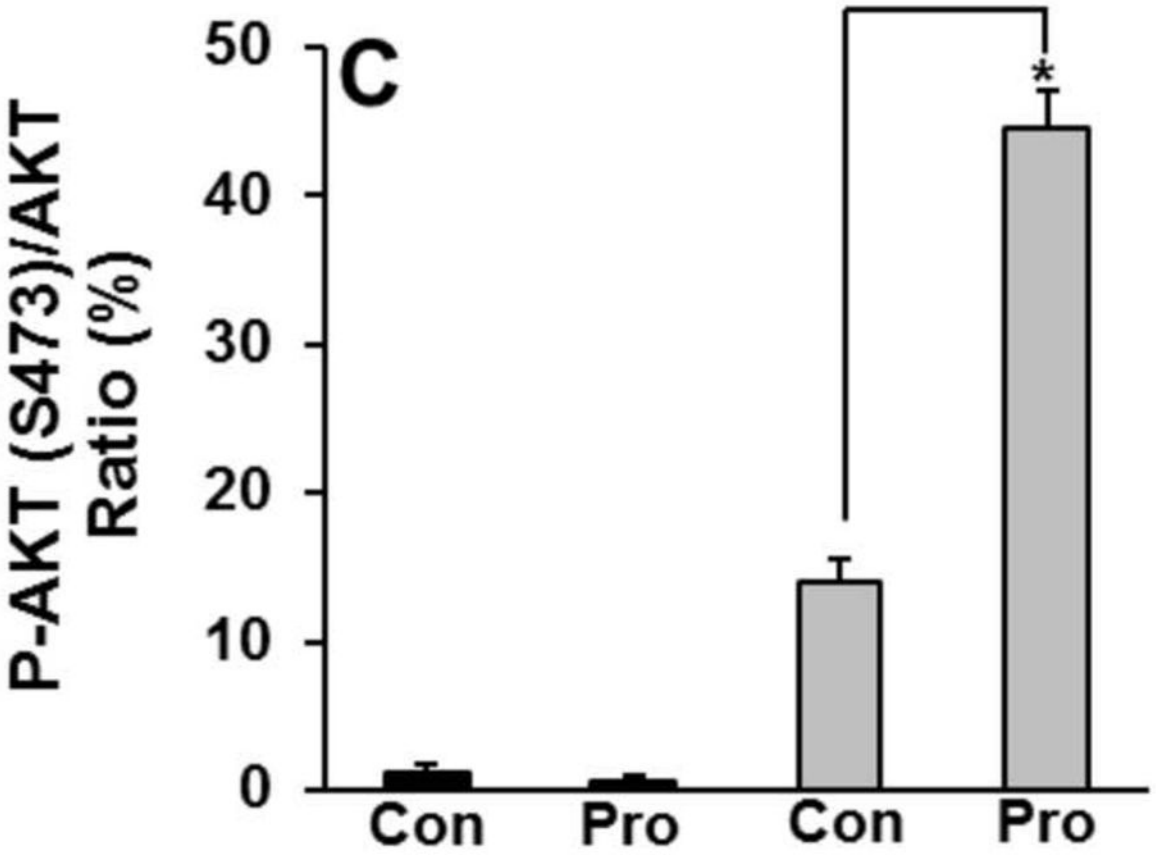


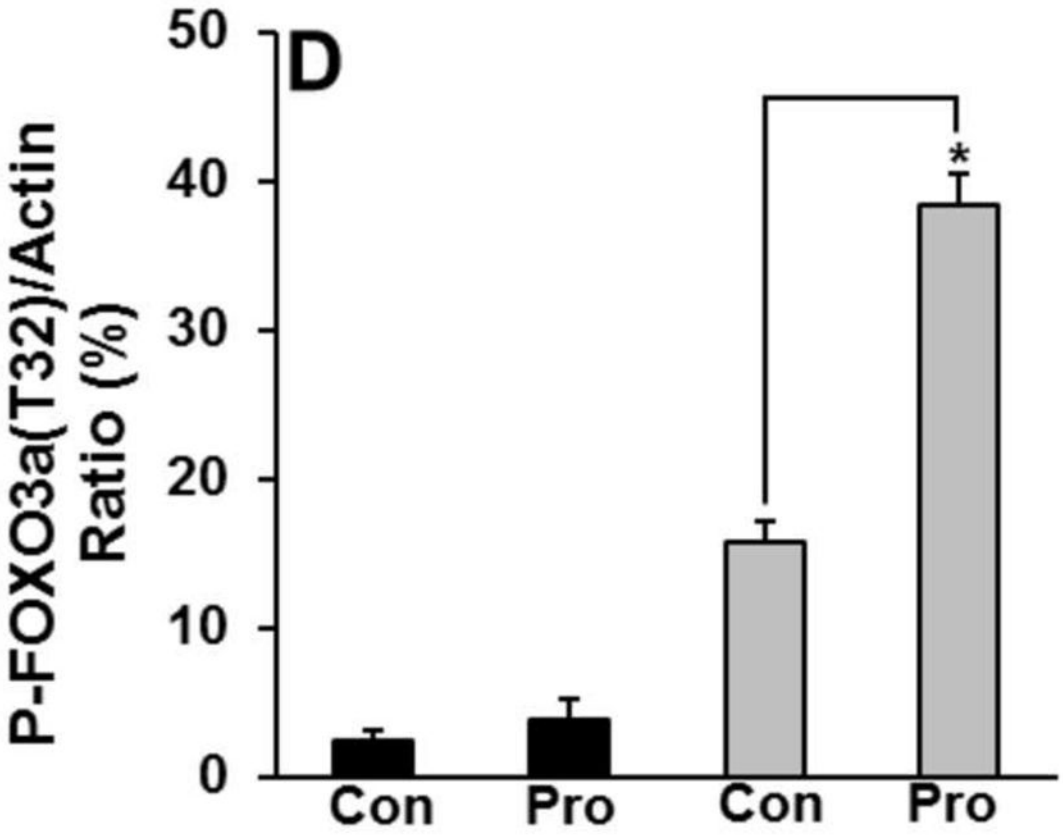
Fig. 6.

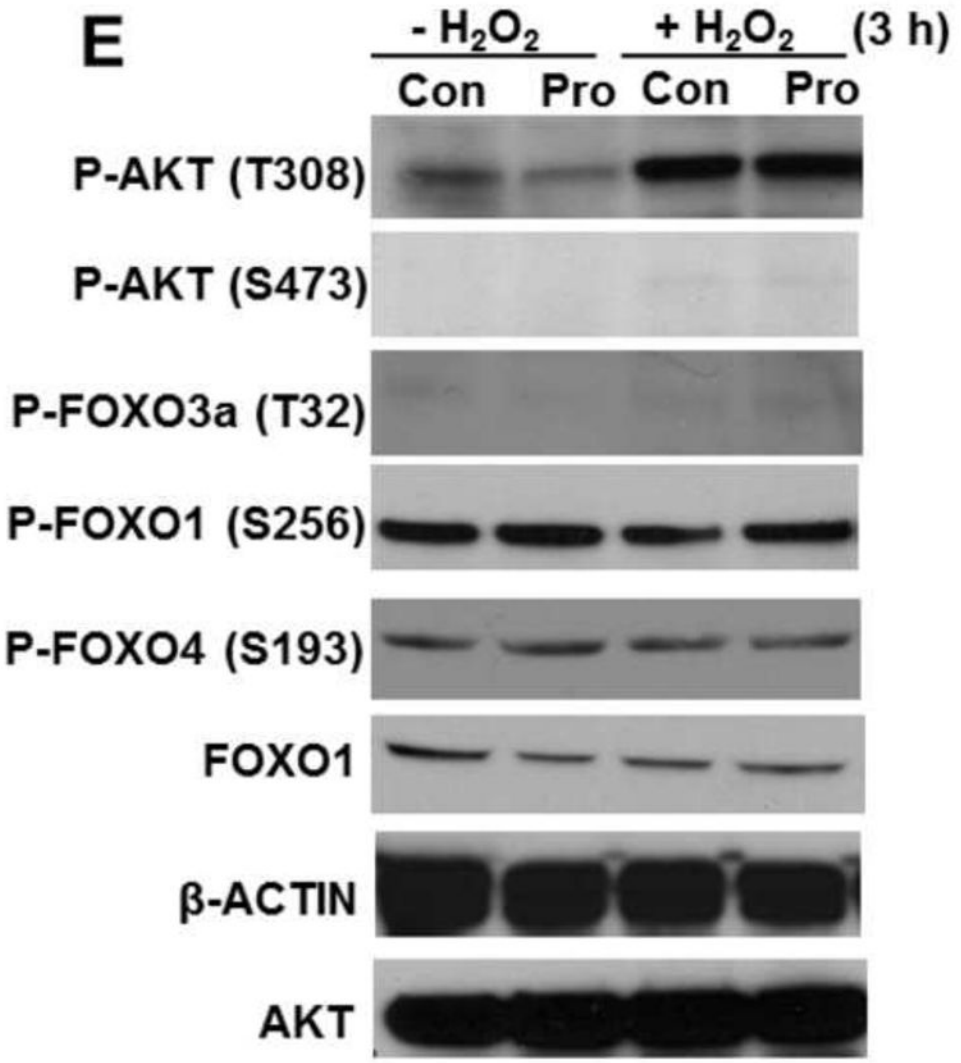
Inhibition of mTORC1 and mTORC2. (A) Cell survival of WM35 cells incubated with rapamycin (10 nM and 100 nM) and treated with and without H_2O_2 stress (3 h) in the absence (control) and presence of proline (5 mM). (B) Cell survival rate of WM35 cells incubated with Ku-0063794 and treated with and without H_2O_2 stress (3 h) in the absence (control) and presence of proline (5 mM). Cells treated with DMSO were used as controls. Percent cell survival was estimated using the MTS cell viability assay. Each value represents mean ± SD of separate experiments (n = 6) (* $P<0.05$).











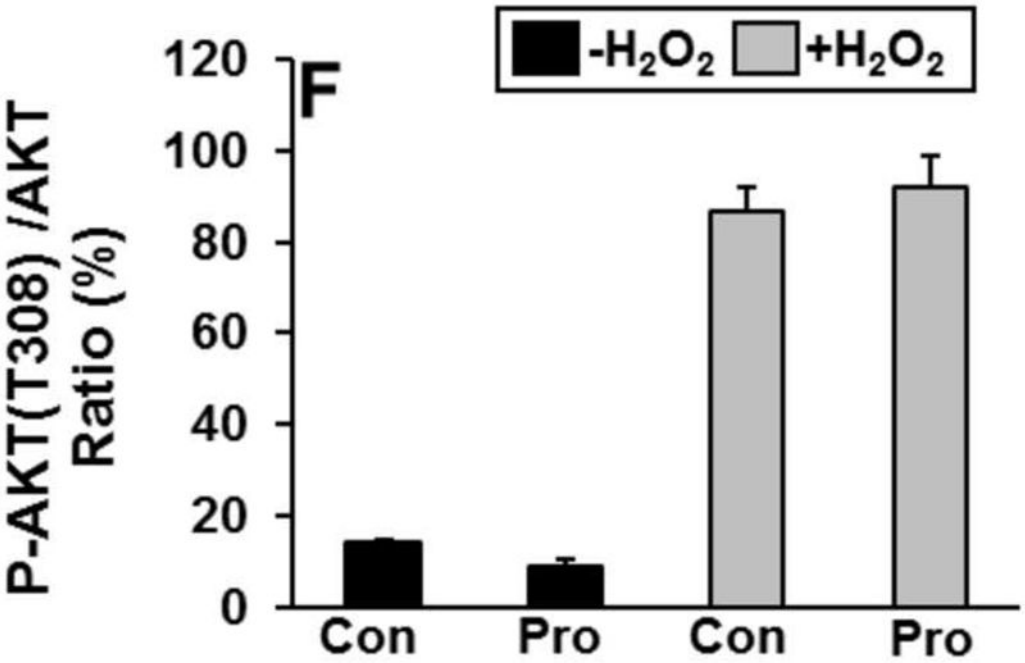
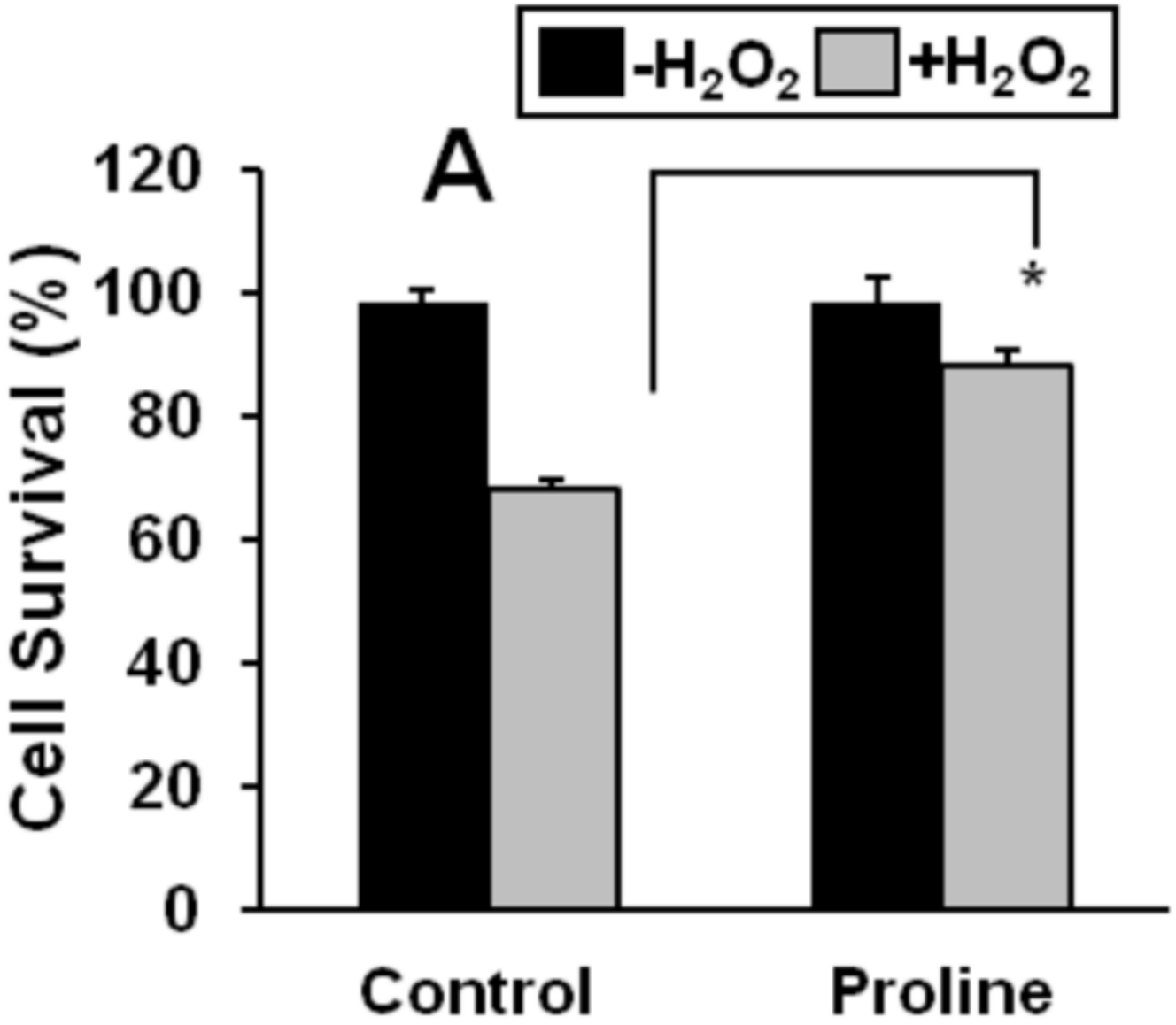
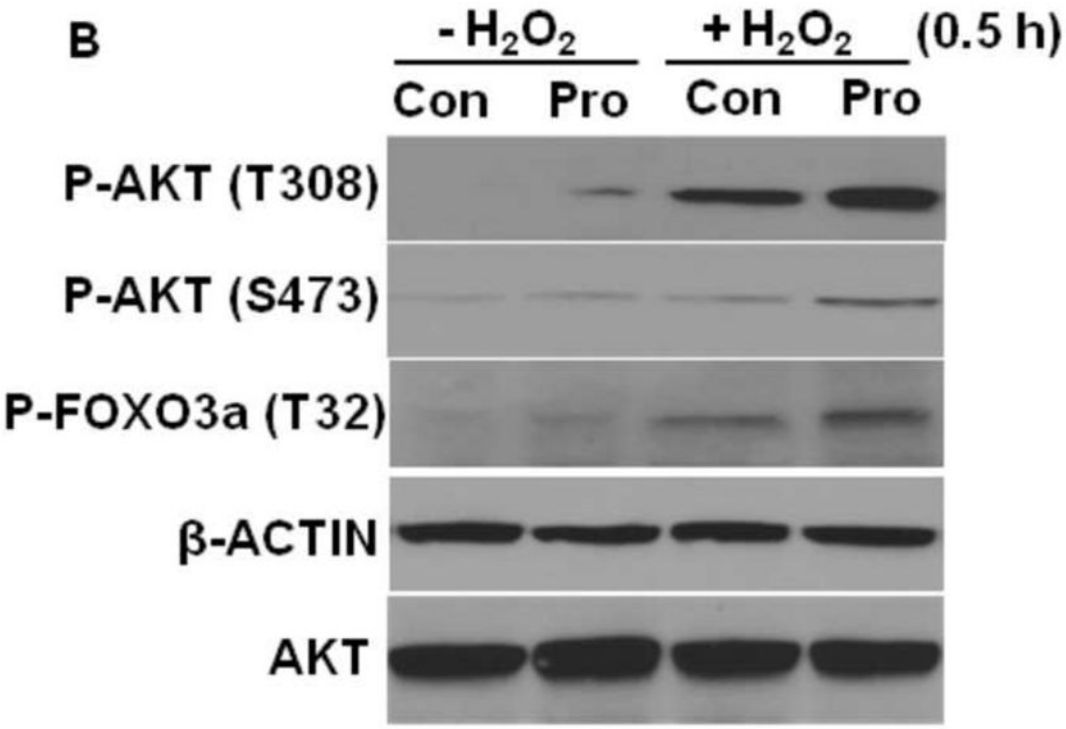
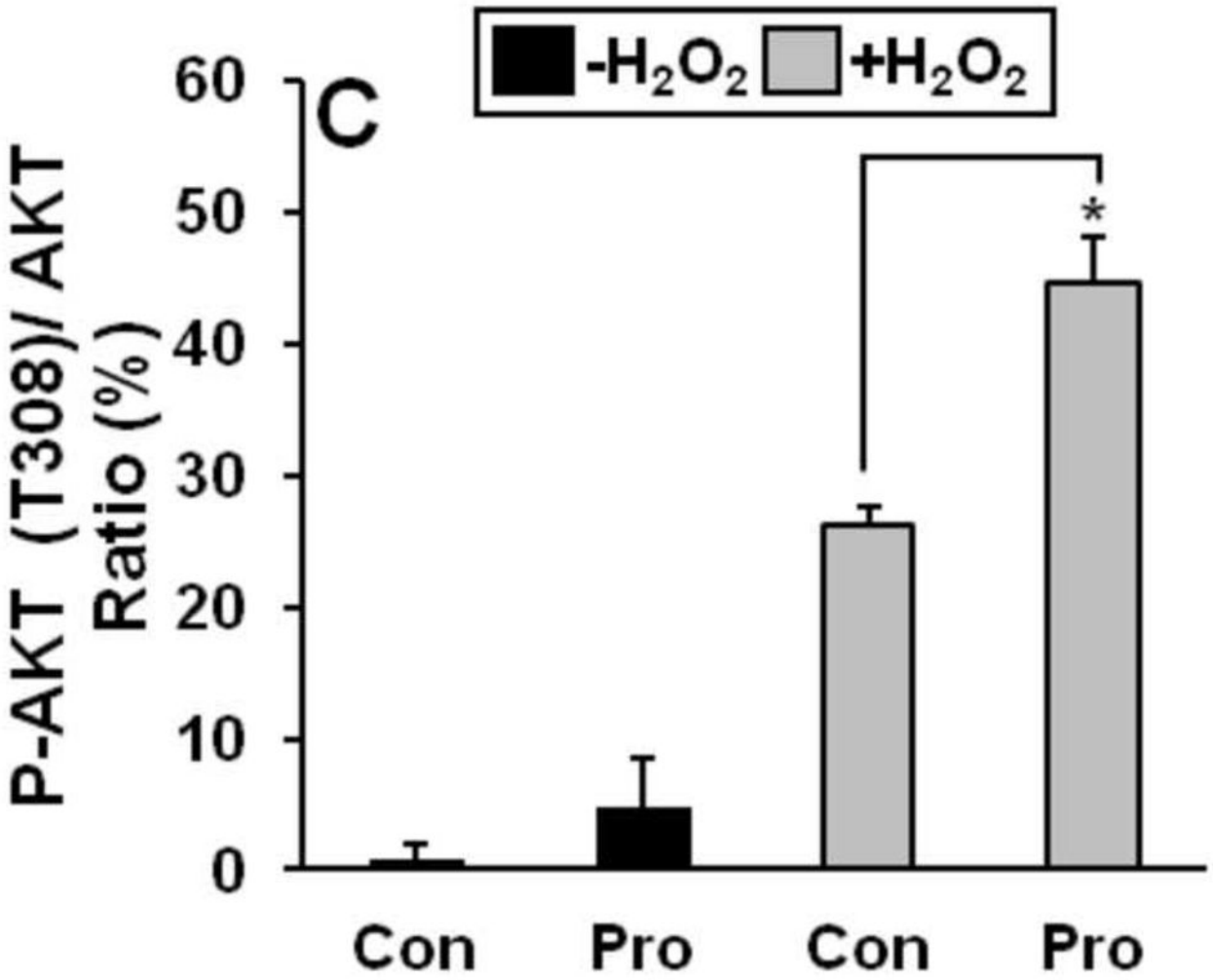


Fig. 7.

Proline upregulates Akt signaling during H_2O_2 stress. (A) Western analysis of P-Akt (T308 and S473), P-FoxO3a (T32), P-FoxO1 (S256) and P-FoxO4 (S193) in WM35 cells treated with and without proline (5 mM) for 12 h and incubated with and without 0.5 mM H_2O_2 (0.5 h) in serum free medium. Controls are FoxO1, β-actin, and Akt. Quantified levels of (B) P-Akt-T308 and (C) P-Akt-S473 relative to total Akt (P-Akt/Akt). (D) Quantified levels of P-FoxO3a-T32 relative to β-actin. Each value represents mean ± SD of separate experiments (n = 4) (*$P < 0.05$). (E) Same as panel A except cells were incubated with and without 0.5 mM H_2O_2 for 3 h. (F) Quantified levels of P-Akt-T308 relative to total Akt (P-Akt/Akt). Each value represents mean ± SD of separate experiments (n = 4).







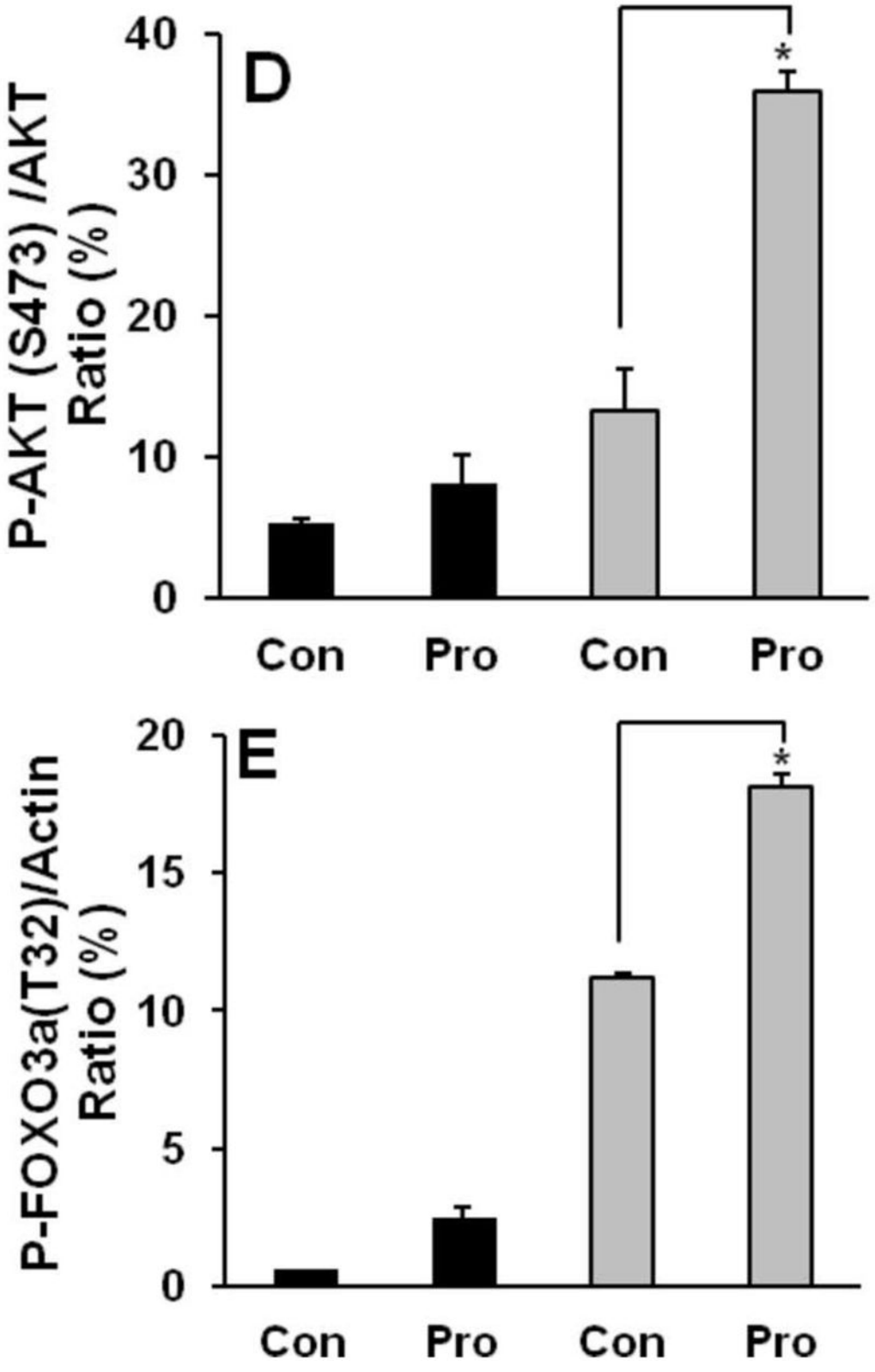
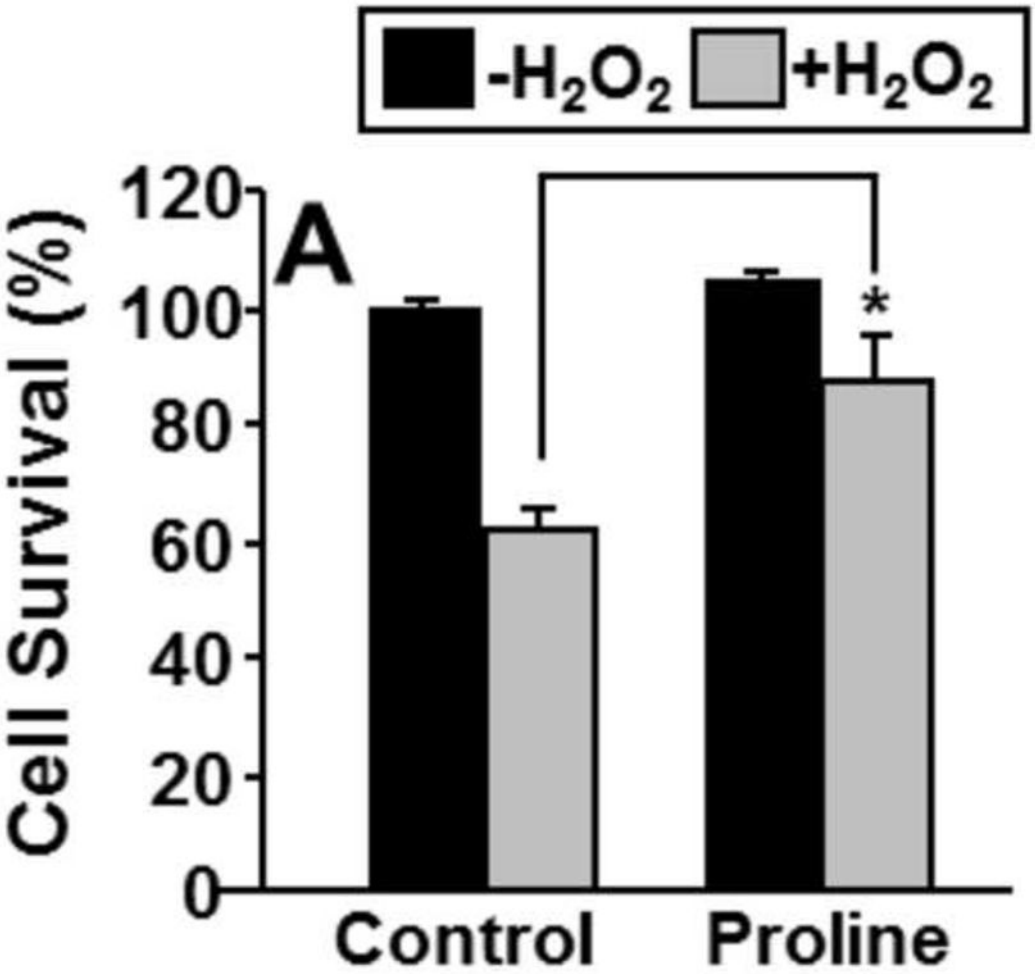
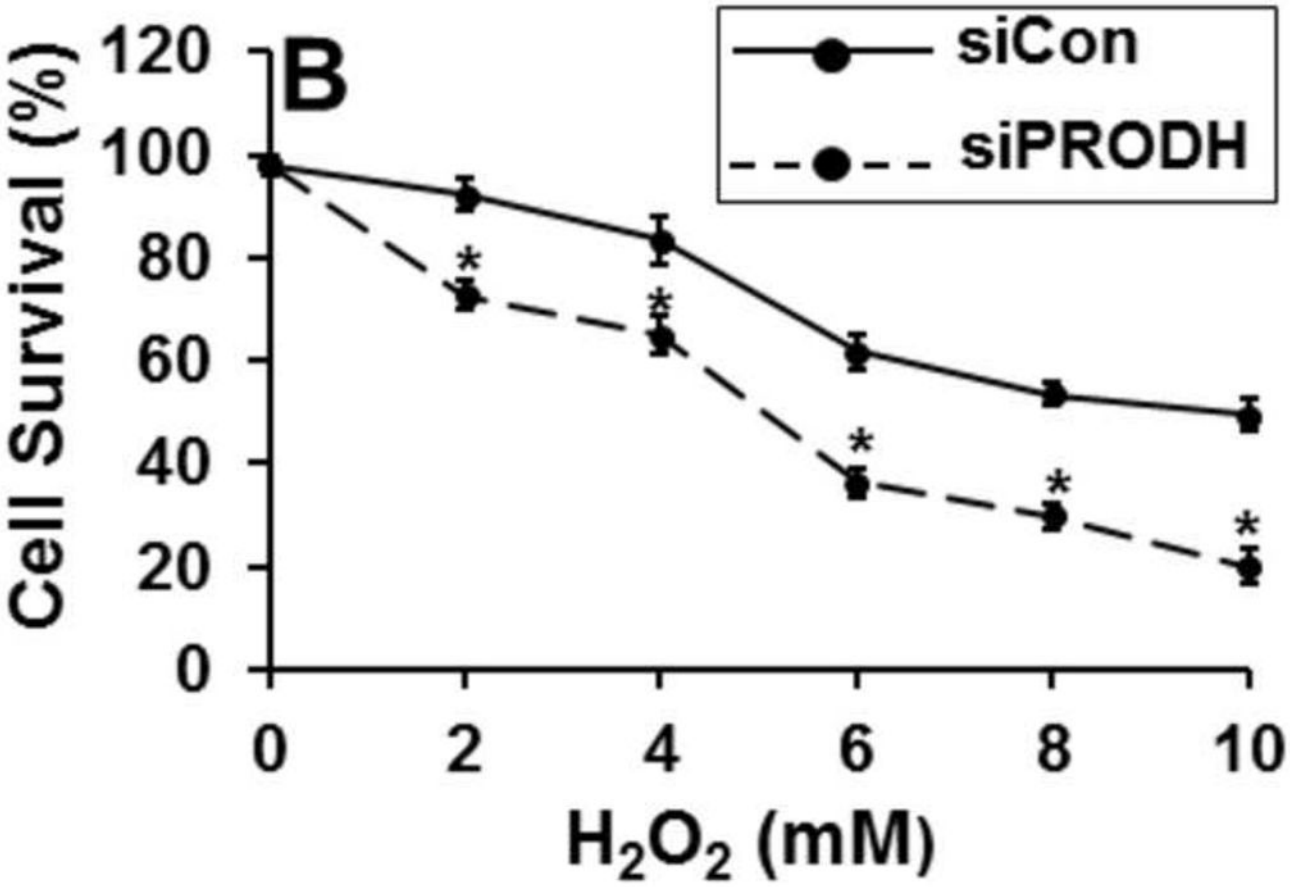
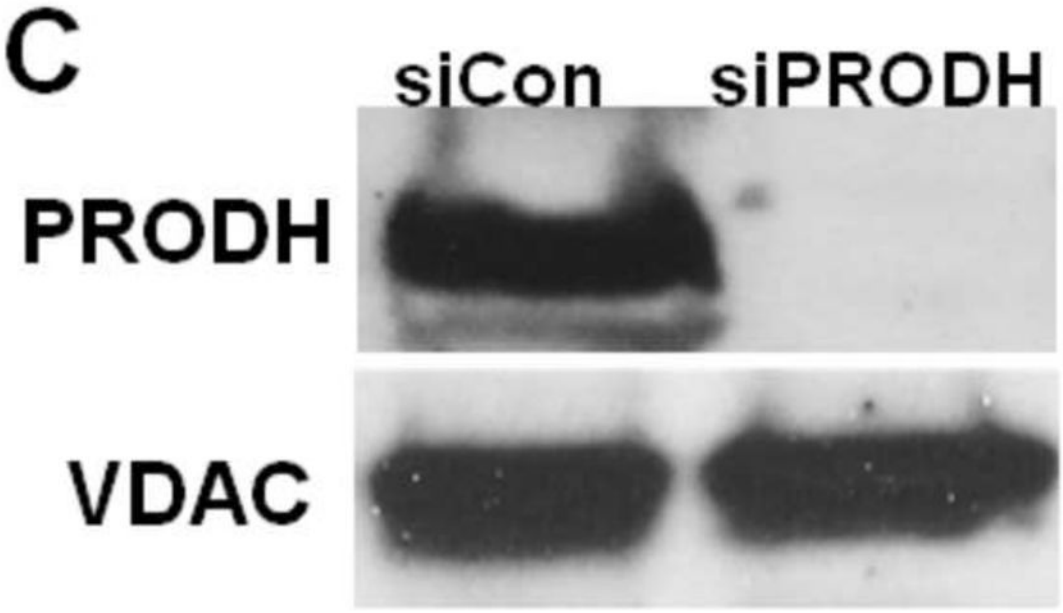


Fig. 8.

Proline protection and Akt signaling at lower H_2O_2 levels. (A) WM35 cells were treated with (proline) and without (control) proline (5 mM) for 12 h and incubated with and without 50 μM H_2O_2 (3 h) in serum free medium. Percent cell survival was estimated using the MTS cell viability assay. (B) Western analysis of P-Akt (T308 and S473) and P-FoxO3a (T32) in WM35 cells treated with and without proline (5 mM) for 12 h and incubated with and without 50 μM H_2O_2 (0.5 h) in serum free medium. Controls are β-actin, and Akt. Quantified levels of (C) P- Akt-T308 and (D) P-Akt-S473 relative to total Akt (P-Akt/Akt). (E) Quantified levels of P- FoxO3a-T32 relative to β-actin. Each value represents mean ± SD of separate experiments (n = 4) (*$P < 0.05$).







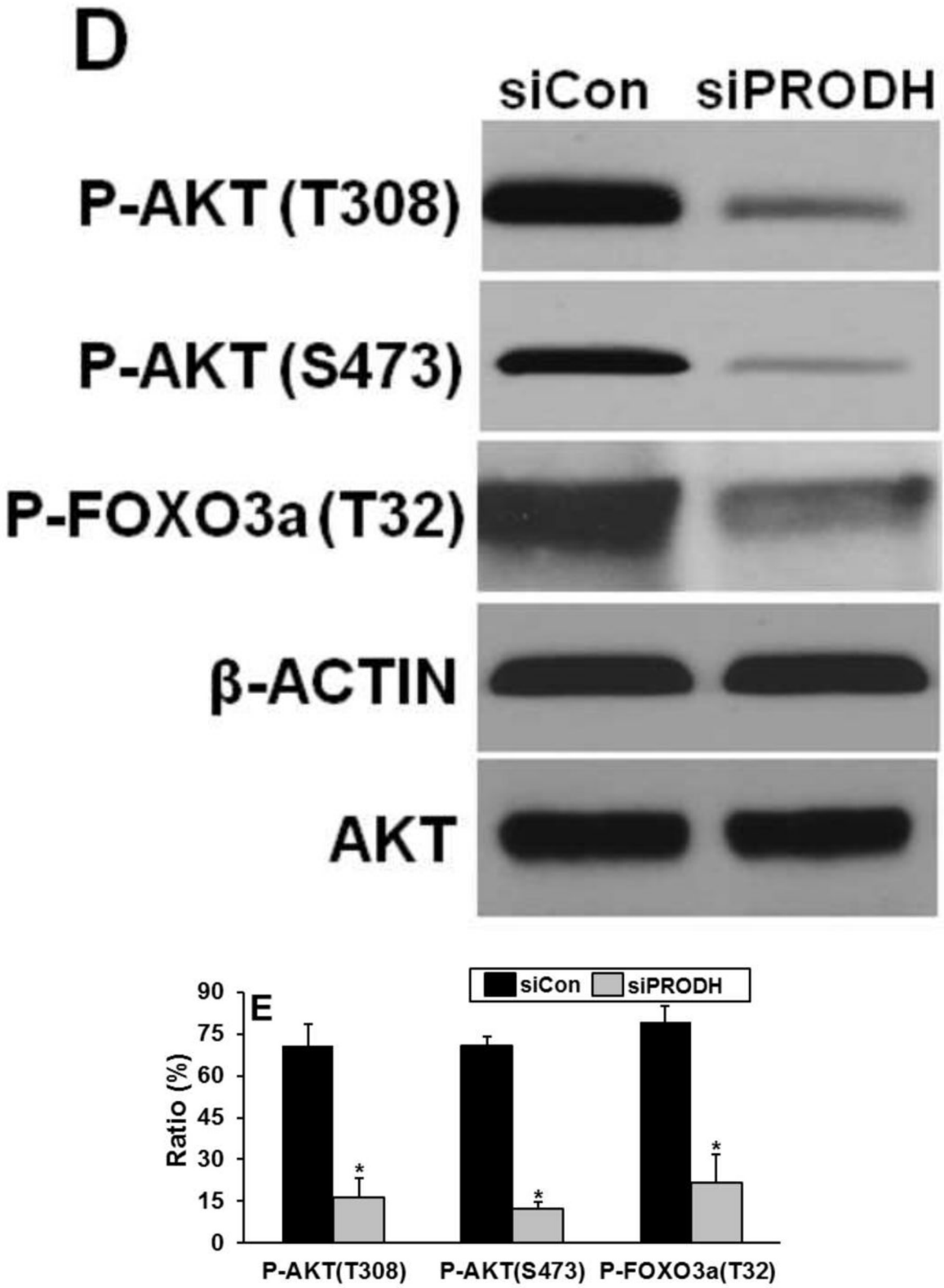


Fig. 9.

Knockdown of PRODH and Akt signaling pathway in prostate cancer cells. (A) RWPE-1 cells were treated with and without proline (5 mM) for 12 h and then incubated with 0.5 mM H_2O_2 for 3 h in serum free medium. Percent cell survival was estimated using the MTS cell viability assay. (B) Survival rates of PC3 cells transfected (48 h) with control siRNA (siCon) and PRODH siRNA (siPRODH) and incubated with different concentrations of H_2O_2 (0–10 mM) for 3 h. (C) Western blot analysis of PRODH in PC3 cells transfected with siCon (50 nM) and siPRODH (50 nM) for 48 h. VDAC is shown as a control. (D) Western blot analysis of P-Akt (T308 and S473), P-FoxO3a (T32), and β-actin in PC3 cells transfected with siCon and siPRODH (48 h). (E) Quantification of P-Akt-T308 and P-Akt-S473 relative to total AKT (P-Akt/Akt) and P- FoxO3a-T32 relative to β-actin. Each value represents mean ± SD of separate experiments (n = 4) (* $P < 0.05$).

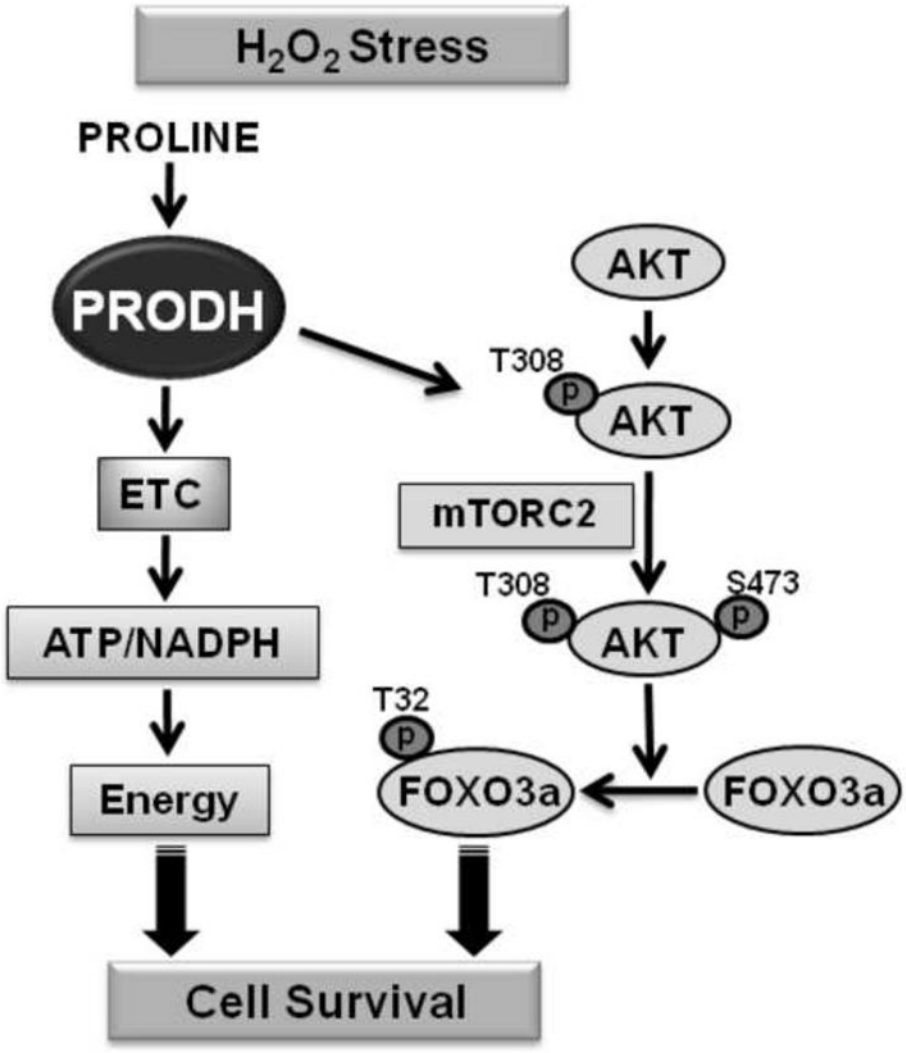


Fig. 10.

Model of proline protection against oxidative stress induced cell death. Proline oxidation in the mitochondrion is coupled to reduction of the electron transport chain (ETC) by PRODH. PRODH activity helps support oxidative phosphorylation and ATP formation and prevents decreases in NADPH/$NADP^+$ which sustains cells during oxidative stress. Through an unknown mechanism, proline/PRODH further activates Akt during H_2O_2 stress which leads to inhibition of FoxO3a and blocks cell death. Activation of Akt by proline and PRODH may involve mTORC2 or other factors not yet identified.