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Regulation of protein phosphatase 2A by hydrogen peroxide and glutathionylation

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Abstract

The regulation of protein phosphatase 2A (PP2A) and protein threonine phosphorylation by H_2O_2 was determined in Caco-2 cell monolayer. Incubation with H_2O_2 (20 μM) resulted in threonine phosphorylation of a cluster of proteins at the molecular mass range of 170–250 kDa. PKC activity and plasma membrane localization of several isoforms of PKC were not affected by H_2O_2. However, H_2O_2 reduced 80–85% of okadaic acid-sensitive protein phosphatase activity. Immunocomplex protein phosphatase assay demonstrated that H_2O_2 reduced the activity of PP2A, but not that of PP2C or PP1. Oxidized glutathione inhibited PP2A activity in plasma membranes prepared from Caco-2 cells and the phosphatase activity of an isolated PP2A. PP2A activity was also inhibited by *N*-ethylmaleimide, iodoacetamide, and *p*-chloromercuribenzoate. Inhibition of PP2A by oxidized glutathione was reversed by reduced glutathione. Glutathione also restored the PP2A activity in plasma membranes isolated from H_2O_2-treated Caco-2 cell monolayer. These results indicate that PP2A activity can be regulated by glutathionylation, and that H_2O_2 inhibits PP2A in Caco-2 cells, which may involve glutathionylation of PP2A.

Keywords: Signal transduction; Protein phosphorylation; Oxidative stress; Protein kinase C; Glutathione; PP2C; PP1

Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radical, are generated in cells as a result of normal aerobic metabolism [1]. Excessive generation and reduced inactivation of ROS play an important role in pathogenesis of a number of diseases [2,3]. A significant body of evidence indicates that ROS alters intracellular signal transduction, including protein kinase C (PKC)-mediated signaling. ROS and antioxidants regulate PKC signaling in various cell systems by interacting with the N-terminal Zn^{2+} binding and cysteine-rich motifs and C-terminal cysteines of PKC isoforms [4]. Oxidative stress-induced expression of ornithine decarboxylase [5], loss of colonocyte integrity [6], induction of proto-oncogenes in vascular smooth muscle [7], mitochondrial dysfunction [8], and prevention of transient outward current in atrial myocytes [9] were mediated by PKC activity. Oxidants and antioxidants are also shown to regulate protein phosphatases, such as calcineurin [10,11] and PP2C [12]. However, very little is known about the regulation of PP2A by oxidative stress in the cell.

PP2A is a family of tightly regulated serine/threonine phosphatases. It is a multimeric protein with a highly conserved catalytic protein (C subunit), a scaffolding protein (A subunit) and a regulatory protein (B subunit). Subunit B is a diverse family of proteins, which determines the substrate specificity and cellular localization of the holoenzyme complex [13]. The catalytic subunit C is regulated by phosphorylation and methylation [13]. A few studies also suggest that PP2A may be regulated by modification of cysteine residues [14]. Studies in a variety of models demonstrated an important role for PP2A in the regulation of cell cycle, cell morphology, and development [15]. PP2A also interacts with a number of cellular proteins, such as L-type calcium channels [16], to specifically regulate the function of these proteins.

In the present study we evaluated the effect of H_2O_2 on PP2A activity in Caco-2 cell monolayers, and determined the role of glutathionylation in inhibition of

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PP2A activity. This study shows that (i) H_2O_2 increases threonine phosphorylation of a cluster of proteins in Caco-2 cell monolayer, (ii) H_2O_2 rapidly inhibits the activity of PP2A, but not that of PP2C and PP1, and (iii) PP2A activity is regulated by glutathionylation, which may be involved in the mechanism of H_2O_2-mediated inhibition of PP2A.

Materials and methods

Cell culture. Caco-2 cells, purchased from American Type Culture Collection, Rockville, MD, were maintained under standard cell culture conditions at 37 °C in medium containing 10% (v/v) fetal bovine serum. Cells were grown on polycarbonate membranes in Transwells (24 mm; Costar, Cambridge, MA), and experiments were performed at 20–22 days after seeding.

H_2O_2 treatment. Confluent monolayers were bathed in PBS (Dulbecco's saline containing 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.6% BSA). H_2O_2 (20 μM) was administered (to both apical and basal compartments). Control cell monolayers were incubated in PBS without H_2O_2.

Preparation of plasma membrane and soluble fractions. Cell monolayers were washed twice with ice-cold PBS, and the plasma membrane and soluble fractions were prepared as described previously [17].

Detection of phosphoproteins. After incubation with or without H_2O_2, cells were lysed in hot lysis buffer-D (20 mM Tris, pH 7.4, containing 0.3% SDS, 0.1 mM sodium fluoride, and 1 mM PMSF). Proteins were extracted by heating for 10 min at 100 °C. Serine and threonine phosphorylated proteins were detected by immunoblot analysis using rabbit polyclonal anti-phosphoserine and anti-phosphothreonine antibodies.

Immunoblot analysis. Denatured protein extract or plasma membrane fraction was mixed with equal volume of Laemli's sample buffer (2× concentrated) and heated at 100 °C for 10 min. Phosphoproteins, PKC isoforms, and PPase isoforms were separated by SDS–PAGE and transferred to PVDF membranes. Membranes were probed with rabbit polyclonal anti-phosphoserine and anti-phosphothreonine antibodies or mouse monoclonal anti-PKC and anti-PPase antibodies, followed by corresponding HRP-conjugated secondary antibodies. The blot was developed using ECL chemiluminescence method (Amersham, Arlington Heights, IL).

Assay of PKC activity. PKC activity in plasma membrane fractions was measured by using the PKC assay kit (Upstate Biotechnology, Lake Placid, NY) according to vendor's instructions. Reaction terminated by spotting the reaction mixture on P81 filter discs. After washing discs were counted for phosphorylated peptide substrate.

Immunoprecipitation. After H_2O_2 treatment, Caco-2 cell monolayers were washed with ice-cold 20 mM Tris (pH 7.4) and the proteins extracted in lysis buffer-N (20 mM Tris, pH 7.4, containing 150 mM NaCl, 0.5% NP40, and protease inhibitors as described above for lysis buffer-F) at 4 °C for 15 min. Each cell monolayer was extracted in 0.5 ml lysis buffer-N, and extracts from two monolayers were pooled for each value for each experimental condition. Immunoprecipitation was performed as described before [17] using 2 μg mouse monoclonal anti-PP2A, anti-PP2C, or anti-PP1 antibodies. For controls, cell extracts were subjected to identical procedure using preimmune mouse IgG (isotype specific) instead of anti-PPase antibodies.

PPase assay. Plasma membrane, soluble fraction or the immunocomplex, was diluted in PPase buffer (50 mM hepes, pH 7.2, 60 mM NaCl, 60 mM KCl, and protease inhibitors as described above) to a final volume of 30 μl and incubated with 5 μl phosphopeptide substrate, KRpTIRR (5 μg). After incubation at 30 °C for 10 min, free phosphate was assayed by using malachite green reagent [18] in a 96-well microtiter plate and a microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA). Zero minute incubation was used for control assay. Assay was also performed in the presence of 10 nM okadaic acid to determine PP2A-specific activity. PPase activity in complexes prepared using preimmune IgG was used as the control activity and subtracted from the activities associated with specific immunocomplexes. The unit (U) of PPase activity represents picomole of free phosphate generated in 1 h under assay conditions.

Treatment with GSSG and GSH. Isolated plasma membranes from Caco-2 cells or purified PP2A (Upstate Biotechnology, Lake Placid, NY) were incubated with varying concentrations of oxidized glutathione (GSSG) for varying times. For reversibility of GSSG effect, membranes were washed two times with assay buffer to remove GSSG, followed by incubation with reduced glutathione (GSH). In case of purified PP2A, GSSG was washed two times by filtration in Microcon concentrators (Millipore, Allen, TX). To determine the restoration of H_2O_2-induced inhibition of PP2A activity, plasma membranes isolated from control and H_2O_2-treated cell monolayers (for 1 h) were incubated in the absence or presence of 5 mM GSH for up to 90 min. At varying times aliquots of membrane suspensions were assayed for PP2A activity.

Chemicals. Cell culture media and related reagents were purchased from Gibco BRL (Grand Island, NY). GSH, GSSG, H_2O_2, malachite green, *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (PCMB), okadaic acid, and protein-G Sepharose were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were of analytical grade purchased either from Sigma Chemical Company or Fisher Scientific (Tustin, CA). PP2A and substrate peptides were purchased from Upstate Biotechnology (Lake Placid, NY). [γ-^{32}P]ATP was purchased from Amersham Radiochemicals (Arlington Heights, IL).

Antibodies. Mouse monoclonal anti-PKCα, anti-PKCβ, anti-PKCδ, anti-PKCε, anti-PKCγ, anti-PKCλ, anti-PP2A, anti-PP2C, anti-PP1, and HRP-conjugated secondary antibodies were purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-phosphoserine and anti-phosphothreonine antibodies were from Zymed Laboratories (South San Francisco, CA).

Statistics. Comparison between two groups was made by Student's *t* tests for grouped data. The significance in all tests was derived at the 95% or greater confidence level.

Results

H_2O_2 induces threonine phosphorylation of proteins in Caco-2 cells

The effect of H_2O_2 on serine and threonine phosphorylation of proteins was determined by immunoblot analysis. A number of threonine phosphorylated proteins were detected in control cell monolayers. Treatment with H_2O_2 increased threonine phosphorylation of a cluster of proteins with the molecular mass range of 170–250 kDa (Fig. 1A). A maximal increase in phosphorylation was achieved by 30 min and sustained until 120 min. A number of serine phosphorylated proteins were also detected in control cell monolayers. However, serine phosphorylation of proteins was not affected by H_2O_2 treatment (data not shown).

Effect of H_2O_2 on PKC activity

To determine whether H_2O_2-induced increase in phosphorylation was caused by the activation of PKC

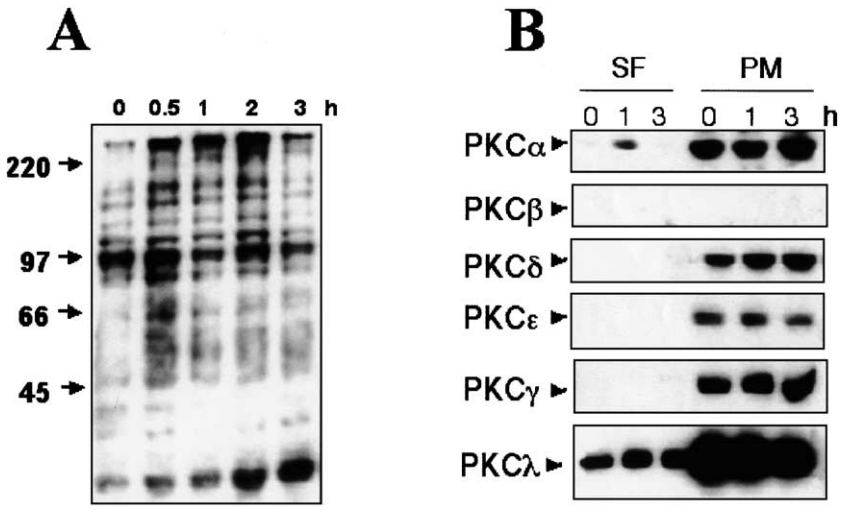

Fig. 1. Effect of H_2O_2 on protein threonine phosphorylation (A) and membrane localization of PKC isoforms (B). (A) Caco-2 cell monolayers were treated with H_2O_2 (20 μM) for varying times. Following treatment, proteins were extracted under denaturing conditions, and phosphothreonine was examined by immunoblot analysis using rabbit polyclonal anti-phosphothreonine antibodies. Number with arrows on the left side of blot indicates the migration of molecular weight markers. (B) Caco-2 cell monolayers were treated with H_2O_2 (20 μM) for 0, 1, or 3 h. Plasma membranes and soluble fractions were isolated. PKC isoforms in plasma membrane and soluble fractions were examined by immunoblot analysis using specific antibodies.

isoforms, we determined the effect of H_2O_2 on PKC activity and translocation of PKC isoforms into plasma membranes by immunoblot analysis. Results show that high activity of PKC was present in plasma membranes of Caco-2 cells, and that H_2O_2 treatment did not alter the PKC activity (data not shown). The PKCα, PKCδ, PKCε, PKCγ, and PKCλ were predominantly localized in the plasma membrane fraction of control cell monolayer (Fig. 1B). Negligibly low levels of PKC isoforms were detected in the soluble fraction, except for PKCλ. Treatment with H_2O_2 produced no considerable effect on plasma membrane localization of PKC isoforms, except for a slight, but time-dependent increase in PKCδ. PKCβ was undetectable in membrane and soluble fractions.

H_2O_2 treatment acutely reduces PP2A activity

To determine the role of PPases in H_2O_2-induced protein threonine phosphorylation, the PPase activity was measured in plasma membrane and soluble fractions of untreated and H_2O_2-treated Caco-2 cell monolayers. PPase activity was measured in the absence and presence of 10 nM okadaic acid to determine the PP2A (okadaic acid-sensitive) and non-PP2A (okadaic acid-insensitive) protein phosphatase activities. Okadaic acid-sensitive (Fig. 2A) and okadaic acid-insensitive (Fig. 2B) PPase activities were present in both plasma membrane and soluble fractions of control cell monolayers. Treatment with H_2O_2 for 1 h resulted in an acute reduction (80–85%) of okadaic acid-sensitive PPase activity in both plasma membrane and soluble fractions (Fig. 2A). Okadaic acid-insensitive PPase activity was not significantly altered by H_2O_2 (Fig. 2B).

Inhibition of specific PPases by H_2O_2 was determined by measuring the activity of PP2A, PP2C, and PP1 by immunocomplex PPase assay. Relatively high activity of PP2A was present in control cell monolayer. Treatment with H_2O_2 resulted in a time-dependent decrease in PP2A activity (Fig. 2C); about 40% decline in activity was achieved at 1 h, and 60% decline at 3 h. The activities of PP2C and PP1 were found to be lower than the activity of PP2A in control cell monolayers (Fig. 2D). Treatment with H_2O_2 failed to alter the activities of PP2C and PP1. Immunoblot analysis of immunocomplexes used for PPase assay showed that similar amounts of PPases were precipitated in control and H_2O_2-treated cell monolayers (inset for Fig. 2D). To determine the effect of H_2O_2 on association of different PPases with plasma membrane, we analyzed their localization in plasma membrane and soluble fractions by immunoblot analysis. PP2A was localized in both plasma membrane and soluble fractions (Fig. 3). PP2C was predominantly localized in plasma membrane fraction, while PP1 localized in the soluble fraction. H_2O_2 treatment produced no alteration in the distribution of PP2A, PP2C, or PP1 in membrane and soluble fractions.

GSSG inhibits PP2A activity

Our previous study demonstrated that H_2O_2 induces glutathione (GSH) oxidation resulting in accumulation of oxidized glutathione (GSSG) in Caco-2 cells [19]. To

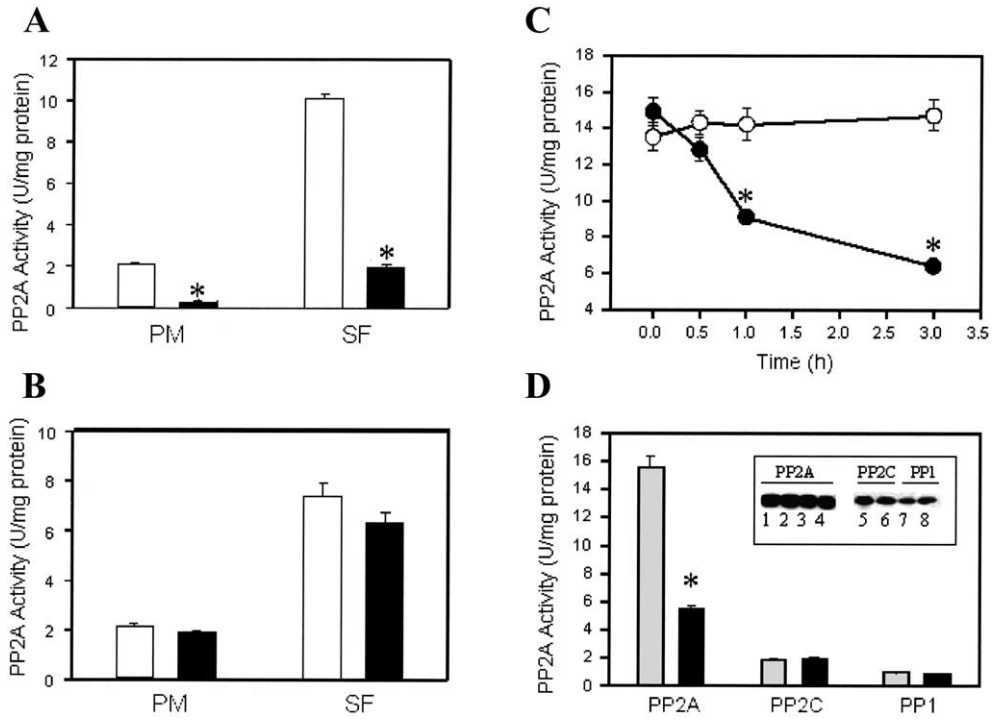

Fig. 2. Effect of H_2O_2 on PPase activity. (A,B) Caco-2 cell monolayers were incubated without (open bar) or with 20 μM H_2O_2 (closed bar) for 1 h. Plasma membrane (PM) and soluble fractions (SF) were isolated. Okadaic acid-sensitive (A) and okadaic acid-insensitive (B) PPase activities were measured in membrane and soluble fractions. Values are means ± SEM ($n = 6$). Asterisk indicates the values that are significantly different ($p < 0.05$) from corresponding values for cell monolayers incubated without H_2O_2. (C) Time course of the effect of H_2O_2 on PP2A activity. Proteins were extracted under native conditions from cell monolayers incubated in the absence (○) or presence (●) of H_2O_2 (20 μM) for varying times. PP2A was immunoprecipitated, and PPase activity measured in the immunocomplex as described in Materials amd methods. Complexes prepared using species and isotype specific preimmune-IgG from control cell monolayers and H_2O_2-treated cell monolayers were prepared as controls and subtracted from the activity in corresponding immunocomplexes. Values are means ± SEM ($n = 5$). Asterisks indicate the values that are significantly different ($p < 0.05$) from corresponding values for cell monolayers incubated without H_2O_2. (D) Effect of H_2O_2 on PP2A, PP1, and PP2C activities. For these experiments, immunocomplexes of PP2A, PP2C, and PP1 were prepared from cell monolayers incubated without (grey bar) or with 20 μM H_2O_2 (black bar) for 3 h. PPase activity was measured in immunocomplexes. Values are means ± SEM ($n = 5$). Asterisk indicates the value that is significantly different ($p < 0.05$) from corresponding value for cell monolayers incubated without H_2O_2. Inset: immunocomplexes after PPase assay were washed and proteins extracted in Laemmli's sample buffer, and immunoblotted for respective PPase isoform. Results from two independent experiments are provided for PP2A, and one experiment for PP2C and PP1. Lanes 1, 3, 5, and 7 represent immunocomplexes from control cell monolayers, while lanes 2, 4, 6, and 8 represent immunocomplexes from H_2O_2-treated cell monolayers.

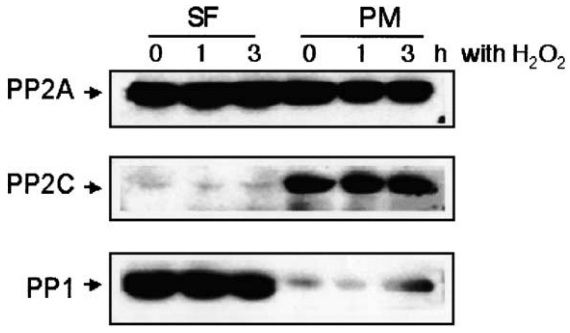

Fig. 3. Effect of H_2O_2 on distribution of PPase isoforms in plasma membranes. Plasma membrane and soluble fractions were isolated from cell monolayers treated with H_2O_2 (20 μM) for 0, 1, or 3 h. Fractions were immunoblotted for PP2A, PP2C, and PP1 by using specific antibodies.

determine the effect of GSSG on plasma membrane-associated PP2A activity the plasma membranes isolated from Caco-2 cells were incubated with varying concentrations of GSSG for varying time periods. GSSG reduced okadaic acid-sensitive PPase activity in plasma membrane in a concentration-related (Fig. 4A) and time-dependent (Fig. 4B) manner. This effect of GSSG was reversed by GSH in a concentration-related manner (Fig. 4C). To confirm the presence of a sensitive cysteine residue in okadaic acid-sensitive PPase, the effect of thiol blockers on plasma membrane-associated PPase activity was evaluated. NEM, iodoacetamide, and PCMB reduced the activity of PP2A in plasma membrane (Fig. 4D). GSSG also inhibited the activity of isolated PP2A in a concentration-related (Fig. 5A) and time-dependent (Fig. 5B) manner. GSSG effect was reversed by GSH (Fig. 5C), and the activity of isolated PP2A was also inhibited by thiol-blockers (Fig. 5D).

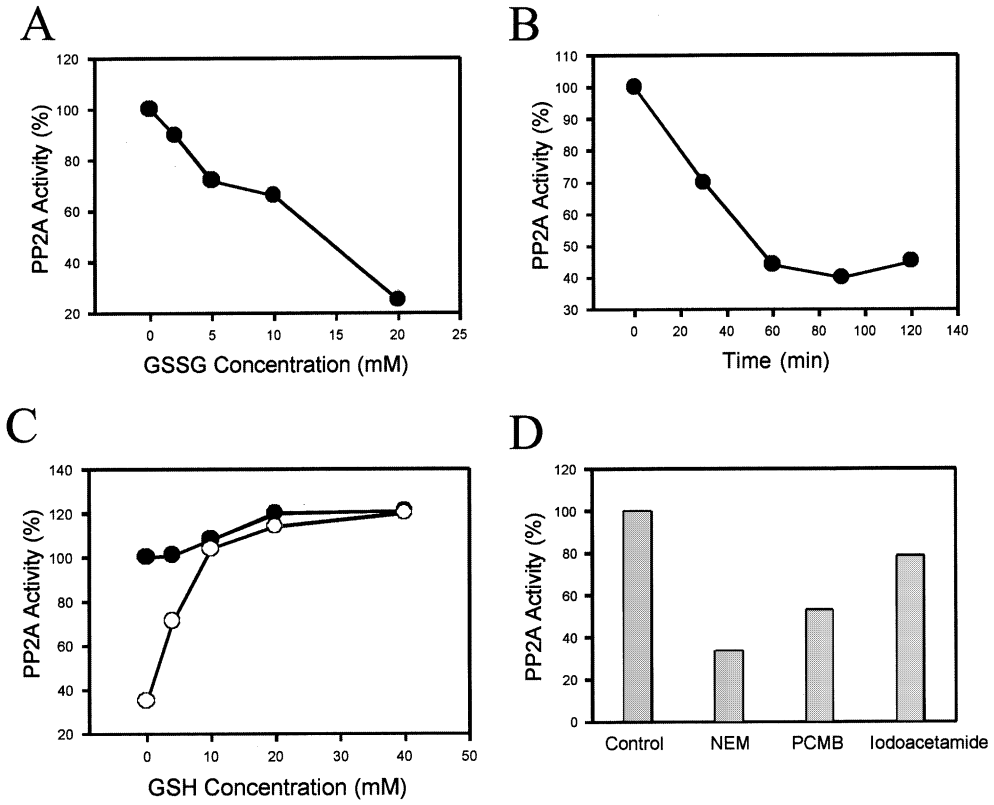

Fig. 4. Inhibition of PP2A activity in plasma membranes by oxidized glutathione. (A) Plasma membranes isolated from Caco-2 cell monolayers were incubated with varying concentrations of GSSG for 90 min. PP2A activity was evaluated by measuring PPase activity in the absence and presence of okadaic acid (10 nM). (B) Time course of the effect of GSSG on PP2A activity. PP2A activity measured in plasma membranes treated with 5 mM GSSG for varying times. (C) Reversibility of GSSG-induced inhibition of PP2A activity by GSH. After incubation without (●) or with 5 mM GSSG (○) for 90 min, plasma membranes were incubated with varying concentrations of GSH for 90 min. (D) Inhibition of PP2A activity by thiol-blockers. Plasma membranes were incubated without (control) or with 5 mM NEM, PCMB, or iodoacetamide. Values are means ± SEM ($n = 3$). All experiments were repeated at least three times with similar results.

GSH restores the activity of PP2A in H_2O_2-treated Caco-2 cell monolayers

To determine whether reduced GSH reverses the H_2O_2-mediated reduction of PP2A activity in Caco-2 cells, plasma membranes isolated from control and H_2O_2-treated cells were incubated in the absence or presence of 5 mM GSH for varying times. Incubation with GSH increased PP2A activity in H_2O_2-treated cells in a time-dependent manner (Fig. 6B). GSH induced only a minor change in PP2A activity in plasma membranes isolated from control cell monolayers (Fig. 6A). Incubation with GSSG (5 mM), on the other hand, further reduced the PP2A activity in plasma membranes isolated from H_2O_2-treated cell monolayers (Fig. 6B).

Discussion

Our previous studies showed that H_2O_2 inhibits proliferation of Caco-2 cells [20] and disrupts the tight junctions of confluent cell monolayers [19,21,22]. The present study shows that H_2O_2 induces serine/threonine phosphorylation of proteins in Caco-2 cells. H_2O_2-treatment resulted in an increase in threonine phosphorylation of a cluster of proteins at the molecular mass range of 170–250 kDa. Interestingly enough, H_2O_2 did not alter serine phosphorylation of proteins.

H_2O_2-induced increase in threonine phosphorylation may be caused by the activation of one or more PKC isoforms, and/or inhibition of PPases. Localization of PKCα, PKCδ, PKCε, PKCγ, and PKCλ in the plasma membrane of control cell monolayer indicated that these PKC isoforms are present in an active state in control cell monolayers. This was supported by the detection of high levels of PKC activity in the plasma membrane fractions. H_2O_2 produced no significant influence on the plasma membrane-localization of PKCα, PKCε, PKCγ, and PKCλ, except for an increase of PKCδ in plasma membranes. H_2O_2 also failed to alter the overall PKC activity in plasma membranes. The active state of different PKC isoforms prior to H_2O_2 treatment suggests that the basal activity of one or more of these PKC

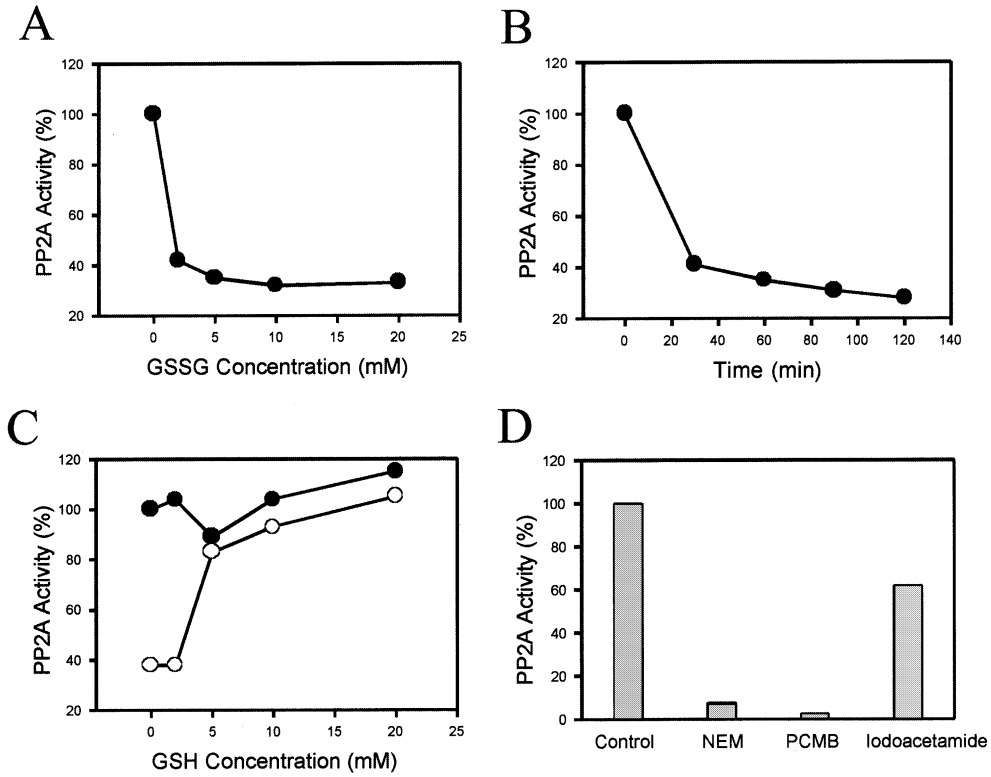

Fig. 5. Inhibition of isolated PP2A activity by oxidized glutathione: (A) Isolated PP2A was incubated with varying concentrations of GSSG for 90 min and PPase activity was measured. (B) Time course of effect of GSSG on PP2A activity. PPase activity measured in PP2A samples treated with 20 mM GSSG for varying times. (C) Reversibility of GSSG-induced inhibition of PP2A activity by GSH. After incubation without (●) or with 20 mM GSSG (○) for 90 min, PP2A was incubated with varying concentrations of GSH for 90 min. (D) Inhibition of PP2A activity by thiol-blockers. PP2A was incubated without (control) or with 5 mM NEM, PCMB, or iodoacetamide. Values are means ± SEM ($n = 3$). Asterisks indicate values that are significantly ($p < 0.05$) different from control values. All experiments were repeated at least three times with similar results.

isoforms may be involved in H_2O_2-induced threonine phosphorylation of proteins.

The results described above also suggest that inhibition or subcellular redistribution of PPases may be involved in H_2O_2-induced increase in protein threonine phosphorylation in Caco-2 cell monolayers. Almost equal levels of okadaic acid-sensitive and okadaic acid-insensitive activities were present in the plasma membranes of control cell monolayer. A greater than 80% reduction in okadaic acid-sensitive PPase activity in both plasma membrane and soluble fractions by 1 h after H_2O_2 administration indicates that inhibition of PPases, rather than the activation of PKC isoforms, is the major mechanism involved in H_2O_2-induced threonine phosphorylation of proteins. A greater than 80% inhibition of okadaic acid-sensitive activity with no significant inhibition of okadaic acid-insensitive PPase activity by H_2O_2 suggests that PP2A may be the major PPase affected by H_2O_2. Immunocomplex PPase assay confirmed that PP2A, but not PP2C or PP1 was inhibited by H_2O_2 treatment, supporting the suggestion that H_2O_2-induced inhibition of PP2A may play a major role in the H_2O_2-induced threonine phosphorylation of proteins. On the other hand, H_2O_2 did not alter the membrane localization of PP2A, PP2C, or PP1.

In a previous study we showed that H_2O_2 induces oxidation of GSH to GSSG resulting in a decrease in the level of protein thiols [19]. To determine the role of GSSG in the mechanism of H_2O_2-induced reduction in PP2A activity we evaluated the effect of GSSG on PP2A activity in plasma membranes prepared from Caco-2 cells. A rapid and concentration-related reduction in the activity of PP2A by GSSG, and the reversal of this GSSG effect by GSH demonstrate that PP2A activity can be regulated by glutathionylation. Results in this study also show that GSSG can inhibit the activity of isolated PP2A. Presence of a sensitive thiol residue in PP2A was further demonstrated by the reduction of PP2A activity in plasma membrane and isolated PP2A by thiol blockers, such as NEM, PCMB, and iodoacetamide. Results also demonstrate that PP2A activity in plasma membrane fraction of H_2O_2-treated cells can be restored by in vitro incubation with GSH.

In summary, this study demonstrates that H_2O_2 inhibits PP2A activity and increases threonine phosphorylation of proteins in Caco-2 cell monolayer. This study

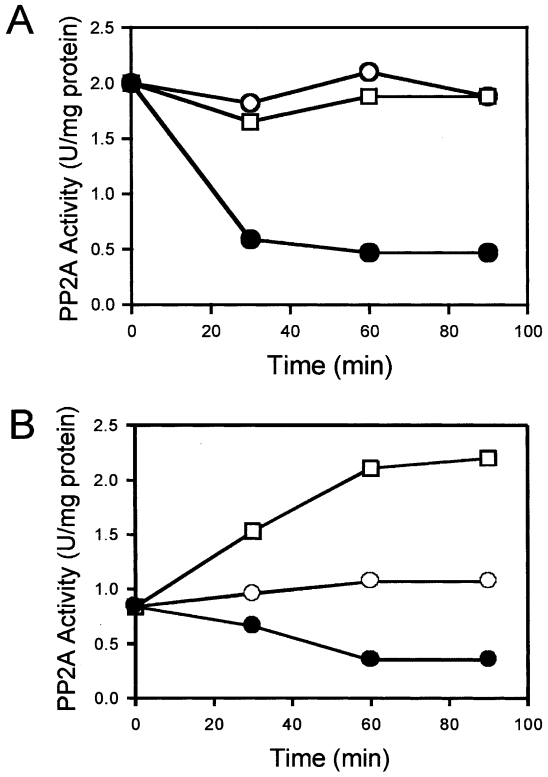

Fig. 6. GSH-mediated reversibility of H_2O_2-induced inhibition of PP2A activity in plasma membranes of Caco-2 cell monolayer. Plasma membranes were isolated from cell monolayers incubated in the absence (A) or presence (B) of 20 μM H_2O_2. Plasma membranes were incubated without (○) or with 5 mM GSH (□) or 5 mM GSSG (●) for varying times, and PP2A activity was measured. Result of representative experiment is presented. Similar result was obtained in an independent experiment.

also demonstrates that PP2A activity can be regulated by glutathionylation, and that H_2O_2-induced inhibition of PP2A in Caco-2 cells may involve glutathionylation of PP2A.

Acknowledgments

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