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Reversible formation of *S*-D-3-phosphoglyceroyl glutathione contributes to cellular protection from acylation by cyclic 3-phosphoglyceric anhydride

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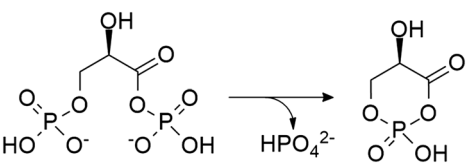
A spontaneous cyclization of 1,3-bisphosphoglycerate produces a highly reactive cyclic 3-phosphoglyceric anhydride (cPGA), which damages cellular nucleophiles through indiscriminate acylation. Although evidence suggests that most of the cPGA is inactivated by DJ-1, a highly efficient cPGA hydrolase, alternative routes of cPGA detoxification have not been explored. Here, we use a kinetic approach to show that a thiol group of glutathione (GSH) reacts with cPGA to produce the corresponding thioester *S*-D-3-phosphoglyceroyl glutathione (pgGS). We found that pgGS is unstable and decomposes back to GSH and cPGA with a half-life of 80 minutes providing DJ-1 with another chance to inactivate cPGA. Apart from spontaneous decomposition, pgGS is efficiently hydrolyzed by Glyoxalase II (GlxII); therefore a significant fraction of cPGA that escapes hydrolysis by DJ-1 is trapped by GSH and subsequently detoxified. Experiments with DJ-1-null cells revealed that depletion of GSH causes a multi-fold increase in the cellular level of *N*-glyceroyl glutamine, confirming that reversible formation of pgGS in reaction of cPGA with GSH serves as a second line of defense against acylation of biomolecules by cPGA.

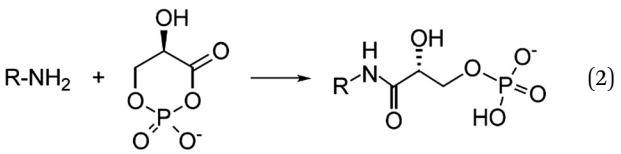
Introduction

The glycolytic intermediate 1,3-bisphosphoglycerate (1,3-BPG) has recently been found to undergo a spontaneous intramolecular acyl transfer reaction (eqn (1)),



(1)

to produce cyclic 3-phosphoglyceric anhydride (cPGA) – an emerging reactive metabolite.[1,2] Unlike parent 1,3-BPG, cPGA readily acylates primary amino groups in metabolites and proteins, resulting in stable phosphoglyceroyl amides (eqn (2)),



(2)

where R is any biomolecule with the exposed –NH_2 group.[1,2] In the cell, most of the resulting phosphoglyceroyl amides are dephosphorylated by an unknown phosphatase to produce the corresponding glyceroyl amides.[1] The physiological ramifications of protein and metabolite acylation by glyceric and phosphoglyceric acids are unknown, but it is likely that these stable modifications are detrimental and call for various protection and repair mechanisms to maintain cell homeostasis. Apart from amines, cPGA readily acylates thiols to produce thioesters.[2] However, the formation of glyceroyl or phosphoglyceroyl thioesters inside the cell has not been reported yet, probably because thioesters are unstable,[3,4] complicating their detection by LC–MS/MS.

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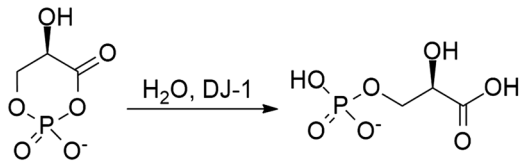
Human protein DJ-1 has been shown to hydrolyze cPGA to yield a non-reactive 3-phosphoglyceric acid (3PG) according to eqn (3),



(3)

with a room temperature k_{cat}/K_M of 5.9×10^6 M^{-1} s^{-1}.[2] A quick DJ-1-catalyzed hydrolysis likely serves as the cell's primary defense mechanism against indiscriminate acylation of biomolecules by cPGA since knockout of DJ-1 in human cells, mice, and fruit flies leads to a dramatic increase in intracellular levels of *N*-glyceroyl and *N*-phosphoglyceroyl adducts with the most abundant metabolites (*e.g.* glutamine and glutathione) and proteins.[1] The cPGA hydrolase activity relies upon a highly nucleophilic catalytic cysteine (Cys106) that is likely to perform the first step in a catalytic cycle by attacking the carbonyl's carbon of cPGA.[1]

Multiple studies reported that mutations in DJ-1 cause early-onset Parkinson's disease due to the premature death of dopaminergic neurons.[5,6] The neuroprotective effects of DJ-1 have been established in various model systems;[7–9] however since DJ-1 is reported to have a large number of different functions, the mechanism underlying this neuroprotection has not been established. For example, the glyoxalase/deglycase activity of DJ-1 has been suggested to underlie its neuroprotective effects[10], but was subsequently ruled out by several studies based, in part, on the fact that the anticipated effects in knockout or knockdown models were modest or absent.[1,11,12] In contrast, proteins from lysates of DJ-1 deficient cells are highly susceptible to acylation by endogenous and exogenous cPGA.[1,2,13,14] This, combined with the very high cPGA hydrolase activity of DJ-1 confirmed by several groups,[2,14,15] establishes a unique protective function for DJ-1. This function may partially or entirely explain the well-known neuroprotective effects of DJ-1, resolving a long-standing puzzle. However, a more detailed picture of the cellular fates of cPGA, including its detoxification pathways, is required to understand whether and how it is involved in neurodegeneration.

In this study, we identify a thiol group of glutathione as a major cellular target of cPGA. The reaction of cPGA with glutathione (GSH) produces a corresponding thioester (*S*-D-3-phosphoglyceroyl glutathione). We show that this thioester is unstable under physiological conditions due to the reverse reaction that produces cPGA and GSH, providing DJ-1 with another chance to inactivate cPGA by hydrolysis. Apart from spontaneous decomposition, pgGS is efficiently hydrolyzed by Glyoxalase II (GlxII), suggesting a new physiological function for this enzyme. Depletion of GSH in DJ-1 deficient cells resulted in increased accumulation of *N*-glyceroyl glutamine, indicating a direct involvement of GSH in the detoxification of cPGA. Thus, the present study advances our understanding of cellular fates of cPGA by establishing that glutathione protects metabolites and proteins against acylation by cPGA.

Results

cPGA is exceptionally reactive toward the SH group of glutathione

Although the reactivity of cPGA was partially characterized at room temperature and pH 7.0,[2] its reactivity toward the most abundant intracellular nucleophiles (proteins, primary amine metabolites and the thiol group of glutathione) under physiological conditions is not known. To characterize cPGA reactivity at 37 °C and pH 7.4, we monitored the kinetics of thioester formation in a series of reactions of cPGA with *N*-acetyl cysteine (NAC). Global curve fitting yielded the rate constants of both the cPGA-NAC reaction and spontaneous cPGA decomposition (27 ± 1 M^{-1} s^{-1} and 0.0073 ± 0.0015 s^{-1} respectively, Fig. 1A). In a similar experiment, we determined the rate constant of cPGA reaction with the thiol group of GSH to be 120 ± 2 M^{-1} s^{-1} at 37 °C and pH 7.4 (Fig. 1B), much higher than the value of 27 M^{-1} s^{-1} determined for NAC under the same conditions (Fig. 1A). Given their structural similarity, such a difference in rate constants of reactions of NAC and GSH is unexpected, however, we confirmed that the thiol group of GSH is ~4 times more reactive than that of NAC by an independent competition experiment (Fig. 1C). Next, using a continuous competition assay with NAC and global fitting of kinetic curves, we determined the rate constant of cPGA reaction with the amino group of glutamine and GSH to be 2.5 ± 0.5 M^{-1} s^{-1} and 10.5 ± 0.2 M^{-1} s^{-1}, respectively, at 37 °C and pH 7.4 (Fig. 1D and E). Finally, to characterize the reactivity of cPGA with proteins, we determined the apparent rate constant of the reaction of cPGA with bovine serum albumin (BSA) to be 130 ± 7 M^{-1} s^{-1} (Fig. 1F). Of note, despite the presence of 30–35 solvent-exposed lysines and an accessible single cysteine in a molecule of BSA, its apparent rate constant of reaction with cPGA is about the same as the rate constant of the cPGA–GSH reaction. This underscores the remarkable potential of GSH as a scavenger of cPGA. In order to demonstrate that GSH is an efficient scavenger of cPGA, we acylated BSA (6 mg ml^{-1}) with 1 mM cPGA in the presence and absence of GSH. GSH at 1 mM nearly completely prevented the acylation of BSA by cPGA (Fig. 1G). In contrast, glutamine showed no protective effect at a concentration of 1 mM and protected BSA only at a much higher concentration of 10 mM (Fig. 1G). Overall, our data suggest that glutathione is a highly efficient scavenger of cPGA, and that its thiol group is likely to be the major target of intracellular acylation by cPGA. This prompted us to synthesize the product of the acylation of the thiol group of glutathione by cPGA (*S*-D-3-phosphoglyceroyl glutathione, or pgGS) and study its chemical properties.

pgGS decomposes to produce cPGA

We synthesized pgGS by allowing cPGA to react with GSH, isolated the product with an overall yield of 70% and identified it as a thioester by NMR (Fig. S1–S3). An optical absorbance spectrum of the pure thioester showed an expected single peak at 235 nm with a corresponding molar extinction coefficient of 3.4 mM^{-1} cm^{-1}, representing a convenient way to monitor its concentration (Fig. 2A). Pure pgGS was found to be stable under

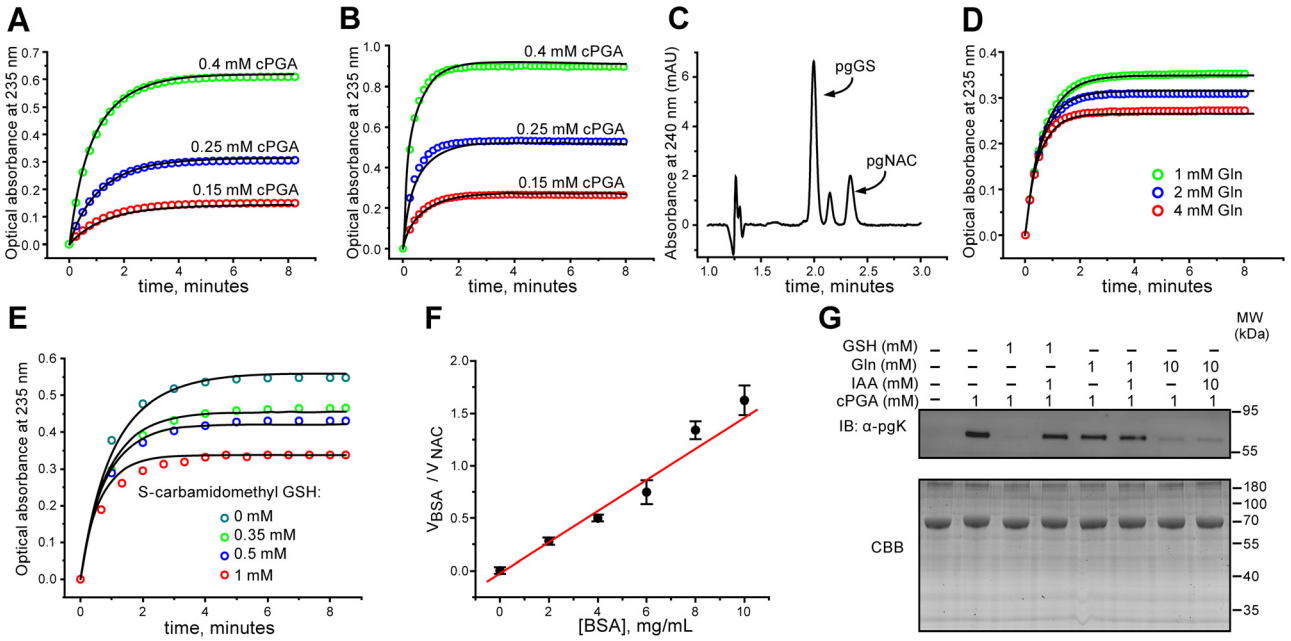


Fig. 1 Thiol group of GSH is highly reactive toward cPGA. All experiments were conducted in 50 mM phosphate buffer, pH 7.4, at 37 °C. (A) and (B) Kinetics of thioester formation in the reaction of 0.15–0.4 mM (A) NAC or 0.15-0.4 mM of GSH (B) with equimolar concentrations of cPGA was monitored by optical absorbance at 235 nm. Global fits to kinetic data are shown as solid lines. (C) GSH and NAC were mixed (0.5 mM each), reacted with 0.5 mM of cPGA and resolved by HPLC. Peaks corresponding to thioesters were identified based on retention times of synthetic standards. (D) and (E) Kinetics of thioester formation in the reaction of 0.5 mM of NAC with 0.3 mM of cPGA in the presence of indicated concentrations of glutamine (D) or *S*-carbamidomethyl glutathione (E) was monitored by optical absorbance at 235 nm. The data were fitted globally (solid lines) to derive rate constants of the cPGA reaction with competing amino groups. (F) The rate constant of the cPGA-BSA reaction was determined by a competition experiment with NAC. The amount of NAC thioester forming in the presence of varying concentrations of competing BSA was quantified by HPLC (mean ± S.E.M., $n = 3$). (G), BSA (6 mg ml^{-1}) was acylated by 1 mM cPGA for 30 minutes in the presence of GSH, glutamine and iodoacetamide at the indicated concentrations. Immunoblot with α-pgK/α-gK antibodies and Coomassie-stained gel (0.1 and 2 μg of BSA, respectively) demonstrate a strong protective effect of GSH at 1 mM, which was decreased by an equimolar amount of iodoacetamide (IAA), suggesting the involvement of the thiol group.

acidic conditions (Fig. 2B) but decomposed at neutral pH with a half-life of about 3 hours at 37 °C and pH 7.4 (Fig. 2B). Monitoring the concentration of free thiol groups in the reaction with Ellman's reagent revealed that decomposition of pgGS was accompanied by the stoichiometric increase in concentration of free thiol groups, indicating that glutathione is one of the decomposition products (Fig. 2C). In order to determine if a second product of pgGS decomposition is cPGA, we repeated the experiment in the presence of DJ-1, a highly efficient cPGA hydrolase.[2] In the presence of 0.2 μM DJ-1, decomposition of pgGS accelerated significantly, so that the half-life of decomposition decreased more than two times to about 80 minutes (Fig. 2D). A five-fold increase in DJ-1 concentration did not accelerate the decomposition any further, indicating that pgGS is not a substrate for DJ-1 (Fig. 2D). Apparent acceleration of pgGS decomposition caused by DJ-1 is best explained by assuming that DJ-1 hydrolyses the cPGA released upon spontaneous decomposition of pgGS at neutral pH. Destruction of cPGA by DJ-1 precludes the restoration of pgGS through a reverse reaction of GSH with cPGA, thereby accelerating the decay of pgGS. We tested this hypothesis directly by monitoring the decomposition of pgGS in the presence of added GSH. Glutathione dose-dependently decreased the rate of pgGS decay (Fig. 2E) allowing us to fit the kinetic data and calculate the true rate constant of pgGS decomposition ($(1.44 \pm 0.03) \times 10^{-4}$ s^{-1}). Since all kinetic curves could be fitted well by considering only the reversible cPGA–GSH reaction, spontaneous cPGA decay and acylation of the NH_2 group of GSH by cPGA, other mechanisms of spontaneous decay such as the irreversible hydrolysis of its thioester bond are unlikely to significantly contribute to pgGS decomposition under physiological conditions (Fig. 2F). Combined, our data indicate that the formation of pgGS in the reaction of cPGA with GSH is a highly favorable and fully reversible process with an equilibrium constant of 8.6×10^5 M under physiological conditions (Fig. 2F). Apart from pgGS, we studied the decomposition of the 3-phosphoglyceroyl thioester of NAC and found that it decomposes much slower compared to pgGS (Fig. S4). The decomposition could be accelerated by adding DJ-1 (Fig. S4), therefore a reversible decomposition accompanied by the release of cPGA is likely a common feature of all 3-phosphoglyceroyl thioesters.

pgGS acylates biomolecules *via* the formation of cPGA but not in direct reactions

To probe the reactivity of pgGS toward nucleophiles, we monitored the spontaneous decomposition of pgGS in the presence of glycine. Glycine at 0.3–20 mM gradually accelerated the decomposition of pgGS (Fig. 3A). At higher concentrations the

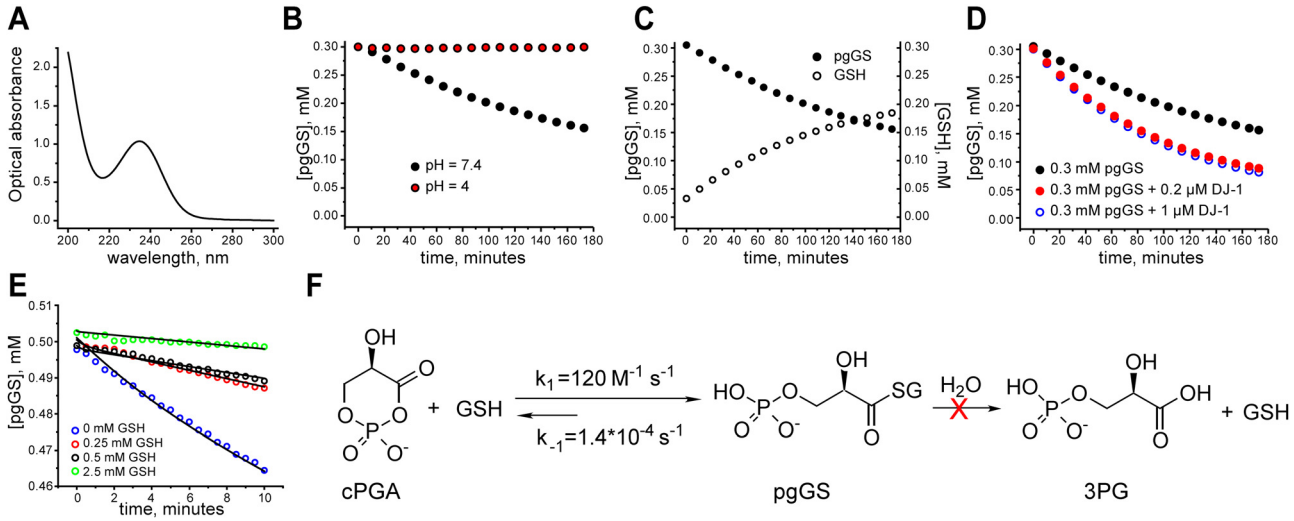


Fig. 2 pgGS decomposes to produce cPGA. Unless indicated otherwise, all experiments were conducted in 50 mM phosphate buffer, pH 7.4, at 37 °C. Thioester decomposition was monitored by optical absorbance at 235 nm. (A) Absorbance spectrum of pgGS features a characteristic thioester peak at 235 nm. (B) Kinetics of pgGS decomposition at pH 7.4 and pH 4. (C) Decomposition of pgGS is accompanied by stoichiometric formation of free GSH measured by Ellman's reagent at 412 nm. (D) Decomposition of pgGS is accelerated by 0.2 μM and 1 μM of DJ-1, suggesting that decomposition is accompanied by the release of cPGA. (E) The effect of GSH on the rate of spontaneous decomposition of pgGS has been monitored spectrophotometrically. Decomposition of pgGS dramatically slows down in the presence of 0.25 and 2.5 mM of glutathione due to the accelerated reverse reaction that restores pgGS. Solid lines represent global fits to the kinetic data used to derive a true rate constant for pgGS decomposition. (F) The combined data suggest that under physiological conditions pgGS decomposes through a reversible reaction, releasing cPGA and GSH, but not through an irreversible reaction of spontaneous hydrolysis. All experiments were repeated at least three times with identical results.

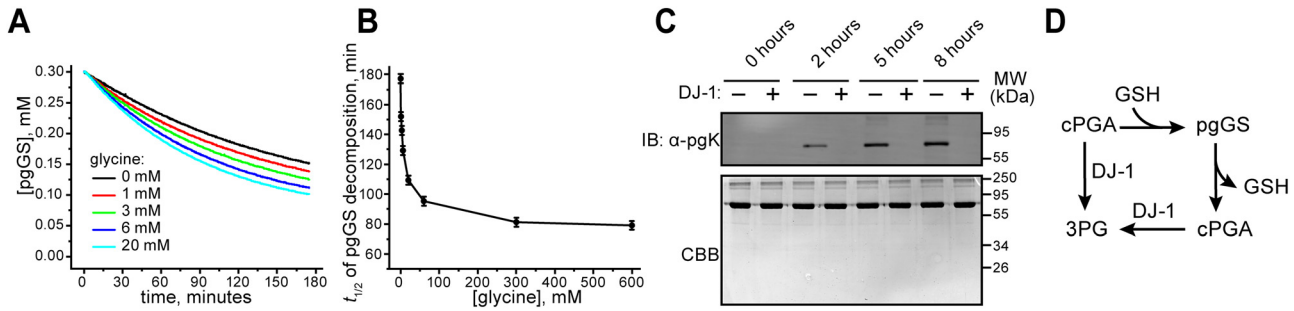


Fig. 3 pgGS does not acylate nucleophiles in direct reactions. All experiments were conducted in 50 mM phosphate buffer, pH 7.4, at 37 °C. (A) Kinetics of decomposition of pgGS in the presence of indicated concentrations of glycine. Glycine seems to accelerate the decomposition of pgGS, but acceleration is not directly proportional to glycine concentration. (B) The dependence of half-life of pgGS in solution on the concentration of glycine. The inability of high concentrations of glycine to accelerate the decomposition of pgGS indicates that pgGS does not acylate the amino group of glycine in a single-step bimolecular reaction. (C) Albumin (20 mg ml^{-1}) was incubated with 10 mM pgGS for the indicated amounts of time in the presence or absence of 1 μM DJ-1. Immunoblot with α-pgK/α-gK antibodies and Coomassie-stained gel (0.1 and 2 μg of BSA, respectively) demonstrates that acylation is completely prevented by 1 μM DJ-1, indicating that acylation is not direct but mediated by cPGA released as a result of pgGS decomposition. (D) cPGA that escaped inactivation by DJ-1 catalyzed hydrolysis and reacted with glutathione will be slowly released in reverse reaction, giving DJ-1 another chance to inactivate it by hydrolysis.

accelerating effect of glycine faded since a 10-fold increase in glycine concentration from 60 mM to 600 mM decreased the half-life of pgGS by less than 20%, from 95 to 80 min (Fig. 3B). These data clearly indicate that even at very high glycine concentrations, the reaction of pgGS with glycine is not a rate limiting step of pgGS decay. Instead, since glycine is readily acylated by cPGA,[2] the apparent acceleration of pgGS decomposition by low mM concentrations of glycine can be explained by scavenging of cPGA by glycine. This conclusion is further supported by the fact that both glycine and DJ-1 fail to accelerate the pgGS decomposition beyond a half-life of ~80 minutes. Based on these considerations, we conclude that pgGS does not acylate glycine in a direct one-step reaction under physiological conditions.

Next, we studied the acylation of BSA (20 mg ml^{-1} or ~300 μM) by excess pgGS (10 mM). Immunoblot analysis with anti-phosphoglyceroyl lysine/anti-glyceroyl lysine (α-pgK/α-gK) antibodies revealed that pgGS robustly acylates BSA (Fig. 3C). This acylation of BSA by pgGS was completely prevented by 1 μM of DJ-1, indicating that pgGS does not acylate BSA in a direct reaction but does so *via* a rate-determining step of cPGA formation (Fig. 3C). Combined, our data establish that pgGS is

relatively unstable and decomposes in solution with the release of cPGA, but the direct acylation of biological nucleophiles by pgGS is unlikely. Furthermore, cPGA that escaped hydrolysis by DJ-1 and was trapped in reaction with the thiol group of GSH will be slowly released in the reverse reaction, giving DJ-1 another chance to inactivate it by catalyzing its hydrolysis (Fig. 3D).

Glyoxalase II hydrolyses pgGS

Glyoxalase II enzymes (GlxII) are ubiquitously expressed hydrolases that demonstrate a high catalytic efficiency not only toward its major physiological substrate *S*-D-lactoyl glutathione but also a wide range of other glutathione-based thioesters.[16,17] Therefore, it is likely that pgGS formed in the reaction of cPGA with GSH will be quickly hydrolyzed by cytoplasmic GlxII further increasing the efficiency of pgGS-mediated cPGA detoxification. Using a standard protocol for spectrophotometric quantitation of enzymatic activity of human GlxII, we found that GlxII quickly hydrolyzed pgGS in a dose-dependent manner (Fig. 4A). The dependence of initial reaction rates on concentration of pgGS could be fit well using the Michaelis–Menten model and yielded values of K_M = 0.13 ± 0.03 mM and k_{cat} = 68 ± 6.6 s^{-1} (Fig. 4B). The calculated k_{cat}/K_M of 5.2 × 10^5 M^{-1} s^{-1} is about an order of magnitude lower than the value of 5 × 10^6 M^{-1} s^{-1} previously reported for D-lactoyl glutathione.[18] Therefore, to make comparisons, we determined the k_{cat}/K_M value for *S*-D-lactoyl glutathione (Fig. 4C) and obtained a similar value of 6.0 × 10^5 M^{-1} s^{-1}, indicating that our preparation of recombinant human GlxII catalyzes the hydrolysis of pgGS and *S*-D-lactoyl glutathione with the same efficiency. Combined, our data indicate that GlxII hydrolyzes pgGS with high efficiency, suggesting that pgGS formed in the cytoplasm *via* the reaction of cPGA with GSH will be quickly hydrolyzed by GlxII completing the detoxification.

Depletion of GSH in DJ-1 deficient cells causes increased formation of *N*-glyceroyl glutamine

Since our results indirectly suggest a protective role of GSH in the detoxification of cPGA, we sought to obtain further evidence of GSH involvement in cPGA detoxification. To demonstrate that GSH protects cellular nucleophiles from acylation by cPGA, we studied the effect of GSH depletion on the intracellular level of *N*-glyceroylated glutamine (gQ). Given that DJ-1 is hypersensitive to oxidation,[13,19,20] we used DJ-1-deficient HCT116 cells in these experiments to exclude a possible effect of oxidative stress caused by GSH depletion on DJ-1 activity. In accordance with the previous study[1], we were able to reliably detect and identify gQ by LC–MS/MS in DJ-1-deficient cells (Fig. 5A). Treatment of DJ-1-deficient HCT116 cells with buthionine sulfoximine (BSO) dose dependently reduced intracellular GSH content (Fig. 5B). The several-fold reduction in GSH content in cells treated with BSO was accompanied by about a ten-fold increase in the level of gQ (Fig. 5C), confirming that GSH exerts strong protective effects against acylation of biomolecules by cPGA.

Discussion

1,3-BPG, a high-energy glycolytic intermediate, undergoes a slow spontaneous transformation into cPGA with an as-yet unknown rate constant. Although the fraction of 1,3-BPG that is lost in this side reaction is likely very small, the resulting cPGA is so reactive that (in the absence of DJ-1) it will be immediately and completely spent on random acylation of intracellular nucleophiles. To mitigate the damage from these acylation reactions nearly all organisms express DJ-1, a protein that destroys cPGA with a previously reported room temperature k_{cat}/K_M value of 5.9 × 10^6 M^{-1} s^{-1}.[2]

Due to a remarkably high rate constant of reaction with cPGA and its high abundance, the thiol group of GSH represents a major acylation target of cPGA in the cell. The resulting thioester, pgGS, is stable under acidic conditions (Fig. 2B), so that its chemical synthesis and handling are facilitated by isolating it as a free acid. A spontaneous reversible decomposition of pgGS at physiological pH (Fig. 2B) represents a convenient way to generate cPGA *in situ* at a known rate. Prior to

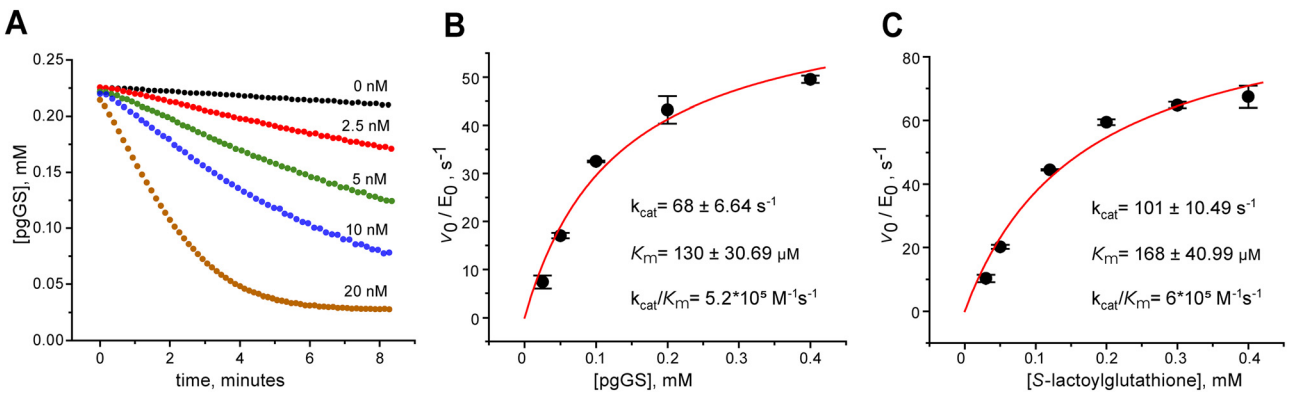


Fig. 4 pgGS is a substrate for Glyoxalase II. Glyoxalase activity was assayed in 20 mM Tris buffer, pH 7.4, at 37 °C. (A) Human GlxII at indicated concentrations dose-dependently catalyzes the hydrolysis of pgGS. (B) and (C) To determine kinetic parameters, the initial rate (v_0) is divided by enzyme concentration E_0 and plotted *versus* concentration of pgGS (B) or S-lactoyl glutathione (C). Rates were measured a minimum of three times with standard deviation shown in bars, and best fits with the Michaelis–Menten equation are shown as red lines.

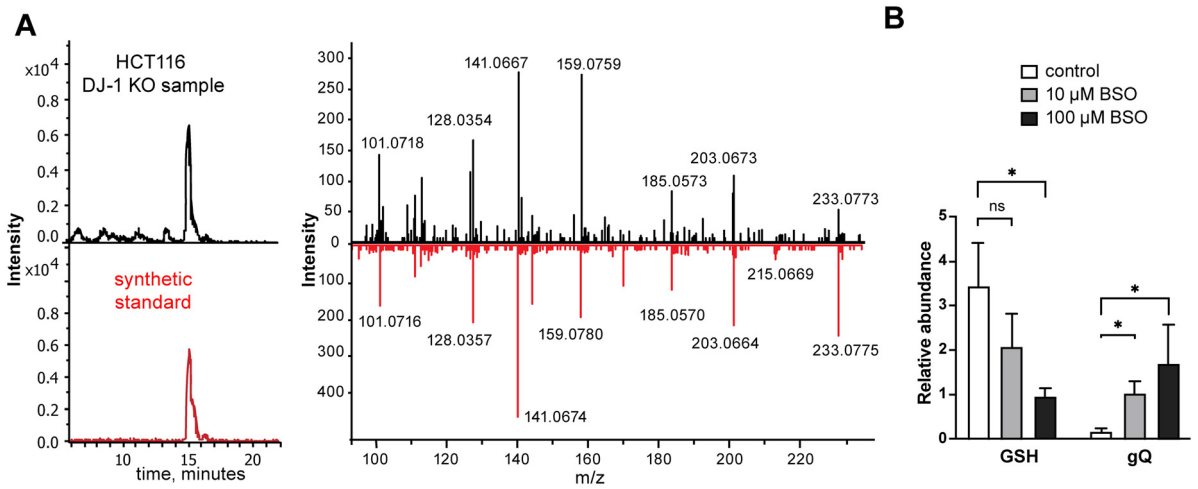


Fig. 5 Glutathione prevents glyceroylation of glutamine in DJ-1 deficient cells. (A) Chromatograms (left) and mirror plot of MS/MS spectra (right) showing the synthetic standard of *N*-glyceroyl glutamine (bottom, red) aligned with the corresponding peak and spectrum from HCT116 DJ-1 KO cells (top, black). (B) Quantification of glutathione (GSH) and *N*-glyceroyl glutamine (gQ) in HCT116 DJ-1 KO cells cultured with 0, 10, or 100 μM BSO for 24 hours. Values are mean ± SEM from three independent experiments, normalized to the median of the 100 μM BSO-treated group independently for each metabolite. Asterisks indicate statistical significance between groups as determined by two-tailed unpaired Student's *t*-test (*$p < 0.05$); ns, not significant.

this work, the only way to synthesize cPGA was to react 3-phosphoglycerate with the dehydrating agent EDC.[2,13] However, cPGA's half-life at neutral pH is ~4 min at 25 °C[2] and less than 2 min at 37 °C (Fig. 1A), making it difficult to model physiological conditions where cPGA is being constantly produced from 1,3-BPG over much longer periods of time. Using pgGS for a constant and slow release of cPGA allows for significantly better modeling of physiological conditions in various samples such as purified proteins (Fig. 3C) or cell lysates. The only caveat in using of pgGS as a donor of cPGA in biological systems is that it can be hydrolyzed by GlxII. However, pgGS can be potentially replaced with other 3-phosphoglyceroyl thioesters that cannot be hydrolyzed by GlxII but still produce cPGA at an adequate rate under physiological conditions. In any case, the ability of 3-phosphoglyceroyl thioesters to constantly produce cPGA established in this study opens new possibilities in cPGA and DJ-1 research.

Acylation of proteins by 3-phosphoglycerate, discovered by Moellering and Cravatt, was attributed to a direct reaction of 1,3-BPG with lysine's side chains.[21] Since many of these modifications were found near the active sites of glycolytic enzymes, it was suggested that these modifications play a regulatory role, redirecting glycolytic intermediates into alternative biosynthetic pathways.[21] However, recent reports provide strong evidence that acylation by 1,3-BPG is not direct but mediated by a rate-limiting step of cPGA formation[1,2] followed by quick reactions of cPGA with the most abundant and reactive proteins and metabolites. Although glutathione's thiol group is predicted to be the primary target of acylation by cPGA, we were unable to detect the formation of pgGS in DJ-1-deficient cells (data not shown). This is expected since, in addition to its loss due to chemical instability, any pgGS forming in the cell will be quickly hydrolyzed by GlxII (Fig. 4).

Given the short-lived nature of pgGS in the cell, the contribution of GSH to the detoxification of cPGA is difficult to demonstrate unless intracellular GSH levels are manipulated, which in turn is likely to reduce the activity of redox-sensitive DJ-1. Since DJ-1 inactivates the vast majority of cPGA molecules, even modest changes in its activity will lead to outsized effects on glyceroylation levels of cellular nucleophiles. Therefore, we chose DJ-1-deficient cells to demonstrate the involvement of GSH in the detoxification of cPGA. We found that a strong reduction of GSH content in DJ-1-deficient cells is accompanied by a roughly 10-fold increase in the cellular level of gQ (Fig. 5B), suggesting a strong protective role for GSH. We conclude that while detoxification of cPGA *via* GSH/GlxII is negligible in healthy cells with normal DJ-1 expression levels, any large drop in DJ-1 activity due to oxidation of its catalytic cysteine in the course of aging[8,22] and/or neurodegeneration[23,24] will proportionally increase the contribution of the GSH/GlxII pathway in detoxification of cPGA. In individuals with early onset of Parkinson's disease due to inactivating mutations in the *PARK7* gene, the GSH/GlxII pathway described in this study is the only known route of cPGA detoxification and should be investigated for potential therapeutic interventions.

To summarize, we established that the sulfhydryl group of GSH is a primary target of cPGA acylation. This acylation reaction redirects cPGA away from damaging proteins and metabolites and allows for quick recycling of GSH and 3-phosphoglycerate, representing a previously unrecognized and chemically efficient way of cPGA detoxification (Fig. 6). These insights provide a better understanding of the molecular mechanisms underlying the damage of biomolecules by cPGA and may inform the development of strategies aimed at preventing and mitigating the development of Parkinson's disease.

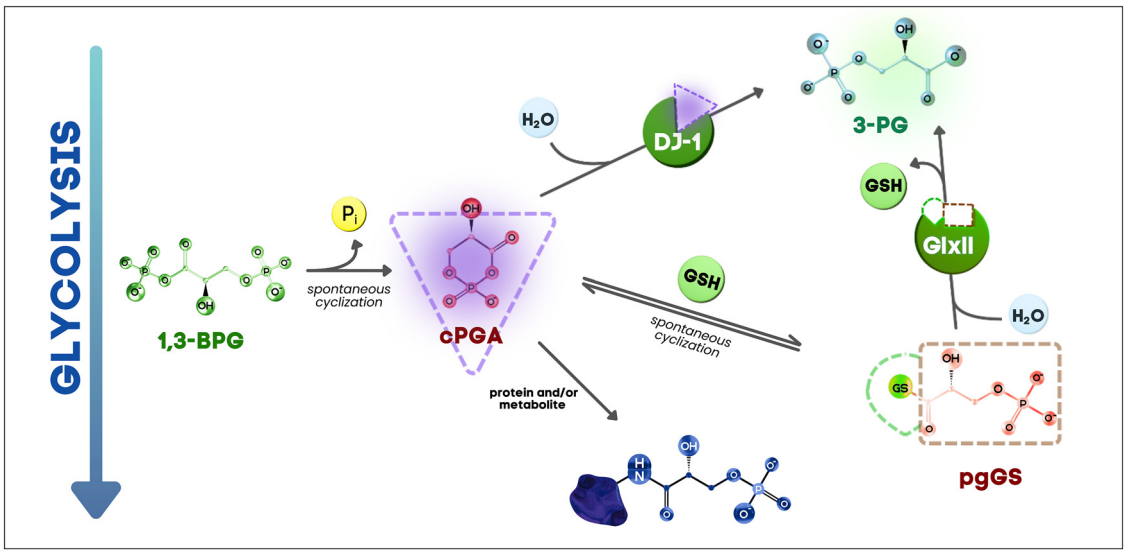


Fig. 6 Schematic representation of the metabolic fate of cPGA in human cells. cPGA is formed by the slow decomposition of 1,3-BPG. The vast majority of cPGA is hydrolyzed by DJ-1 to 3PG, while a fraction forms a thioester with GSH. The GSH thioester is hydrolyzed by GlxII to GSH and 3PG, but also decomposes back to GSH and cPGA. The remaining cPGA acylates proteins and metabolites.

Experimental procedures

Preparation and spectrophotometric quantitation of cPGA

An optimized procedure for the preparation of cPGA was published recently.[13] In brief, solid EDC was dissolved in a freshly prepared, ice-cold solution of 3PG and HCl to obtain a solution with 50 mM final concentration of all reactants. After incubation for 3 minutes on ice, the solution was flash-frozen in 50–500 µl aliquots. Spectrophotometric quantitation of cPGA was performed as described.[2] Samples of cPGA in 50 mM phosphate buffer (pH 7.0) were quickly mixed with NAC and NaOH (5 and 20 mM final concentrations, respectively) and incubated for 1 minute, after which HCl was added to a final concentration of 60 mM. OD_{235} values were recorded for 20 seconds and averaged. Concentrations of cPGA were calculated using ε_{235} = 3.0 mM^{-1} cm^{-1} after accounting for ~7% dilution due to the addition of NAC, NaOH and HCl.

Synthesis of glutathione thioester of 3-phosphoglyceric acid

3-Phosphoglyceric acid (48.53 mg, 0.21 mmol) was dissolved in ice-cold water and then HCl (0.21 mmol) was added, and this solution (3.2 ml) was used to dissolve solid EDC (40.45 mg, 0.21 mmol). The reaction was quickly mixed and incubated on ice for 2.5 minutes. The resulting cPGA solution was quickly mixed with a solution of GSH (84.2 mg, 0.274 mmol, 1.3 equiv.) in a 0.1 M Na_2CO_3/$NaHCO_3$ buffer of pH 9.16 (4.2 mL). The pH of the reaction mixture was adjusted to 8–9 with 0.1 M NaOH. The mixture was stirred for 30 minutes at room temperature. The product was diluted to 30 mL with H_2O and purified on a 1 mL Resource Q anion exchange column in 3 batches using a gradient of 0 to 100% of 60 mM HCl. Collected fractions were immediately pooled, frozen and lyophilized to obtain a clear gel solid (70 mg, 0.19 mmol, 70%). ^{1}H NMR (500 MHz, D_2O): δ 4.49 (dd, J = 7.5, 4.9 Hz, 1H, Cys C*H*CH_2S), 4.36–4.33 (m, 1H, CH_2CHOH), 4.07–4.0 (m, 1H, Glu C*H*NH_2), 3.97–3.92 (m, 2H, C*H*$_2$CHOH), 3.82 (s, 2H, Gly C*H*$_2$COOH), 3.30 (dd, J = 14.2, 4.9 Hz, 1H, Cys CHC*H*$_2$S), 3.06 (dd, J = 14.2, 7.5 Hz, 1H, Cys CHC*H*$_2$S), 2.46–2.32 (m, 2H, Glu CH_2CH_2CONH), 2.12–1.98 (m, 2H, Glu C*H*$_2$$CH_2$CONH). ^{13}C NMR (126 MHz, D_2O): δ 204.1 (C-1, *C*O), 174.4 (C-2, *C*O), 173.1 (C-3, *C*O), 172.2 (C-4, *C*O), 171.8 (C-5, *C*O), 76.4 (C-6, d, J = 8.1 Hz, *C*H$_2$OP), 66.9 (C-7, CO*C*OH), 52.8 (C-8, Cys *C*HCH$_2$S), 52.4 (C-9, Glu *C*HNH$_2$), 41.3 (C-10, Gly *C*H$_2$COOH), 30.9 (C-11, CH*C*H$_2$S), 29.1 (C-12, Glu CH_2*C*H$_2$CONH), 25.5 (C-13, Glu *C*H$_2$CH$_2$CONH). ^{31}P NMR (202 MHz, D_2O): δ 0.53. UV/vis: λ_{max} 235 nm.

Synthesis of *N*-3-phosphoglyceroyl glutamine and *N*-3-glyceroyl glutamine

Glutamine was reacted with cPGA and purified by anion exchange chromatography. Detailed procedures are described in supplementary Methods (SI).

Production of anti-glyceroyl-lysine/anti-phosphoglyceroyl lysine (α-gK/α-pgK) antibodies

Human GAPDH (5 mg mL^{-1} in 0.1 M sodium phosphate buffer, pH 7.6) was modified with 17 mM cPGA for 60 min, followed by desalting into PBS. Half of the resulting sample was dephosphorylated with 5 U ml^{-1} of calf intestinal phosphatase to produce glyceroyl adducts. For immunization, the resulting modified proteins were diluted to 0.6 mg mL^{-1} with PBS and mixed at a 1 : 1 ratio with Freunds' adjuvant. These solutions were injected subcutaneously (~0.3 mg of GAPDH) into two different 4-month-old New Zealand male rabbits. Booster immunizations were performed at 7, 14, 21, and 28 days. Six weeks after the first immunization rabbits were deeply anesthetized with isoflurane followed by exsanguination/euthanization by intracardiac blood collection. Antibodies were partially purified from the obtained plasma by two-step precipitation

with ammonium sulfate (precipitate from 35% saturation was discarded and precipitate from 45% saturation was used further). Precipitated antibodies were collected by centrifugation, re-suspended in 12 mL of PBS, and dialyzed against PBS plus 0.05% of sodium azide overnight. These antibodies were mixed and affinity-purified on immobilized gK/pgK-labelled BSA to obtain antibodies that recognize both gK and pgK modified proteins. The detailed procedure is described in SI Methods. Antibody production was carried out at the National Center for Biotechnology (Astana, Kazakhstan) and approved by the Institutional Animal Care and Use Committee (IACUC) of the National Center for Biotechnology (IRB00013497). The authors assert that all experiments were performed in accordance with relevant guidelines and regulations. All research work with laboratory animals was performed in accordance with generally accepted ethical standards and complied with the rules adopted by the European Convention for the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes.

Cell culture

HCT116 cells were purchased from ATCC and maintained at 37 °C and 5% CO_2. Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 2 mM glutamine, and antibiotics.

Western blotting

Protein samples were separated by 10% SDS-PAGE and transferred onto PVDF membranes. Blots were blocked overnight at 4 °C in 5% non-fat dry milk and 2% gelatin in TBS and 0.1% Tween-20. Blots were incubated with primary antibodies (1 : 2000) for 1 h and with secondary antibodies (1 : 20 000) for 45 min at room temperature. The secondary antibody signal was measured using the Odyssey CLx infrared imaging system (LI-COR).

Determination of rate constants of reactions of cPGA with nucleophiles

To determine rate constants of reactions of cPGA with GSH and NAC, 2 mL reactions were set up in spectrophotometer cuvettes at 37 °C in 50 mM sodium phosphate buffer (pH 7.4) containing 0.3 mM thiols. To initiate the reaction, an aliquot of cPGA was thawed and immediately added to the reaction mixture. Kinetic data were collected for 5–10 min by monitoring optical absorbance at 235 nm and 37 °C on a Shimadzu UV-1900i spectrophotometer. To determine the rate constant of the cPGA reaction with thiols, absorbance data from at least 3 reactions with varying concentrations of cPGA were globally fitted using KinTek global kinetic explorer software (version 11.01). The model included two reactions: a second-order one-step reaction of cPGA with NAC or GSH and a first-order reaction of cPGA decomposition. Both rate constants were extracted by globally fitting the model to a series of reaction kinetics.

The rate constant for cPGA reaction with glutamine and the amino group of GSH was obtained in a similar manner by including glutamine (1–4 mM) and S-carbamidomethyl glutathione (0.35–1 mM) into the reaction mixture. A corresponding reaction step was added to the fitting model to derive the reaction constant while the rate of spontaneous decomposition of cPGA was fixed at 0.0073 s^{-1}.

Expression and purification of DJ-1 and Glyoxalase II

His-tagged, full-length human DJ-1 and His-tagged human glyoxalase II were expressed and purified on a Ni-NTA column as described,[4] immediately desalted on a PD-10 column into a storage buffer (20 mM Tris, 0.5 M NaCl, 1 mM 2-mercaptoethanol) and frozen in 50 μl aliquots.

LC–MS analysis of metabolites

The HCT116 DJ-1 KO cells[2] were cultured in a 10 cm plate to ~ 80% confluency and treated with 10 μM or 100 μM of buthionine sulfoximine for 24 hours. The cells were washed three times with PBS, quenched with liquid nitrogen, and extracted with 1 mL of 80% methanol. The suspension was vortexed at 4 °C for 10 min and centrifuged at 22 000 × *g* for 10 min. The supernatant was dried in a speed vac and resuspended in 100 μl of 80% methanol before the LC–MS analysis.

Metabolite separation was performed using a 5 μm Hypersil GOLD™ HILIC column (100 × 2.1 mm; Thermo Scientific) coupled to a UHPLC ELITE+ at a flow rate of 0.3 mL min^{-1}. Mobile phase A consisted of 5 mM ammonium formate (Sigma-Aldrich) with 0.1% formic acid. Mobile phase B consisted of 100% acetonitrile (Sigma-Aldrich) with 0.1% formic acid. The gradient program was as follows: 0–10 min, 80% to 70% B; 10–18 min, 70% to 40% B; 18–20.5 min, 40% to 30% B; and 20.5–22 min, 30% to 80% B. The autosampler was maintained at 4 °C, and the column temperature was set to 30 °C.

Metabolites were analyzed using a Q-TOF Impact II VIP mass spectrometer (Bruker) equipped with an electrospray ionization (ESI) source operated in negative ion mode. The ESI parameters were as follows: spray voltage, 3000 V; nebulizer pressure, 3.0 bar; drying gas, 7.0 L min^{-1} at 220 °C. Spectra were acquired over an *m*/*z* range of 50–1300 at a frequency of 2 Hz by averaging 9929 transients. Compound identification was based on accurate *m*/*z* values and retention times compared with authentic standards. The areas under the curve of extracted-ion chromatograms of the $[M–H]^-$ forms were integrated using Bruker Compass DataAnalysis (Version 6.1) and normalized to the mean peak areas of all detected metabolites per sample ('total ion current'). *N*-Glyceroyl glutamine was further characterized by MS/MS fragmentation at collision energies of 5, 10, 20, and 30 eV.

Statistical analysis

Data were analyzed and plotted using GraphPad Prism 10. Statistical analysis was performed on log-transformed data. Values are presented as mean ± SEM from three independent experiments. Comparisons between groups were made using two-tailed unpaired Student's *t*-tests, with $p < 0.05$ considered statistically significant. For metabolite comparisons between groups, normalized values were further scaled independently

for each metabolite to the median of the 100 μM BSO-treated group (= 1) before statistical analysis.

Author contributions

D. U. designed the research; A. Y., A. B., A. A., E. S., Z. S. and Z. I. performed the experiments; A. A. and D. U. analyzed the data; D. U. wrote the paper with the other authors' help. D. U. and D. S. directed the research.

Conflicts of interest

The authors have no relevant financial or non-financial interests to disclose.

Abbreviations

3PG	3-Phosphoglyceric acid
cPGA	Cyclic 3-phosphoglyceric anhydride
gQ	*N*-Glyceroyl glutamine
GSH	Glutathione
LC–MS/MS	Liquid chromatography mass–spectrometry
NAC	*N*-Acetylcysteine
pgGS	*S*-D-3-Phosphoglyceroyl glutathione
pgK	3-Phosphoglyceroyl lysine

Data availability

All metabolomics data generated and analyzed in this study can be accessed at the Metabolomics Workbench[25] under Study ID ST004322. The data can be accessed directly *via* its Project DOI: https://dx.doi.org/10.21228/m80p0s.[26] The remaining data are available within the article and supporting information (SI). Supplementary information is available. See DOI: https://doi.org/10.1039/d6cb00085a.

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