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The MEK inhibitor PD98059 Elevates Primary Cultured Rat Hepatocyte Glutathione Levels Independent of Inhibiting MEK

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Abbreviations: MAPK, mitogen activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; MEK, MAPK kinase; GSH, glutathione; GST, glutathione S-transferase; GCL, gamma-glutamylcysteine ligase; GCLC, GCL catalytic subunit; ARE, antioxidant response element.

Abstract

The antioxidant activity of flavonoids, directly through scavenging oxidizing species and indirectly through modulating drug-metabolizing enzyme activities, is associated with chemopreventive and chemotherapeutic effects. However, little published information is available concerning the effect of flavonoids on gluthathione (GSH) homeostasis. We previously demonstrated that PD98059 (2'-amino-3'-methoxyflavone), a flavone derivative and selective MEK1 inhibitor, enhanced the insulin-mediated increase in GSH levels (Kim et al, 2004). To determine whether the PD98059-mediated increase in GSH levels was associated with MEK inhibition, primary cultured rat hepatocytes were treated with PD98059, the MEK inhibitor U0126, which is not a flavone derivative, or flavone. PD98059 increased GSH levels in a concentration-dependent manner in hepatocytes cultured in the presence or absence of insulin. In contrast, GSH levels were not affected by U0126 at concentrations sufficient to inhibit insulin-mediated ERK1/2 phosphorylation. Flavone, however, markedly increased GSH levels without inhibition of ERK1/2 phosphorylation. The concentration of GSH in the culture medium was also elevated by PD98059 or flavone, suggesting that the cellular GSH elevation could not be accounted for by the inhibition of GSH efflux into medium. Interestingly, PD98059 and flavone increased cellular cysteine levels, which may be responsible for the PD98059- and flavone-mediated elevation of GSH levels. These results provide evidence that PD98059 and flavone produce dramatic changes in GSH homeostasis in hepatocytes, through a mechanism(s) unrelated to MEK inhibition. Moreover, the

current study implies that flavonoid-induced chemopreventive and chemotherapeutic effects may be mediated by regulation of redox state through the stimulation of GSH synthesis.

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PD98059 (2'-amino-3'-methoxyflavone) was identified as the first synthetic inhibitor of the mitogen activated protein kinase (MAPK) pathway (Dudley et al., 1995) and has been extensively used for identifying the physiological roles of the extracellular signal-regulated kinase 1/2 (ERK1/2). PD98059 binds to the inactive form of MAPK kinase (MEK), the kinase immediately upstream of ERK1/2, preventing its activation by Raf-1 and other upstream activators (Alessi et al., 1995). The inhibitor neither competes with ATP nor inhibits the phosphorylation of MEK, and thus is likely to have a distinct binding site on MEK. In a comparison of multiple kinase inhibitors, PD98059 appeared to be the most specific kinase inhibitor tested (Davies et al., 2000), although the inhibitor has been shown to inhibit activation of the ERK5 pathway through direct effects on MEK5 (Karihaloo et al., 2001).

We previously used PD98059 as a MEK1 inhibitor in studies of the signaling pathways responsible for the stimulation of glutathione (GSH) synthesis in response to insulin (Kim et al., 2004). In that study we found that PD98059, at a concentration commonly used for complete inhibition of ERK1/2 activation, enhanced the insulin-mediated increase in GSH levels in primary cultured rat hepatocytes. However, whether the PD98059-mediated inhibition of MEK/ERK was responsible for the elevation of GSH levels was unknown.

PD98059 is a derivative of flavone, which belongs to the chemical class of flavonoids, naturally occurring compounds found in fruits and vegetables that may exert common biological functions.

Flavonoids have attracted attention as potential chemopreventive and chemotherapeutic agents in inflammatory diseases, cardiovascular diseases and cancers (Harborne and Williams, 2000; Middleton et al., 2000; Havsteen, 2002). The possible mechanisms of action of flavonoids include an increase in antioxidant capacity by directly scavenging oxidizing species and indirectly modulating drug metabolizing enzyme activities (Middleton et al., 2000; Frei and Higdon, 2003). In fact, flavone, 2'-amino-3'-methoxyflavone (PD98059), naphthoflavone and 3'-methoxy-4'-nitroflavone induce a G_1 arrest in cell cycle, increase Ah receptor-mediated gene expression and activate CCAAT/enhancer-binding protein β resulting in alpha-class glutathione S-transferase (GST) induction (Reiners et al., 1999; Kang et al., 2003). However, little is known concerning the effect of flavonoids on GSH homeostasis.

GSH serves several vital intracellular functions, including detoxifying electrophiles, scavenging free radicals, maintaining the essential thiol status of proteins, and providing a reservoir for cysteine (Lu, 1998). Thus, maintenance of GSH levels is pivotal for cellular defense against oxidative injury and for cellular integrity. The maintenance of cellular GSH is a dynamic process involving its synthesis, utilization and export to blood and bile (Lu, 1998; Ookhtens and Kaplowitz, 1998). γ-Glutamylcysteine ligase (GCL), which consists of a regulatory subunit and a catalytic subunit (GCLC), is a major determinant of GSH synthesis capacity, as it is the rate-limiting step in GSH synthesis. It has been suggested that regulation of GCLC expression is critical for GSH homeostasis (Cai et al, 1997).

The objective of this study was to determine whether MEK inhibition is responsible for the PD98059-mediated elevation of GSH levels. The MEK inhibitor U0126 which does not have a flavone structure failed to affect GSH levels. Flavone, on the other hand, elevated GSH levels, but was without effect on ERK Phosphorylation. Together, these results suggest that the PD98059-mediated elevation of GSH levels occurs via mechanism(s) unrelated to MEK inhibition. Neither PD98059 nor flavone had any significant effect on GCL activity or GCLC protein levels. In contrast, PD98059 and flavone resulted in significant elevation of cellular cysteine levels, suggesting a role of the increased cysteine levels in the PD98059- and flavone-mediated increase in GSH levels.

Materials and Methods

Materials. Modified Chee's medium and L-glutamine were obtained from Invitrogen (Carlsbad, CA). Insulin (Novolin R) was purchased from Novo-Nordisk (Princeton, NJ). Collagenase (type I) was purchased from Worthington Biochemicals (Freehold, NJ). Vitrogen (95-98 % type I collagen, 2-5% type III collagen) was obtained from Cohesion Technologies (Santa Clara, CA). GCLC antibody was purchased from NeoMarkers, Inc. (Freemont, CA). Antibodies against ERK1/2 and phospho-ERK1/2 (Thr202, Tyr204) were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit antibody was obtained from BioRad Laboratories (Hercules, CA). Enhanced chemiluminescence reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). PD98059 and U0126 were obtained from Calbiochem (La Jolla, CA). Flavone and all other reagents were purchased from Sigma (St. Louis, MO).

Primary rat hepatocyte culture. Hepatocytes were isolated from the livers of male Sprague-Dawley rats (200-300 g) using collagenase perfusion as described previously (Woodcroft and Novak, 1997). Hepatocytes were plated onto dishes covalently coated with Vitrogen, and modified Chee's medium was fortified as described (Woodcroft and Novak, 1997) and supplemented with 0.1 μM dexamethasone and 1 μM insulin. Cells were plated at a density of $3x10^6$ cells/60 mm dish. Four hours after plating, cells were washed with insulin-free medium several times and cultured in insulin-free medium thereafter. Cells were treated with PD98059 (0-100 μM), U0126 (0-50 μM) or flavone (0-50

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μM) for up to 24 h. These chemicals inhibitors were dissolved in DMSO and the final DMSO concentration in the medium was 1 μl/ml (0.1%). This concentration of DMSO did not affect the GCLC protein level, GCL activity or GSH level relative to untreated hepatocytes. In some instances PD98059, U0126 or flavone was added 1.5 h prior to addition of insulin (10 nM) for up to 24 h. None of the chemicals resulted in increased cell toxicity, as compared to untreated cells, at the concentrations used in this study. The Wayne State University Animal Investigation Committee approved all experimental procedures involving animals.

Determination of GSH and cysteine levels. The concentration of GSH was measured as described previously (Kim et al, 2003b). Reaction mixtures (final volume of 1 ml) contained 5 mM EDTA, 0.6 mM 5,5′-dithiobis(2-nitrobenzoic acid), 0.2 mM NADPH, 0.2 ml supernatant and 3 unit GSH reductase in 0.1 M potassium phosphate buffer (pH 7.5). Reaction mixtures were incubated for 4 min followed by the addition of GSH reductase to the mixtures. The rate of formation of 2-nitro-5-thiobenzoic acid was measured immediately following the addition of GSH reductase at 412 nm. None of the protein kinase inhibitors interfered with the determination of GSH. Cysteine concentration was determined using the HPLC method of Carducci et al. (1999). HPLC equipment was from Shimadze: pump, model LC-10AT; system controller, model SCL-10A; injector, Rheodyne equipped with a 20-μl loop; RF-10A fluorescence detector. Following the reduction of cysteine by 2-mercaptoethanol, cysteine was then

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derivatized with *O*-phthalaldehyde/iodoacetate and quantified using a HPLC equipped with a fluorescence detector and a 3.5 μm Kromasil C18 column (4.6×100 mm; Eka, Bohus, Sweden).

GCL activity determination. The activity of GCL was measured as described previously (Kim et al., 2003b). Reaction mixtures contained 0.1 M Tris–HCl buffer (pH 8.0), 150 mM KCl, 5 mM ATP, 2 mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 20 mM $MgCl_2$, 2 mM EDTA, 0.2 mM NADH, 17 μg pyruvate kinase, 17 μg lactate dehydrogenase and 0.1 mg protein of enzyme solution in a final volume of 1 ml. Absorbance at 340 nm was monitored for 5 min. The enzyme activity was calculated using a molar extinction coefficient of 6.3×10^2 mol^{-1} mm^{-1}.

Immunoblot analysis. Whole cell lysates were prepared as described previously (Kim et al, 2003d). For immunoblot analysis of GCLC, lysates (20 μg) were resolved by 10% SDS-PAGE, transferred to nitrocellulose (Bio-Rad, Hercules, CA), blocked in 5% milk powder in PBS-T (0.05% Tween 20 in PBS) and incubated with anti-rat GCLC (1:10,000 in 5% milk powder in PBS-T) overnight at room temperature. To determine the phosphorylation state of ERK, cell lysates were prepared by scraping cells directly into 500 μL SDS-PAGE sample buffer (Kim et al., 2003c). Lysates (10 μl) were separated by 10% SDS-PAGE, transferred to nitrocellulose, blocked in 5% milk powder in TBS-T (0.05% Tween 20 in Tris-HCl buffered saline) and probed with phospho-specific antibodies (1:250 in 5% bovine serum albumin in TBS-T) overnight at 4°C. Blots were stripped and re-probed with phosphorylation

state-independent antibodies to ERK. Proteins were detected by enhanced chemiluminescence on Kodak X-OMAT film (Sigma) and quantified by densitometry with a Molecular Dynamics scanning laser densitometer and ImageQuant analysis program (Amersham Biosciences, Piscataway, NJ).

Statistical analysis. Significant differences between groups were determined by ANOVA followed by the Newman-Keuls multiple comparison test ($p<0.05$). Statistical analysis was performed on 3-5 cell lysates from a single hepatocyte preparation. Reproducibility of results was confirmed in two to four separate hepatocyte preparations.

Results

Effects of PD98059, U0126 and flavone on ERK1/2 phosphorylation and GSH level.

Effects of PD98059 and U0126 on GSH levels were examined in rat hepatocytes cultured in the presence or absence of 10 nM insulin (Fig. 1). The inhibitory action of PD98059 and U0126 on MEK1 was confirmed by monitoring EKR1/2 phosphorylation in response to 10 nM insulin (Fig. 1A). Insulin treatment for 5 min resulted in a 4- to 5-fold increase in ERK1/2 phosphorylation. PD98059 and U0126 inhibited insulin-mediated EKR1/2 phosphorylation in a concentration-dependent manner, with complete inhibition occurring at 50 μM PD98059 or 25 μM U0126. Thus, U0126 appears to be a more potent inhibitor of MEK1 than PD98059.

Insulin treatment for 22.5 h resulted in a ~43% increase in GSH levels relative to controls lacking insulin (Fig. 1B, C), which is consistent with our previous findings (Kim et al., 2004). PD98059 pretreatment substantially augmented the insulin-mediated increase in GSH levels in a concentration-dependent manner (Fig. 1B). GSH levels in hepatocytes cultured in the absence of insulin were increased to 106, 130, 159 or 152%, relative to untreated cells, in the presence of 1, 10, 50 or 100 μM PD98059, respectively, (Fig. 1B, panel B1), while cells treated with PD98059 and cultured in the presence of insulin also exhibited a progressive increase in GSH levels, except at the highest concentration used in this study (Fig. 1B, panel B2).

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Treatment of cells with U0126 revealed a different affect on GSH levels. In contrast to PD98059, U0126, up to 25 μM (sufficient to inhibit the insulin-mediated ERK1/2 phosphorylation), failed to increase GSH levels in hepatocytes cultured in either the presence or absence of insulin (Fig. 1C, panels C1 and C2). GSH levels were slightly but significantly elevated (~124%, relative to untreated cells) only by the highest concentration of U0126 (50 μM) employed in this study (Fig. 1C). These results suggest that PD98059, but not U0126, increases GSH levels in primary cultured rat hepatocytes, independent of the presence of insulin and independent of their inhibition of ERK1/2 phosphorylation.

We next investigated whether the flavone structure of PD98059 (Fig. 2A) might be responsible for the elevated GSH levels in response to PD98059 treatment. In the absence of insulin, GSH levels were increased to 106, 122, 167 or 200%, relative to untreated cells, in the presence of 0.1, 1, 10 or 50 μM flavone, respectively (Fig. 2B). Thus flavone appears to be more potent than PD98059 (Fig. 1B) at increasing GSH levels in cultured hepatocytes. The presence of PD98059 (100 μM) or flavone (100 μM) in the GSH samples did not change the rate of increasing absorbance caused by reduction of 5,5'-dithiobis-2-nitrobenzoic acid (data not shown), indicating that neither PD98059 nor flavone interfered in the determination of GSH.

To determine whether flavone has MEK inhibitory activity, the phosphorylation of ERK1/2 was examined in hepatocytes cultured in the absence of insulin for 1.5 h following addition of 50 μM

flavone, 50 μM PD98059 or 25 μM U0126 (Fig. 2C). Basal ERK1/2 phosphorylation was decreased by 50 μM PD98059, and more markedly by 25 μM U0126, but was unaffected by flavone at concentrations up to 50 μM (Fig. 2C). Insulin-mediated phosphorylation of ERK1/2 was marginally decreased (maximally 15%) by 50 μM flavone (data not shown). Thus, it is apparent that the flavone-mediated elevation of GSH levels is not associated with MEK1 inhibition. These results do not rule out the possibility that the MEK/ERK cascade modulates GSH levels in hepatocytes; however, these data do demonstrate that PD98059 produces dramatic changes in GSH homeostasis in hepatocytes through a mechanism(s) unrelated to MEK inhibition.

Effects of PD98059 and flavone on GCLC expression, GCL activity, GSH efflux and cellular cysteine levels.

In order to investigate possible mechanisms for the GSH elevation by PD98059 and flavone, the effects of PD98059 and flavone on GCLC protein level and GCL activity were examined (Fig. 3). Treatment of hepatocytes with 10 nM insulin increased both GCLC protein level and GCL activity to ~200% and ~160%, respectively, relative to untreated hepatocytes, which is consistent with our previous findings (Kim et al., 2004). However, neither flavone, up to 50 μM, nor PD98059, up to 100 μM, altered GCLC protein levels or GCL activity in hepatocytes (Fig. 3). These data suggest that the mechanism(s) for PD98059- or flavone-induced elevation of GSH levels differs from that of insulin.

Under normal circumstances, GSH synthesized by hepatocytes is mainly removed by sinusoidal and canalicular effluxes (Ookhtens and Kaplowitz, 1998). The possible involvement of GSH efflux in the PD98059- or flavone-induced effect on GSH homoestasis was examined by determining the GSH concentration in hepatocyte culture medium (Fig. 4A). GSH levels in the culture medium were also increased in the presence of PD98059 or flavone in a concentration-dependent manner. These results indicate that PD98059 and flavone do not prevent GSH efflux from hepatocytes and suggest that the elevated cellular GSH levels induced by PD98059 or flavone may be associated with increased GSH synthesis.

Because pro-oxidants and oxidants that result in an initial depletion of GSH content cause the elevation of cellular GSH (Lu, 1998), GSH levels were determined at 1, 4, 12 and 24 h following treatment with 50 μM PD98059 or 50 μM flavone (Fig. 4B). Neither PD98059 nor flavone caused a decrease in GSH levels, and the PD98059- or flavone-mediated increases in GSH levels were observed as early as 4 h following initiation of treatment. These results suggest that the mechanism(s) of PD98059- or flavone-induced elevation of GSH synthesis differs from that of oxidative stress.

Since the supply of cysteine, a limiting substrate for GSH biosynthesis, is directly related to the synthesis of GSH, cysteine levels in hepatocytes were determined at 1, 4, 12 and 24 h following treatment with 50 μM PD98059 and 50 μM flavone (Fig. 4C). PD98059 and flavone increased cellular

cysteine levels as early as 1 to 4 h following the initiation of treatment. These data suggest that one possible mechanism for the PD98059- and flavone-mediated elevation of GSH synthesis in hepatocytes is the increased cellular cysteine levels, which occurred in response to PD98059 and flavone treatment.

Discussion

Numerous flavonoids have cytostatic, apoptotic, anti-inflammatory, anti-angiogenic and estrogenic effects (Harborne and Williams, 2000; Middleton et al., 2000; Havsteen, 2002). The possible cancer chemopreventive effects of flavonoids, some of which have proven effective in clinical trials (Ferry et al., 1996), may result from the modulation of antioxidant enzyme activity as well as inhibition of enzymes that generate reactive metabolites (Middleton et al., 2000; Frei and Higdon, 2003). A number of studies have demonstrated that hepatic GST and UDP-glucuronyltransferase enzyme activities are induced in rats fed dietary flavone (Brouard et al., 1988; Nijhoff et al., 1995). Siess et al. (1989) showed that flavone was the most potent inducer of drug-metabolizing enzymes among the tested flavonoids. In the present study we demonstrate that flavone and PD98059 regulate GSH homeostasis in primary cultured rat hepatocytes. To our knowledge, this is the first report of increased GSH synthesis induced by PD98059.

PD98059 treatment resulted in increased GSH levels in hepatocytes cultured in both the presence and absence of insulin. However, U0126, at concentrations sufficient to completely inhibit ERK1/2 phosphorylation in response to insulin, failed to affect GSH levels. Moreover, flavone did not inhibit basal ERK1/2 phosphorylation, but GSH levels were more markedly increased by flavone than PD98059, indicating that elevation of GSH levels by flavone is not due to MEK1 inhibition. Taken together, these data suggest that PD98059 produces dramatic changes in GSH homeostasis in

hepatocytes through a mechanism(s) unrelated to MEK inhibition.

PD98059 and flavone caused the elevation of GSH levels in medium as well as in hepatocytes, suggesting that the cellular GSH elevation could not be accounted for by the inhibition of GSH efflux into medium. The elevation of GSH levels in response to both PD98059 and flavone was observed as early as 4 h, without an initial depletion of GSH content. In addition, these chemicals did not affect GCLC protein level or GCL activity, suggesting that the mechanism(s) for PD98059- or flavone-induced GSH synthesis is different from that of chemicals causing oxidative stress. Cellular cysteine levels were elevated by both flavone and PD98059 as early as 1 to 4 h. Hepatic synthesis of GSH is limited by the availability of cysteine as well as by the activity of GCL (Lu, 1998). We have reported that betaine treatment and protein-calorie malnutrition result in a marked decrease in hepatic GSH levels through alteration of cysteine availability, although GCL activity was increased (Kim et al., 2003b and 2003e). Our previous study also suggested that the reduction of cysteine catabolism to taurine could salvage the cysteine supply needed for the synthesis of GSH in mice supplemented betaine for 2 weeks (Kim and Kim, 2005). The Chee's medium used in this study contains 62.6 mg/l L-cystine 2HCl (~0.2 mM) and 60 mg/l L-methionine (~0.4 mM), but does not contain any cysteine. It has been reported that GSH levels in hepatocytes are increased by the addition of cysteine or methionine in a concentration-dependent manner up to 2 mM (Wang et al., 1997). These results, in conjunction with the results of the present study, suggest that the elevation of cellular cysteine levels

induced by PD98059 and flavone may be responsible for the increased GSH synthesis in primary cultures of rat hepatocytes. These results also warrant further studies to elucidate the effect(s) of PD98059- and flavone on the cellular transport and metabolism of cysteine.

GSH, the predominant cellular low molecular weight thiol (normal cellular levels of 0.5−10 mmol/L), scavenges free radicals and other reactive oxygen/nitrogen species (e.g., hydroxyl radical, lipid peroxyl radical, peroxynitrite, and H_2O_2) directly, and indirectly through enzymatic reactions mediated by GSH peroxidase and GSTs. GSH also reacts with various electrophiles to form mercapturates, a reaction initiated by GSTs. Thus, maintenance of GSH levels is critical for cellular defense against oxidative injury and for cellular integrity. Moreover, it is important to note that shifting the GSH redox state toward the oxidizing state regulates several signaling pathways including MAPKs, phosphatidylinositol 3-kinase/Akt, protein tyrosine kinases, nuclear factor κB, apoptosis signal-regulated kinase 1 and receptor tyrosine kinases (Kang et al., 2000; Chiarugi and Cirri, 2003; Torres and Forman, 2003; Haddad, 2004). These results raise the possibility that flavone and PD98059 shift the cellular redox state by elevating GSH synthesis, which may be associated with regulation of cell proliferation and apoptosis.

It has been reported that oxidative stress resulting in decreased GSH availability may produce the transcriptional induction of antioxidant enzymes such as GST, heme oxygenase-1, quinone reductase

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and GCLC, which is associated with an increase in transcription factor binding to the antioxidant response element (ARE) (Masuya et al., 1998; Yu et al., 1999; Kang et al., 2000; Zipper and Mulcahy, 2000; Nguyen et al., 2003). Protein phosphorylation is of major importance in the response to oxidative stress that stimulates ARE-mediated transcription (Nguyen et al., 2003). A number of studies employing PD98059 for ERK inhibition suggested that transcription factor binding to the ARE is a downstream consequence of ERK activation, and that elevation of antioxidant enzyme expression induced by oxidative stress is inhibited by pretreatment with PD98059 through inhibition of ERK activation (Masuya et al., 1998; Zipper and Mulcahy, 2000; Owuor and Kong, 2002). Considering that GSH plays a critical role in regulation of cellular redox state and detoxification of reactive oxygen/nitrogen species and electrophiles, the suppressive effect of PD98059 on antioxidant enzyme induction mediated by oxidative stress may be due to PD98059-induced elevation of GSH levels. In fact, GSH and *N*-acetylcysteine, a GSH precursor, inhibit ARE-related gene expression in response to oxidative stress (Day et al., 2002; Buckley et al., 2003; Kim et al., 2003a)

In summary, the results of the present study indicate that PD98059 and flavone produce dramatic changes in GSH homeostasis in hepatocytes, through a mechanism(s) unrelated to MEK inhibition. Furthermore, the present study suggests that the increase in cysteine induced by PD98059 and flavone may be responsible, at least partially, for the elevation of GSH synthesis. These results, in conjunction with previous studies (Brouard et al., 1988; Siess et al. 1989; Nijhoff et al., 1995), suggest that flavone-

like flavonoids result in elevation of GSH synthesis as well as modulation of drug metabolizing enzyme expression, which may be associated with flavonoid-induced chemopreventive and chemotherapeutic effects. These results also raise the possibility that the suppressive effect of PD98059 on ARE-mediated gene expression in response to oxidative stress may be due to inhibition of oxidative stress through the PD98059-induced elevation of GSH levels.

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Fig. 1. Effects of PD98059 or U0126 on insulin-mediated ERK1/2 phosphorylation and GSH levels in primary cultured rat hepatocytes. A: hepatocytes were treated with PD98059 or U0126 for 1.5 h before addition of 10 nM insulin for 5 min. B and C: hepatocytes were treated with PD98059 or U0126 in the absence of insulin for 24 h, or for 1.5 h before addition of 10 nM insulin for 22.5 h. Untreated (UT) hepatocytes were cultured in the absence of insulin and inhibitor. In UT hepatocytes, GSH levels were 30.4 ± 0.7 nmol/mg protein. Data are means ± SD of six preparations of cell lysates from a single hepatocyte preparation. **,***Significantly different than levels monitored in untreated hepatocytes cultured in the absence of insulin and inhibitors, $p<0.01$ or $p<0.001$, respectively. #,##,###Significantly different than levels monitored in hepatocytes treated with insulin only, $p<0.05$, $p<0.01$ or $p<0.001$, respectively.

Fig. 2. Effects of flavone on GSH levels and ERK1/2 phosphorylation in primary cultured rat hepatocytes. A: structures of PD98059 and flavone. B: hepatocytes were treated with flavone for 24 h. Data are means ± SD of six preparations of cell lysates from a single hepatocyte preparation. **,***Significantly different than levels monitored in untreated hepatocytes cultured in the absence of flavone, $p<0.01$ or $p<0.001$, respectively. C: hepatocytes were treated with flavone (50 μM), U0126 (25 μM) or PD98059 (50 μM) for 1.5 h. Untreated (UT) hepatocytes were cultured in the absence of PD98059, U0126 and flavone.

Fig. 3. Effects of PD98059, flavone or insulin on GCLC protein levels (A) and GCL activity (B) in primary cultured rat hepatocytes. Hepatocytes were treated with PD98059, flavone or 10 nM insulin for 24 h. GCLC protein levels are plotted as a percentage of the level monitored in untreated hepatocytes cultured in the absence of insulin and flavones (100%). Data are means ± SD of 3-5 preparations of cell lysates from a single hepatocyte preparation. Untreated (UT) hepatocytes were cultured in the absence of insulin and inhibitor.

Fig. 4. Effects of PD98059 or flavone on GSH levels in culture medium (A) and time-dependent effects of PD98059 or flavone on cellular hepatocyte GSH (B) and cysteine (C) levels. A: hepatocytes were treated with PD98059 or flavone for 24 h and GSH levels monitored as described in Materials and Methods. B and C: hepatocytes were treated with 50 μM PD98059 or 50 μM flavone for 1, 4, 12 or 24 h and levels of GSH and cysteine determined as described in Materials and Methods. Data are means ± SD of 4-5 preparations of cell lysates from a single hepatocyte preparation. *, **, ***, significantly different from levels monitored in hepatocytes cultured in the absence of PD98059 or flavone, at $p<0.05$, $p<0.01$ or $p<0.001$, respectively. .

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Fig. 1

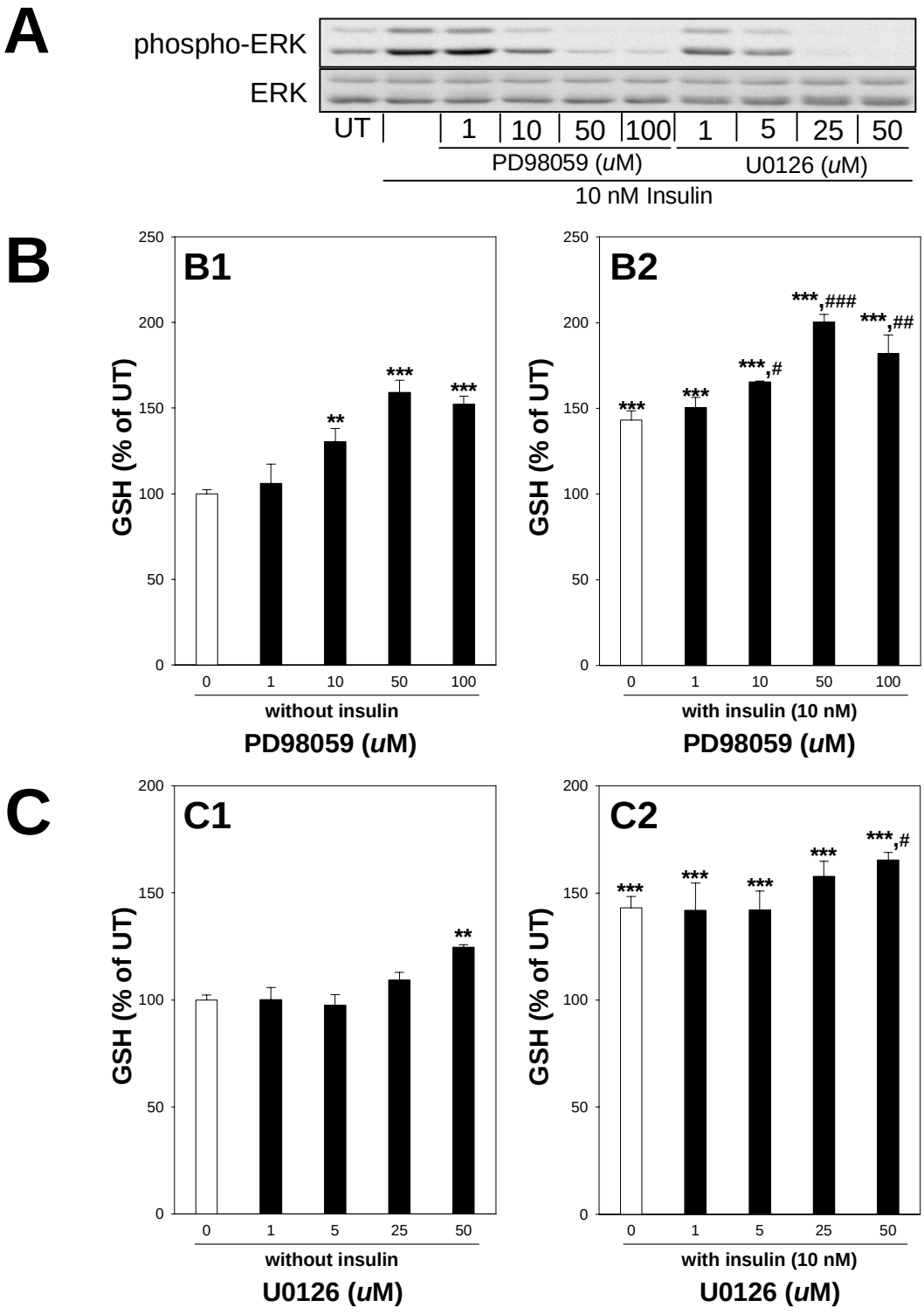

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Fig. 2

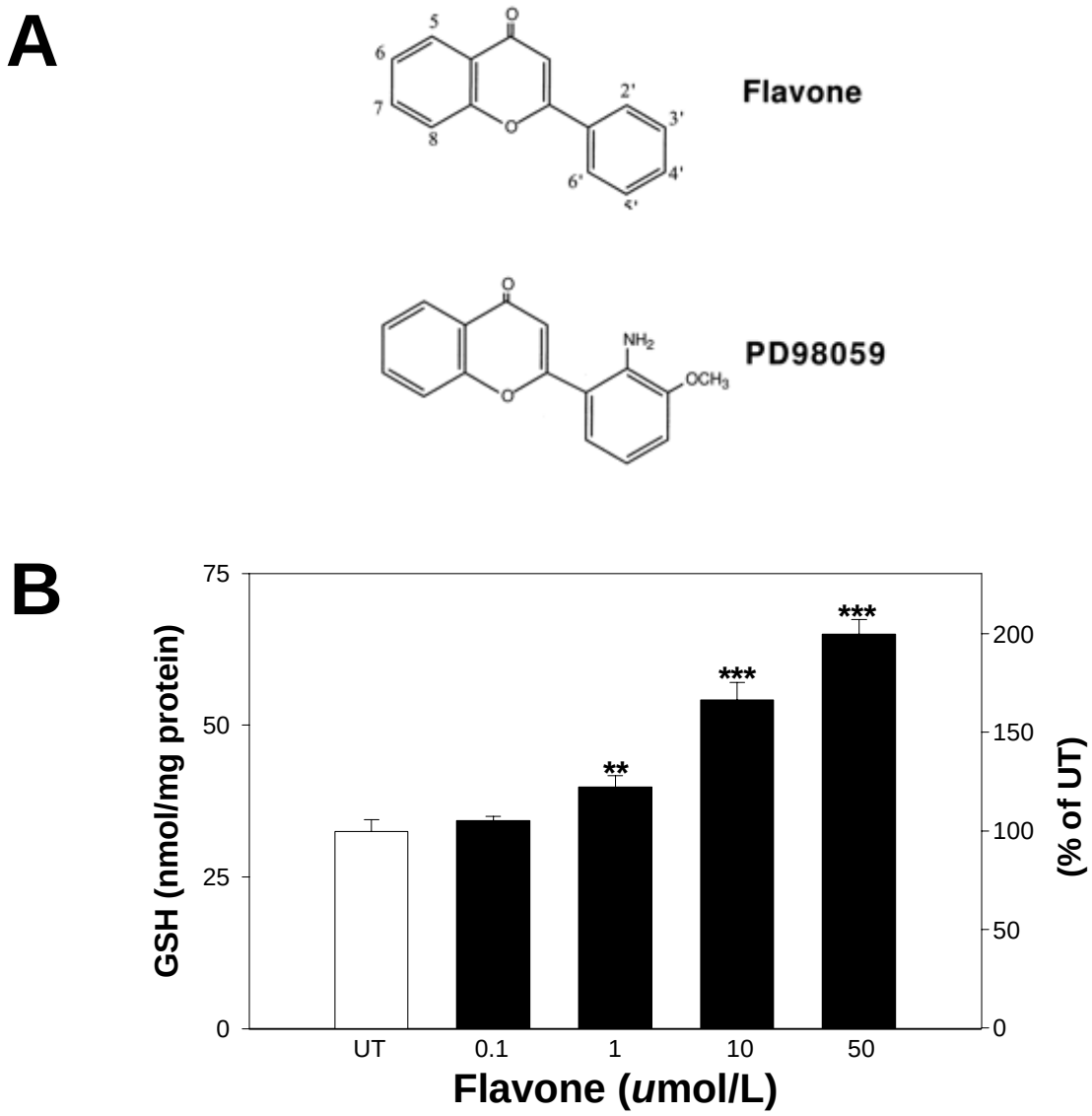

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Fig. 3

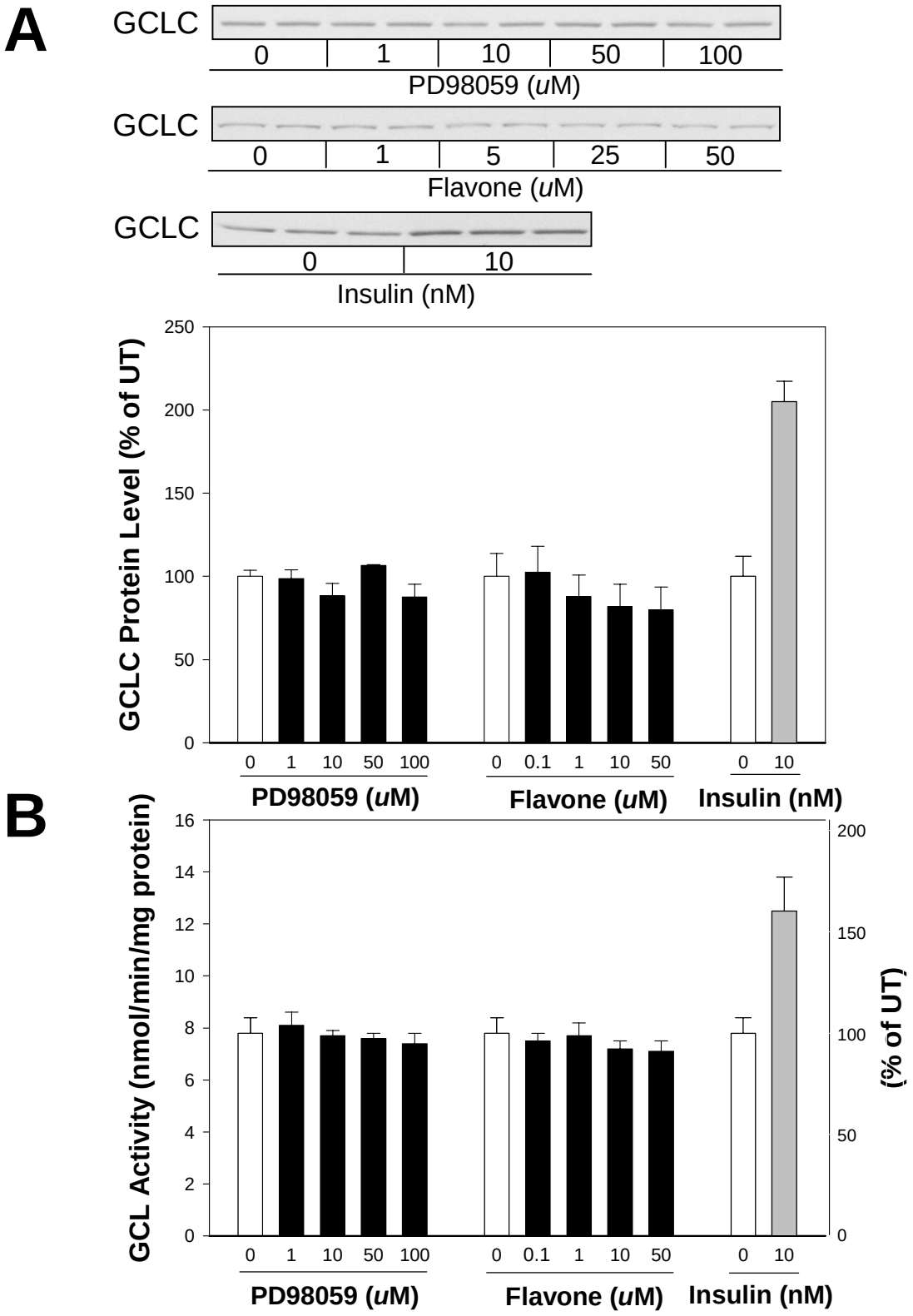

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Fig. 4