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**Suppression of phosphoenolpyruvate carboxykinase gene expression by reduced endogenous glutathione level**

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Keywords: Glutathione, PEPCK, hepatocytes, ageing and diabetes, PI-3 kinase.

Running title: Reduced cellular glutathione level may directly suppress PEPCK mRNA level through a PI-3 kinase dependent pathway.

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## Summary

Glutathione is a small tripeptide to maintain overall reducing environment *in vivo*. Reduced endogenous glutathione level has been associated with aging, obesity and diabetes. In this study, the direct impact of low endogenous glutathione level on energy homeostasis is investigated at molecular level. Depletion of endogenous glutathione in rat primary hepatocytes by BSO, an inhibitor of  $\gamma$ -glutamylcysteine synthase, leads to reduced mRNA levels of several key enzymes in energy homeostasis, including phosphoenolpyruvate carboxylkinase (PEPCK), the rate-limiting enzyme in gluconeogenesis. Supplementation of various reducing reagents, including N-acetylcysteine, DTT and glutathione, reverses the inhibitory effect of BSO on PEPCK mRNA level. The suppressive effect of BSO on PEPCK mRNA level is also reversed through co-treatment with either SB210290, a specific p38 kinase inhibitor, or wortmannin and LY294002, the well-established PI-3 kinase inhibitors, suggesting the involvement of these kinases in this process. These observations correlate well with the observations that reduced endogenous glutathione level and reduced gluconeogenesis coincide with aging process, implying a causal relationship between these changes in aged population. More importantly, this study suggests that endogenous glutathione level tightly associates with energy homeostasis at molecular level, identifying reduced endogenous glutathione level as a potential contributing factor to dysregulated metabolic processes in aging, obese and diabetic populations. In addition, the different responses of PEPCK expression to the alteration of endogenous glutathione level in rat hepatoma cells from primary hepatocytes raises caution against using established cell lines in examining the dysregulated metabolic process related to altered endogenous glutathione level.

## 1. Introduction

Ageing is tightly associated with myriad changes of metabolic processes, as demonstrated by the high prevalence of various metabolic diseases including Alzheimer disease, diabetes, and cardiovascular diseases. Understanding the dysregulated metabolic processes of ageing becomes critical to future prevention and intervention of various ageing-associated metabolic diseases.

A leading theory of aging is the *Free Radical Theory of Aging* [1]. This theory proposes that free radicals, initiated by the mitochondria, interfere with various biological processes owing to their high reactivity. The excessive free radicals, commonly defined as oxidative stress, are suggested to be actively involved in signaling pathway mediating multiple biological processes including aging.

One direct consequence of the accumulation of free radicals is the disturbance of the overall reducing environment of the cell. This reducing environment is maintained by various redox couples, including glutathione system as the leading contributor [2]. The tripeptide glutathione is the most abundant low molecular weight thiol *in vivo*. It is maintained at mM level, 500 to 1000 fold higher than other redox couples. Glutathione exists in either reducing (GSH) or oxidized forms (GSSG and GS-R) [3]. The endogenous level of the reducing form of glutathione is 10 to 100 fold higher than that of the oxidized forms. Together with superoxide dismutase, glutathione eliminates free radicals accumulation *in vivo*, while itself is being converted to the oxidized form, GSSG.

Clearly, maintaining constant level of endogenous glutathione is critical for normal cellular activities.

Low cellular glutathione level has been observed in liver, kidney, and blood of aged animals [4, 5]. Similar observations have also been made in diabetic and obese animals [6, 7]. The cause of low glutathione level under these pathophysiological conditions is still unclear, and it is also unclear if low endogenous glutathione level contributes directly to the dysregulated metabolic process commonly associated with aging, obesity and diabetes.

L-buthionine-[S,R]-sulfoximine (BSO) is a potent inhibitor of  $\gamma$ -glutamylcysteine synthetase, the key enzyme in glutathione synthesis. Treatment of BSO depletes endogenous glutathione in cell culture and animal studies [8]. In this study, endogenous glutathione level was lowered by BSO treatment, and the effect of low glutathione level on cellular metabolism was investigated. The results suggested that low glutathione level might contribute directly to dysregulated metabolic processes *in vivo*.

## 2. Materials and Methods

*2.1 General agents:* Restriction and modifying enzymes were obtained from New England Biolabs; Taq DNA polymerase was from Promega; RediPrimer DNA labeling kit, [ $\alpha$ -<sup>32</sup>P] dCTP and N-bond membrane were from Amersham Biosciences; and Topo TA Cloning kit, Trizol reagent were from Invitrogen. ExpressHyb Hybridization Solution was purchased from BD Clontech. Insulin, Triiodothyronine was purchased from Sigma. Dexamethasone was purchased from BioMol. All the kinase inhibitors were purchased from EMD biosciences. Liver digestion medium, liver perfusion medium, M199, DMEM medium and fetal bovine serum were purchased from invitrogen. All the culture dishes were purchased from BD Discover Labware. CM-H<sub>2</sub>DCFDA was purchased from Invitrogen. All media were supplemented with Penicillin/streptomycin according to manufacturer's instructions.

*2.2 Isolation of rat primary hepatocytes:* Male Sprague-Dawley rats (250g-300 g) were obtained from Harlan World Headquarters (Indianapolis, Indiana). Animals were housed in colony cages, maintained on a 12-h light/ 12-h dark cycle, and fed Teklad 4% chow diet. Rat primary hepatocytes were isolated from nonfasting rats by collagenase method [9]. Briefly, animals were anesthetized with halothane. Rat liver was perfused *in situ* via the portal vein with 100 ml liver perfusion medium at a rate of ~ 7 ml/min. The liver was then perfused with Liver Digest Medium containing collagenase for 20 mins at a flow rate of 5 ml/min. After perfusion, the liver was transferred to a 100 mm culture dish with Liver Digest Medium and the hepatic capsules were stripped and shaken to dissociate

cells in the medium. Cells were filtered through a gauze and washed twice with ice cold DMEM (invitrogen) supplemented with 5% fetal bovine Serum before they were seeded in rat collagen I-coated dishes (BD Labware) in M199 medium supplemented with 5% fetal bovine serum. The cells were washed twice with DPBS after 4 hours, and were cultured in serum free M199 medium supplemented with 100 nM T<sub>3</sub>, 100 nM Dexamethasone and 1 nM insulin overnight. Before treatment, cells were washed twice with DPBS and changed into plain M199 medium supplemented with 100 nM dexamethasone.

*2.3 Northern Blot Analysis:* Procedure has been described elsewhere [10]. In brief, Total RNA was extracted using Trizol reagent, and separated in 1% formaldehyde agarose gel before they were transferred to nylon membrane for hybridization using probes labeled with [ $\alpha$ -<sup>32</sup>P] dCTP.

*2.4. Glutathione Measurement:* Endogenous glutathione level was measured using modified Griffith method [11]. In brief, cells were harvested and homogenized in glutathione reaction buffer (125 mM Na<sub>3</sub>PO<sub>4</sub>, 6.3 mM EDTA, pH 7.5). Cytosol was separated from pellet after centrifugation at 3000 rpm for 5 mins in microcentrifuge tube, and 4 $\mu$ l cytosol was collected for protein measurement. The rest of the cytosol was transferred to another tube and treated with half volume of 10% 5-sulfosalicylic acid to precipitate soluble proteins. The treated cytosol was spinned using microcentrifuge at 3000 rpm for another 5 mins. The supernatant was transferred into another tube and was neutralized using 4 M Triethanolamine (50  $\mu$ l/ml). Aliquot of 40  $\mu$ l from each sample

was added into reaction mixture (0.6 mM DTNB, 50 unit/ml bovine glutathione reductase, 0.3 mM NADPH in 1 ml GSH buffer). The reaction was monitored using spectrophotometer at 412 nm. The endogenous glutathione level was derived from comparison of reaction kinetics with a reference curve.

*2.5. Quantitative Real Time PCR:* Total RNA was prepared from rat primary hepatocytes using TriZol reagent (Invitrogen). Equal amounts of RNA were treated with DNase I (DNA-free<sup>TM</sup>; Ambion, Inc.). First strand cDNA was synthesized from 2 µg of DNase I-treated total RNA with random hexamer primers using the ABI cDNA synthesis kit (Applied Biosystems). The reactions contained, in a final volume of 20 µl, 20 ng of reverse transcribed total RNA, 167 nM forward and reverse primers, and 10 µl of 2× SYBR Green PCR Master Mix (catalog no. 4312704; PerkinElmer Life Sciences). PCR was carried out in 384-well plates using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). All reactions were done in triplicate. The relative amount of all mRNAs was calculated using the comparative C<sub>T</sub> method [12]. 36B4 mRNA expression was used as the invariant control for all studies.

*2.6. Measurement of Reactive Oxygen Species:* The intracellular production of reactive oxygen species was determined by measurement of the oxidation of CM-H<sub>2</sub>DCFDA to the fluorescent compound CM-H<sub>2</sub>DCF with a Cytofluor II multiplate fluorimeter (Millipore). Rat primary hepatocytes were seeded in a 96 well collagen-coated plate in M199 medium supplemented with 100 nM Dexmethasone. Cells were treated as indicated in the figure overnight, and washed once with M199 medium supplemented

with 100 nM before they were changed into M199 medium containing 5  $\mu$ M CM-H<sub>2</sub>DCFDA for 30 minutes. Fluorescence was monitored at excitation and emission wavelengths of 485 and 530 nm, respectively.

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### 3. Results

To test the direct effect of low glutathione level on energy homeostasis at molecular level, endogenous glutathione was depleted by the use of BSO in rat primary hepatocytes. Cells were treated with BSO at 250  $\mu$ M for 16 hours, alongside with 100 nM insulin as positive control. The mRNA levels of several key enzymes in energy homeostasis, including those involved in glucose homeostasis (glucose kinase, GK; phosphoenolpyruvate kinase, PEPCK; glucose-6-phosphatase, G-6-Pase; insulin growth factor binding protein 1, IGFBP-1) and lipid metabolism (Fatty acid synthase, FAS; and sterol regulatory element binding protein 1c, SREBP-1c), were measured using real time PCR method (RT-PCR) (Fig. 1A). Consistent with previous reports, insulin treatment significantly reduced mRNA levels of PEPCK, G-6-P, IRS-2 and IGFBP-1, and increased mRNA levels of GK, FAS, and SREBP-1c. BSO treatment, on the other hand, also significantly reduced mRNA levels of PEPCK, IRS-2 and IGFBP-1. However, it had minimal effects on those of G-6-Pase, GK, FAS, and SREBP-1c. The expression level of Insulin receptor (IR) did not change under either insulin or BSO treatment, thus serving as an internal control of the experiment. This observation suggested that reduced endogenous level of glutathione might directly interfere with energy homeostasis at molecular level. The real time-PCR result was also confirmed using northern blot analysis on selected genes (Fig. 1B).

The suppressive effect of BSO on PEPCK gene expression was further investigated to understand the molecular mechanism underlying the regulatory effect of low glutathione level on cellular metabolic processes. Endogenous glutathione level was altered either

through depletion of endogenous glutathione (BSO treatment) or supplementation of exogenous glutathione, and their effects on PEPCK expression were analyzed using northern blot analysis (Fig. 2). Supplementation of exogenous glutathione (GSH) for 4 hours significantly increased PEPCK mRNA level. Supplementation of exogenous GSSG also slightly increased PEPCK mRNA level (data not shown). The expression level of ApoE was used here and elsewhere as the loading control and was not changed in these treatments (Fig. 2A). BSO-treated cells incubated with several other reducing agents, like DTT (5 mM) or N-acetylcysteine (5 mM), also reversed BSO effect on PEPCK mRNA level in rat primary hepatocytes (data not shown).

The suppressive effect of BSO on PEPCK mRNA level was also demonstrated by both time course and dose response studies (Fig. 2B and 2C). Incubation of cells with 250  $\mu$ M BSO in the initial 8 hours had minimum effect on PEPCK mRNA level. The suppressive effect of BSO was first observed after 12 hours of incubation, and was evident after 16 hours (Fig. 2B). The slow response of the mRNA level of PEPCK to BSO treatment agreed well with the hypothesis that depletion of endogenous glutathione, rather than BSO itself, suppresses PEPCK expression at transcriptional level. Dose response experiment in Fig. 2C showed that after 16 hours of treatment, BSO at 50  $\mu$ M already significantly reduced PEPCK mRNA level, and from 250  $\mu$ M on, BSO reduced PEPCK mRNA to undetectable level.

The depleting effect of BSO on endogenous glutathione level in rat primary hepatocytes was verified using glutathione assay based on the method of Griffith [11]. Rat

hepatocytes were treated with increasing doses of BSO for 16 hours, and endogenous glutathione level was measured and normalized to cytosolic protein level. Consistent with northern blot analysis (Fig. 2C), 10  $\mu$ M BSO had minimal effect on endogenous glutathione level. From 50  $\mu$ M BSO on, BSO significantly depleted endogenous glutathione, and at 250  $\mu$ M, BSO reduced endogenous glutathione level by 80%. Overall, these observations suggested that reduced endogenous glutathione level leads to reduced PEPCK expression in rat primary hepatocytes.

This idea was further tested in the next experiment (Fig. 3A). Exogenous glutathione was used to supplement low endogenous glutathione level in BSO-treated cells, and the expression level of PEPCK was examined. Consistent with previous results, incubating cells with exogenous glutathione increased PEPCK mRNA level, while BSO treatment suppressed its expression. Co-incubation of cells with exogenous glutathione reversed BSO effect on PEPCK mRNA level significantly (compared lane 2 with lane 4), further supporting the hypothesis that reduced endogenous glutathione level was the direct cause of reduced mRNA level of PEPCK in rat primary hepatocytes.

Several other known reducing reagents, including N-Acetylcysteine (NAC) and ascorbic acid, were also co-incubated with BSO in rat primary hepatocytes (Fig. 3B). Supplementation of NAC significantly increased PEPCK mRNA level in BSO-treated cells. Ascorbic acid, on the other hand, failed to reverse BSO effect on PEPCK expression. In addition, DTT at 5 mM was as effective as glutathione in reversing the inhibitory effect of BSO on PEPCK expression level (data not shown). These results

were consistent with the redox potentials of these reducing reagents, indicating that altered reducing environment of the cell resulted from the depletion of endogenous glutathione modulates PEPCK mRNA level.

The suppressive effect of BSO on PEPCK mRNA level was also investigated in a rat hepatoma cell line, H4IIE cells (Fig. 3C). As a positive control, treatment of insulin led to significantly reduced PEPCK expression level. In contrast to rat primary hepatocytes (Fig. 2A), however, incubation of 5 mM glutathione with H4IIE cells had little effect on PEPCK mRNA level (compare lane 1 with lane 3). What is more, BSO treatment at 250  $\mu$ M for 16 hours had no suppressive effect on PEPCK mRNA level (compare lane 1 with lane 4). In fact, consistent with a previous report [13], BSO treatment slightly increased basal level expression of PEPCK, and co-incubation of glutathione had minimal effect on this stimulatory effect of BSO on PEPCK expression. This experiment clearly demonstrated drastic differences between immortalized cells and rat primary hepatocytes in response to BSO treatment. The implications of this observation will be discussed later.

To understand the molecular mechanism underlying the suppressive effect of low glutathione level on PEPCK mRNA level, a group of well-known kinase inhibitors were used to treat cells to investigate the putative involvement of these kinase cascades in this process. These inhibitors included PD 98059 (MEK inhibitor), Wortmannin and LY 294002 (PI-3 Kinase inhibitors), rapamycin (p<sup>70</sup>S6 Kinase inhibitor) and SB-202190 (p38 inhibitor) (Fig. 4A). Treatment of PD 98059 had minimal effect on the regulatory effect

of BSO on PEPCK mRNA level. Treatment of SB-202190 on the other hand, potent inhibited BSO effect on PEPCK mRNA level. Furthermore, both LY 294002 and Wortmannin increased basal PEPCK mRNA level significantly. Co-treatments of BSO and wortmannin or LY 294002 effectively abolished the suppressive effect of BSO on PEPCK mRNA level. Treatment of rapamycin also significantly increased the basal level expression of PEPCK. However, the suppressive effect of BSO was not inhibited in the presence of rapamycin (Compare Lane 9 and Lane 10). The involvement of PI-3 kinase in this process was further confirmed in a dose response study (Fig. 4B). Wortmannin at 100 nM significantly abolished BSO effect at both 250  $\mu$ M and 500  $\mu$ M on PEPCK mRNA level. These experiments strongly suggested that the suppressive effect of BSO on PEPCK mRNA level was a regulated process, and several kinases, including PI-3 kinase and p38 kinase, were possibly involved in this process.

One important role of glutathione *in vivo* is to protect cells from the accumulation of reactive oxygen species. Low endogenous glutathione level may fail to prevent the accumulation of free radicals and their toxic effect on cellular metabolism. The possibility that the suppressive effect of BSO on PEPCK expression was the result of the accumulation of free radicals was also investigated in the following studies. Total level of reactive oxygen species was measured using Dichlorodihydrofluorescein derivatives (CM-H<sub>2</sub>DCFDA), a cell-permeable indicator for reactive oxygen species (Fig.5). Its fluorescence intensity is used as the indicator of the endogenous level of reactive oxygen species. Cells incubated with exogenous glutathione alone showed reduced signal intensity. However, compared with cells treated with DMSO, BSO treatment did not lead

to significantly increased signal intensity. These results raised doubt on the direct involvement of the free radicals in the suppressive effect of BSO on PEPCK mRNA level. This conclusion was also supported by the minimal effects of both MnTBAP, a cell permeable superoxide dismutase mimetic [14] and catalase (from bovine liver) on the suppressive effect of BSO on PEPCK mRNA level (data not shown), suggesting that superoxide is unlikely directly involved in the BSO effect on PEPCK mRNA level. Ascorbic acid, a powerful agent to detoxify superoxide, also had minimal effect on the suppressive effect of BSO on PEPCK mRNA level (Fig.3B).

Furthermore, as a major component of reactive oxygen species, hydrogen peroxide has been suggested to mediate a variety of biological processes. Hydrogen peroxide was used to treat rat primary hepatocytes for 16 hours, alongside with BSO in Fig. 5B. Clearly, hydrogen peroxide up to 3 mM had minimal effect on the expression level of PEPCK, while BSO significantly suppressed PEPCK expression at mRNA level. This experiment further suggested the unlikely involvement of ROS in the suppressive effect of BSO on PEPCK mRNA level..

#### 4. Discussion

The data presented here describe for the first time the regulatory effect of low endogenous glutathione level on energy homeostasis at molecular level. Through investigation of its suppressive effect on PEPCK expression level, low endogenous glutathione level is suggested to modulate cellular metabolism in a PI-3 kinase-dependent pathway, without direct involvement of reactive oxygen species. Thus, low endogenous glutathione level, commonly associated with obesity, aging and diabetes, may contribute directly to these dysregulated metabolic processes. In addition, results from this study indicates that while reactive oxygen species may be involved in multiple biological processes, the putative direct impact of persistent disturbed reducing environment on overall cellular metabolism cannot be discounted. While further studies are required to verify these findings, continuing investigation of the molecular mechanism underlying the regulatory effect of endogenous glutathione level on energy homeostasis is needed for the prevention and treatment of these pathphysiological states.

The finding that low endogenous glutathione level suppresses PEPCK expression at mRNA level is also consistent with reports where reduced PEPCK expression is commonly observed in aged rodent models. PEPCK is the rate-limiting enzyme in gluconeogenesis. Its expression is primarily regulated at transcriptional level, subjecting to multiple controls including hormones and dietary carbohydrates [15]. In aged rodent models, both mRNA level and enzymatic activity of PEPCK are reduced significantly [16-20]. Considering that reduced endogenous glutathione level is also commonly observed in aging population, observations from this study suggest a causal relationship

between low endogenous glutathione level and reduced PEPCK expression level, and possibility of alleviation of aberrant energy homeostasis in aged population by increasing endogenous glutathione level.

Antioxidants, as popular nutritional supplements, have been suggested to prevent several diseases including cancer, diabetes and aging. However, the beneficial effects of antioxidants are evaluated mainly through population studies, and the results remain controversial [21]. In this study, incubation of cells with glutathione, DTT or N-acetylcysteine all significantly increase basal level expression of PEPCK, while incubation cells with ascorbic acids has no detectable effect on PEPCK expression. These observations imply that some of nutritional values of certain antioxidants may lie in their effects in restoring aberrant expression levels of key enzymes resulting from low endogenous glutathione level commonly associated with aging and diabetes.

The drastic difference at PEPCK mRNA level in established cell line vs. primary cells in response to altered glutathione level was also unexpected (Fig. 3A and 3C). Observations from this study indicate that up-regulation of PEPCK mRNA level in response to BSO treatment in rat hepatoma cells may not be directly related to the alteration of endogenous glutathione level. Rather, it may be the result of increased oxidative stress, as suggested in the paper. More importantly, the different responses of PEPCK mRNA level to BSO treatment in established cells vs. rat primary hepatocytes further emphasizes the importance of using primary cells or animal tissues to investigate metabolic process associated with the alteration of endogenous glutathione level. While

the underlying mechanism of this difference is still unclear, this sharp difference could also be observed in their response to hydrogen peroxide treatment. H4IIE cells died within hours in the presence of 100  $\mu$ M Hydrogen Peroxide, while rat primary hepatocytes were resistant to hydrogen peroxide treatment at mM range for couple of days (data not shown). Presumably, compared with established cell line, primary cells are more resistant to oxidative stress. This may also explain why depletion of endogenous glutathione in rat primary hepatocytes was not associated with dramatic increase of ROS (Fig. 5). Nonetheless, these differences raise concerns about studies based on established cell lines to investigate biological process associated with altered endogenous glutathione level [13]. Although compared with established cell line, primary cells are more troublesome to manipulate, and difficult for genetic manipulations, it remains necessary experimental material for future investigation of biological processes associated with alteration of endogenous glutathione level.

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## Figure Legends

Figure 1: Depletion of endogenous glutathione leads to global changes of cellular metabolism. Rat primary hepatocytes were isolated as described in the Material and Method section. **1A.** Cells were treated with either 100 nM insulin or 250  $\mu$ M BSO for 16 hours before the total RNA was extracted for quantitative real time PCR analysis using primers described elsewhere [22]. The results are average of three independent experiments in triplicates. \*  $p < 0.05$  based on student t-test. All the results were normalized using rat 36B4 as internal control. The dashed line indicates the expression level of the indicated gene in control cells. **1B.** Cells were setup as in 1A and treated with either DMSO, 100 nM insulin, or BSO at either 250  $\mu$ M or 500  $\mu$ M respectively. Total RNA was extracted for northern blot analysis using probes against PEPCK, G-6-Pase, IGFBP-1, GK and ApoE.

Figure 2: Low endogenous glutathione level suppresses PEPCK expression at mRNA level. **2A:** Incubation of cells with reduced form of glutathione significantly increased mRNA level of PEPCK. Cells were treated with reducing form of glutathione (GSH) for 4 hours, and total RNA was extracted for northern blot analysis using probes against either PEPCK or ApoE. **2B:** Time course study of BSO effect on PEPCK expression. Cells were treated for 4, 8, 12, 16 hours with 250  $\mu$ M BSO before total RNA was extracted for northern blot analysis using specific probes against PEPCK and ApoE. **2C;** Dose response study of BSO effect on PEPCK expression. Rat primary hepatocytes were treated with BSO at final concentration of 10  $\mu$ M, 50  $\mu$ M, 250  $\mu$ M, and 500  $\mu$ M for 16 hours. Total RNA was extracted for northern blot analysis using specific probes for

PEPCK and ApoE. **2D**: Measurement of endogenous glutathione level in cells treated with BSO at concentrations indicated in the figure. Rat primary hepatocytes were seeded at  $6 \times 10^6$ /100 mm dish in M199 medium supplemented with 100 nM dexamethasone, and treated with BSO at concentrations indicated in the figure for 16 hours. Endogenous glutathione level was measured using method of Griffith [11] as described in the material and method. The results from three experiments were averaged and expressed as the percentage of the endogenous glutathione level of untreated cells. The endogenous level of glutathione in untreated cells was around 7.5 nmole/mg cytosolic protein. \*.p<0.05 based on student t-test.

Figure 3: Antioxidants inhibit suppressive effect of BSO on PEPCK expression. **3A**: Supplementation of reduced form of glutathione abolished suppressive effect of BSO on PEPCK mRNA level. Cells were treated with 250  $\mu$ M BSO for 16 hours with and without co-treatment of 6 mM glutathione (GSH, reducing form). Total RNA was extracted for northern blot analysis. **3B**: N-acetylcysteine, like glutathione, reversed inhibitory effect of BSO on PEPCK expression. Cells were treated with BSO for 16 hours with and without co-treatments of glutathione (GSH, 6 mM), N-acetylcysteine (NAC, 5 mM) and ascorbic acid (5 mM) respectively for 16 hours. Total RNA was extracted for northern blot analysis. Results are the representatives of at least three experiments. **3C**: Rat hepatoma H4IIE cells were setup at  $2 \times 10^6$ /60 mm dish in DMEM medium supplemented with 10% fetal bovine serum overnight before they were changed into plain DMEM medium, with and without co-treatments of BSO, glutathione, or insulin, as indicated in the figure. Cells were treated overnight, and total mRNA was

collected for northern blot analysis using probes against PEPCK or ApoE, as described in Fig. 2.

Figure 4: PI-3 kinase activation is required for the suppressive effect of BSO on PEPCK expression. **4A:** Wortmannin abolished BSO effect on PEPCK expression. Rat primary hepatocytes were treated with BSO at 250  $\mu\text{M}$ , alongside with PD98059 (50  $\mu\text{M}$ ), SB202190 (20  $\mu\text{M}$ ), Wortmannin (100 nM), rapamycin (50  $\mu\text{M}$ ) and LY294002 (20  $\mu\text{M}$ ) for 16 hours. Total RNA was extracted for northern blot analysis (20 $\mu\text{g}/\text{lane}$ ). The PEPCK mRNA level under these treatments were also quantified using ImageJ program. The result is the average of three independent experiments. \*:  $p < 0.05$ . The dashed bar indicates the basal level expression of PEPCK in control cells. **4B:** Dose responses study. Cells were treated with BSO at 250  $\mu\text{M}$  or 500  $\mu\text{M}$ , alongside with wortmannin (100 nM) for 16 hours, and PEPCK mRNA level was analyzed using northern blot analysis (20 $\mu\text{g}/\text{lane}$ ). All the results were repeated at least three times.

Figure 5: BSO treatment is not associated with increased level of reactive oxygen species (ROS). **5A.** Rat primary hepatocytes were seeded at  $5 \times 10^4/\text{well}$  in 96 well plate. Cells were treated with BSO (250  $\mu\text{M}$ ) and glutathione (5mM) as indicated in the figure for 16 hours and the overall reactive oxygen species levels were measured as described in Materials and Methods. The fluorescence intensity of cells treated with DMSO alone was arbitrary set as 1. The dashed line indicates the basal level fluorescence intensity. This experiment has been repeated at least three times. **5B.** Rat primary cells were setup as described in Fig. 2, and treated with either Hydrogen Peroxide at indicated

concentrations or BSO at 250  $\mu$ M for 16 hours. Total RNA was prepared for northern blot analysis as described in Fig. 2.

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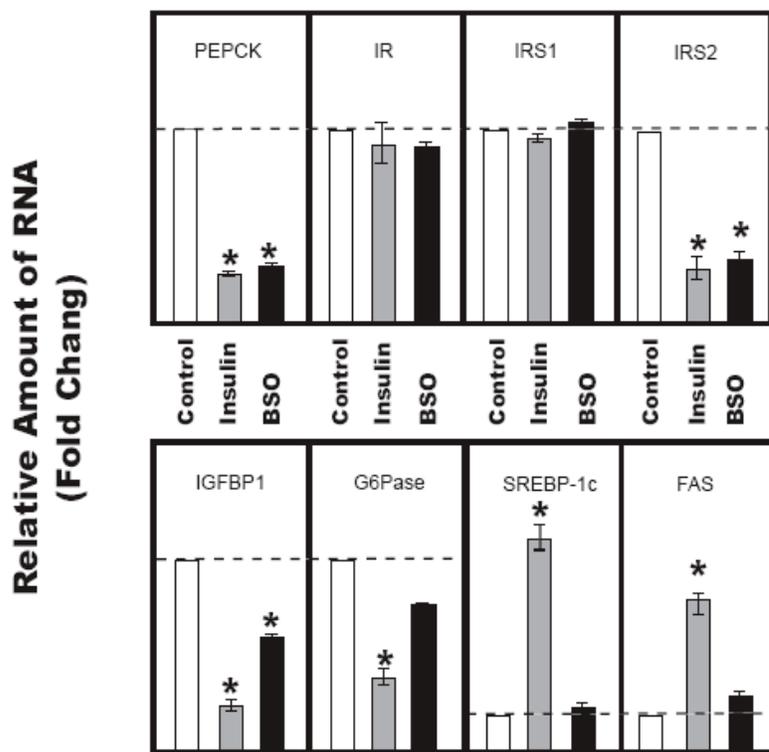
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Figure 1

A



B

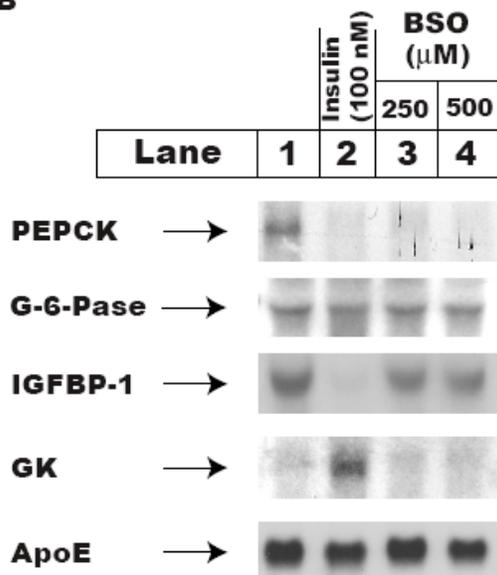
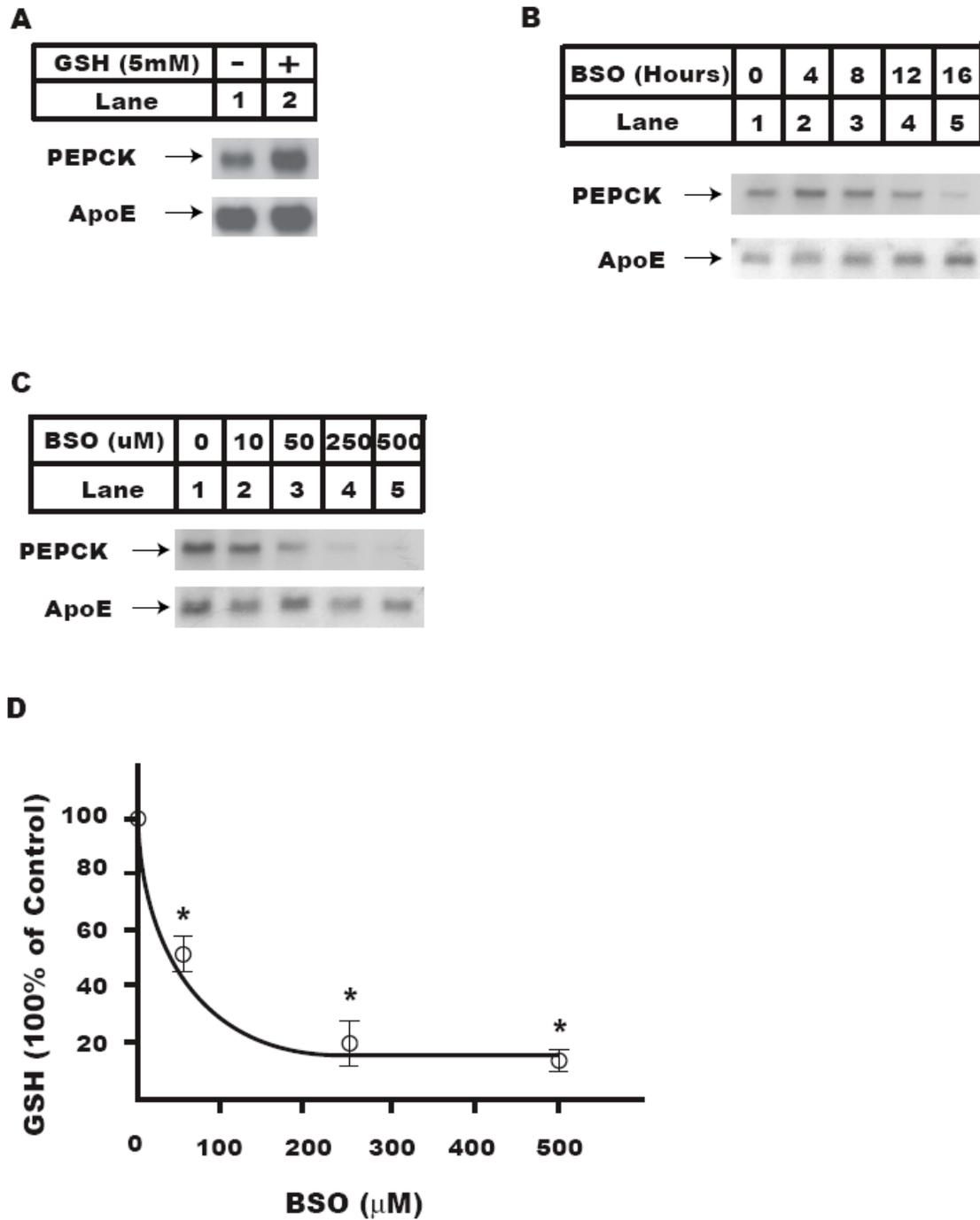


Fig. 2



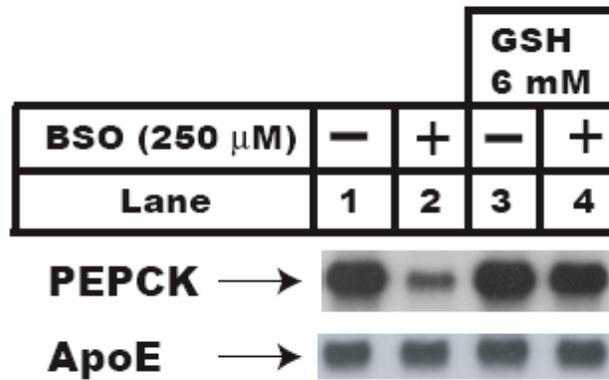
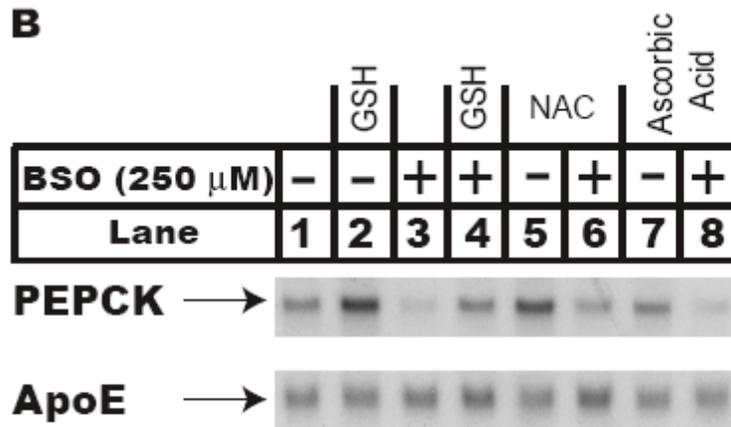
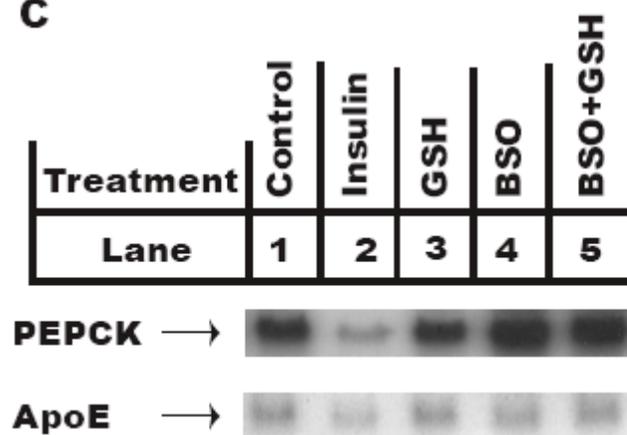
**Fig. 3****A****B****C**

Fig. 4

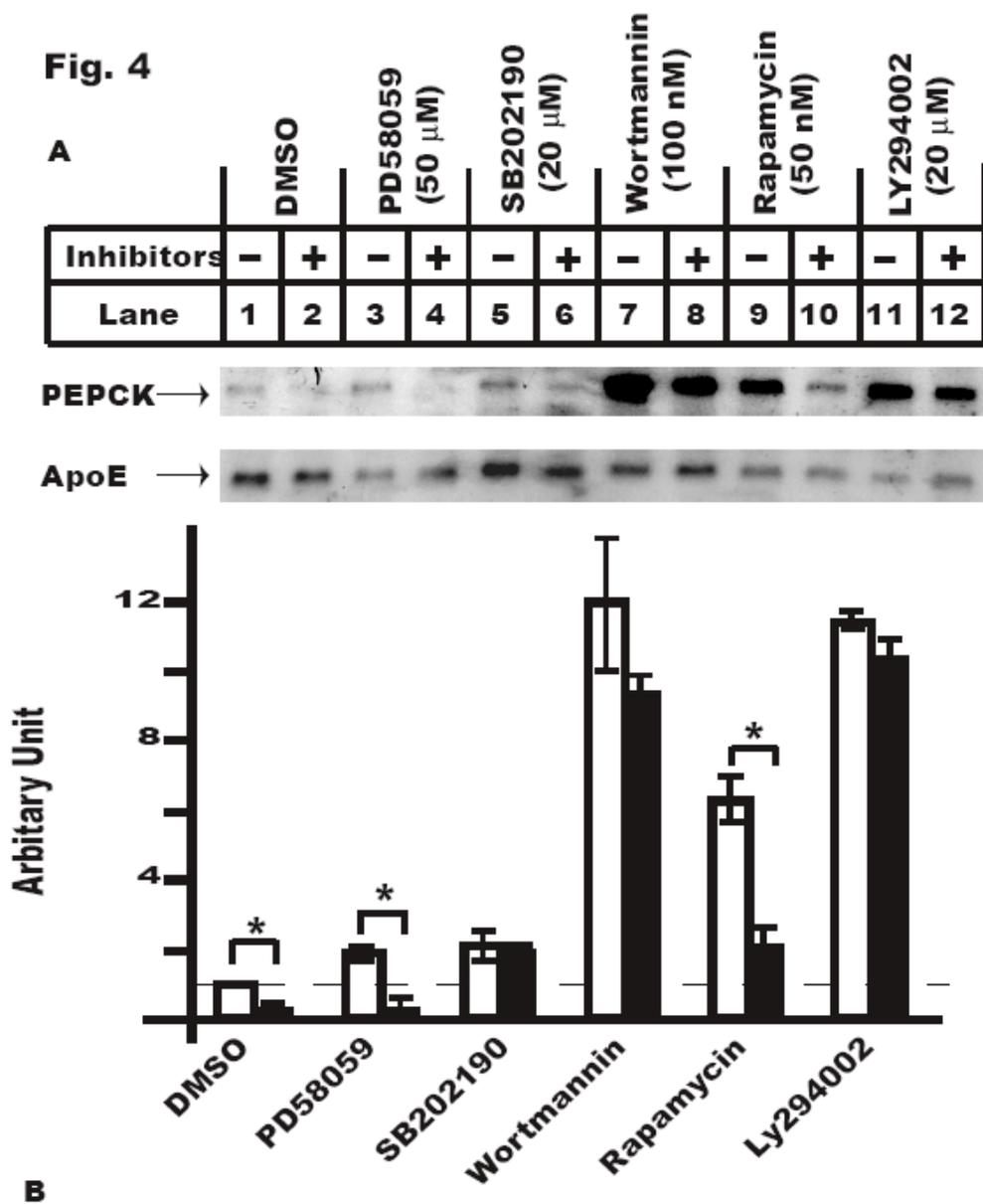
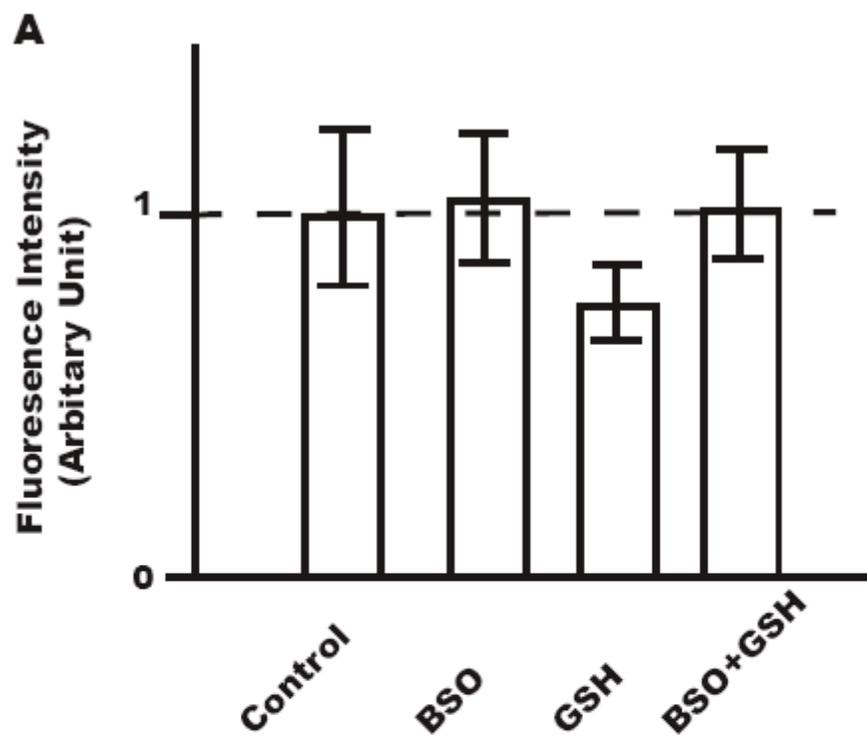


Fig. 5

**B**