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Repression of γ-Glutamylcysteine Synthetase and Glutathione S-Transferases by Metformin, an Anti-diabetic Agent, in H4IIE Rat Hepatocytes

Eun Ju Bae, Min Joo Cho and Sang Geon Kim

College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, Korea

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Metformin is a drug used to lower blood sugar levels in patients with type 2 diabetes via activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK). The primary objective of this study was to investigate whether metformin at the pharmacologically effective concentrations affects the expressions of γ-glutamylcysteine synthetase and phase II antioxidant genes in the H4IIE cell. Treatment of the cells with either metformin or 5-aminoimidazole-4-carboxamide riboside (AICAR) abrogated *tert*-butylhydroxyquinone (*t*-BHQ) induction of γ-glutamylcysteine synthetase, a rate limiting enzyme of GSH synthesis. The ability of *t*-BHQ to induce glutathione S-transferases (GSTs), a major class of phase II detoxifying enzymes that play a critical role in protecting cells from oxidative stress or electrophiles, was also inhibited by the agents. Transcriptional gene repression by metformin was verified by the *GSTA2* promoter luciferase assay. Moreover, either metformin or AICAR treatment significantly decreased *t*-BHQ-dependent induction of other GSTs (i.e., GSTμ and GSTπ forms). Taken together, our data indicate that metformin treatment may result in the repression of γ-glutamylcysteine synthetase and glutathione S-transferase genes possibly via AMPK activation.

Key words: Metformin, AMPK, Oxidative stress, γGCS, GSTA2, GSTμ, GSTπ, Transcription

INTRODUCTION

The incidence rate of type 2 diabetes has risen significantly worldwide and the metabolic diseases become a leading health problem. Metformin, the most widely used drug for type 2 diabetes, ameliorates hyperglycemia by improving peripheral insulin sensitivity and by reducing the gastrointenstinal glucose absorption and hepatic glucose production. AMP-activated protein kinase (AMPK), a target capable of mediating the beneficial metabolic effects of metformin, has been intensely investigated as a therapeutic target for type 2 diabetes.

Correspondence to: Sang Geon Kim, College of Pharmacy, Seoul National University, Sillim-dong, Kwanak-gu, Seoul 151-742, Korea
E-mail: sgk@snu.ac.kr

Abbreviations: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide riboside; AMPK, AMP-activated protein kinase; BSO, buthionine sulfoximine; DCFH, 2',7'-dichlorofluorescin; FBS, fetal bovine serum; γGCS, γ-glutamylcysteine synthetase; GSH, glutathione; GST, glutathione S-transferase; Met, metformin; *t*-BHQ, *tert*-butylhydroquinone; ROS, reactive oxygen species.

AMPK, which functions as a heterotrimer composed of a catalytic α-subunit and two non-catalytic subunits, β and γ, is activated by an increase in intracellular ratio of AMP to ATP during metabolic stress caused by exercise, hypoxia, nutrient deficiency and hormones (e.g. adiponectin and leptin) (Kemp *et al.*, 2003; Hardie 2003). Once activated, AMPK-mediated phosphorylation events switch cells from active ATP consumption (e.g. fatty acid and cholesterol biosynthesis) to active ATP production (e.g. fatty acid and glucose oxidation). So, AMPK is an energy sensor to metabolic stress for cellular adaptation, glucose regulation and lipid homeostasis. The essential role of AMPK in energy metabolism in cells is supported by a number of findings such as the induction of abnormal insulin sensitivity by AMPKα2 gene knockout (Viollet *et al.*, 2003) and the association of glycogen storage disease with mutations of human AMPK (Polekhina *et al.*, 2003).

The adverse-effect profile of metformin has been well established in the literature (Stephen, 2006). Lactic acidosis is a rare, but potentially life-threatening, adverse effect associated with metformin. The more prevalent

adverse effects that occur with metformin are gastrointestinal disturbances such as diarrhea, abdominal discomfort, nausea and anorexia. Furthermore, the use of the drug is contraindicated to hepatic diseases. In the experimental models, there are controversial reports on the effects of metformin on cell apoptosis. Some reports insist that metformin or AMPK activation caused apoptosis (Kefas *et al.*, 2003; Kefas *et al.*, 2004; Meisse *et al.*, 2002), and that apoptosis associated with AMPK activation involved c-Jun-NH_2-terminal kinase (JNK) and caspase activation (Kefas *et al.*, 2003; Meisse *et al.*, 2002).

In the previous study, we tested the hypothesis that links GSH deficiency and metformin's deleterious effects. We used a variety of prooxidants such as *tert*-butylhydroquinone (*t*-BHQ) which is autooxidized to *t*-butylquinone that produces reactive oxygen species (ROS) by redox-cycling (Pinkus *et al.*, 1996), buthionine sulfoximine (BSO, a GSH depleting agent) and doxorubicin (anticancer agent causing cytotoxicity by ROS generation). Our data showed that either metformin or 5-aminoimidazole-4-carboxamide riboside (AICAR) increased dichlorofluorescein oxidation and prevented an adaptive increase in cellular GSH in H4IIE cells, and thereby induced apoptosis under the conditions of GSH deficiency (Bae *et al.*, 2007). Furthermore, compound C, a selective AMPK inhibitor, reversed apoptosis, which suggests the possible involvement of metformin's activation of AMPK in the cellular process.

The phase II antioxidant enzymes linked with the homeostatic regulation of the redox-state are induced in cells as part of the adaptive responses to oxidative stress. Given the deleterious effects of metformin on apoptosis, we were tempted to investigate whether metformin at the concentrations pharmacologically achievable in the diabetic rat liver (Hermann, 1979; Wilcock *et al.*, 1991; Wilcock and Bailey, 1994) inhibits the induction of phase II antioxidant genes which are responsible for the homeostatic regulation of GSH. The effect of metformin's activation of AMPK on cellular adaptations to oxidative stress was also addressed. In this experimental design, we further examined the effects of AICAR in combination with *t*-BHQ on the induction of phase II antioxidant enzymes.

MATERIALS AND METHODS

Chemicals and reagents. Metformin was supplied from Dalim Corporation (Seoul, Korea). AICAR and *t*-BHQ were obtained from Sigma Chemical (St. Louis, MO, USA). Anti-murine γ-glutamylcysteine synthetase (γGCS) antibody was purchased from Lab Vision (Fremont, CA, USA). Anti-GSTA2, anti-GSTπ, and anti-GSTμ antibodies were supplied from Detroit R&D (Detroit, MI, USA). Horseradish peroxidase-conjugated goat anti-rabbit and rabbit anti-goat IgGs were obtained from Zymed Laboratories (San Francisco, CA, USA).

Cell culture. H4IIE rat hepatocyte cell line obtained from American Type Culture Collection (Rockville, MD, USA) was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA), 50 units/ml penicillin and 50 μg/ml streptomycin, and maintained at 37°C in humidified atmosphere with 5% CO_2. Cells (1×10^6) were plated in a 10-cm^2 plastic dish for 2~3 days (i.e. 80% confluency) and serum-starved for 24 h. The cells were incubated with 30~1000 μM metformin dissolved in sterile water, AICAR and/or *t*-BHQ dissolved in DMSO for the indicated time period.

Preparation of cell lysates. Total cell lysates were prepared according to the reported method (Park *et al.*, 2004). In brief, H4IIE cells in dishes were washed twice with ice-cold PBS, scraped from the dishes with PBS, and then transferred to microtubes. To prepare the cell lysates, the cells were centrifuged at 3000 *g* for 3 min and allowed to swell after the addition of the lysis buffer containing 10 mM Tris-HCl (pH 7.1), 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Triton X-100, 0.5% Nonidet P-40, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The samples were centrifuged at 10,000 *g* for 10 min and the recovered lysate fractions were stored at -70°C pending use.

Immunoblot analysis. SDS-polyacrylamide gel electrophoresis and immunoblot analyses were performed as described previously (Park *et al.*, 2004). Protein samples were fractionated by 7% or 12% gel electrophoresis and transferred to nitrocellulose membrane by electroblotting. The membranes were incubated with the antibodies directed against γGCS, GSTA1/2, GSTμ and GSTπ, followed by incubation with alkaline phosphatase or horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized by 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium or by an enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK). Equal loading of proteins was verified by actin immunoblotting (Santa Cruz Biotechnology). At least three separate experiments were performed with different samples. Relative changes in the protein levels were determined via scanning densitometry by using an Image Scan & Analysis System (Alpha-Innotech, San Lean-

dro, CA, USA). The area of each lane was integrated using the software AlphaEase™ version 5.5, followed by background subtraction.

pGL-1651 luciferase assay. The pGL-1651 reporter construct was generated by ligating the region -1.65 kb upstream of the transcription start site of rat *GSTA2* gene to the firefly luciferase gene coding sequence. The activity of luciferase expressed from pGL-1651 was measured described as previously (Park *et al.*, 2004). Briefly, the cells (5×10^5/well) were re-plated in six-well plates overnight, serum-starved for 6 h, and transiently transfected with 1 μg pGL-1651 and 0.3 μg pCMV-β-galactosidase (Invitrogen, Carlsbad, CA, USA) in the presence of Lipofectamine® Reagent (Life Technologies, Gaithersburg, MD) for 3 h. pCMV-β-galactosidase plasmid was used to evaluate the transfection efficiency. Transfected cells were incubated in DMEM containing 1% FBS for 3 h and exposed to metformin with or without *t*-BHQ for 18 h at 37°C. For β-galactosidase activity, 10 μg of cell lysates was added to the solution containing 0.88 mg/ml *o*-nitrophenyl-β-D-galactopyranoside, 100 μM $MgCl_2$, and 47 mM β-mercaptoethanol in 100 mM sodium phosphate buffer. The reaction mixture was incubated for 12 h at 37°C and the absorbance was determined at 420 nm. The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of β-galactosidase.

Statistical analysis. One-way analysis of variance was used to assess the significant differences among treatment groups. For each significant effect of treatment, the Newman-Keuls test was used for comparisons of multiple group means. All data are reported as means ± SD. The criterion for statistical significance was set at $p < 0.05$ or $p < 0.01$.

RESULTS

Inhibitory effect of metformin on γGCS induction by t-BHQ. A variety of stresses including ROS (e.g., NO) stimulate *de novo* GSH synthesis as part of the adaptive responses in cells. Biosynthesis of GSH is catalyzed by γGCS, a rate limiting enzyme, and glutathione synthetase. It was reported that *t*-BHQ induced γGCS in mouse Hepa1c1c7 cells (Jeyapaul and Jaiswal, 2000). We asked if the lack of increase in the GSH content by metformin involved the inhibition of γGCS induction. *t*-BHQ treatment (30 μM, 24 h) induced γGCS (catalytic heavy subunit) in H4IIE cells, which was completely abrogated by concomitant treatment with metformin or AICAR (Fig. 1). Either metformin or AICAR

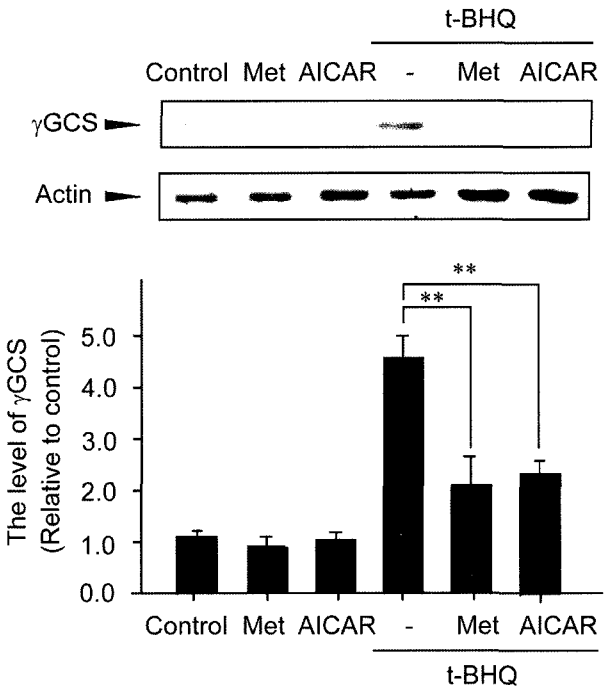

Fig. 1. The expression of γGCS. γGCS heavy chain was immunoblotted in the lysates of H4IIE cells incubated in a serum-free medium for 24 h and subsequently treated with metformin (Met, 1 mM) or AICAR (300 μM) in the presence or absence of *t*-BHQ (30 μM) for 24 h. Relative changes were assessed by scanning densitometry of the band intensities. Equal loading of proteins was verified by probing the replicate blot for actin. Data represent mean ± SD with 3 separate experiments (significant compared to *t*-BHQ, **P < 0.01, control = 1).

treatment alone unchanged the constitutive expression of γGCS. Hence, decreases in intracellular GSH by metformin under prooxidative condition might result at least in part from the lack of adaptive γGCS induction.

The inhibitory effect of metformin on the t-BHQ inducible expression of GSTA2. Glutathione *S*-transferases (GSTs) as the members of the major phase II detoxifying enzymes display broad substrate specificity and play a critical role in protecting cells from oxidative stress or electrophiles. The effect of metformin on the constitutive or *t*-BHQ-inducible expression of GSTA2, a representative GSTα form, were evaluated in this experiment. Treatment of H4IIE cells with metformin alone unchanged the constitutive GSTA2 expression (Fig. 2A). However, GSTA2 induction by *t*-BHQ (30 μM, 24 h) was repressed by simultaneous treatment of the cells with varying concentrations of metformin (Fig. 2B). Metformin at 300 μM or above completely abolished the ability of *t*-BHQ to induce GSTA2. AICAR treatment

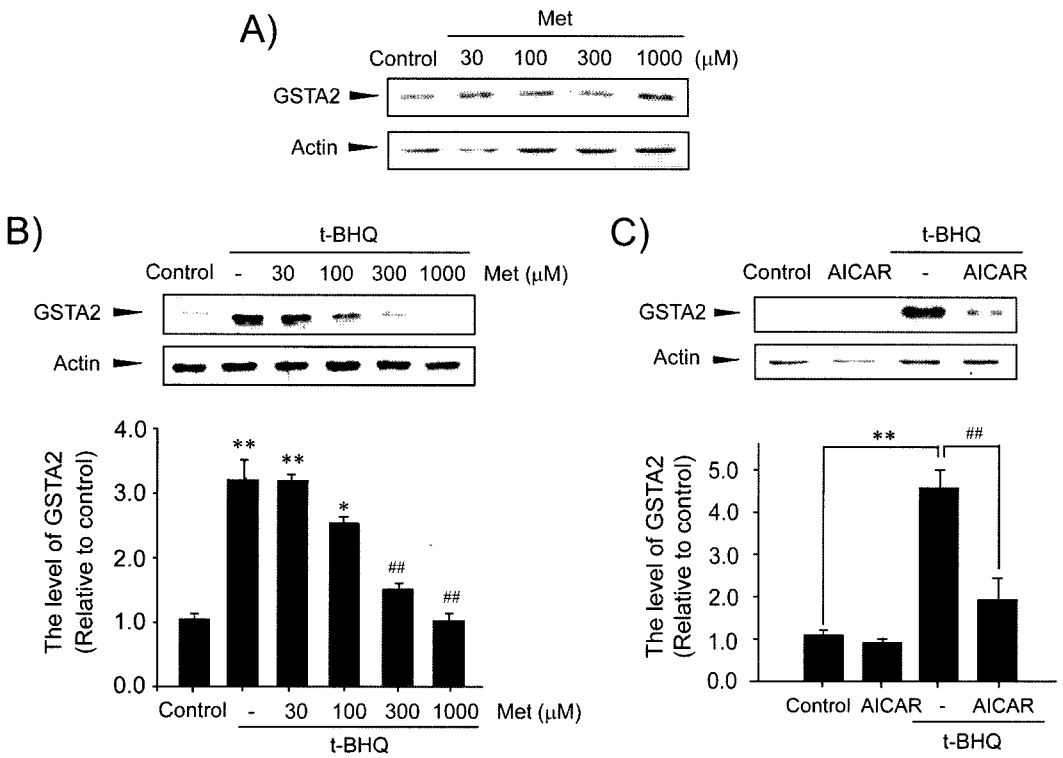

Fig. 2. The effects of metformin or AICAR on GSTA2 expression. (A) GSTA2 expression in H4IIE cells treated with metformin. The cells were treated with 30 μM~1 mM metformin (Met) for 24 h and subjected to immunoblotting for GSTA2. (B) GSTA2 expression in cells treated with metformin + *t*-BHQ. GSTA2 was immunoblotted in the lysates of cells treated with 30 μM *t*-BHQ plus varying concentrations of metformin for 24 h. (C) GSTA2 expression in cells treated with AICAR + *t*-BHQ. GSTA2 was immunoblotted in cells treated with *t*-BHQ + AICAR for 24 h. Data represent mean ± SD with 3 separate experiments (significant compared to control, *P < 0.05, **P < 0.01, significant compared to *t*-BHQ, ##P < 0.01, control = 1).

caused a similar inhibitory effect (Fig. 2C), suggesting the involvement of AMPK activation in the repression of GSTA2 by metformin.

Metformin inhibition of GSTA2 transactivation by t-BHQ. The *GSTA2* gene is a representative member of class α GSTs that contains xenobiotic response element, phenobarbital-responsive element, and glucocorticoid-responsive element. Analyses of the 5'-flanking region of the *GSTA2* gene from our and other research laboratories revealed the specific sequences defining the location of regulatory regions, namely the CCAAT/enhancer binding protein (C/EBP) response element and the antioxidant response element (ARE) (Kang *et al.*, 2001, 2003). Also, the HNF binding site was found to contribute to the maximum basal expression of the *GSTA2* gene (Paulson *et al.*, 1990), and we showed that HNF1 interacts with the HNF binding element in the promoter region of the *GSTA2* gene (Park *et al.*, 2004).

To assess whether the repression of GSTA2 by metformin or AICAR was due to transcriptional inhibition, luciferase reporter assays were performed in cells trans-

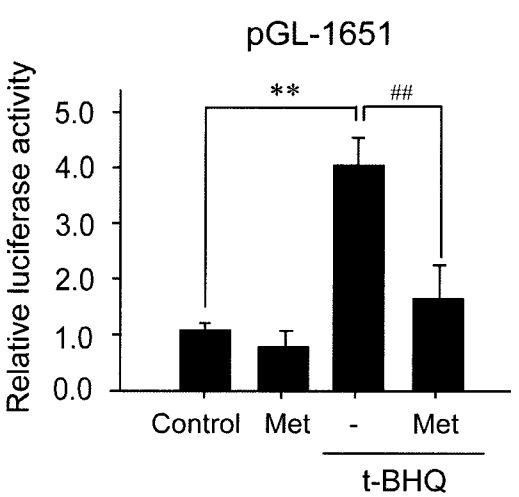

Fig. 3. pGL-1651 *GSTA2* promoter luciferase assays. The effect of metformin on *t*-BHQ-inducible luciferase expression from pGL-1651 was assessed in the lysates of cells treated with vehicle (DMSO), metformin (1 mM), *t*-BHQ (30 μM) or metformin + *t*-BHQ for 24 h. Data represent mean ±SD with 4 separate experiments (significant compared to control, **P < 0.01, significant compared to *t*-BHQ, ##P < 0.01, control = 1).

fected with pGL-1651. Consistent with the results of immunoblotting, luciferase induction from pGL-1651 by *t*-BHQ was significantly decreased by metformin treatment (Fig. 3). Our data confirmed that metformin treatment inhibited the GSTA2 gene transactivation.

Effects of metformin on the expression of other GSTs. As part of the complete studies on the effects of metformin on other GST expression, we monitored GSTμ and GSTπ forms in cells incubated with metformin in the presence or absence of *t*-BHQ. The expression of GSTμ or GSTπ form was enhanced 24 h after *t*-BHQ treatment (Fig. 4). Metformin or AICAR treatment completely abrogated the induction of the GST forms by *t*-BHQ. These results indicate that metformin prevents the induction of other GSTs by *t*-BHQ. MTT and flow cytometric assays showed that either metformin or AICAR treatment with or without *t*-BHQ did not inhibit the viability of cells, verifying that the repression of γGCS or GST by metformin at the time point examined was not due to cytotoxicity. Since we observed decreased cell viability at prolonged incubation (i.e, 48 h) with metformin in the presence of *t*-BHQ, the lack of adaptive induction of γGCS and phase II GSTs at 24 h after *t*-BHQ treatment might be associated with entering of the cells into pro-apoptotic pathway.

Taken together, these results indicate that metformin treatment abrogates adaptive transactivation of antioxidative phase II genes in H4IIE cells, which might be due to its activation of AMPK.

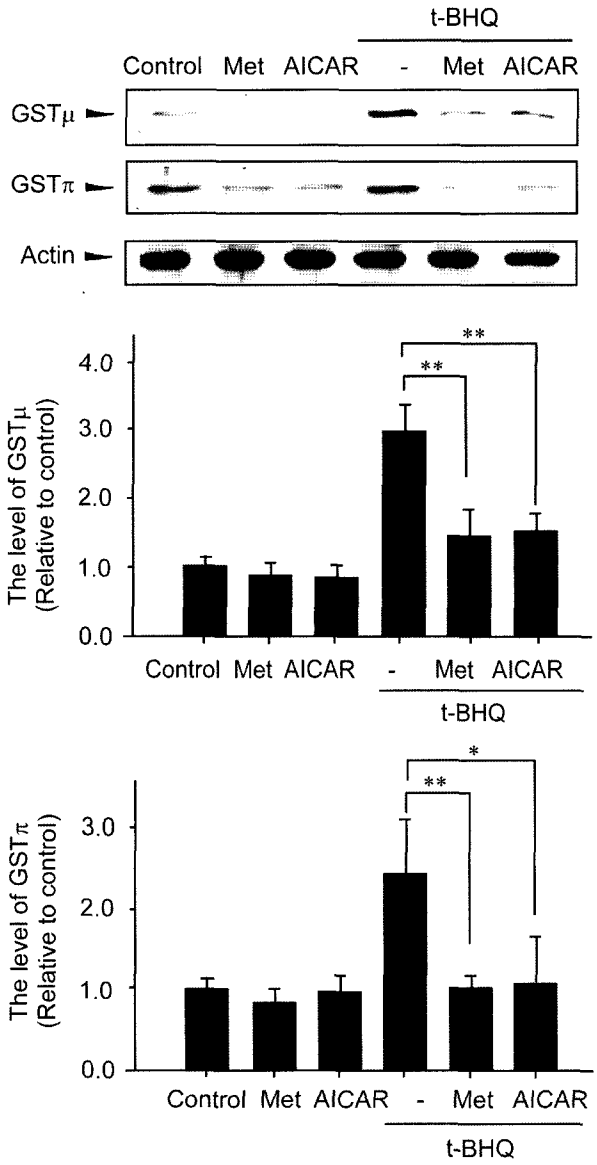

Fig. 4. The expression of GSTμ and GSTπ forms. GSTμ and π forms were immunoblotted in the lysates of cells treated, as described in the legend to Fig. 1, for 24 h. Relative levels of GSTs were assessed by scanning densitometry. Data represent mean ± SD with 3 separate experiments (significant compared to *t*-BHQ, *P < 0.05, **P < 0.01, control = 1).

DISCUSSION

Previously, we showed that treatment of H4IIE cells with metformin or AICAR impaired the adaptive increase in cellular GSH in response to *t*-BHQ although treatment of the cells with each agent alone did not alter the reduced GSH content (Bae *et al.*, 207). The results of the present study demonstrate that metformin treatment inhibits the expression of γGCS and major forms of GST enzymes. The repression of the antioxidant genes by metformin may be helpful in understanding its adverse effects. In a previous study, we found that metformin treatment increased the DCFH oxidization in H4IIE cells, which suggest that metformin at certain specified circumstances could activate AMPK and generate ROS. Furthermore, we observed for the first time that metformin abrogated the ability of prooxidant to adaptively increase the intracellular GSH content and decreasing the GSH below the level of control. GSH protects cells from oxygen free radicals (e.g., H_2O_2 and lipid hydroperoxides), and detoxifies electrophiles and xenobiotics through direct thiol conjugation or by enzyme-mediated catalytic reactions. Thus, GSH depletion in cells or animal tissues increases their susceptibilities to free radical-induced damage. A similar inhibitory effect on the GSH content by AICAR renders us to infer that AMPK activation prevents an increase in intracellular GSH, which occurs as an adaptive response to prooxidant. Hence, the prooxidative condition in combi-

nation with metformin may elicit cytotoxicity as a consequence of decrease in GSH possibly via its AMPK activation. The adverse effects of metformin observed in clinical situations may be linked with the potential cytotoxic effect of the drug. The essential role of decrease in GSH for the induction of apoptosis by metformin is further strengthened by the observation that metformin treatment enhances apoptosis of cells deficient of GSH. We found that metformin increased apoptosis of cells under oxidative stress, although metformin treatment alone did not do so at an earlier time presumably because the cells might have some remaining GSH capacity. Since metformin does not undergo hepatic metabolism (Klepser and Kelly, 1997), reactive metabolic intermediate is unlikely to be produced from the drug. Therefore, cell death induced by prolonged metformin treatment under prooxidative condition may result from sustained GSH depletion due to AMPK-mediated repression of antioxidant enzymes.

Deficiency of GSH decreases the antioxidant capacity of cells, leading to the accumulation of ROS. Hence, oxidative stress causes cells to promote transactivation of the adaptive genes, whose protein products detoxify free radicals or reactive intermediates of xenobiotics. Intracellular GSH levels are controlled primarily by biosynthesis and also by other regulatory mechanisms such as recycling (GSSG reductase), utilization (GST, GPxs and glutaredoxin), breakdown (GGT) and transport (MRPs and OATs) (Ogasawara *et al.*, 1985). This study determined the expression of γGCS as a representative sulfhydryl regulatory enzyme. We found here that metformin treatment abrogated the induction of γGCS by *t*-BHQ, which accounts at least in part for the lack of the adaptive increase in GSH in cells treated with metformin+*t*-BHQ. The role of AMPK activation in the inhibition of γGCS induction by metformin was supported by the result obtained with AICAR. Our finding that metformin activation of AMPK prevents the adaptive increase in GSH content due to γGCS repression lends support to the contention that metformin therapy may augment oxidative stress under the condition of GSH deficiency.

GSTs catalyze GSH conjugation of the reactive intermediates of xenobiotics (Bolton *et al.*, 1993). GSTs also have noncatalytic binding properties as ligandins, possessing the capability to sequester nonsubstrate drugs and hormones. GST expression is a crucial factor in determining the sensitivity of cells to a broad spectrum of toxicants. Hence, the induction of GSTs is a protective adaptive response to oxidative stress (Primiano *et al.*, 1995). In addition, GST serves as a regulatory molecule for cellular signaling pathways and may affect cell proliferation and cell cycle control, as indicated by the observations that GST inhibits formation of JNK complex and blocks mitogenic signaling induced by Ras-p21 (Villafania *et al.*, 2000). Previously, we showed that GST was induced by oxidative stresses such as sulfur amino acids deficiency and *t*-BHQ (Kang *et al.*, 2000; Kang *et al.*, 2002). Currently, we reported that either metformin or AICAR abolished the induction of major GST forms by *t*-BHQ. In addition, the result of pGL-1651 luciferase assay provided the evidence that metformin represses GST gene transcription. A question as to how metformin inhibits the transactivation of certain antioxidant genes remains to be clarified.

In conclusion, metformin inhibits the expression of a battery of antioxidant enzymes and induce apoptosis under the condition of GSH deficiency due to enhanced susceptibility to oxidative stress. This investigation holds an implication that metformin therapy may exert deleterious effects in the patients who have gone or will go through GSH deficiency.

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