Oxidative Stress Induces Phosphoenolpyruvate Carboxykinase Expression in H4IIE Cells

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Oxidative stress is closely associated with diabetes and is a major cause of insulin resistance. Impairment of hepatic insulin action is thought to be responsible for perturbations in hepatic glucose metabolism. In this study, we found that oxidative stress is involved in the dysregulation of gene expression of phosphoenolpyruvate carboxykinase (PEPCK), a key gluconeogenic enzyme, by a mechanism independent of insulin. Elevation of oxidative stress by injection of ferric nitrilotriacetate in rats increased the expression of hepatic PEPCK mRNA. To examine the direct action of oxidative stress on PEPCK expression, we treated H4IIE hepatoma cells with buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis. BSO increased intracellular oxidative stress and the expression of PEPCK mRNA. Inhibition of p38 mitogen-activated protein kinase (p38 MAP kinase), which mediates responses to oxidative stress, suppressed the induction of PEPCK mRNA by BSO. These results suggest that oxidative stress dysregulates hepatic PEPCK expression by an insulin-independent mechanism.

Key words: diabetes; oxidative stress; phosphoenolpyruvate carboxykinase (PEPCK); p38 mitogen-activated protein kinase (p38 MAP kinase); gluconeogenesis

Alterations in hepatic glucose metabolism play an important role in the development of the hyperglycemia associated with diabetic states. Increased gluconeogenesis is responsible for excess hepatic glucose output and contributes to both fasting hyperglycemia and exacerbated postprandial hyperglycemia. Studies in animal models of type 2 diabetes and obesity suggest that phosphoenolpyruvate carboxykinase (PEPCK), a rate-controlling gluconeogenic enzyme, contributes to this elevation of gluconeogenesis in that they show 2- to 3-fold increases in PEPCK mRNA levels as compared to nondiabetic animals. Also, transgenic mice that overexpress PEPCK display hyperglycemia. These animals also show insulin resistance, suggesting that elevation of blood glucose impairs insulin action.

There is considerable evidence that hyperglycemia results in the generation of reactive oxygen species (ROS), ultimately leading to increased oxidative stress. Accumulation of oxidative stress is involved not only in the development of diabetic complications but also in the progression of insulin resistance, because it interferes with insulin signaling. Oxidative stress can activate the stress-activated signaling pathways, including p38 mitogen-activated protein (MAP) kinase, which modulates factors in the insulin signaling pathway. 

In vitro studies using a variety of cell lines have demonstrated that low levels of ROS, such as hydrogen peroxide ($H_2O_2$), can be produced in the presence of elevated glucose and that micromolar concentrations of $H_2O_2$ can inhibit insulin signaling and glucose transport by activating the stress-activated pathways. Thus an increase in glucose levels raises ROS production, increases oxidative stress, and activates the stress-activated signaling pathways. These events, in turn, reduce insulin action, accelerating the progression to severe diabetic states.

The elevation of PEPCK expression under diabetic states is thought to be due in part to impairment of the suppressive effect of insulin, but it has been shown that PEPCK expression is induced by a mechanism independent of insulin action. In kidney cells, activation of p38 MAP kinase by culture in acidic media induces PEPCK mRNA expression. Like the kidney, the liver is a major gluconeogenic organ, but it is unclear whether oxidative stress has a role independent of its actions on insulin signaling in the increased expression of the hepatic PEPCK gene.

Therefore, in the current study, we investigated the possibility that oxidative stress regulates PEPCK gene expression. We found that oxidative stress induces

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Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; p38 MAP kinase, p38 mitogen-activated protein kinase; ROS, reactive oxygen species; BSO, buthionine sulfoximine; FeNTA, ferric nitrilotriacetate; TBARS, 2-thiobarbituric acid-reactive substance; DCFH-DA, dichlorodihydrofluorescein diacetate; MDA, malondialdehyde


PEPCK gene expression in two models with increases in ROS, viz., rats treated with ferric nitrotriacetate (FeNTA) and H4IE rat hepatoma cells treated with buthionine sulfoximine (BSO). In addition, the p38 MAP kinase pathway, which is activated by oxidative stress, is involved in the elevation of PEPCK mRNA in H4IE cells. These results suggest that oxidative stress is responsible for the dysregulation of hepatic glucose metabolism by a mechanism independent of effects on insulin action.

Materials and Methods

**Materials.** Dulbecco’s modified Eagle’s medium (DMEM) and SB203580 were purchased from Sigma (St. Louis, MO). Fetal bovine serum was from Equitech-Bio (Kerrville, TX). Bovine serum albumin and BSO, an inhibitor of γ-glutamylcysteine synthetase, were obtained from Wako (Osaka, Japan). MnTBAP was obtained from Alexis Biochemicals (Lausen, Switzerland). Other chemicals were of the best grade commercially available.

**Animal experimental procedures.** Five-week-old male Wistar rats, purchased from CLEA Japan (Tokyo), were individually housed in stainless steel wire cages and maintained at 22°C and 55% relative humidity under a 12-h light-dark cycle (06:00–18:00). They were allowed free access to water and a 20% casein diet according to AIN93[20] for 7 d. On day 7, they were divided randomly into two groups of six animals each, and one group of rats received an intraperitoneal injection of FeNTA at a dose of 8.4 mg Fe/kg body weight, prepared as described previously.21) The rats in the other group, designated control animals, were given saline. Rats were anesthetized with diethylether and killed 6 h after injection. The animal care protocol for this experiment was approved by the Iwate University Animal Research Committee under the Guidelines for Animal Experiments of Iwate University.

**Measurement of 2-thiobarbituric acid-reactive substance (TBARS).** Liver tissues were homogenized in 10 volumes of cold 1.15% KCl. Liver TBARS was determined according to the method of Mihara and Uchiyama.[22] The protein concentration was measured by the method of Markwell et al.[23]

**Northern blotting.** For the detection of mRNA transcripts by Northern blotting, specific cDNA probes were synthesized using the following oligonucleotides: rat PECK, 5'-TGTAGGAGCCGCGCATGAGAT-3' and 5'-CTATTTTCGTAAGGGAGGTCGG-3'; GAPDH, 5'-AC-CACGATCCATGCCATCAC-3' and 5'-TCCACCCACCCTGGTGGCTGA-3'. Total RNA was prepared by the AGPC method.[24] Twenty μg of total RNA from rat liver or 12 μg of total RNA from H4IE cells were separated on a 1.2% agarose-formaldehyde gel and transferred to a positively charged nylon membrane (Amersham Biosciences, Tokyo). After UV cross-linking, membranes were hybridized with digoxigenin-labeled cDNA probes generated by Taq DNA polymerase reaction for 12 to 16 h at 50°C in hybridization solution (5 × SSC, 50% formamide, 50 mM sodium phosphate buffer [pH 7.0], 7% sodium dodecyl sulfate [SDS], 2% blocking reagent [Roche Diagnostics, Tokyo], and 0.1% N-laurysarcosine). Membranes were washed twice 2 × SSC-0.1% SDS for 15 min at room temperature and twice with 0.1 × SSC-0.1% SDS for 15 min at 68°C. Specific hybridization was detected with an anti-digoxigenin antibody conjugated with alkaline phosphatase, and blots were developed with the CDP-star reagent (Tropix, Bedford, MA). To estimate the relative intensity of each band, X-ray films were scanned and analyzed using NIH Image.

**Cell culture.** H4IE cells (American Type Culture Collection no. CRL1600) were grown to confluence in DMEM supplemented with 10% fetal bovine serum at 37°C under an atmosphere of 5% CO2 in air in a humidified incubator. The cells were washed three times with phosphate buffered saline (PBS), and then incubated in DMEM containing 0.1% bovine serum albumin with or without various concentrations of BSO and/or test reagents for 24 h.

**Measurements of intracellular peroxides.** Oxidative activity was detected by flow-cytometric analysis using a fluorescein-labeled dye, dichlorodihydrofluorescein diacetate (DCFH-DA; Wako). The acetoxymethyl ester derivative readily permeates cell membranes and is trapped within the cell after cleavage by esterases. Oxidation by ROS converts the dye from its non-fluorescent form to a fluorescent form. In brief, H4IE cells were cultured with 500 μM BSO for 24 h and incubated with 50 μM DCFH-DA for the last 30 min. After incubation with the dye, the cells were washed with PBS, removed with trypsin, and suspended in ice-cold PBS. Intracellular peroxide levels were measured with a FACScan (Becton Dickinson, Tokyo), and the results were calculated with Kolmogorov-Smirnov Statistic module in Cell Quest software (Becton Dickinson).

**Glucose production assay.** The cells were washed three times with PBS to remove glucose and then incubated for 16 h in 1 ml of glucose production medium (glucose- and phenol red-free DMEM containing gluconeogenic substrates, 20 mM sodium lactate and 2 mM sodium pyruvate) in the presence or absence of BSO and/or test reagents in a 60 mm dish. Three hundred μl of medium was sampled for measurement of glucose concentration using a glucose assay kit (Sigma GAGO-20). The glucose concentration was normalized with cellular protein concentration measured by the method of Markwell et al.[23]
Results

FeNTA injection induces PEPCK gene expression in rat liver

We first investigated the effect of oxidative stress on PEPCK gene expression in rat liver. Iron overload with FeNTA effectively generates ROS, superoxide, and/or hydrogen peroxide through an iron-catalysed free radical reaction and induces oxidative stress in various tissues.25,26) The TBARS levels in liver, the reactive product of 2-thiobarbituric acid and malondialdehyde (MDA) caused by lipid peroxidation, increased at 6 h after injection of FeNTA (Fig. 1A). Concomitantly, the PEPCK gene was expressed higher in the liver of rats treated with FeNTA than in those treated with saline (Fig. 1B).

Intracellular peroxide levels and PEPCK expression in BSO-treated H4IIE cells

Free radicals have the potential to modify signaling molecules in the insulin signal pathway because they attack lipids, proteins, and nucleotides. Moreover, a single injection of FeNTA has been shown transiently to increase the plasma glucose and corticosteroid, and repeated injection of FeNTA can cause pancreatic damage and hepatocellular injury, degranulation from islet cells, fibrosis, and cirrhosis.26,27) Thus it is difficult to distinguish between a direct effect of oxidative stress on the expression of the PEPCK gene and an indirect one, such as impairment of insulin action or changes in hormonal factors and metabolites (e.g., insulin, corticosteroid, or blood sugar) due to pancreatic damage. Furthermore, FeNTA decreases the viability of a variety of cells because it strongly induces radicals.28) Hence we analyzed the direct role of oxidative stress in the expression of PEPCK in rat H4IIE hepatoma cells treated with BSO, which markedly decreases cellular glutathione levels and increases reactive oxygen species without any effect on cell viability.29,30)

H4IIE cells were incubated with 100 or 500 μM of BSO for 24 h and then analyzed by flow cytometry using DCFH-DA. ROS produced in cells causes the oxidation of dichlorodihydrofluorescein, yielding a fluorescent product.14) As expected, incubation with BSO did not affect the morphology or the viability of H4IIE cells (data not shown), but elevated intracellular peroxide levels in a dose-dependent manner (Fig. 2).

Treatment of the cells with BSO increased their PEPCK mRNA levels in a dose-dependent manner (Fig. 3). As shown in Fig. 4, the effect of BSO was suppressed by MnTBAP, a synthetic cell-permeable compound possessing both superoxide dismutase and catalase-like activity.31) These results indicate that oxidative stress induced by BSO enhances the expression of PEPCK.

Oxidative stress up-regulates PEPCK expression and increases glucose production through activation of the p38 MAP kinase pathway

Oxidative stress is known to activate p38 MAP kinase. In H4IIE cells, glutathione reduction by sulfur amino acid deprivation has been shown to induce oxidative stress and to activate the p38 MAP kinase pathway.32) We examined the role of the p38 MAP kinase pathway in BSO-induced PEPCK expression. When cells were incubated with BSO in the presence of
SB203580, a selective p38 MAP kinase inhibitor, BSO-induced PEPCK mRNA expression was blunted, whereas the vehicle, DMSO, had no effect (Fig. 5). Next, we investigated whether the expression of PEPCK induced by oxidative stress leads to a functional consequence, elevation of glucose production. As shown in Fig. 6, treatment with BSO increased glucose production. The elevation of glucose production by BSO was suppressed by in the presence of the inhibitor of p38 MAP kinase. Taken together, these results indicate that the p38 MAP kinase pathway is involved in the oxidative stress-induced expression of PEPCK and glucose production in H4IIE cells.

**Discussion**

Hyperglycemia is a typical feature of diabetic states and can cause the generation of ROS, which in turn activate the stress-activated signaling pathways, resulting in insulin resistance. This impairment of hepatic insulin action is a major reason for the increased expression of PEPCK. Recent studies, however, have shown that prolonged exposure of Fao hepatoma cells to hyperglycemia augments cAMP stimulation of PEPCK gene expression and that stress conditions, acidic or chemical stress, induce expression of PEPCK mRNA independent of insulin action. These studies raise
the possibility that oxidative stress increased by hyperglycemia might be involved in the dysregulation of the hepatic PEPCK gene in diabetic states by a mechanism independent of effects on insulin signaling. Hence, in this study, we used two models of increased oxidative stress, viz., the livers from rats injected with FeNTA and cultured hepatoma cells treated with BSO, to investigate the role of oxidative stress in the regulation of the liver PEPCK gene. We found that oxidative stress induces hepatic PEPCK expression and glucose production. Thus oxidative stress might contribute to the dysregulation of hepatic glucose metabolism independent of effects on insulin action.

Several lines of evidence indicate that oxidative stress contributes to regulation of the PEPCK gene. Hydroxyl radicals have been shown to enhance glucagon-dependent expression of PEPCK mRNA in rat hepatocytes.\textsuperscript{36} Davies \textit{et al.} showed that troglitazone, which is not only a ligand for peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)) but also has an \(\alpha\)-tocopherol moiety, decreases oxidative stress in rat hepatocytes and inhibits PEPCK gene expression and, furthermore, that these effects are unique to troglitazone and not shared by other ligands of PPAR\(\gamma\). Hence they concluded that troglitazone inhibits expression of the PEPCK gene by a PPAR\(\gamma\)-independent, antioxidant-related mechanism.\textsuperscript{37} But it is unclear from these studies whether oxidative stress can directly induce PEPCK expression. Here, we demonstrated that treatment of H4IIE cells with BSO increased intracellular oxidative stress and induced expression of PEPCK and glucose production. To our knowledge, this is the first report that oxidative stress induces hepatic gluconeogenesis independent of its effects on insulin action, but we cannot rule out the possibility that there are different mechanisms underlying the up-regulation of PEPCK expression between FeNTA injection and BSO treatment, because the manner of generation of ROS is different, and FeNTA injection induces radicals more rapidly and strongly than BSO treatment. Also, which radicals are responsible for the up-regulation of PEPCK is difficult to determine. Indeed, ROS induce intracellular lipid peroxidation and lipid peroxides or their end products, such as aldehydes. These products are known to activate the stress-activated pathway.\textsuperscript{38} Further studies are necessary to determine which pathways are responsible for the up-regulation of PEPCK gene expression by oxidative stress under diabetic states.

Oxidative stress activates several signaling pathways, including the p38 MAP kinase and NF-\(\kappa\)B pathways. NF-\(\kappa\)B has been shown to play a role in the development and pathogenesis of diabetes and regulates the expression of genes associated with diabetic complications.\textsuperscript{39} but NF-\(\kappa\)B is not likely to be involved in the induction of PEPCK expression upon oxidative stress, because Walter-Law \textit{et al.} demonstrated that NF-\(\kappa\)B represses PEPCK gene expression through interaction with a coactivator, CREB binding protein.\textsuperscript{40} Some reports have shown that aberrant activation of p38 MAP kinase is associated with diabetic states. In type 2 diabetic patients, the basal phosphorylation of p38 MAP kinase increases and its insulin-stimulated phosphorylation is blunted in skeletal muscle.\textsuperscript{41} Similarly, it has been shown that increased phosphorylation of p38 MAP kinase in the basal state contributes to insulin-stimulated down-regulation of Glut4 in adipocytes from type 2 diabetic patients.\textsuperscript{42} Recently, Cao \textit{et al.} showed that phosphorylation of p38 MAP kinase
and expression of PEPCK in the liver are elevated in streptozotocin-induced diabetic mice and blocked by inhibition of p38 MAP kinase. In the present study, we found that p38 MAP kinase is involved in the induction of PEPCK expression and the elevation of glucose production by oxidative stress. On the basis of these studies, we speculate that activation of p38 MAP kinase is caused by accumulation of oxidative stress associated with hyperglycemia. Moreover, activation of the p38 MAP kinase pathway has been shown to regulate several transcriptional components controlling the induction of the PEPCK gene. Activation of p38 MAP kinase leads to phosphorylation of ATF2, which might be involved in the control of the PEPCK promoter activity in hepatoma cells. The transcriptional coactivator PGC-1α (PPARγ coactivator 1α) has been shown to be important mediator of PEPCK expression, and it is activated upon phosphorylation by p38 MAP kinase. Taken together with these previous studies, our findings suggest that activation of the p38 MAP kinase pathway by oxidative stress might play an important role in the regulation of PEPCK expression in diabetes.

A number of studies have shown that antioxidants ameliorate or reverse altered physiological and metabolic parameters in diabetes. For example, vitamin E therapy prevents glucose-induced changes in physiological and metabolic parameters in diabetes. For example, vitamin E ameliorates or reverses altered physiological and metabolic parameters in diabetes. For example, vitamin E ameliorates or reverses altered physiological and metabolic parameters in diabetes.

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