Regular Article

Regulation of Soluble Epoxide Hydrolase (sEH) in Mice with Diabetes: High Glucose Suppresses sEH Expression

Ami OGURO, Natsuko FUJITA and Susumu IMAOKA*

Nanobiotechnology Research Center and Department of Bioscience, School of Science and Technology, Kwansei Gakuin University, Sanda, Japan

Summary: Soluble epoxide hydrolase (sEH) is a xenobiotic-metabolizing enzyme that metabolizes epoxides to produce vicinal diols. Diabetes is a common pathological condition which affects drug metabolism. This study, investigates changes in the levels of sEH in mice with diabetes induced by streptozotocin (STZ). Diabetes reduced the amount of sEH protein in the liver and insulin restored the level of protein. The kidneys are a target of diabetes. Diabetes significantly decreased levels of sEH protein and also mRNA. The distribution of sEH in the kidney was studied with immunostaining. There was distinct staining in the proximal tubules but not in the glomerulus or other regions. Diabetes is characterized by high glucose concentrations that lead to increased production of reactive oxygen species (ROS). High glucose suppressed sEH mRNA and protein expression in Hep3B cells. NADPH oxidase is the main source of ROS generation in high glucose condition. The NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPIC), inhibited decrease in sEH expression at high glucose and hydrogen peroxide suppressed sEH expression. These findings indicate that diabetes reduces sEH expression by inducing ROS and may have important effects on the metabolism of xenobiotics and endogenous substrates of sEH.

Keywords: soluble epoxide hydrolase; epoxyeicosatrienoic acid (EET); Cytochrome P450; diabetes; high glucose

Introduction

Soluble epoxide hydrolase (sEH) is a phase I xenobiotic-metabolizing enzyme that produces vicinal diols from epoxides.1) Epoxides are produced through the oxidation of double bonds by cytochrome P450 (P450) or through a non-enzymatic pathway including oxidation by reactive oxygen species. Aromatic hydrocarbons such as styrene and benz[a]pyrene can be oxidized to form mutagenic epoxides by P450s.2) The hydration of epoxides is a pathway of detoxification by epoxide hydrolase. The endogenous substrates for sEH are epoxyeicosatrienoic acids (EETs).3) EETs are products of arachidonic acid formed by the NADPH-dependent drug-metabolizing enzyme, cytochrome P450s (P450s).4,5) EETs were initially identified as endothelium-derived hyperpolarizing factors (EDHF) and have attracted much interest.6,7) However, EETs are more than just vasodilators, influencing a variety of biological processes including ion channel regulation, mitogenesis, and inflammation.8,9) EETs have 4 regioisomers, 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET, which are reported to have different biological functions.10) sEH metabolizes 14,15-EET most efficiently and this is thought to be the pathway for inactivation of EETs.11,12) Therefore, sEH is a therapeutic target for enhancing the beneficial functions of EETs.

Diabetes is a pathological condition which affects the metabolism of drugs by P450s. In our previous studies, diabetes induced the expression of CYP2C6, 2E1, 3A2, and 4A2 in the rat liver, and CYP2E1 and 4A2 in the rat kidney.13,14) Changes in CYP2C6, 2E1, and 3A2 expression caused by diabetes are regulated by increase in ketone bodies and growth hormones.15–18) Diabetes also af-
fects the metabolism of fatty acids which may increase CYP4A2 expression. Peroxisome proliferator receptors play important roles in lipid metabolism in diabetes. Peroxisome proliferators such as clofibrate induce CYP4A2 expression in rat liver and kidney and induce \( \omega \)-oxidation of fatty acids. Clofibrate also induces sEH expression in mouse liver but the physiological roles of sEH in the induction are unknown. Ohtoshi et al. reported polymorphisms of the sEH gene to be associated with insulin resistance in type 2 diabetic patients. However, physiological roles of sEH in diabetes are also unknown.

In this study, diabetes was induced in mice by treatment with streptozotocin (STZ) and the expression of sEH in the liver and kidney was investigated. Diabetes significantly suppressed levels of sEH protein in the liver and kidney. We found that a high concentration of glucose but not ketone bodies suppressed the expression of sEH and the decrease was due to ROS generation caused by high glucose.

**Materials and Methods**

**Materials:** Streptozotocin (STZ), insulin from bovine pancreas, penicillin (100 units/mL), streptomycin (100 \( \mu \)g/mL), cycloheximide, diphenylleuiodionium chloride (DPIC) and 2',7'-dichlorofluorescin diacetate were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum was from Biological Industries (Vilnius, Lithuania). Nitrocellulose membrane, horseradish peroxidase conjugated to goat anti-rabbit IgG, and 4-chloro-1-naphthol were obtained from Bio-Rad Laboratories (Hercules, CA). Go Taq Green Master Mix was acquired from Promega (Madison, WI). Dulbecco’s modified Eagle’s medium (DMEM high glucose), 2-oxobutyric acid and hydrogen peroxide were purchased from Wako Pure Chemicals (Tokyo, Japan).

**Induction of diabetes in mice:** Male BALB/c mice were obtained from Japan SLC (Hamamatsu, Japan). All experiments were conducted in accordance with guidelines on the welfare of experimental animals and with the approval of the Ethics Committee on the use of animals of Kwansei Gakuin University. Diabetes was induced in male BALB/c mice (7 weeks of age, 21–26 g in body weight) with an intravenous injection of STZ (150 mg/kg body weight). STZ was dissolved in 10 mM sodium citrate (pH 4.5) just before use. Control mice were injected with buffer only. Blood glucose levels of mice were measured on the 6th day after the injection of STZ with Glucose CII-test Wako (Wako). Two weeks after the injection, some diabetic mice were treated with insulin (2 units) twice a day for 3 weeks.

**Cell Culture:** The human hepatoma cell line, Hep3B, was obtained from the Cell Resource Center for Biomedical Research at the Institute of Development, Aging and Cancer of Tohoku University, Japan. Hep3B cells were cultured in DMEM containing 10% FCS, penicillin (100 units/ml), streptomycin (100 \( \mu \)g/ml) and maintained at 37°C in 5% CO\(_2\) and 95% air. Cells were cultured in media containing normal glucose (25 mM), high glucose (75 mM) and mannitol (25 mM glucose \(+ 50\) mM mannitol) and the media were renewed every 48 h. The mannitol was used as an osmotic control. Cycloheximide (10 \( \mu \)M), DPIC (0.5 \( \mu \)M) was added to cells under high glucose. Cells were treated with 2-oxobutyric acid (5 mM or 10 mM) every 24 h for 72 h or hydrogen peroxide (50 \( \mu \)M, 100 \( \mu \)M, and 500 \( \mu \)M) for 24 h.

**Isolation of cDNA and construction of plasmid:** The entire coding region of human sEH (GenBank accession no. NM001197) was isolated by PCR. sEH-coding regions comprising a 1.0 kb upstream fragment and 1.0 kb downstream fragment were amplified using each primer set. The primers for the upstream fragment were 5'-AAGGATCCCGCATGACCTGCAGCGGCG-3' (forward primer; underlined, BamHI site; double underline, start codon) and 5'-AGAAGAGAGCCATGTACCAC-3' (reverse primer; 1023–1042nd nucleotide). The primers for downstream fragment were 5'-ATCCTGTCCAGGACAAGTGA-3' (forward primer; underlined, BamHI site; double underline, start codon) and 5'-TGAATTCGGGGCACACGCGTT-3' (reverse primer; underlined, EcoRI site; stop codon is upstream of this primer). The amplified upstream fragment was digested with BamHI and Spl and the amplified downstream fragment was digested with Spl and EcoRI. These two fragments were ligated into pBluescript II cut with BamHI and EcoRI. The entire coding region of human \( \beta \)-actin (GenBank accession no. NM001197) was isolated by PCR. The primers for PCR were 5'-AAGGATCCCATGATGATGATATCGC-3' (forward primer; underlined, BamHI site; double underline, start codon) and 5'-AATCTGCAAGAAGCCATGCCTACATC-3' (reverse primer; underlined, PstI site; stop codon is upstream of this primer). The amplified full-length cDNA of \( \beta \)-actin was digested with BamHI and PstI and ligated into pBluescript II.

**Preparation of antibodies:** The antibodies against rat CYP2E1 and human NADPH-P450 reductase (fp2) were prepared with rabbits as described previously. The antibodies against human \( \beta \)-actin and human sEH were prepared in this study. The sEH cDNA in pBluescript II was digested with BamHI and SalI and subcloned into the pCold I DNA vector (Takara, Shiga, Japan). The \( \beta \)-actin cDNA in pBluescript II was subcloned into pQE80 (Qiagen, Hilden, Germany). sEH and \( \beta \)-actin proteins were expressed in E.coli (BL21 codon plus and DH5\( \alpha \), respectively). sEH was purified by preparative electrophoresis with Mini Prep Cell (Bio Rad). \( \beta \)-actin was purified with a Ni-NTA agarose column.
Antibodies against human sEH and β-actin were prepared with rabbits using a method described previously.  

**Immunoblotting:** Whole homogenate, cytosol, and microsomes of cells and tissues were subjected to immunoblotting with the antibodies against CYP2E1, fp2, sEH, and β-actin. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with the primary antibodies. Each protein was visualized using horseradish peroxidase conjugated to goat anti-rabbit IgG (the secondary antibody) and with 4-chloro-1-naphthol. Band intensity was quantified using the NIH Image program.

**Isolation of RNA and real-time PCR:** Total RNA was extracted from the tissue of mice or cells with Isogen according to the manufacturer’s instructions. After treatment with DNase, total RNA was converted to cDNA by reverse-transcription reaction as described previously.  

Real-time PCR analysis was performed with KAPA SYBR FAST qPCR Kit (KAPA BIOSYSTEMS) according to the manufacturer’s instructions. The PCR reaction was performed in a Smart Cycler II system according to the manufacturer’s instructions. The PCR with KAPA SYBR FAST qPCR Kit (KAPA BIOSYSTEMS) was amplified using the NIH Image program.

**Results**

**Expression of sEH protein in the liver of mice treated with STZ:** The administration of STZ raised the concentration of glucose in sera and the concentration remained high for 5 weeks (Fig. 1A). The administration of insulin significantly decreased the serum glucose level. As described previously, diabetes induced CYP2E1 expression in the liver (Fig. 1B). Diabetes reduced the level of sEH protein in the liver and insulin restored the level (Fig. 1C).

**Effects of diabetes on the expression and distribution of sEH in the kidney:** Diabetes is one of the main factors responsible for end-stage renal disease caused by impaired endothelium.  

EETs, substrates of sEH, have important roles in renal function such as ion transport and the proliferation of cells and the action of EETs is important for maintaining renal and vascular homeostasis.  

First, the effects of diabetes on sEH expression in mouse kidney were investigated (Fig. 2). Diabetes significantly decreased the level of protein and mRNA. Insulin restored the levels of sEH and serum glucose. The distribution of sEH in the kidney was investigated by immunostaining (Fig. 3). sEH protein was detected in proximal tubules but not the glomerulus. This is reasonable because EET-producing P450 enzymes such as CYP2C23 are expressed in proximal tubules.

**Mechanism regulating sEH:** Diabetes increases glucose and ketone bodies in sera. Ketone bodies induce the expression of P450s such as CYP2E1 in diabetes.  

The human hepatoma cell line Hep3B was cultured in media containing high glucose and the expression of sEH was investigated (Fig. 4A). The high glucose significantly suppressed sEH protein in Hep3B cells, although mannitol used as an osmotic control did not change sEH levels.  

2-Oxobutyric acid, a ketone body found in diabetic serum, did not change the level of sEH (Fig. 4B), although P450 was induced under these conditions (data not shown). sEH mRNA expression in Hep3B cells was also suppressed by high glucose and 2-oxobutyric acid did not change sEH mRNA (Fig. 4C). Cycloheximide inhibited decrease in sEH, suggesting protein synthesis to be required for the regulation of sEH (Fig. 4D).

**sEH regulation mediated by ROS generation under high glucose condition:** Hyperglycemia is known to increase the generation of ROS and it is one of
Fig. 1. sEH expression in the liver of mice with diabetes induced by STZ
Mice were treated with STZ. Treatment with insulin started in the 2nd week. Mice treated with STZ and STZ + insulin were sacrificed in the 5th week. (A) Blood glucose levels in diabetic mice. (B) CYP2E1 levels in diabetic mice. Microsomal fractions of liver (25 µg of protein for CYP2E1 and 13 µg for fp2 used as a microsomal marker protein) were analyzed by SDS-PAGE with a 10% polyacrylamide gel. The ratio (CYP2E1/fp2) for control mice was set at 1.0. (C) sEH expression in the liver of diabetic mice. The cytosolic fraction (15 µg of protein for sEH and 20 µg for β-actin) was analyzed by SDS-PAGE with a 10% polyacrylamide gel. STZ: streptozotocin Values are given as the mean ± S.D. of 5 or 6 sample preparations.
*significantly different from control or STZ treatment, p < 0.05. **significantly different from control or STZ treatment, p < 0.01.

Fig. 2. Expression of sEH in the kidney of diabetic mice
(A) Expression of sEH protein in kidney of diabetic mice. The cytosolic fraction (20 µg of protein for sEH and 20 µg for β-actin) was analyzed by SDS-PAGE with a 10% polyacrylamide gel. The ratio (sEH/β-actin protein) for control mice was set at 1.0. (B) Expression of sEH mRNA in the kidneys of diabetic mice. Total RNA was isolated from the kidney and real-time PCR was done. The ratio (sEH/histone mRNA) for control mice was set at 1.0. STZ: streptozotocin Values are given as means ± S.D. for 5 or 6 sample preparations.
*significantly different from control or STZ treatment, p < 0.05. **significantly different from control or STZ treatment, p < 0.01.

The activation of NADPH oxidase has been reported as a main source of ROS generation by high glucose. ROS was detected by 2',7'-dichlorofluorescin diacetate in Hep3B cells cultured in high glucose conditions (Fig. 5A). Fluorescence intensity increased by high glucose and reduced by DPIC, NADPH oxidase inhibitor. Under the same conditions, changes in the sEH protein levels were investigated (Fig. 5B). DPIC inhibited decrease in sEH expression under high glucose. The addition of hydrogen peroxide to Hep3B cells suppressed sEH expression in dose-dependent manner (Fig. 5C), suggesting increase in ROS generation caused by high glucose to suppress sEH expression.

Discussion
Physiological and pathophysiological conditions affect the activity of xenobiotic-metabolizing enzymes, P450s and phase II enzymes. These effects may cause unfavorable drug-drug interactions and side effects of ther-
Fig. 3. Distribution of sEH in mouse kidney
Sections of kidney from control mice were immunostained with anti-sEH antibody or normal serum. The sections were counterstained with hematoxylin.

Fig. 4. Effects of high levels of glucose or ketone bodies on sEH expression in cells
(A) Effects of high glucose on sEH expression in Hep3B. Cells were cultured in media containing 25 mM glucose (normal glucose), 75 mM glucose (high glucose) or 25 mM glucose + 50 mM mannitol for 72 h. Whole cell lysate (30 μg of protein for sEH and 2.5 μg of protein for β-actin) was analyzed by SDS-polyacrylamide gel electrophoresis with a 10% acrylamide gel. (B) Effects of ketone bodies on sEH expression. Hep3B cells were treated with 5 mM or 10 mM 2-oxobutyric acid (2-OA) every day for 72 h, and sEH protein levels were analyzed by western blotting. (C) sEH mRNA levels under high glucose or in the presence of 2-oxobutyric acid were analyzed by real-time PCR. (D) Western blot analysis of Hep3B cells treated with 10 μM CHX at a normal or high concentration of glucose. CHX: cycloheximide The ratio (sEH/β-actin protein or sEH/histone mRNA) for normal glucose was set at 1.0. Values are given as means ± S.D. for three separate experiments. *significantly different from normal glucose, p < 0.05.

Ami OGURO, et al.

apeutic drugs and also change physiologically important substances produced by these enzymes. Diabetes is a worldwide concern as a risk factor for metabolic syndromes and changes in drug metabolism in the liver and kidney.33) Diabetes induces CYP2E1 and CYP4A2 expression and reduces CYP2C11 expression by altering levels of insulin and growth hormones and increases levels of glucose, ketone bodies, and free fatty acids in serum.13) Ketone bodies stabilize CYP2E1 mRNA expression leading to an increase in CYP2E1 protein in diabetes and fatty acids induce CYP4A2 expression via the peroxisome proliferator activated receptor (PPAR) alpha.
CYP2C11 is regulated by insulin or growth hormone.\(^{13,35}\) In this study, we found that glucose suppresses sEH expression at both mRNA and protein levels and is required for the inducement of a new protein because cycloheximide inhibited the decrease. CYP4A2 expression is induced by peroxisome-proliferators such as clofibrate, which also induce sEH expression.\(^{36}\) However, diabetes suppressed sEH expression.

sEH metabolizes xenobiotic compounds such as styrene oxide and endogenous compounds such as EETs and leukotoxin.\(^1\) EETs are vasodilators and candidates for EDHF.\(^6,7\) In the spontaneously hypertensive rat (SHR), sEH was highly expressed and the inhibition of sEH activity efficiently decreased blood pressure.\(^{33}\) Furthermore, male mice lacking the sEH gene had lower systolic blood pressure.\(^{38}\) These results are consistent with EETs being vasodilators that contribute to the regulation of blood pressure.

The kidneys are a target organ of diabetes. Therefore, we focused on sEH in mouse kidney. Mouse kidney segments were immunostained with anti-sEH antibody. There was distinct staining in proximal tubules but not in the glomerulus or other regions. These stained areas matched the renal distribution of EET formation and P450 which forms EET.\(^{27,28,39}\)

High glucose suppressed sEH mRNA and protein expression in Hep3B cells but these may be indirect effects because cycloheximide inhibited them and treatment with high glucose for 48 h was not enough to decrease sEH (data not shown). Diabetes is characterized by high glucose concentrations that lead to increased production of reactive oxygen species via several mechanisms including autoxidation of glucose, the polyol pathway and activation of NADPH oxidase.\(^40\) We showed the increase in ROS generation by high glucose in Hep3B cells. Reactive oxygen species are related to the activation of transcription factors such as protein kinase C, NF-kappaB, and AP-1.\(^{31}\) Inhibition of NADPH oxidase inhibited the decrease in sEH by high glucose and the addition of hydrogen peroxide to Hep3B cells suppressed the expression of sEH. Increase in ROS generation by high glucose would thus appear to suppress sEH expression. sEH may be regulated by reactive oxygen species via the transcription factors described above. Further study is necessary to elucidate the regulatory mechanism for sEH.
Acknowledgments: This study was supported in part by a Grant-in-aid for Scientific Research (c) and a special Grant-in-aid of the Advanced Program of High Profile Research for Academia-Industry Cooperation, sponsored by the Ministry of Education, Science, Culture, Sports and Technology of Japan. Support was also provided by a Grant-in-aid from Kwansei Gakuin University.

References


29) Kraner, J. C., Lasker, J. M., Corcoran, G. B., Ray, S. D. and Rau-
Regulation of sEH in Diabetes


