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DNA methylation of the promoter of soluble epoxide hydrolase silences its expression by an SP-1-dependent mechanism

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ABSTRACT

Epoxyeicosatrienoic acids, derived from arachidonic acid, function as antihypertensive and antihypertrophic mediators in the cardiovascular system. They are hydrolyzed by soluble epoxide hydrolase (sEH). Pharmacological inhibition of sEH increases the level of epoxyeicosatrienoic acids, which may have a cardiovascular protective effect. However, the regulation and function of sEH in cancer are largely unknown. The present study investigated whether DNA methylation regulates the expression of sEH in carcinoma HepG2 cells. The mRNA and protein expressions of sEH in HepG2 cells were lower than those in transformed human embryonic kidney cells and in primary cultured human endothelial cells. Bioinformatic analysis revealed a putative CpG island and 5 SP-1 binding sites located in the promoter region of the sEH gene. Furthermore, the sEH expression was significantly enhanced by demethylation treatment with 5-Aza-CdR, a DNA methyltransferase inhibitor, and the sEH promoter was transformed from hypermethylation to hypomethylation as detected by methylation-specific PCR and bisulfite sequencing. Transient transfection assays showed that the activity of the human sEH promoter was increased in HepG2 cells in response to 5-Aza-CdR. Five SP-1 binding sites in the promoter region responding to treatment with 5-Aza-CdR were identified by construct deletion and mutation analysis and chromatin immunoprecipitation assay. Interestingly, adenoviral overexpression of sEH in HepG2 cells decreased cell proliferation. Thus, SP-1 is involved in the decrease in the transcription of sEH as a result of DNA methylation in HepG2 cells, which might contribute to epigenetic mechanism-induced carcinogenesis in hepatocytes.

1. Introduction

Soluble epoxide hydrolase (sEH, EC 3.3.2.3), a member of the α/β-hydrolase family, is a multifunctional enzyme with an N-terminal phosphatase activity in addition to a C-terminal hydrolase activity [1,2]. In humans, the activity and expression of sEH are highest in the liver and kidney but also occur in other organs and tissues, including the lung, heart, and gut [3,4]. In the metabolism of epoxy fatty acids, sEH can enzymatically hydrolyze epoxyeicosatrienoic acids (EETs) to dihydroxyeicosatrienoic acid derivatives under physiological conditions [5]. EETs are important signaling molecules derived from arachidonic acid through metabolism by cytochrome P450s (including CYP2C8, CYP2C9, and/or CYP2J2 in humans); they are involved in cell signaling, proliferation and apoptosis [6,7]. Thus, inhibitors of sEH have been shown to be potent anti-inflammatory compounds and can lower blood pressure [8,9], so sEH is a potential therapeutic target in the management of hypertension and inflammation [10].

Interestingly, the altered distribution of sEH expression was detected in several human malignant neoplasms. The sEH expression was decreased or lost in malignant liver neoplastic and surrounding margin tissues but showed a high expression in non-neoplastic liver tissue samples [11]. Similar results were found in renal, colon, and prostate cell carcinomas. Conversely, testicular seminoma samples showed a marked increase in the levels of sEH but a low expression in normal tissues [12]. The activity and expression of sEH were also lower in certain human carcinoma cell lines, including HepG2, ACHN, SN12C, Caco-2, DU145, and HeLa cells, than in normal cells (293T cells) [13–19]. The loss of sEH in specific tissues and cell lines could contribute to carcinogenesis through the loss of hydrolase or phosphatase activity. The lack of C-terminal epoxide hydrolase activity may result in accumulation of EETs, which were recently suggested to promote a neoplastic phenotype in carcinoma cells [20] and stimulate angiogenesis [21]. In addition, products of the N-terminal phosphatase activity such as farnesol and geranylgeraniol were previously described as anti-carcinogenic perhaps by inducing apoptosis [22–24]. Hence, loss of sEH expression may result in disequilibrium of cancer-promoting EETs and "anti-carcinogenic"

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isoprenols with a net effect in favor of carcinogenesis. Similarly, downregulation of sEH expression could represent a mechanism to promote wound repair and tissue regeneration.

In the past decade, epigenetic regulation has been frequently found in tumorigenesis. The hypermethylation of CpG islands in gene promoters is associated with the silencing of tumor-suppressor genes and tumor-related genes by subsequent downregulation of mRNA expression. Epigenetic-silenced genes are involved in important molecular pathways of carcinogenesis such as cell cycle regulation, apoptosis, DNA repair, or cell adhesion. Aberrant methylation of several tumor-suppressor and tumor-related genes such as P16, COX-2, RASSF1A, hMLH1, and SOCS1 is frequently observed in hepatocellular carcinomas [19].

Our previous study identified a GC-rich region (−374 and +28 nt), not typical TATA or CAAT box motifs, in the 5′-flanking region of the sEH gene. Furthermore, this GC-rich region was found to contain the minimal essential sequence for the basic transcriptional regulation of sEH [25]. However, the underlying mechanism of sEH regulation and its role in human malignant neoplasms are not clear. Here, we confirmed the decrease in the sEH level in HepG2 cells. The hypermethylation status of sEH was identified, and an SP-1 transcription factor was found to induce sEH expression through demethylation. Furthermore, overexpression of sEH by a recombinant adenovirus in HepG2 cells could inhibit cell growth and induce cell cycle arrest. Thus, DNA methylation could be an important epigenetic mechanism leading to the decrease in sEH level in hepatocellular carcinoma cells, which might contribute to cancer cell growth.

2. Materials and methods

2.1. Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were isolated and maintained as described [26]. All experiments were performed with HUVECs up to passage 3 and cultured to confluence before treatment. Human liver carcinoma HepG2 and human embryonic kidney (HEK) 293 cell lines were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were cultured for 24 h and then treated with different concentrations of the DNA methyl transferase inhibitor 5-aza-2′ deoxycytidine (5-Aza-CdR, Sigma) or a specific sEH inhibitor 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea (TUPS) [27] for various times.

2.2. Western blot analysis

Cell lysates were resolved by 10% SDS–PAGE and transferred to a PVDF membrane. The sEH protein was detected by the use of a polyclonal anti-sEH antibody (Santa Cruz Biotechnology, Santa Cruz, CA) then horseradish peroxidase-conjugated secondary antibody. The level of β-actin protein was also measured as an internal control. The protein bands were visualized by the Enhanced Chemiluminescence (ECL) detection system (Amersham, Arlington Heights, IL), and the densities of the bands were quantified and normalized to that of β-actin by the use of the Scion Image software (Scion Corp., Frederick, MD).

2.3. Quantitative real-time RT–PCR

Total RNA was isolated from cells with TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA (1 μg) was converted to cDNA by the use of the SuperScript II Reverse Transcriptase (Invitrogen). Quantitative RT–PCR involved the use of the Mx3000P™ Real-Time PCR System (Stratagene, California, USA). Briefly, the PCR conditions were 40 cycles at 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec. Quantitative RT–PCR of cDNA with the Brilliant SYBR Green QPCR system involved β-actin as an internal control. The nucleotide sequences of the primers are in Table 1.

2.4. Site-directed mutagenesis, transfection, and luciferase assay

The plasmids of the human sEH promoter sEH-1091-Luc [28] and deletion constructs were used for transient transfection. Primers containing the mutation of the SP-1 binding site (shown in Table 1) contained 3 mutations (bold) in the GC box consensus sequence. Mutagenesis involved the use of the TaKaRa MutanBEST Kit (TaKaRa, Biotechnology Co., China) according to the manufacturer's protocol, with the deletion of plasmids used as the template for each PCR amplification and the resulting mutants confirmed by sequencing.

HepG2 cells were seeded into 24-well tissue-culture plates (Falcon) at 5×10^3 cells/well; the plasmid DNA was transfected by the use of the jetPEI method (Polyplus, San Marcos, CA). CMV–β-gal was co-transfected as a transfection control. After 5-Aza-CdR treatment, cells were lysed, and the cell lysates were collected for the luciferase activity assay (Luciferase Reporter Assay System, Promega).

2.5. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described [29]. In brief, HepG2 cells were treated with or without 5-Aza-CdR (4 μmol/L) for 72 h. Cells were cross-linked, sonicated, and then underwent immunoprecipitation (IP) with polyclonal anti-SP-1 (Santa Cruz Biotechnology). Normal IgG was used as an IP control, and the supernatant was an input control. After digestion with proteinase K, the resulting DNA

Table 1
Complete list of primers used in this work. Sequences are from the GenBank, their numbers were bate-actin NM_001101.2, sEH NM_001979, CYP 450 2J2 NM_000775.2, and CYP450 2C9 NM_000771.3. The bold and shadow fonts were mutations in the SP1 banding sites among the sEH promoter sequence.

Assay	Name	Primer oligonucleotide (5′–3′)	Products (bp)
RT–PCR	sEH	F: CTTCATACCAGCAAATCCC R: CCTCCCGCTTCACAGAC	198
	CYP 450 2J2	F: CGCCCAAAGAACTACCCG R: CTGCTCGAAGTCCACAAGG	75
	CYP 450 2C8	F: CTGCAATAATTTCCCTCTACTC R: TCTCCCTAATGTAACTTCGTGT	95
	CYP 450 2C9	F: TCCCTGACTTCTGTGCTACAT R: AAATTGCCACCTTCATCCA	89
	β-actin	F: TTAGTTGCGTTACACCCTTTC R: GCTGTCACCTTCACCGTTC	156
MSP	sEH methylation	F: TATTTTTTAGGTTAGTTGCGTGTTC R: ACCAAATCTTCAATTTCATAATTCG	290
	sEH unmethylation	F: TTTTTTAGGTTAGTTGTGTGTTTGG R: CAAATCTTCAATTTCATAATTCACA	286
BGS	sEH promoter	F: GGAGGGGGTATAAGAGATTTTGGATTTTT R: CTTCAAAACCAAATCTTCAATTTCATAATT	581
Mutation	pGL3-SP-1 no. 1	F: GGGGAGGAGGC**AAA**CCCAGGGC R: GGTACCTATCGATAGAGAAATGTTC	4916
	pGL3-SP-1 no. 2	F: CCCGGGCAGAGG**AAA**GAGTCCC R: GGTACCTATCGATAGAGAAATGTTC	4951
	pGL3-SP-1 no. 3	F: TCTTTC**AAA**CCAGAGTCCAGCC R: GGTACCTATCGATAGAGAAATGTTC	4978
	pGL3-SP-1 no. 4	F: GGTCC**AAA**CCTTCCCGGCCTCC R: GGTACCTATCGATAGAGAAATGTTC	5045
	pGL3-SP-1 no. 5	F: TCCAGGAAGGC**AAA**GGCCTGGG R: GGTACCTATCGATAGAGAAATGTTC	5179
ChIP	sEH promoter	F: GAGCTGTCAGTCCGTCCAGG R: GGAAAGGGAGTCAGAGGGAGA	197
	Negative control	F: GGAAGGACTCCCAGAA R: TCCCAGCCAAACCA	101