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Metabolomic analysis revealed the role of DNA methylation in the balance of arachidonic acid metabolism and endothelial activation

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ABSTRACT

Arachidonic acid (AA) metabolism plays an important role in vascular homeostasis. We reported that DNA hypomethylation of EPHX2 induced a pro-inflammatory response in vascular endothelial cells (ECs). However, the change in the whole AA metabolism by DNA methylation is still unknown. Using a metabolomic approach, we investigated the effect of DNA methylation on the balance of AA metabolism and the underlying mechanism. ECs were treated with a DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-AZA), and AA metabolic profiles were analyzed. Levels of prostaglandin D2 (PGD2) and thromboxane B2 (TXB2), metabolites in the cyclooxygenase (COX) pathway, were significantly increased by 5-AZA treatment in ECs resulting from the induction of PGD2 synthase (PTGDS) and thromboxane A synthase 1 (TBXAS1) expression by DNA hypomethylation. This phenomenon was also observed in liver and kidney cell lines, indicating a universal mechanism. Pathophysiologically, homocysteine, known to cause DNA demethylation, induced a similar pattern of the change of AA metabolism. Furthermore, 5-AZA activated ECs, as evidenced by the upregulation of adhesion molecules. Indomethacin, a COX inhibitor, reversed the effects of 5-AZA on the levels of PGD2 and TXB2, EC activation and monocyte adhesion. *In vivo*, the plasma levels of PGD2 and TXB2 and the expression of *In vivo* PTGDS and TBXAS1 as well as adhesion molecules were increased in the aorta of the mice injected with 5-AZA. In conclusion, using a metabolomic approach, our study uncovered that DNA demethylation increased AA metabolites PGD2 and TXB2 by upregulating the expression of the corresponding enzymes, which might contribute to the DNA hypomethylation-induced endothelial activation.

1. Introduction

Accumulating evidence has strongly suggested that arachidonic acid (AA) metabolism plays an important role in cardiovascular diseases. AA, catalyzed by phospholipase A2 from membrane phospholipids, can be metabolized to hundreds of metabolites by three pathways: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP). Hundreds of biological active metabolites of AA are related to the progression of diseases. Prostaglandins (PGs) and thromboxanes (TXs) are crucial bioactive molecules derived from the COX pathway and subsequent PG synthase that are implicated in inflammation, fever and pain. Hydroxyeicosatetraenoic acids (HETEs), metabolites in the LOX pathway, have been implicated in numerous biological processes, such as angiogenesis, platelet activation and asthma. CYP enzymes metabolize AA to multiple products including epoxyeicosatrienoic acids (EETs) and ω-hydrolased 20-HETE. EETs act as anti-hypertensive and anti-atherosclerotic mediators for vasculature and are degraded by soluble epoxide hydrolase (sEH), whereas 20-HETE is a pro-inflammatory and pro-fibrotic eicosanoid. Considering that various metabolites of AA play different roles in cardiovascular diseases [1], the metabolic profiling of AA during important physical and pathophysiological processes is needed.

Gene expression is regulated epigenetically by histone modification, DNA methylation, and microRNAs. The mechanism of DNA methylation is well known: the cytosine of CpG dinucleotide adds a methyl to form 5-methylcytosine, which results in changed expression. DNA methylation is an essential component of normal development and transcriptional regulation, while aberrant patterns of DNA methylation are associated with a number of pathological conditions and diseases,

Abbreviations: AA, arachidonic acid; COX, cyclooxygenases; LOX, lipoxygenases; CYP, cytochrome P450; EETs, epoxyeicosatrienoic acids; HETEs, hydroxyeicosatetraenoic acids; PGs, prostaglandins; PGD2, prostaglandin D2; PTGDS, prostaglandin D2 synthase; TXB2, thromboxane B2; TBXAS1, thromboxane A synthase 1; sEH, soluble epoxide hydrolase; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; ECs, endothelial cells; HUVECs, human umbilical vein endothelial cells; Hcy, homocysteine; VSMCs, vascular smooth muscle cells; 5-AZA, 5-aza-2′-deoxycytidine; IND, indomethacin; MSP, methylation-specific PCR; BSP, bisulfite sequencing PCR.

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such as tumor formation, inflammation and cardiovascular diseases [2–7]. Since hypomethylated extracellular superoxide dismutase was found associated with atherosclerosis in 1999 [8], DNA methylation has been implicated as a novel risk factor of cardiovascular disease [9]. In view of the recent findings of global or promoter-aberrant DNA methylation in atherosclerosis [10] and the use of DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-AZA), an FDA-approved drug, reducing atherosclerotic lesion formation and endothelial activation [11], DNA methylation has been closely associated with the development of atherosclerosis. A genome-wide analysis revealed the hypomethylation of chromosomal DNA predominates in atherosclerotic plaques, and two-thirds of genes were up-regulated in comparison to healthy mammary arteries [3]. It was also reported that the atherosclerotic aortas had a tendency for decreased 5-methylcytosine content in atherosclerotic aortas [8]. Further, DNA methylation polymorphisms preceded any histological sign of atherosclerosis in ApoE knockout mice [12]. Our previous work showed that homocysteine (Hcy) upregulated platelet-derived growth factor level via DNA demethylation in endothelial cells (ECs), which affected cross-talk between ECs and vascular smooth muscle cells (VSMCs) and led to VSMC dysfunction [13]. Moreover, DNA demethylation may coordinately contribute to Hcy-induced sEH upregulation and EC activation [14], which implies that DNA methylation associated with AA metabolism. However, whether other enzymes involved in AA metabolism can be methylated, which may result in changed AA metabolic profile and in turn, endothelial dysfunction is unknown. A highly specific approach to determine the AA metabolic profile could be valuable.

As a core analytical technique of metabolomics and lipidomics, mass spectrometry (MS) combined with liquid chromatography (LC–MS) is used to detect biological metabolites at the parts-per-billion (ppb) level in a "global" or "targeted" manner. LC–MS/MS-based targeted metabolomics is a powerful and accurate tool to explore metabolic profiling under aberrant DNA methylation conditions in certain diseases [15]. Aebersold and colleagues revealed the metabolism of AA with a genomics–proteomics–metabolomics approach but did not focus on the balance in the three pathways nor examine the metabolic change related to the epigenetic regulation of genes [16].

In the present study, we used an LC–MS/MS-based metabolomic approach and data analysis to investigate the AA metabolic profile with 5-AZA treatment to elucidate the effect of DNA methylation on the balance of AA metabolism *in vitro* and *in vivo*. Certain AA metabolism-related enzymes, such as prostaglandin D2 synthase (PTGDS) and thromboxane A synthase 1 (TBXAS1), were epigenetically regulated by DNA demethylation, which suppressed gene silencing. Accordingly, levels of their corresponding products, PGD2 and TXB2, were increased in parallel with the upregulation of vascular adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) in ECs. This work shows the crosstalk between DNA methylation and AA metabolism *in vitro* and *in vivo* and reveals aberrant AA metabolism induced by DNA demethylation involved in EC dysfunction.

2. Materials and methods

2.1. Chemicals and reagents

Butylated hydroxytoluene (BHT), 5-AZA, acetic acid and indomethacin (IND) were purchased from Sigma Aldrich Inc. (St. Louis, MO); pGEM-T easy vector was purchased from Promega (Madison, WI); 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl (BCECF-AM) was from Invitrogen (Carlsbad, CA). Antibodies against PTGDS and TBXAS1 were purchased from Abcam (Cambridge, UK). Mobile-phase acetonitrile (LC–MS grade), methanol and n-hexane (HPLC grade) were from Merck (Darmstadt, Germany). Ethyl acetate, formic acid, and glacial acetic acid were from Fisher Scientific (Pittsburgh, PA). Oasis HLB 10 mg SPE cartridges were from Waters Co. (Milford, MA). Centrifuge tube filters were from Corning Co. (Corning, NY). All other chemical reagents were from Sigma.

2.2. Cell culture

Human umbilical vein ECs (HUVECs) were isolated and maintained as described [17]. Experiments were performed with HUVECs between 4 and 6 passages. Human liver carcinoma HepG2 and human embryonic kidney 293T (HEK293T) cell lines were cultured as described [18]. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. For experiments, subconfluent ECs were treated with the DNA methyltransferase inhibitor 5-AZA or COX inhibitor IND as indicated.

2.3. Metabolomic analysis

The method of metabolite detection in cell lysates, cultured medium or plasma and tissues from mice was described in our previous work [19]. The tissues of the aorta, liver or kidney were homogenized before lipid extraction. HUVECs were lysed by repeated freeze–thawing cycles and then pre-processed in the solvent of methanol. After centrifugation, the supernatant was extracted by ethyl acetate twice, and then the upper organic phase was evaporated. The residue was dissolved in 100 μL 30% acetonitrile. The subsequent procedure involved ultra-high-performance liquid chromatography (Waters, Milford, MA) with a 5500 QTRAP hybrid triple-quadruple linear ion trap mass spectrometer (AB Sciex, Foster City, CA) equipped with a Turbo Ion Spray electrospray ionization source (LC–MS/MS) as described [19].

2.4. Quantitative RT-PCR (qPCR)

Total RNA was collected from cells or tissues in QIAzol and purified by the use of the QIAGEN miRNeasy Mini Kit. Total RNA was reverse transcribed into cDNA with SuperScript III and random primers (Invitrogen), as described previously [18]. qPCR for specific genes involved the Brilliant II SYBR Green qPCR Master Mix (Stratagene) with custom-designed primers and the ABI 7900HT Real-Time PCR System (Life Technologies, CA). Results were normalized to β-actin. Primer sequences are in Supplemental Table S1.

2.5. Western blot analysis

The western blot was performed as described previously [18]. Cell lysates were resolved by 12% SDS-PAGE and then transferred to a PVDF membrane. The membrane was incubated with primary antibodies then horseradish peroxidase-conjugated secondary antibody. The level of β-actin was also measured as an internal control. 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).

2.6. Animal experiments

C57BL/6 male mice, 8 weeks old, fed standard laboratory chow and tap water ad libitum and bred in a 12-h light/dark cycle, were divided into 2 groups for treatment (control group, n = 13; 5-AZA group, n = 15). The mice were injected with either phosphate buffered saline (PBS) or 5-AZA (1 mg/kg/d) intraperitoneally for 5 days and then anesthetized with pentobarbital sodium and sacrificed. The blood plasma, liver, kidney and aorta were collected. The investigation conformed to the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (NIH Publication No. 85-23, updated 2011). The animal experimental protocol was approved by the Tianjin Medical University Institutional Animal Care and Use Committee.