Ligands of Peroxisome Proliferator-activated Receptor Inhibit Homocysteine-induced DNA Methylation of Inducible Nitric Oxide Synthase Gene

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Abstract Homocysteine (Hcy) is a risk factor for atherosclerosis. It is generally accepted that inducible nitric oxide synthase (iNOS) is a key enzyme in the regulation of vascular disease. The aim of the present study is to investigate the effects of peroxisome proliferator-activated receptor ligands on iNOS in the presence of Hcy in human monocytes. Foam cells, induced by oxidize low density lipoprotein (ox-LDL) and phorbol myristate acetate (PMA) in the presence of different concentrations of Hcy, clofibrate and pioglitazone in human monocytes for 4 d, were examined by oil red O staining. The activity of iNOS was detected by real-time quantitative reverse transcription-polymerase chain reaction and Western blot analysis. The capability of DNA methylation was measured by assaying endogenous C5 DNA methyltransferase (C5MTase) activity, and the iNOS promoter methylation level was determined by quantitative MethyLight assays. The results indicated that Hcy increased the activity of C5MTase and the level of iNOS gene DNA methylation, resulting in a decrease of iNOS expression. Clofibrate and pioglitazone could antagonize the Hcy effect on iNOS expression through DNA methylation, resulting in attenuation of iNOS transcription. These findings suggested that Hcy decreased the expression of iNOS by elevating iNOS DNA methylation levels, which can repress the transcription of some genes. Peroxisome proliferator-activated receptor α/γ ligands can down-regulate iNOS DNA methylation, and could be useful for preventing Hcy-induced atherosclerosis by repressing iNOS expression.

Key words homocysteine; DNA methyltransferase; PPARα/γ ligand; iNOS DNA methylation

Homocysteine (Hcy) is a sulfur-containing amino acid form during the metabolism of methionine. Hyperhomocysteine (HHcy) is found in 30% of patients with premature atherosclerosis of carotid and peripheral arteries [1]. Elevated plasma Hcy levels have been implicated as an independent risk factor for coronary heart diseases [2]. Evidences have also indicated that inducible nitric oxide synthase (iNOS) is the key mechanism in the regulation of vascular diseases. However, strong associations among HHcy, iNOS and atherosclerosis have been observed in many studies [3].

The precise mechanisms of Hcy-induced atherosclerosis and iNOS anti-atherosclerosis are still to be fully investigated. Much attention has been focused on the impacts of HHcy on dysfunction and injury of vascular cells. Increasing evidence, however, indicates that HHcy might also be involved in the regulation of disturbing the expression of atherosclerosis-related genes through the interference of DNA methylation [4,5].

DNA methylation is a form of epigenetic gene regulation that leads to the suppression of gene expression when occurring in a regulatory region [6]. Some reports showed that it might be important for atherogenesis, which is at least partially regulated by DNA methylation. ApoE knockout mice and the neointima of balloon-denuded New Zealand White rabbit aortas [6] showed that significant
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genomic hypomethylation developed during the first replication of aortic smooth muscle cells (SMCs) in vivo, and that hypomethylation occurred in some specific genes, such as 15-lipoxygenase and extracellular superoxide dismutase, which have been indicated to be deeply involved in atherosclerosis [7,8]. This might result from a direct regulatory effect of hypomethylation on gene expression or from a secondary effect by affecting DNA integrity and function.

Peroxisome proliferator-activated receptors (PPARs) have been implicated in macrophage biology, lipid homeostasis, and atherogenesis. In addition, it is generally accepted that iNOS is a key factor in the regulation of vascular disease [9,10]. Strong associations between PPARα/γ ligands and iNOS in atherosclerosis have been observed in many studies [10–12]. But the precise anti-atherosclerotic mechanisms of PPARα/γ ligands and the relationship between PPARα/γ ligands and iNOS remain obscure.

Taking into account that Hcy interferes with the earliest processes of lipid accumulation and metabolism [13], we examined the capacity of C5 DNA methyltransferase (C5MTase) to transfer the methyl group to DNA and iNOS DNA promoter methylation status and the effects of PPARα/γ ligands on iNOS DNA promoter methylation. The alteration of DNA methylation on iNOS might be an important finding that could point to a mechanism against atherosclerosis in which epigenetic gene silencing is a feature. The iNOS uncovered aberrations of DNA methylation induced by Hcy as well as the effects of PPARα/γ ligands and the PPAR ligands pathways could be a potential target for anti-atherosclerosis therapy. These data for the first time indicate the effect of DNA methylation on the iNOS gene in the context of atherosclerosis.

Materials and Methods

Cell cultures

Human blood from a healthy donor was drawn into heparinized syringes. The whole blood was separated into peripheral blood mononuclear cells and neutrophils using the density gradient from Nycoprep 1.077 (Life Technologies, Chengdu, China), then the monocytes were isolated from peripheral blood mononuclear cells by adherence to a serum-coated culture flask for 2 h. Adherent cells were then detached and resuspended in RPMI 1640 medium containing 5% autologous plasma. Only cell preparations with a 95% viability or greater were used. The cells were planted into 6-well plates and grown to 80% confluence. Serum was deprived for 4 d cell synchronicity. The cells were then cultured with 0.5 μg/ml phorbol myristate acetate (PMA) (Sigma-Aldrich, St. Louis, USA) together with 100 μg/ml oxidize low density lipoprotein (ox-LDL) and different concentrations of Hcy, and clofibrate (5 μM) and pioglitazone (10 μM).

Oil red O stained foam cells

The cultured monocytes were washed with phosphate-buffered saline (PBS) three times and fixed in 2.5% glutardialdehyde for 3 h, dipped in 2.5% potassium dichromate for 16 h, and stained in 1% oil red O (Sigma-Aldrich) for 20 min to identify lipid droplets in cytoplasm. Cell nuclei were then stained in hematoxylin for 15 s. All products were washed with distilled H2O. Monocyte-derived foam cells were observed. Semi-quantitative analysis of foam cells was evaluated by the percentage of positive oil red O staining cells.

Endogenous C5MTase activity

A modification of the assay developed by Hattori et al. [14] was used to determine DNA MTase activity. A total of 1×10³ cells was scraped from plates, pooled into ice-cold PBS and collected by centrifugation. The cells were suspended in 500 μl lysis buffer, then lysed by four cycles of freezing at −70 °C and thawing at 37 °C. Protein concentration was determined by the Bradford assay. Cell lysates containing 5 μg protein were mixed with 0.5 μg poly[dI-dC]·poly[dI-dC] and 1.5 μM S-adenosyl-L-[methyl-3H] methionine in a total volume of 20 μl and incubated at 37 °C for 2 h. The reactions were terminated by adding 300 μl of stop solution (1% sodium dodecyl sulfate, 2 mM EDTA, 5% 2-propanol, 125 mM NaCl, 1 mg/ml Proteinase K, 0.25 mg/ml carrier DNA) for 1 h at 37 °C. The DNA was extracted with phenol-chloroform and ethanol precipitated. The recovered DNA was resuspended in 30 μl of 0.3 M NaOH and incubated for 30 min at 37 °C. DNA was spotted on GF/C filter discs (Whatman, Shanghai, China) and dried. Filters were placed in scintillation vials and incubated for 1 h at 60 °C with 500 ml of 0.5 M perchloric acid. Then 5 ml of scintillation cocktail was added and radioactive 3H incorporation into DNA was assessed using a liquid scintillation counter (Beckman Coulter, Shanghai, China).

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Genomic RNA was isolated from the cultured cells by the E.Z.N.A Tissue RNA Kit (Omega Bio-Tek). RNA was
reversely transcribed by the RevertAid First Strand cDNA Synthesis Kit (MBI) in a final volume of 20 μl containing 5 μl RNA, 3 μl oligo dT primer, and 12 μl DEPC-treated water. The mixture was incubated at 70 °C for 10 min, then put on ice. Four microliters 5×reaction buffer, 1 μl ribonuclease inhibitor (recombinant; 20 U/μl), 2 μl dNTP mix, and 1 μl RevertAid M-MuLV Reverse Transcriptase (200 U/μl) were added and incubated at 20 °C for 10 min, then incubated at 48 °C for 60 min. cDNA was used for PCR.

Primers of iNOS (GenBank accession No. AF440783) were designed with Primer Premier 5.0 software. The forward primer was 5′-CTATGTAGCCCTAGTTTGCC-3′, the reverse primer was 5′-GTGGTAGATTGGCGTGAGC-3′ and the probe primer was 5′-6-FAM-AGTCGTGT-GGGATGGTGA-TAMRA-3′. Real-time PCR was carried out using an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, USA) with the program running for 40 cycles at 95 °C for 45 s, 60 °C for 60 s and 72 °C for 120 s. The melting curve analysis was carried out at the range 55 °C–95 °C by monitoring 6-FAM fluorescence with increasing temperature (0.5 °C increments at 10 s intervals). PCR-specific products were determined by clear single peaks at the melting curves above 80 °C. Real-time PCR was duplicated for each cDNA sample. Each gene RNA level was acquired from the value of the threshold cycle (Ct) of the real-time PCR related to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; calibrator) through the formula

$$ΔCt = Ct_{(calibrator)} - Ct_{(sample)}$$

Final results, expressed as N-fold differences in target gene expression relative to the calibrator, termed “N_target”, were determined as

$$N_{iNOS} = 2^{ΔCt_{(sample)}}/2^{ΔCt_{(calibrator)}}$$

**Western blot analysis of iNOS**

Total protein was extracted from the cultured monococytes and analyzed by Western blotting [15,16]. Briefly, cultured cells were harvested by scraping with a plastic scraper. Extracts of whole cells (5×10⁶) were isolated from cell culture by lysis buffer. Protein concentrations were determined by Coomassie Protein Assay. Polyacrylamide gel electrophoresis (7%–19% polyacrylamide gradient gels) was followed by electrophoretic transfer of proteins from the gel to a nitrocellulose membrane. The membrane was incubated in 10 ml of blocking solution (0.1 ml/cm²) for 2 h at room temperature with gentle agitation on a platform shaker, and washed three times for 5 min each in TBST solution (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20). The membrane was then incubated with a monoclonal anti-iNOS antibody (at 1:250 dilution) in 10 ml primary antibody dilution buffer with gentle agitation at 4 °C, then washed three times with TBST solution, and incubated with secondary antibody goat anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (Jackson ImmunoResearch, West Grove, USA) in PBS at 1:2000 dilution containing 1% bovine serum albumin for 1 h at room temperature. After being washed again three times with TBST solution, the membrane was incubated with 10 ml LumiGLO solution (New England Biolabs, Beverly, USA) with gentle agitation for 1 min at room temperature. Excess developing solution was drained out, but the membrane was not dried. It was wrapped in plastic and exposed to X-ray film.

**iNOS promoter methylation analysis**

**Bisulfite conversion and DNA recovery** Bisulfite modification was carried out using the EZ DNA Methylation Kit (Zymo Research, Orange, USA) following the manufacturer’s instructions. The bisulfite-treated DNA was isolated using the Wizard DNA Clean-Up System (Promega, Madison, USA). The DNA was eluted by 50 μl of warm water and 5.5 μl of 3 M NaOH was added and allowed to stand for 5 min. The DNA was ethanol precipitated with glycogen as a carrier and resuspended in 20 μl of water. **Standard curve** Peripheral blood leukocyte (PBL) DNA was used as a substrate for M.SssI treatment as described by the manufacturer [17]. PBL DNA (0.05 μg/μl) was incubated with M.SssI at a concentration of 1 U/μg DNA (0.05 U/μl) and 0.16 mM AdoMet (Sigma-Aldrich) overnight at 37 °C. Then extra AdoMet (to 0.20 mM) and M. SssI (to 0.065 U/μl) were added followed by a second overnight incubation at 37 °C. The sample was stored at 4 °C. To generate unmethylated human DNA as control samples for testing the MethyLight reactions, sperm and PBL DNA were amplified using a WGA kit (Molecular Staging, New Haven, USA) as described by the manufacturer. The unmethylated and methylated DNA were then treated with bisulfite and recovered as described above. The following ratios were prepared (methylated/unmethylated): 0/100, 10/90, 25/75, 50/50, 75/25, 90/10 and 100/0. Each sample was examined by real-time PCR analysis in duplicate. We correlated the ΔCt values with the predefined prevalence of methylated alleles. The curve had a sigmoid shape with a linear part in the range of 10%–90% of methylated DNA. From this we deduced an algorithm to calculate the methylation ratio of an unknown sample from its ΔCt value.

**MethyLight reactions and methylation calculations** We carried out a novel quantitative analysis of methylated...
alleles that is essentially a major improvement over a
previous method based on real-time PCR (MethyLight)
[18]. We used a VIC-labeled probe that specifically
hybridizes to the sequence derived from the methylated
allele, and a FAM-labeled probe that binds to the sequence
generated from the unmethylated allele. The amount of
fluorescent dye released during PCR is measured by a
real-time PCR system and is directly proportional to the
amount of PCR product generated. The binding site of
the probes covers three differently methylated CpG
dinucleotides. Their improved sequence specificity
facilitates relative quantification of methylated and
unmethylated alleles that are simultaneously amplified in a
single tube.

PCR primers were designed to amplify the bisulfite-
converted antisense strand of the iNOS sequence. The
PCR primers and probes and the strategy for designing
the MethyLight reaction are listed in Fig. 1. The PCR was
carried out using a 96-well optical tray with caps at a final
reaction volume of 20 µl. Samples contained 10 µl of
TaqMan Universal PCR Master Mix (Bio-Rad), 2 µl of
bisulfite-treated DNA, 2.5 µM each of the iNOS forward
primer and PPARα/γ reverse primer and 150 nM each of the
fluorescently labeled probes iNOS met and unmet.

The probes for modified iNOS are indicated by shaded boxes, and the polymerase
chain reaction (PCR) primers for the iNOS sequence are indicated by arrows. The
following equation was deduced from the results generated by the standard curve,
\[ c = \frac{100}{\left[1 + 2 \left(2^{\Delta C_t} - 1\right)\right]} \]
where \(c\) is the methylated/unmethylated ratio, \(\Delta C_t\) is the difference in

cycle time between methylated and unmethylated alleles. The following
expression for the log2 transformation of \(c\) is represented by
\[ \log_2(c) = \frac{c - 1}{c + 1} \]

\[ c = \frac{1}{1 + 2 \left(2^{\Delta C_t} - 1\right)} \]

Statistical analysis

Results are expressed as mean±SEM. The data were
analyzed using one-way ANOVA and additional analysis used
Student-Newman-Keuls’ test for multiple comparisons
within treatment groups or Student’s t-test for between
two groups. \(P<0.05\) was considered significant.

Results

Effect of Hcy on foam cells derived from monocytes

Fig. 2 shows the effects when foam cells were induced
after 4 d incubation with PMA and ox-LDL, 0, 50, 100,
200 or 500 µM Hcy, and clofibrate and pioglitazone in
monocytes. A great number of foam cells were found by
oil red O staining. The foam cells cultured with various
concentrations of Hcy (50 to 500 µM) for 4 d were
significantly elevated compared with the control
\(P<0.05\). Hcy at 100 µM produced the highest stimulation of foam
cells (\(P<0.001\)), indicating that Hcy is important for foam
cell formation. The role of the addition of clofibrate and
pioglitazone were further tested that they can decrease
foam cell formation \(P<0.05\).

Endogenous C5MTase activity

To determine whether Hcy is able to induce changes in
C5MTase in human monocytes, the monocytes were cul-

Fig. 1  Sequences of inducible nitric oxide synthase (iNOS)
primers and probes

The probes for modified iNOS are indicated by shaded boxes, and the polymerase
chain reaction (PCR) primers for the iNOS sequence are indicated by arrows. The
sequence of iNOS after sodium bisulfite modification is also shown. The solid line
arrows indicate the extension direction. Methylated and unmethylated PCR have
the same primers. Fluorescence detection was carried out by two passages. When
PCR was carried out with methylation modified gDNA, the fluorescence of the
methylated probe was detected by the one passage the same as PCR with
unmethylation modified gDNA. The other kind of fluorescence of unmethylated
probes was detected by the other passages. Upper row, bisulfite modified sequence (for display, assume all CpG sites are methylated);
\(+\), CpG site; , non-CpG “C” converted to “T” ; >>>, left primer; <<<, right primer.

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Fig. 2 Effects of homocysteine (Hcy) on foam cells derived from monocytes
(A) The cells were cultured with 0.5 μg/ml PMA (phorbol myristate acetate) and 100 μg/ml ox-LDL (oxidize low density lipoprotein) as well as 0 (control), 50, 100, 200 or 500 μM Hcy for 4 d. (B) After incubation with 100 μM Hcy and clofibrate and pioglitazone, the number of foam cells of cultured monocytes was measured by the percentage of positive oil red O staining cells. Values (mean±SEM) are expressed relative to basal activity, designated as 100% (n=6). *P<0.05, **P<0.01 compared with the control; †P<0.05 compared with the ox-LDL+PMA+Hcy group; #P<0.05 compared with the ox-LDL+PMA group. PMA, phorbol myristate acetate; ox-LDL, oxidize low density lipoprotein.

Fig. 3 Effects of homocysteine (Hcy), clofibrate and pioglitazone on C5 DNA methyltransferase (C5MTase) activity in human monocytes
The activity of C5MTase was expressed as the amount of incorporated [methyl-3H] groups into poly[dI-dC]·poly[dI-dC] in cell homogenates. Data were presented as the mean±SD for four independent experiments with three simultaneous samples each. *P<0.05, **P<0.01 compared with the control; †P<0.05 compared with the ox-LDL+PMA+Hcy group; #P<0.05 compared with the ox-LDL+PMA group. PMA, phorbol myristate acetate; ox-LDL, oxidize low density lipoprotein.

Monocytes cultured with PMA and ox-LDL as well as different concentrations of Hcy, and clofibrate and pioglitazone. The endogenous C5MTase activity is shown in Fig. 3. Hcy up-regulated the activity of C5MTase, with maximum activity at a concentration of 100 μM Hcy (P<0.01, compared with the control group). The other experimental groups also showed significant elevation of C5MTase activities (P<0.05). The cells cultured with clofibrate and pioglitazone decreased the activity of C5MTase in cultured monocytes within 4 d (P<0.05, compared with the ox-LDL+PMA+Hcy group).

Effect of Hcy on iNOS RNA and protein expressions
To determine whether Hcy and PPARα/γ ligands modulate the expression of iNOS mRNA, total RNA was isolated from the cultured monocytes treated with Hcy at different concentrations, clofibrate and pioglitazone. After normalization against GAPDH mRNA, the mRNA level of iNOS was down-expressed in all Hcy-treated groups compared with the control group (P<0.05). The decrease
in the levels of iNOS mRNA after incubation with 100 μM Hcy reached the maximum when cultured monocytes were incubated with Hcy (50–500 μM) for 4 d. But the increasing concentrations of Hcy did not result in dose-dependent decreasing effects on mRNA levels of iNOS. There was no significant difference in mRNA expression of iNOS between the experimental groups. PPARα/γ ligands also increased iNOS production (Fig. 4).

![Fig. 4](image_url)

**Fig. 4**  Inducible nitric oxide synthase (iNOS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) real-time polymerase chain reaction (PCR) graph and effects of homocysteine (Hcy) on iNOS RNA levels in cultured human monocytes

(A) Representative autoradiogram of RNA for iNOS and GAPDH in monocytes treated with Hcy. (B) Representative autoradiogram of RNA for iNOS and GAPDH in monocytes treated with clofibrate and pioglitazone. (C,D) Densitometric analysis of iNOS RNA in control, the ox-LDL+PMA+Hcy group, the PMA+ox-LDL group, clofibrate and pioglitazone group. The fluorescence was plotted versus the PCR cycle number for both reactions and each sample was indicated. The relative mRNA levels of iNOS after normalization were acquired from the value of the threshold cycle (Ct) of the real-time PCR as related to that of GAPDH through the formula

\[ \Delta \Delta Ct = Ct_{GAPDH} - Ct_{target} \]

Final results, expressed as N-fold differences in target gene expression relative to the calibrator, termed “Ntarget”, were determined using the formula:

\[ N_{target} = 2^{\Delta \Delta Ct_{sample}} - 2^{\Delta \Delta Ct_{calibrator}} \]

where \( \Delta \Delta Ct \) values of the calibrator and the sample were determined by subtracting the Ct value of target gene. Data were expressed as mean±SEM of three separate experiments. *P<0.05 compared with the control group; ‡P<0.01 compared with the ox-LDL+PMA+Hcy group; #P<0.05 compared with the ox-LDL+PMA group. PMA, phorbol myristate acetate; ox-LDL, oxidize low density lipoprotein.
The effects of Hcy on the protein levels of iNOS were measured by Western blot analysis, and showed a result similar to that of iNOS mRNA expression. The lowest protein expression of iNOS was also at a concentration of 100 μM Hcy, and there was no significant difference in iNOS protein levels between various dosages of Hcy. PPARα/γ ligands increased iNOS protein expression. These results show that PPARα/γ ligands not only reduced the production of iNOS protein but also iNOS mRNA levels in cultured human monocytes (Fig. 5).

**Hcy-induced iNOS methylation changes**

To explore the possible role of the Hcy and PPARα/γ ligands in iNOS methylation levels, the methylation status of the iNOS promoter region was investigated in cultured monocytes. Using a quantitative TaqMan-based real-time PCR, it was found that an increase in Hcy dose (50, 100, 200 and 500 μM) led to a significant increase in iNOS DNA methylation by 31%, 87%, 32% and 50%, respectively (P<0.05), and decrease in clofibrate and pioglitazone 32% and 64% compared with the control, and 26% and 61% comparred to 100 μM Hcy (Fig. 6) by 32%, 26% and 64%, 61% (P<0.01). These findings suggested that DNA methylation played a role in iNOS expression observed in human monocytes.

**Discussion**

Atherosclerosis has been viewed as bland deposits of excess lipid in the vascular wall, but mounting evidence has redefined them as dynamic sites of chronic inflammation [20,21]. Considerable epidemiological evidence has identified Hcy as a risk factor for arteriosclerosis, but the mechanisms of Hcy-induced atherosclerosis have been linked to inflammation, oxidative stress, and apoptosis. Increasing evidence, however, indicates that HHcy might also be involved in disturbing the expression of atherosclerosis-related genes through the interference of DNA methylation [22,23].

Previous studies have reported the effects of Hcy-induced DNA methylation in vascular SMCs (VSMCs) [24,25]. However, our experiments showed that Hcy not only promoted C5MTase activity but also induced DNA
hypomethylation of the iNOS gene promoter and the downregulation of iNOS expression in foam cells derived from monocytes. We also showed that PPARα/γ ligands decreased activity of DNA MTase and down-regulated iNOS DNA methylation, which repressed iNOS expression, resulting in anti-atherosclerosis. This finding has important clinical implications, and is supported by the following evidence. First, the plasma concentration of Hcy in patients suffering from homocystinuria is as high as 500 μM [26,27]. Patients with plasma Hcy levels >50 μM are at increased risk for vascular disorders [28,29]. This is in the range used in the present study. Second, in the present study, we have also produced insights regarding the mechanisms responsible for Hcy-induced iNOS promoter region DNA methylation in foam cells derived from monocytes.

Although the molecular mechanism underlying Hcy-induced atherosclerosis has been the subject of intensive investigation, most previous studies have focused on the influence of Hcy on endothelial cells and VSMCs [30,31]. Recent studies have shown that Hcy might also act directly on the immune cells to initiate and promote the progression of atherosclerosis [32,33]. Holven et al. [34] suggested that Hcy might exert its atherogenic effect by enhancing the inflammatory response. Similarly, Hcy might work through a new mechanism involving the up-regulation of iNOS DNA methylation, especially in the development of atherosclerotic plaque in patients with vascular disorders [35]. This should provide new insight to our understanding of Hcy-induced atherosclerosis.

We tested C5MTase activity, which acted as a mediator in Hcy-induced DNA methylation, and found that Hcy (50 μM) elevated C5MTase activity. A great difference between Hcy and cysteine is a one-carbon methyl group transfer metabolism, which involves the Hcy, but not the cysteine. The transmethylation modulation of genomic DNA (gDNA) is a very important epigenetic way for the regulation of gene expression, and has been shown to involve the expression of some atherosclerosis-related genes. In this cycle, the methyl-group for gDNA modification is catalyzed by DNA methyltransferase. After the methyl-group is transferred to DNA or other target compounds, the produced S-adenosylhomocysteine is then hydrolyzed to Hcy. Hcy can be recycled to methionine, or be eliminated by other metabolic ways. An abnormal increased Hcy concentration might interfere with this cycle, and result in feedback impacts on DNA MTase and the methylation status of gDNA. In the present study, we have found that C5MTase activity can be triggered by the Hcy range of 50–500 μM. It has been reported that MTase activity in cancer tissue was actually increased in spite of the genome-wide hypomethylation [36]. MTase activity can be seen as a compensatory mechanism to maintain the genomic methylation pattern. Our unpublished data suggest that the Hcy level significantly increases MTase activity both in VSMCs and monocytes.

Second, we have distinguished the expression of iNOS. The present study has provided evidence that iNOS is the major enzyme responsible for Hcy-induced atherogenesis, our results indicating that iNOS protein expression was induced by Hcy in foam cells derived from monocytes. iNOS is believed to produce low amounts of NO to execute physiological and/or anti-inflammatory functions.
The inflammation-associated expression of iNOS and the subsequent overproduction of NO are assumed to be responsible for the cardiovascular failure [37]. The results also showed that iNOS promoter was hypermethylated after Hcy treatment. A reasonable explanation is that increasing concentrations of Hcy increases the intracellular ratio of S-adenosylmethionine (SAM)/S-adenosylhomocysteine and stimulates DNMT activity that the methyl group of SAM transfers to iNOS. Increased iNOS gene promoter hypermethylation is silenced in association with the CpG-island methylation. For this reason, lowered iNOS expression is a reasonable, and easy, explanation [38,39]. But the result of decreased iNOS expression levels contradicts the report by Woo et al. [40]. A potential, yet plausible explanation is that there are differences in iNOS expression between foam cells and macrophages. In many mechanisms involved in the synthesis of iNOS in the process of foam cell development, in which iNOS promoter DNA methylation might be predominant, iNOS replication was repressed by promoter hypermethylation.

Furthermore, some evidence has indicated that PPARα/γ ligands reduce inflammation and anti-atherosclerosis [3]. However, our data have shown that PPARα/γ ligands can partially block Hcy-induced C5MTase activity and are involved in the mechanism of Hcy-induced DNA methylation in cultured human monocytes. This is consistent with the fact that PPARα/γ ligands have an anti-atherosclerotic effect in cultured VSMCs. PPARα/γ ligands play an important role in transferring the methyl group to DNA or other target compounds [41]. We have also examined the effects of PPARα/γ ligands on Hcy-induced response to iNOS, and showed that PPARα/γ ligands clearly inhibited Hcy-induced iNOS in cultured human monocyes. These results suggest that Hcy-induced iNOS can be regulated by peroxisome proliferators [42], and the activation of PPARα/γ might have beneficial effects in patients with atherosclerotic disorders.

The dosage of Hcy used in the present study is clinically relevant, from moderate hyperhomocysteinemia (Hcy concentration of 100 μM, found in up to 40% of patients with myocardial infarction, stroke, or venous thrombosis) to severe hyperhomocysteinemia (Hcy concentration of 500 μM, found in patients with inherited homocystinuria). The impacts of various concentrations of Hcy on the one-carbon methyl group transfer metabolism, however, did not show a dose-effect relationship. The highest effects on aberrant methylation of iNOS and the factors involved in the pathway of DNA methylation and the SAM cycle were at the Hcy concentration of 100 μM. Increased Hcy concentrations, on the contrary, exert weaker effects on the aberrant methylation of iNOS and involved factors. This was unexpected, but might be reasonable. Hyperhomocysteinemia has been found to be associated with many deleterious effects, including pro-apoptosis on endothelial cells, promoting proliferation of VSMCs, activating some inflammatory pathways and coagulation cascades, even mediating cholesterol dysregulation. Hcy concentrations higher than 100 μM might exert more direct injurious effects, such as oxidative stress and apoptosis, whereas moderate hyperhomocysteinemia might have a milder impact on epigenetic modulation of gene expression [43]. There is a proliferation of SMCs at the level of 100 μM Hey, but viable cell counts progressively reduce in Hcy concentrations of 200 μM and above. This phenomenon suggests that the varied detrimental effects of Hey could be attributed to different concentrations by different mechanisms. In mild and moderate hyperhomocysteinemia, Hey might primarily influence the epigenetic regulation of gene expression through the interference of methyl group transfer metabolism, whereas in higher Hey concentrations, the essential impacts might be more directly injurious through oxidative stress, pro-apoptosis, and inflammation.

Our findings uncovered Hcy-induced hypermethylation in the iNOS promoter and increased activity of DNA MTase, similar to the finding in cancer tissues. It is possible that alterations in iNOS promoter hypermethylation play an important role in atherogenesis. However, there is no proven direct relationship between iNOS promoter hypermethylation and atherogenesis, there might simply be an association between these two processes, but our findings also suggested that different concentrations of Hey exert different effects through different mechanisms. Therefore, interventions directed towards HHcy should be more specific, taking into account the Hcy concentration in each case and related mechanisms, for the treatment of vascular disorders in the treatment of vascular disorders. PPARα/γ ligands clearly inhibit Hey-induced iNOS. The induction of iNOS promoter hypermethylation by HHcy and the anti-atherosclerotic effect of PPARα/γ ligands is a new, hitherto unreported element of their mechanisms. These findings reveal a novel role for Hcy in the pathogenesis of human vascular disease.

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