Arch. Immunol. Ther. Exp. (2014) 62:329–340
DOI 10.1007/s00005-014-0271-4

ORIGINAL ARTICLE

Effects of Resveratrol on the Expression and DNA Methylation of Cytokine Genes in Diabetic Rat Aortas

Xu-dan Lou · Hai-dong Wang · Shi-jin Xia · Sven Skog · Jiao Sun

Received: 3 December 2012 / Accepted: 30 July 2013 / Published online: 5 February 2014

Abstract This paper studies the expression of proinflammatory cytokines such as IL-1β, IL-6, TNF-α, and IFN-γ and anti-inflammatory cytokines such as IL-10 in diabetic rat aortas, the effects of resveratrol on these cytokines, and the potential epigenetic mechanisms involved. The experiment was performed on rats divided into four groups: normal group (NC), normal interventional group (NB), diabetic group (DM), and diabetic interventional group (DB). The NB and DB groups were treated with resveratrol. After more than 3 months, the rats' aortas were removed and analyzed for cytokines by using immunohistochemistry, Western blotting, real-time PCR, and methylation-specific PCR. Histological localization of these cytokines was mainly found in the arterial intima of diabetic rats. The protein and mRNA expression levels of IL-1β, IL-6, TNF-α, and IFN-γ were significantly higher in the DM group than in the NC group ($p < 0.05$), whereas in the resveratrol-treated groups (NB and DB), the levels were relatively lower than those in the corresponding groups. The DM group showed reduced levels of DNA methylation at the specific cytosine phosphate guanosine sites of IL-1β, IL-6, TNF-α, and IFN-γ, relative to those in the NC group ($p < 0.01$), and these levels were increased by resveratrol. In contrast, IL-10 was dramatically methylated and showed decreased expression in response to high glucose, and resveratrol reversed this effect. These results demonstrate that the inflammatory response is involved in diabetic macroangiopathy. Resveratrol inhibits the expression of proinflammatory cytokines and thus may have a protective effect on the aorta in hyperglycemia. Thus, DNA methylation, an epigenetic gene silencing signal, may be responsible for these two phenomena.

Keywords Inflammatory cytokines · Resveratrol · DNA methylation

X. Lou · H. Wang (✉) · J. Sun
Endocrinology Department, Huadong Hospital, Fudan University, NO. 221 West of Yan'an Road, Shanghai 200040, China
e-mail: wanghaidong@medmail.com.cn

H. Wang
Diabetes Association of Administration and Education, Shanghai, China

S. Xia
Shanghai Institute of Geriatrics, Huadong Hospital, Fudan University, Shanghai, China

S. Xia
Institute of Respiratory Diseases, Xinqiao Hospital, Third Military Medical University, Chongqing, China

S. Skog
Sino-Swed Molecular Bio-Medicine Research Institute, Shenzhen, China

Introduction

Diabetes mellitus is a clinical syndrome consisting mainly of glucose and lipid metabolism disorders. Its increasing incidence and serious complications pose a great threat to human health and have become one of the main causes of morbidity and mortality burden on the economy. Both type 1 and type 2 diabetes are associated with vascular complications, including microvascular complications such as diabetic nephropathy, retinopathy, and neuropathy and macrovascular complications such as cardiovascular disease, hypertension, and stroke (Villeneuve and Natarajan 2010). Chronic low-grade inflammation is emerging as a conceivable etiological mechanism underlying and a

fundamental pathological process of the disease. Furthermore, a cascade of immune-inflammatory processes initiated in diabetes may activate macrophages to produce numerous proinflammatory cytokines, including interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, which are considered to have cytotoxic, cytostatic or cytocidal effects on pancreatic islets and to mediate vascular damage at cellular or molecular levels (Dogan et al. 2006). Our preliminary studies confirmed that the levels of IL-1β, IL-6, TNF-α, and IFN-γ were notably elevated in NOD/SCID mice that developed diabetes induced by transgenic and diabetogenic T lymphocytes (Wang et al. 2009a, b). In this study, we compared the expression of inflammatory cytokines in both normal and diabetic rat aortas.

A data-mining analysis performed by Wren and Garner (2005) suggested an epigenetic pathogenesis process for type 2 diabetes. Recent studies have further shown that hyperglycemia induces dynamic epigenetic signatures closely associated with diabetic vasculopathy (Pirola et al. 2011). Epigenetics refers to phenotypic changes that are not related to the underlying DNA sequence but in which the alterations in gene expression can possibly be inherited by the next generation (Krause et al. 2009). Among the known covalent modifications of the genome, DNA methylation has been extensively studied. DNA methylation is mediated by DNA methyltransferases that transfer a methyl group from *S*-adenosyl-methionine to the 5′ position of the cytosine ring of DNA, generating 5-methylcytosine (Chen and Riggs 2011). In mammals, this specific activation mainly occurs at the cytosine phosphate guanosine (CpG) dinucleotides. However, parts of sequences that contain at least 200 base pairs with a high quantity of GCs, called CpG islands, mostly remain non-methylated. At the promoters of regulatory genes, the abnormal methylation of CpG islands, generally hypermethylation, can lead to transcriptional repression or gene silencing (Miranda and Jones 2007). Therefore, DNA methylation is associated with gene silencing, whereas non-methylation is associated with gene activation, and demethylation is often correlated with reactivation of a silent gene (Fuks 2005). It should be noted that epigenetic regulatory mechanisms play a vital role in the control of the inflammatory response (Wilson 2008). Changes in DNA methylation patterns, particularly in the promoter regions, can have profound effects on cytokine gene expression (Gopisetty et al. 2006). One of our objectives was to investigate whether the basal methylation profiles of specific CpGs located in the IL-1β, IL-6, TNF-α, and IFN-γ promoters, as measured by methylation-specific PCR, would change in diabetic rat aortas.

Vascular dysfunction is the initial step in cardiovascular disease, which accounts for the majority of the mortality cases in type 2 diabetes. Numerous studies now support that resveratrol (*trans*-3,5,4′-trihydroxystilbene) is the major bioactive ingredient in red wine and contributes to its cardiovascular protective effects (Mukamal et al. 2003). Recent studies have shown that resveratrol, by activating silent mating type information regulation 2 homolog 1 (SIRT1), safely mimics the effects of calorie restriction in laboratory animals and delays the aging process in a silent information regulator 2-dependent manner (Baur et al. 2006; Burnett et al. 2011). In diet-induced obese mice, oral administration of resveratrol has been shown to improve insulin sensitivity, reduce plasma glucose levels, and increase mitochondrial capacity (Lagouge et al. 2006). Furthermore, resveratrol exhibits remarkable inhibition of proinflammatory cytokines, free radicals, and apoptosis processes (Wong et al. 2011). Although resveratrol has been widely used in various studies, the exact mechanism underlying its protective effect has not yet been fully elucidated. It has been established that persistent activation of advanced glycation end products (AGEs)/receptor for AGEs-NF-κB signaling is implicated in diabetes-induced vasculopathy (Jing et al. 2010). This signaling system is believed to have stimulatory effects on vascular smooth muscle cell (VSMC) proliferation, medial collagen deposition, and cross-linking of the extracellular matrix. These cellular events are presumably involved in vessel wall stiffness and thickening. Importantly, in vitro studies have shown that resveratrol inhibits cultured rat VSMC proliferation and induces apoptosis (Poussier et al. 2005). Therefore, we measured rat aortic thickening to test the effects of resveratrol on vascular function, and we further investigated the potential mechanisms underlying this from an epigenetic point of view.

Materials and Methods

Establishment of the Diabetic Rat Model

Two-week-old healthy male Sprague–Dawley rats with similar weights were randomly divided into two groups: the model group and the normal group. In total, 65 rats were enrolled in the model group, and after 4 weeks of a high-fat diet, their islet β cells were injured with streptozotocin (STZ; dissolved in 0.1 mmol/l citrate buffer, pH 4.4). The rats were injected intraperitoneally with a low dose of STZ (30 mg/kg), once a day for 2 days so as to prevent hypoglycemia, as STZ is capable of inducing fatal hypoglycemia because of massive pancreatic insulin release, and to develop a stable diabetic animal model. Age-matched control rats received an equivalent amount of sodium-citrate buffer. Blood samples were obtained from the tail vein at 72 h after the last injection of STZ to measure glucose levels. Rats with non-fasting plasma

glucose levels equal to or >300 mg/dl (16.7 mmol/l) were selected and divided into two groups: one group was given a high-fat diet and the other was given a high-fat diet along with resveratrol. The remaining 35 normal group rats were also divided into two groups: rats given a basal diet and rats given a basal diet along with resveratrol.

Resveratrol Treatment

Resveratrol was freshly prepared in 25 % ethanol and administered intraperitoneally at a dose of 10 mg/kg/day, at a volume not exceeding 0.1 ml/100 g rat weight. The choice of the dose was based on the results of several studies showing that 5–20 mg/kg of resveratrol provided beneficial results (Palsamy and Subramanian 2011; Schmatz et al. 2012; Soufi et al. 2012). Furthermore, taking into account that red wine is the main dietary source of resveratrol and assuming that the average concentration of resveratrol in wine is 5 mg/l and moderate daily consumption of wine is 250 ml, the mean daily intake of resveratrol under these conditions is 0.02 mg/kg. Therefore, we selected a dose (10 mg/kg) that was 500 times higher than this estimate in order to provide a sufficiently large safety margin (Juan et al. 2002). Therefore, the study population was then divided into four groups: normal group (NC group, $n = 19$), normal interventional group (NB group, $n = 16$), diabetic group (DM group, $n = 29$), and diabetic interventional group (DB group, $n = 25$). The NB and DB groups were treated with resveratrol, whereas the NC and DM groups received equal volumes of 25 % ethanol without resveratrol. During the observation period, weight increase, food and water intake, and appearance changes for each rat were noted. After approximately 10 weeks, the rats were decapitated, and the aorta specimens from each group were separated for cryopreservation and reserved for further experiments. The animal experiments were approved by the Fudan University Institutional Animal Care and Use Ethical Committee.

Immunohistochemistry Analysis and Aortic Thickness Measurement

Rats were selected randomly from the NC, NB, DM, and DB groups, with four rats being chosen from each group. The aortas were processed for paraffin embedding, sliced into 2- to 3-μm-thick cryosections, and adhered to Superfrost Plus slides. Twenty-five sections of abdominal aortas from each rat (five for each cytokine) at 200-μm intervals were stained using a SuperPolymer rabbit and mouse horseradish peroxidase (HRP) kit (CoWin Bioscience Co. Ltd, Beijing, China). Endogenous peroxidase activity was blocked using 3 % hydrogen peroxide for 10 min and 2 % bovine serum albumin for 20 min at room temperature to reduce non-specific absorption of the antibodies. After xylene dewaxing, gradient ethanol hydration, and antigen retrieval in citrate buffer, the tissues were washed three times with 0.01 M phosphate-buffered saline (PBS), pH 7.4, and incubated with primary antibodies (1:50 dilution; Cell Signaling Technology, USA) for 1 h at 37 °C. The secondary antibody with the streptavidin–biotin–HRP complex was then added to the system and incubated for 30 min at 37 °C. After coloration with 3,3-diaminobenzidine, the slides were counterstained with hematoxylin, mounted, and visualized using light microscopy. On high-power observation (40 × 4 magnification), the thickness of the thickest wall of each section was measured, and the mean value of all the measurements in each group was taken as the thickness of the aorta.

Western Blotting

To determine the protein levels of each cytokine, 50 mg of the aorta of each rat from the four groups was collected and 100-μl protein lysates containing 1 % phenylmethanesulfonyl fluoride were prepared. The protein concentrations of the tissue extracts (30 μg) were determined using the bicinchoninic acid assay system, according to the manufacturer's instructions (Uptima; Interchim, Montlucon, France). The proteins were diluted at least 20 times before the assay, and 500 ng of protein per lane was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 5 % for stacking gels and 8 % for separating gels) and then transferred onto nitrocellulose membranes. The membranes containing the proteins were preincubated in blocking buffer (5 % [w/v] skimmed milk powder in PBS/0.1 % Tween 20) for 1 h and then incubated with primary antibodies (1:1,000) against IL-1β, IL-6, IL-10, TNF-α, IFN-γ, or β-actin (overnight, 4 °C). After the membranes were washed three times with blocking buffer, they were incubated with alkaline phosphatase-conjugated secondary antibodies (1:10,000) for 120 min and finally washed in 0.1 M Tris, pH 9.5, containing 0.05 M $MgCl_2$ and 0.1 M NaCl. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (toluidine salt; Pierce, Rockford, IL, USA) were used as substrates to detect alkaline phosphatase-conjugated specific antigen–antibody complexes. The density (specific binding) of each band was measured by densitometry using Quantity One (Bio-Rad Laboratories Inc., Munich, Germany).

Real-Time qPCR Assay

Total RNA was isolated from rats aortas using Trizol reagent (Invitrogen Life Technologies Inc., Carlsbad, CA, USA), and β-actin was used as the internal control. The

Table 1 Primer sequences and annealing temperatures for real-time PCR

Gene	Primer sequence	Annealing temperature (°C)	Product size (bp)
IL-1β-F	5′-AGCAGCTTTCGACAGTGAGGAGAA-3′	55	182
IL-1β-R	5′-TCTCCACAGCCACAATGAGTGACA-3′		
IL-6-F	5′-GATGTTGTTGACAGCCACTGCCTT-3′	56	112
IL-6-R	5′-TAAGCCTCCGACTTGTGAAGTGGT-3′		
TNF-α-F	5′-CTGGCCAATGGCATGGATCTCAAA-3′	56	148
TNF-α-R	5′-ATGAAATGGCAAATCGGCTGACGG-3′		
IFN-γ-F	5′-AAAGACAACCAGGCCATCAGCAAC-3′	56	133
IFN-γ-R	5′-TCTGTGGGTTGTTCACCTCGAACT-3′		
IL-10-F	5′-ATAACTGCACCCACTTCCCAGTCA-3′	55	168
IL-10-R	5′-ACAAGGCTTGGCAACCCAAGTAAC-3′		
β-actin-F	5′-TTGCTGACAGGATGCAGAAGGAGA-3′	56	159
β-actin-R	5′-ACTCCTGCTTGCTGATCCACATCT-3′		

F forward, *R* reverse

RNA was dissolved in 0.1 % diethyl pyrocarbonate. The RNA content was determined from the ratio of optical density (OD260/280), and the RNA quality was analyzed by agarose gel electrophoresis. RNA (1 μg) was reverse-transcribed using a Moloney murine leukemia virus reverse transcription system (Promega, Madison, WI, USA), as previously described (Petro 2005). The cDNA was then used for PCR. The PCR products and a 100-bp DNA ladder were run on a 2 % agarose gel stained with ethidium bromide to determine whether the PCR products showed only one specific amplified band. Real-time PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR Green fluorescence, and melting curves were obtained to analyze the specificity of amplification and to check for contamination with genomic DNA. Primer sequences and annealing temperatures are shown in Table 1. The cDNA PCR products were tenfold gradient-diluted, from 1×10^{-1} to 1×10^{-9}. Real-time PCR was performed and the ABI Prism 7300 SDS software was used to obtain the standard curve (C_t, threshold value). Relative mRNA expression was calculated after normalization to β-actin by using the comparative $2^{-\Delta\Delta C_t}$ method, as described previously (Kubista et al. 2006).

Methylation-Specific PCR

Genomic DNA was extracted from diabetic rat aortas by using the QIAamp DNA Mini kit (Qiagen Gmbh, Hilden, Germany), and 500 ng of DNA was modified using an EpiTect bisulfite kit (Qiagen Gmbh, Hilden, Germany) to convert the cytosine into uracil. DNA was amplified using a methylation-specific primer set and using another non-methylation-specific primer set for IL-1β, IL-6, TNF-α, IFN-γ, and IL-10 promoters, which were designed using MethPrimer (Li and Dahiya 2002) (Table 2). The primer sequences included the −229 CpG site for IL-1β (Hashimoto et al. 2013), −174 CpG site for IL-6 (Pereira et al. 2011), −245 and −239 CpG sites for TNF-α (Campión et al. 2009), −186 and −54 CpG sites for IFN-γ (Bonilla-Henao et al. 2005), and −185 and −110 CpG sites for IL-10 (Viana et al. 2011). The PCR conditions used were as follows: initial denaturation at 95 °C for 10 min, 38 cycles each of denaturation at 95 °C for 30 s, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min; followed by stabilization for 7 min at 72 °C. To ensure the linearity of the PCR reactions and to validate the DNA quantification, CpG methylase (M.SssI) (NEB, Ipswich, MA, USA) treated and untreated normal DNA was used as positive control in amplification with primers against methylated and unmethylated sequences. 5-Methyl-2′-deoxycytidine (Chemos GmbH, Regenstauf, Germany) was used as an internal standard. The results of the methylation-specific polymerase chain reaction (MSP) were visualized on a 1.5 % agarose gel; a 100-bp ladder was used, and the intensity of the bands was measured in triplicate using the Adobe Photoshop software (Adobe, San José, CA, USA). The promoter methylation levels were analyzed as described elsewhere (Keyes et al. 2007).

Statistical Analysis

All the results have been expressed as mean ± SD values, $p < 0.05$ was considered statistically significant. Comparisons between groups were performed by Student's t test after normality assessment. Pearson's correlation coefficient was calculated to determine the statistical relationships among variables. Statistical analyses were conducted using the SPSS 15.0 software for Windows.

Table 2 Promoter regions for MSP

Gene	M-forward primer (5′ → 3′)	M-reverse primer (5′ → 3′)
IL-1β	TGGTAGTTATTTATGTTTTGTTCGT	AAACATTTTTATTATTCATCTCGAA
IL-6	TGGTAGTTATTTATGTTTTGTTCGT	AAACATTTTTATTATTCATCTCGAA
IL-10	TTTTAAGTTTGGGACGTTATAAAAC	ATAAAAATTCTCAAATTCCAACGTA
TNF-α	TTGAGATGTGTTGTAATTAAGACGA	ATAAACTCAACCCTAAAAATTCACG
IFN-γ	TATATTGGTTTTTGTAATCGAACGT	ACCCTTACAATATAAATTTCTCGTC
Gene	U-forward primer (5′ → 3′)	U-reverse primer (5′ → 3′)
IL-1β	GTGGTAGTTATTTATGTTTTGTTTGT	AAACATTTTTATTATTCATCTCAAA
IL-6	TGGTAGTTATTTATGTTTTGTTTGT	AAACATTTTTATTATTCATCTCAAA
IL-10	TTAAGTTTGGGATGTTATAAAATGG	ATAAAAATTCTCAAATTCCAACATA
TNF-α	TTGAGATGTGTTGTAATTAAGATGA	AAACTCAACCCTAAAAATTCACAAA
IFN-γ	TATATTGGTTTTTGTAATTGAATGT	AACCCTTACAATATAAATTTCTCATC

M methylated, *U* unmethylated

Results

Immunohistochemical Staining Localization and Aortic Thickness

IL-1β is the principal circulating isoform of IL-1, which shows increased synthesis in arterial plaques. Representative images for IL-1β and IL-10 and data pertaining to measured abdominal aorta thickness are shown in Fig. 1. IL-1β was mainly expressed in the arterial intima of diabetic rats, and its concentration greatly increased in the DM group, whereas the IL-1β concentration in the NC and NB groups appeared to be minimal and that in the DB group was between that of the NC and DM groups (Fig. 1a–d). The IL-10 concentration was lower in the DM group than in the NC, NB and DB groups (Fig. 1e–h). The wall thickness was found to be 150.52 ± 6.40 μm in the abdominal aortas in the DM group, 90.61 ± 4.62 μm in the age-matched NC group ($p < 0.05$ with respect to DM rats, $n = 4$ in each group), 89.7761 ± 4.03 μm in the NB group ($p > 0.05$ compared with NC rats), and 108.84 ± 7.19 μm in the resveratrol-treated DB group; the values for the other groups were significantly lower than that of DM rats ($p < 0.05$, $n = 4$ in each group).

Expression of Each Cytokine in Four Groups of Rat Aortas

Western blotting analysis was performed using antibodies against each cytokine, as shown in Fig. 2. The protein levels of IL-1β, IL-6, TNF-α, and IFN-γ in the DM group were notably elevated compared with those in the NC group, which suggests that inflammatory factors play a role in both diabetes and vascular diseases. In contrast to the expression of these proinflammatory cytokines, the expression of IL-10 greatly decreased in rat aortas with glucose toxicity, which demonstrates that Th2-type cytokines, such as IL-10, may play a central role in the control of inflammation and thus prevent the progression of diabetes. Synthesis of the housekeeping protein β-actin remained unaffected. IL-1β, IL-6, TNF-α, and IFN-γ mRNA levels measured by real-time-qPCR significantly increased in the DM group, in parallel with reduction in the level of IL-10 (Fig. 3). The outcome was highly consistent with western blotting analysis, which indicates that the overexpression of inflammatory genes may play a vital role in diabetic vasculopathy. The NB and DB groups were treated with resveratrol, and the expression of proinflammatory cytokines decreased notably compared to the levels in the NC and DM groups, whereas resveratrol exhibited an opposite effect on IL-10, as confirmed by both western blotting and real-time PCR (Figs. 2, 3). Here, we hypothesized that at least part of the regulatory mechanism underlying the onset of diabetic complications and the positive effects of resveratrol may occur at the gene expression level.

Modification of DNA Methylation at the Promoter of Each Cytokine

As described in the Introduction, a high degree of DNA methylation mainly located at the CpG islands is associated with low gene expression. Analysis of the methylation levels by MSP showed that the DM group had lower methylation levels at the specific CpG sites of IL-1β, IL-6, TNF-α, and IFN-γ than the NC group, whereas certain CpG sites of IL-10 were dramatically methylated in response to high glucose levels (Fig. 4a–e). Among the resveratrol-treated groups, proinflammatory cytokines in the NB and DB groups showed greater methylation of specific CpG

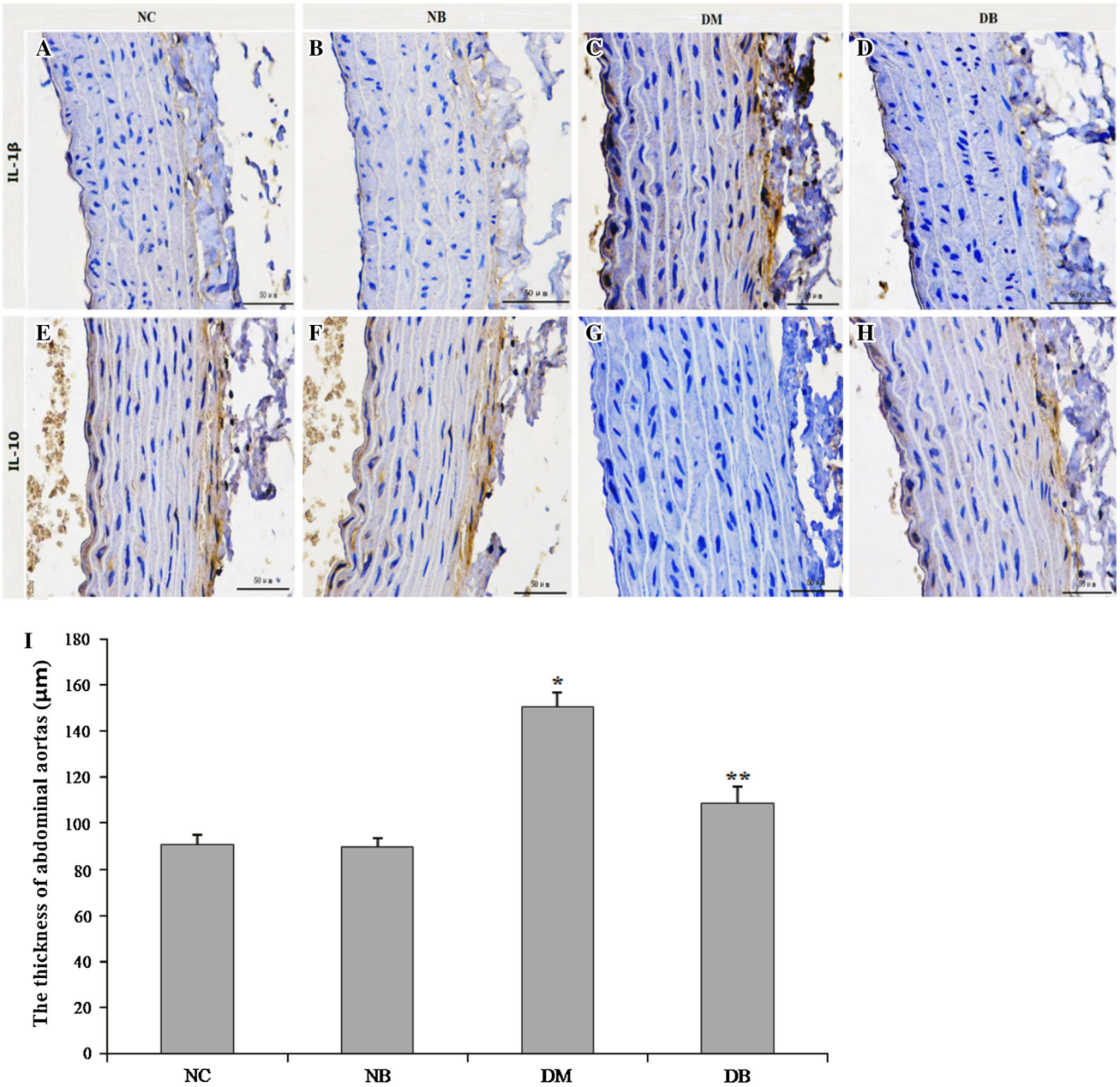

Fig. 1 Immunohistochemical staining of IL-1β (**a**–**d**) and IL-10 (**e**–**h**) in rat aortas from the NC, NB, DM and DB groups ($n = 4$ in each group). **i** The wall thickness of the abdominal aorta was measured as described in the "Materials and Methods", *$p < 0.05$ vs. NC group, **$p < 0.05$ vs. DM group

sites than the NC and DM groups, respectively. However, this epigenetic modification of IL-10 also presented a great difference between the interference groups (NB and DB) and the non-interference groups (NC and DM), which demonstrates that resveratrol may hinder the methylation of anti-inflammatory cytokines, such as IL-10, and thus promote their gene expression. The ratios of U (unmethylated) and M (methylated) are illustrated in Fig. 4f, which describes an intuitive and statistically significant correlation among the DNA methylation levels of each cytokine in the four groups. Table 3 shows that the U/M values of all cytokines in the DM group were significantly different from those in the NC group ($p < 0.01$), whereas the values in the NB and DB groups were lower (proinflammatory cytokines) or higher (IL-10) than those in the NC and DM groups ($p < 0.05$ and $p < 0.01$, respectively). As expected, the analysis of protein and mRNA expression levels supported the DNA methylation changes, which indicates that DNA methylation at the specific CpG sites of each gene is associated with diabetic vascular diseases.

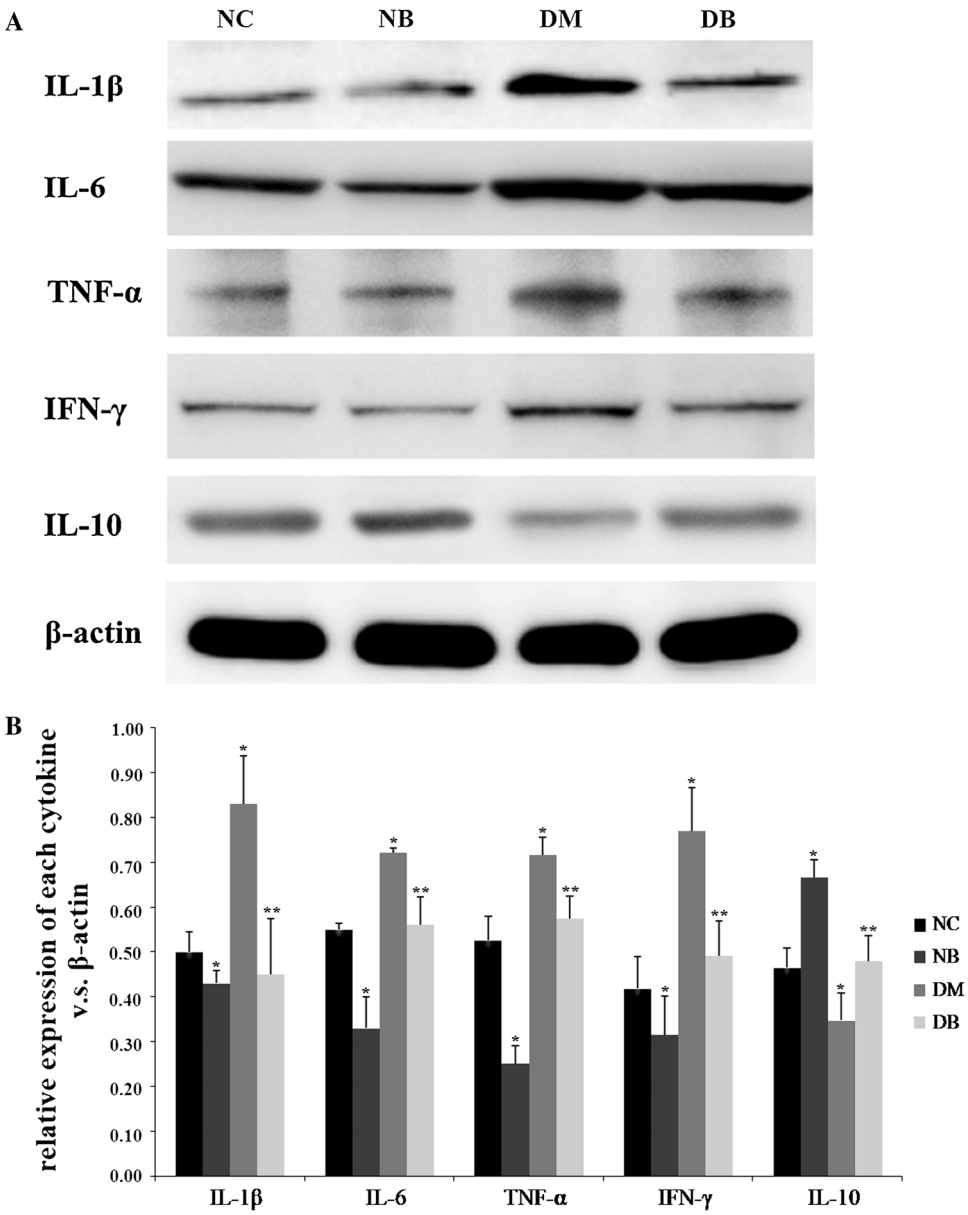

Fig. 2 Western blotting analysis was performed to determine the protein level of each cytokine in the NC ($n = 15$), NB ($n = 12$), DM ($n = 25$) and DB ($n = 21$) groups. **a** The density of each band of β-actin, as measured by densitometry, was used as an internal control. **b** Relative expression of each cytokine vs. β-actin in the four groups; *$p < 0.05$ vs. NC group, **$p < 0.05$ vs. DM group

Discussion

It is widely accepted that inflammatory factors participate in the entire process of diabetes and are involved in its complications. Interleukins are regulatory proteins with the ability to enhance or inhibit inflammatory processes and can be classified based on their role in diabetes and atherosclerosis. The interleukins are clustered into three groups: noxious, comprising IL-1β and IL-6; protective, comprising IL-10, and aloof (Fisman et al. 2008). Infected macrophages, monocytes, and endothelial cells have been reported to express and secrete an increased amount of IL-1β when exposed to high glucose levels in vitro (Liu et al. 2012). Our immunohistochemistry findings showed that the activation and upregulation of proinflammatory cytokines such as IL-1β mainly occurred in the arterial intima of diabetic rats, which provides a potential mechanism underlying endothelial dysfunction and vasculopathy in diabetes. IL-1β expression and the aortic wall thickness significantly increased in the DM group but were minimal in the NC and NB groups, which indicate that an increase in the amount of proinflammatory cytokines in the intima may be the initial step of diabetic atherosclerosis (shown in Fig. 1). IL-10 is expressed in advanced human atherosclerosis and is associated with low inducible nitric oxide synthase expression and low levels of apoptosis, implying a protective role (Mallat et al. 1999). In NOD mice, recombinant IL-10 protein, IL-10 fusion protein, an IL-10-

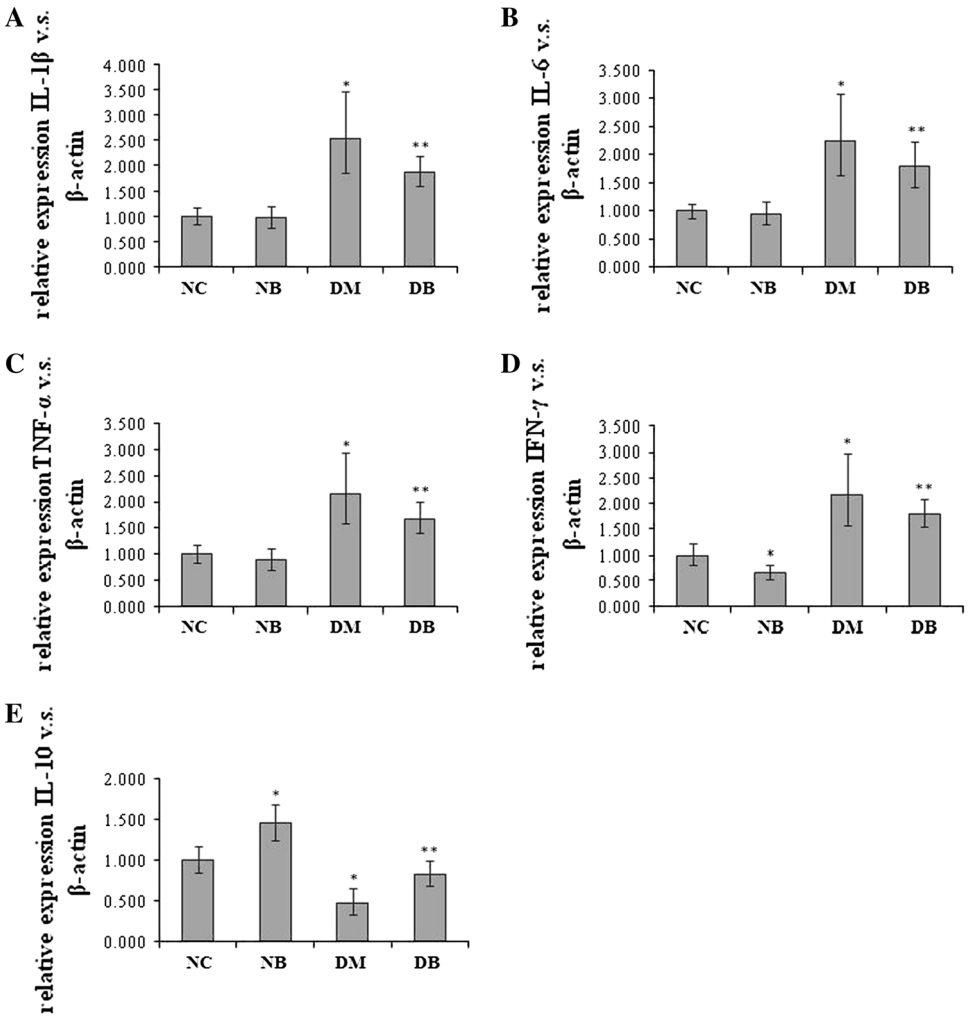

Fig. 3 mRNA at the transcriptional level was detected by real-time PCR. The relative expression of each cytokine in the NC ($n = 15$), NB ($n = 12$), DM ($n = 25$) and DB ($n = 21$) groups was calculated after normalization to β-actin by using the comparative $2^{-\Delta\Delta C_t}$ method. **a**–**e** Present the results for IL-1β, IL-6, TNF-α, IFN-γ, and IL-10, respectively. In figures **a**, **b**, and **c**, the mRNA expression levels in the NB group were lower than those in the NC group, but the difference was not statistically significant. $^{*}p < 0.05$ vs. NC group, $^{**}p < 0.05$ vs. DM group

expressing plasmid, and adoptive transfer with IL-10-cDNA-transduced islet-specific T cell clones have all been shown to prevent the onset of spontaneous diabetes (Yang et al. 2002). Figure 1 presented histologic evidence that the expression of IL-10 was lower in the DM group than in the NC and NB groups. Our in vitro experiments (Figs. 2, 3) showed a large increase in Th1-derived cytokine levels (IL-1β and IL-6) in diabetic rat aortas, whereas levels of a Th2-typed cytokine (IL-10) were negatively correlated with vasculopathy in hyperglycemic status, which agrees with other previous studies and supports the concept of the inflammatory element in the development of diabetic complications. Some studies have indicated that reduced IL-10 levels constitute a marker of not only plaque instability favoring the development of diabetic vascular diseases but also poor prognosis even after the occurrence of an acute cardiovascular event caused by vessel injury (Fisman et al. 2008). Our studies further demonstrate that IFN-γ was sensitive to the inflammatory response induced by high blood glucose levels. A recent study showed that IFN-γ disrupts the expression of genes critical for cellular metabolism and energy expenditure by repressing the activity of SIRT1 at the transcription level (Li et al. 2012). The NAD^{+}-dependent deacetylase SIRT1 has emerged as a critical coordinator of the intracellular environment primarily by affecting deacetylation levels and thus fine-tuning the activation of key transcription factors involved in inflammatory processes. Thus, this mechanism of IFN-γ provides a link between resveratrol and epigenetics.

To date, increasing evidence has shown that the level of DNA methylation varies under diabetic conditions. A recent study found that insulin gene expression is regulated

Fig. 4 DNA methylation levels at the specific CpG sites of each cytokine in the NC ($n = 15$), NB ($n = 12$), DM ($n = 25$) and DB ($n = 21$) groups were measured using the MSP technique. **a**–**e** Show the results of the MSP reactions visualized on an agarose gel with the U and M primer combination. **f** Coefficient ratios of U/M were used to compare the methylation levels of each cytokine in the four groups. $^{*}p < 0.05$ vs. NC group, $^{**}p < 0.01$ vs. NC group, $^{***}p < 0.01$ vs. DM group. *U* unmethylated, *M* methylated

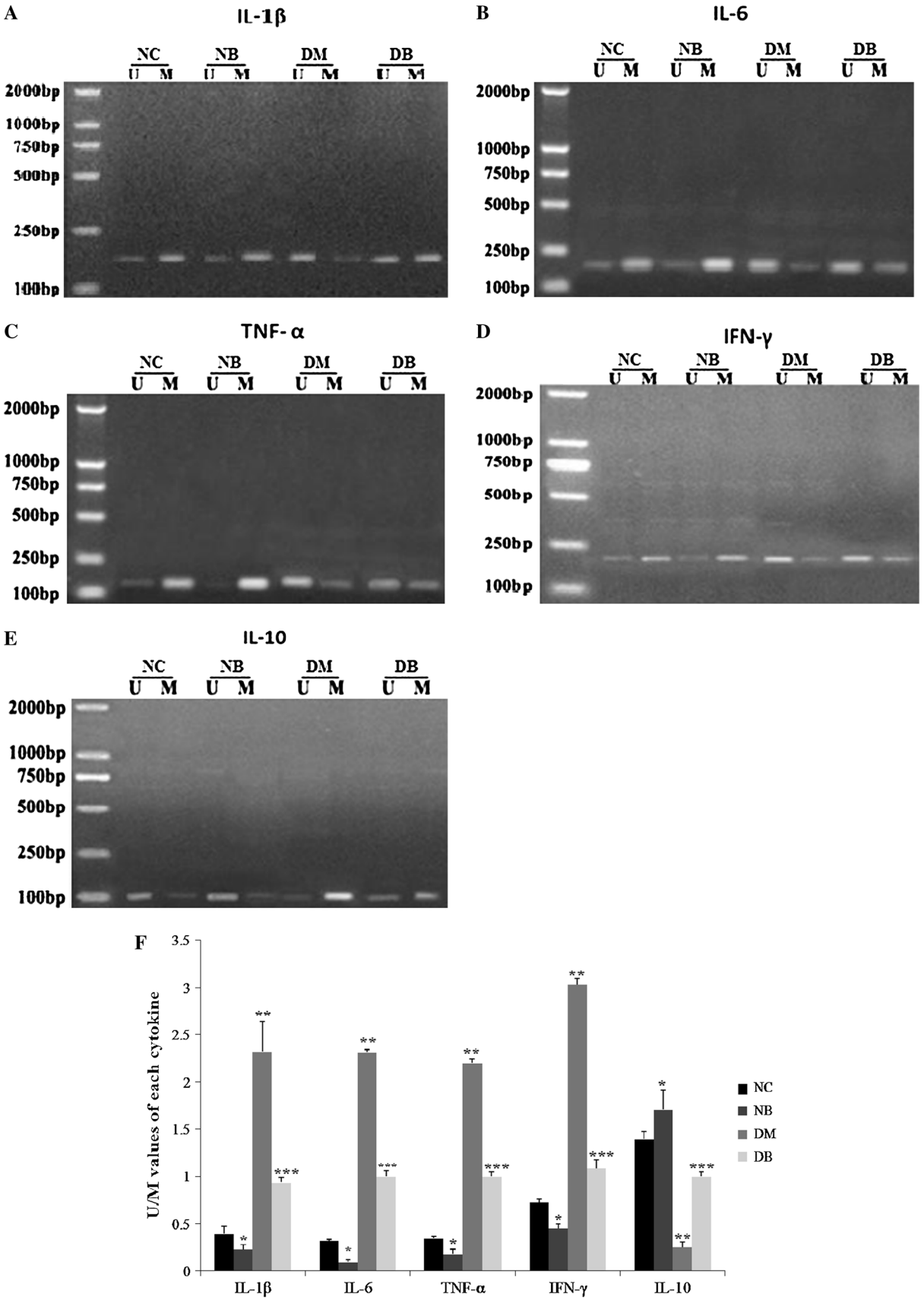
A
IL-1β
NC NB DM DB
U M U M U M U M
2000bp
1000bp
750bp
500bp
250bp
100bp
B
IL-6
C
TNF-α
D
IFN-γ
E
IL-10
F
U/M values of each cytokine
3.5
3
2.5
2
1.5
1
0.5
0
IL-1β
IL-6
TNF-α
IFN-γ
IL-10
NC
NB
DM
DB

Table 3 U/M values and standard deviations for each cytokine in the four groups

Group	IL-1β	IL-6	TNF-α	IFN-γ	IL-10
NC	0.39 ± 0.08	0.32 ± 0.02	0.34 ± 0.02	0.73 ± 0.03	1.39 ± 0.08
NB	0.23 ± 0.05	0.09 ± 0.03	0.18 ± 0.05	0.45 ± 0.06	1.71 ± 0.21
DM	2.32 ± 0.32	2.31 ± 0.02	2.20 ± 0.05	3.03 ± 0.06	0.25 ± 0.06
DB	0.93 ± 0.06	1.00 ± 0.06	1.00 ± 0.04	1.09 ± 0.09	1.00 ± 0.04

by DNA methylation (Kuroda et al. 2009). In another case, progressive epigenetic silencing through DNA methylation at the promoter of Pdx1, a transcription factor that affects β cell differentiation and gene activation, was shown to lead to the development of diabetes (Park et al. 2008). In damaged diabetic islets, peroxisome proliferator-activated receptor-γ, which is closely related to the mechanisms of thiazolidinediones, is highly methylated at DNA CpG islands in its promoter (Ling et al. 2008). In addition, DNA methylation is considered to be related to diabetes-associated cardiovascular diseases (CVDs) and chronic kidney diseases (CKDs) (Keating and El-Osta 2012; Villeneuve et al. 2011). Homocysteine has been increasingly identified as a risk factor for both diabetes-related CVDs and CKDs, mainly through inhibition of DNA methyltransferases, as reported in previous studies (Ekstrom and Stenvinkel 2009; Ingrosso and Perna 2009; Zhang et al. 2009a). Several inflammatory factors relevant to atherosclerosis show chromatin methylation pattern alterations in VSMCs and endothelial cells (Reddy and Natarajan 2011), which indicates that inflammatory gene expression may be regulated by epigenetic modifications. In fact, DNA methylation mechanisms have already been evaluated for some cytokine genes, such as those encoding IL-1β, IL-6, IL-8, IL-10, and TNF-α (Armenante et al. 1999; Cordero et al. 2011; Szalmás et al. 2008; Tekpli et al. 2013). These events may be important in understanding the pathogenesis of inflammatory diseases in which cytokine expression is not regulated. Our MSP studies showed that the genes encoding proinflammatory cytokines, such as IL-1β, IL-6, TNF-α, and IFN-γ, in diabetic rat aortas had low levels of DNA methylation at specific CpG sites, whereas the genes encoding protective cytokines, such as IL-10, were hypermethylated in response to high glucose levels, leading to the corresponding changes in protein and mRNA expression levels (Figs. 2, 3 and 4). Although none of the experiments alone can explain these phenomena, when taken together, the evidence strongly suggests that DNA methylation may be one of the mechanisms regulating cytokine genes and is thus implicated in diabetic vascular complications. In our follow-up investigations, CpG island prediction and primer selection were performed using the MethPrimer software. IL-1β, IL-6, and TNF-α had one CpG island in their promoter sequences, whereas IFN-γ and IL-10 had two CpG islands. The IL-1β gene contained nine CpG sites in the region analyzed within the CpG island, whereas IL-6 contained thirteen, TNF-α and IL-10 contained eleven, and IFN-γ contained ten CpG sites. MSP can be used to rapidly assess the methylation status of almost any cytosine of CpG sites within a CpG island and is sensitive to 0.1 % methylated alleles of a given CpG island locus, but the exact quantity and location of the specific sites cannot be identified by MSP (Ku et al. 2011). Primers that included some specific CpG sites were selected for our study because the methylation patterns of these CpG sites had been evaluated as previously described. However, whether these sites are the specific methylated sites for the cytokine genes in the conditions used in the present study remains unclear and needs more investigations.

A recent study by Zhang et al. (2009b) demonstrated that chronic resveratrol administration rescued aortic dysfunction in diabetic mice and that the vascular protective role of resveratrol may be related to its TNF-α inhibitory properties. Our study showed that resveratrol can not only effectively inhibit TNF-α mRNA and protein expression but also dramatically reduce IL-1β, IL-6, and IFN-γ levels, in parallel with a large enhancement of IL-10 expression, particularly between the DM and DB groups, which suggests profound anti-inflammatory effects of resveratrol in diabetic macroangiopathy. Moreover, resveratrol-treated groups showed decreased aortic thickening (seen in Fig. 1), which indicates that resveratrol may alleviate arterial intimal lesions and may have the potential to preserve vascular health when multiple cardiovascular risk factors are present. These results contribute to our understanding of the effects of resveratrol in diabetic vasculopathy. Further studies should explore the mechanisms underlying the inactivation of inflammatory signaling pathways involved in this complex system. Studies have shown that in STZ-induced diabetic rats, resveratrol may ameliorate vasculopathy through attenuation of AGE formation and prevention of NF-κB signaling (Jing et al. 2010). AGEs are triggered to accumulate at vascular injured sites during chronic events (Vlassara and Palace 2002), which results in persistent vascular inflammation even if the blood glucose level is controlled at an ideal level, which suggests the existence of metabolic memory or a legacy effect (Tonna

et al. 2010). NF-κB is one of the major transcription factors activated in inflammatory pathways under diabetic conditions, and it allows monocytes and macrophages to accumulate in blood vessels and induces macrovascular atherosclerosis (Miao et al. 2004). Notably, both factors (AGEs and NF-κB) can be modulated by epigenetic modifications (Brasacchio et al. 2009). As illustrated in Fig. 4 and Table 3, the two interventional groups (NB and DB) showed hypermethylation at the specific CpG sites of each proinflammatory cytokine and hypomethylation of IL-10 relative to that in the NC and DM groups. Thus, our present results strongly indicate that DNA modification may be one of the protective mechanisms of resveratrol, which may be used as a therapeutic tool targeting diabetic vascular complications. However, further investigations are still required to elucidate the exact molecular pathways.

Acknowledgments This study was supported by Shanghai Committee of Science and Technology Development Funds for Basic Research under Grant No. 10JC1404800 and the National Natural Science Foundation of China under Grant No. 31171129.

References

Armenante F, Merola M, Furia A et al (1999) Repression of the IL-6 gene is associated with hypermethylation. Biochem Biophys Res Commun 258:644–647

Baur JA, Pearson KJ, Price NL et al (2006) Resveratrol improves health and survival of mice on a high-calorie diet. Nature 444:337–342

Bonilla-Henao V, Martínez R, Sobrino F et al (2005) Different signaling pathways inhibit DNA methylation activity and up-regulate IFN-gamma in human lymphocytes. J Leukoc Biol 78:1339–1346

Brasacchio D, Okabe J, Tikellis C et al (2009) Hyperglycemia induces a dynamic cooperativity of histone methylase and demethylase enzymes associated with gene-activating epigenetic marks that coexist on the lysine tail. Diabetes 58:1229–1236

Burnett C, Valentini S, Cabreiro F et al (2011) Absence of effects of Sir2 over-expression on lifespan in *C. elegans* and drosophila. Nature 477:482–485

Campión J, Milagro FI, Goyenechea E et al (2009) TNF-alpha promoter methylation as a predictive biomarker for weight-loss response. Obesity 17:1293–1297

Chen ZX, Riggs AD (2011) DNA methylation and demethylation in mammals. J Biol Chem 286:18347–18353

Cordero P, Campion J, Milagro FI et al (2011) Leptin and TNF-alpha promoter methylation levels measured by MSP could predict the response to a low-calorie diet. J Physiol Biochem 67:463–470

Dogan Y, Akarsu S, Ustundag B et al (2006) Serum IL-1β, IL-2, and IL-6 in insulin-dependent diabetic children. Mediators Inflamm 2006:59206

Ekstrom TJ, Stenvinkel P (2009) The epigenetic conductor: a genomic orchestrator in chronic kidney disease complications? J Nephrol 22:442–449

Fisman EZ, Adler Y, Tenenbaum A (2008) Biomarkers in cardiovascular diabetology: interleukins and matrixins. Adv Cardiol 45:44–64

Fuks F (2005) DNA methylation and histone modifications: teaming up to silence genes. Curr Opin Genet Dev 15:490–495

Gopisetty G, Ramachandran K, Singal R (2006) DNA methylation and apoptosis. Mol Immunol 43:1729–1740

Hashimoto K, Otero M, Imagawa K et al (2013) Regulated transcription of human matrix metalloproteinase 13 (MMP13) and interleukin-1β (IL1B) genes in chondrocytes depends on methylation of specific proximal promoter CpG sites. J Biol Chem 288:10061–10072

Ingrosso D, Perna AF (2009) Epigenetics in hyperhomocysteinemic states. A special focus on uremia. Biochim Biophys Acta 1790:892–899

Jing YH, Chen KH, Yang SH et al (2010) Resveratrol ameliorates vasculopathy in STZ-induced diabetic rats: role of AGE–RAGE signalling. Diabetes Metab Res Rev 26:212–222

Juan ME, Vinardell MP, Planas JM et al (2002) The daily oral administration of high doses of trans-resveratrol to rats for 28 days is not harmful. J Nutr 132:257–260

Keating ST, El-Osta A (2012) Chromatin modifications associated with diabetes. J Cardiovasc Transl Res 5:399–412

Keyes MK, Jang H, Mason JB et al (2007) Older age and dietary folate are determinants of genomic and p16-specific DNA methylation in mouse colon. J Nutr 137:1713–1717

Krause B, Sobrevia L, Casanello P (2009) Epigenetics: new concepts of old phenomena in vascular physiology. Curr Vasc Pharmacol 7:513–520

Ku JL, Jeon YK, Park JG (2011) Methylation-specific PCR. Methods Mol Biol 791:23–32

Kubista M, Andrade JM, Bengtsson M et al (2006) The real-time polymerase chain reaction. Mol Aspects Med 27:95–125

Kuroda A, Rauch TA, Todorov I et al (2009) Insulin gene expression is regulated by DNA methylation. PLoS One 4:e6953

Lagouge M, Argmann C, Gerhart-Hines Z et al (2006) Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell 127:1109–1122

Li LC, Dahiya R (2002) MethPrimer: designing primers for methylation PCRs. Bioinformatics 18:1427–1431

Li P, Zhao Y, Wu X et al (2012) Interferon gamma (IFN-γ) disrupts energy expenditure and metabolic homeostasis by suppressing SIRT1 transcription. Nucleic Acids Res 40:1609–1620

Ling C, Del Guerra S, Lupi R et al (2008) Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion. Diabetologia 51:615–622

Liu Y, Biarnés Costa M, Gerhardinger C (2012) IL-1β is upregulated in the diabetic retina and retinal vessels: cell-specific effect of high glucose and IL-1β autostimulation. PLoS One 7:e36949

Mallat Z, Heymes C, Ohan J et al (1999) Expression of interleukin-10 in advanced human atherosclerotic plaques: relation to inducible nitric oxide synthase expression and cell death. Arterioscler Thromb Vasc Biol 19:611–616

Miao F, Gonzalo IG, Lanting L et al (2004) In vivo chromatin remodeling events leading to inflammatory gene transcription under diabetic conditions. J Biol Chem 279:18091–18097

Miranda TB, Jones PA (2007) DNA methylation: the nuts and bolts of repression. J Cell Physiol 213:384–390

Mukamal KJ, Conigrave KM, Mittleman MA et al (2003) Roles of drinking pattern and type of alcohol consumed in coronary heart disease in men. N Engl J Med 348:109–118

Palsamy P, Subramanian S (2011) Resveratrol protects diabetic kidney by attenuating hyperglycemia-mediated oxidative stress and renal inflammatory cytokines via Nrf2–Keap1 signaling. Biochim Biophys Acta 1812:719–731

Park JH, Stoffers DA, Nicholls RD et al (2008) Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of Pdx1. J Clin Invest 118:2316–2324

Pereira DS, Garcia DM, Narciso FM et al (2011) Effects of 174 G/C polymorphism in the promoter region of the interleukin-6 gene

on plasma IL-6 levels and muscle strength in elderly women. Braz J Med Biol Res 44:123–129

Petro TM (2005) Disparate expression of IL-12 by SJL/J and B10.S macrophages during Theiler's virus infection is associated with activity of TLR7 and mitogen-activated protein kinases. Microbes Infect 7:224–232

Pirola L, Balcerczyk A, Tothill RW et al (2011) Genome-wide analysis distinguishes hyperglycemia regulated epigenetic signatures of primary vascular cells. Genome Res 21:1601–1615

Poussier B, Cordova AC, Becquemin JP et al (2005) Resveratrol inhibits vascular smooth muscle cell proliferation and induces apoptosis. J Vasc Surg 42:1190–1197

Reddy MA, Natarajan R (2011) Epigenetic mechanisms in diabetic vascular complications. Cardiovasc Res 90:421–429

Schmatz R, Perreira LB, Stefanello N et al (2012) Effects of resveratrol on biomarkers of oxidative stress and on the activity of delta aminolevulinic acid dehydratase in liver and kidney of streptozotocin-induced diabetic rats. Biochimie 94:374–383

Soufi FG, Vardyani M, Sheervalilou R et al (2012) Long-term treatment with resveratrol attenuates oxidative stress pro-inflammatory mediators and apoptosis in streptozotocin-nicotinamide-induced diabetic rats. Gen Physiol Biophys 31:431–438

Szalmás A, Bánáti F, Koroknai A et al (2008) Lineage-specific silencing of human IL-10 gene expression by promoter methylation in cervical cancer cells. Eur J Cancer 44:1030–1038

Tekpli X, Landvik NE, Anmarkud KH et al (2013) DNA methylation at promoter regions of interleukin 1B, interleukin 6, and interleukin 8 in non-small cell. Cancer Immunol Immunother 62:337–345

Tonna S, El-Osta A, Cooper ME et al (2010) Metabolic memory and diabetic nephropathy: potential role for epigenetic mechanisms. Nat Rev Nephrol 6:332–341

Viana MB, Cardoso FP, Diniz MG et al (2011) Methylation pattern of IFN-γ and IL-10 genes in periodontal tissues. Immunobiology 216:936–941

Villeneuve LM, Natarajan R (2010) The role of epigenetics in the pathology of diabetic complications. Am J Physiol Renal Physiol 299:F14–F25

Villeneuve LM, Reddy MA, Natarajan R (2011) Epigenetics: deciphering its role in diabetes and its chronic complications. Clin Exp Pharmacol Physiol 38:451–459

Vlassara H, Palace MR (2002) Diabetes and advanced glycation endproducts. J Intern Med 251:87–101

Wang H, Sun J, Xia S (2009a) Construction of the retrovirus vectors carrying the IL-17 or siRNA-IL-17 genes and expression of IL-17 gene in the transgenic diabetogenic T cells. Chin Gerontol J 29:2873–2877

Wang H, Sun J, Xia S (2009b) Interleukin-17 mediates the pathogenesis of diabetes induced by diabetogenic T lymphocytes in NOD mice. Chin J Endocrinol Metab 25:539–543

Wilson AG (2008) Epigenetic regulation of gene expression in the inflammatory response and relevance to common diseases. J Periodontol 79(8 Suppl):1514–1519

Wong YT, Gruber J, Jenner AM et al (2011) Chronic resveratrol intake reverses pro-inflammatory cytokine profile and oxidative DNA damage in ageing hybrid mice. Age 33:229–246

Wren JD, Garner HR (2005) Data-mining analysis suggests an epigenetic pathogenesis for type 2 diabetes. J Biomed Biotechnol 2005:104–112

Yang Z, Chen M, Wu R et al (2002) Suppression of autoimmune diabetes by viral IL-10 gene transfer. J Immunol 168:6479–6485

Zhang D, Jiang X, Fang P et al (2009a) Hyperhomocysteinemia promotes inflammatory monocyte generation and accelerates atherosclerosis in transgenic cystathionine beta-synthase-deficient mice. Circulation 120:1893–1902

Zhang H, Zhang J, Ungvari Z et al (2009b) Resveratrol improves endothelial function role of TNF-α and vascular oxidative stress. Arterioscler Thromb Vasc Biol 29:1164–1171