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Journal of Nutritional Biochemistry 21 (2010) 872–880
Journal of Nutritional Biochemistry

Folic acid consumption reduces resistin level and restores blunted acetylcholine-induced aortic relaxation in obese/diabetic mice☆

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Received 23 February 2009; received in revised form 17 June 2009; accepted 30 June 2009

Abstract

Folic acid supplementation provides beneficial effects on endothelial functions in patients with hyperhomocysteinemia. However, its effects on vascular functions under diabetic conditions are largely unknown. Therefore, the effect(s) of folic acid (5.7 and 71 μg/kg/day for 4 weeks) on aortic relaxation was investigated using obese/diabetic (*+db/+db*) mice and lean littermate (*+db/+m*) mice. Acetylcholine-induced relaxation in *+db/+db* mice was less than that observed in *+db/+m* mice. The reduced relaxation in *+db/+db* mice was restored by consumption of 71 μg/kg folic acid. Acetylcholine-induced relaxation (with and without folic acid treatment) was sensitive to N^G-nitro-L-arginine methyl ester, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one, geldanamycin and triciribine. In addition, acetylcholine-induced relaxation was attenuated by resistin. The plasma level of resistin in *+db/+db* mice was sevenfold higher than that measured in *+db/+m* mice, and the elevated plasma level of resistin in *+db/+db* mice was reduced by 25% after treatment with 71 μg/kg folic acid. Folic acid slightly increased the ratio of reduced glutathione to oxidized glutathione in *+db/+db* mice. Moreover, folic acid caused a reduction in PTEN (phosphatase and tensin homolog deleted on chromosome 10) expression, an increase in the phosphorylation of endothelial nitric oxide synthase ($eNOS^{Ser1177}$) and Akt^{Ser473}, and an enhanced interaction of heat shock protein 90 (HSP90) with eNOS in both strains, with greater magnitude observed in *+db/+db* mice. In conclusion, folic acid consumption improved blunted acetylcholine-induced relaxation in *+db/+db* mice. The mechanism may be, at least partly, attributed to enhancement of PI3K/HSP90/eNOS/Akt cascade, reduction in plasma resistin level, down-regulation of PTEN and slight modification of oxidative state.

Keywords: Folic acid; Relaxation; Acetylcholine; PTEN; eNOS; *+db/+db* mice

1. Introduction

Type 2 diabetes mellitus is one of the major risk factors for the development of cardiovascular diseases [1]. Alteration of vascular responsiveness to various vasoconstrictors/vasodilators is considered as one of the complications of diabetes mellitus. It has been demonstrated by our group, as well as by other groups, that blood vessels obtained from diabetic animal models exhibited impaired endothelium-dependent relaxation to acetylcholine [2,3], which may be important for the development of diabetes-mellitus-associated hypertension.

Previous studies have demonstrated that folic acid supplementation provides beneficial effects on endothelial functions in patients with hyperhomocysteinemia [4–6]. However, recent studies suggested that folic acid consumption could provide direct beneficial effects on endothelial functions beyond homocysteine-lowering properties [7,8]. Acute intravenous administration of 5-methyltetrahydrofolate (5-MTHF) improved endothelial functions via a reduction in intracellular endothelial superoxide formation without altering the homocysteine level in patients with coronary artery diseases [9]. In heterozygous cystathionine-β-synthase-deficient mice, folic acid (400 μg/70 kg/day and 5 mg/70 kg/day; regimens same as those used in our study) reversed endothelial dysfunction

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doi:10.1016/j.jnutbio.2009.06.015

via both homocysteine-dependent and homocysteine-independent manner [10]. In addition to direct superoxide-scavenging effects, 5-MTHF improves nitric oxide (NO)-mediated endothelium-dependent vascular responses by preventing peroxynitrite-mediated tetrahydrobiopterin (BH_4) oxidation and by improving endothelial nitric oxide synthase (eNOS) coupling in human isolated saphenous vein and internal mammary artery [11]. Interestingly, folic acid consumption (5 mg/day for 4 weeks) improved flow-mediated vasodilation in patients with coronary artery diseases by promoting eNOS dimerization [12].

Resistin, a 12.5-kDa cysteine-rich peptide secreted primarily from adipose tissue, is a unique signaling molecule that contributes to insulin resistance [13–15]. An increased plasma level of resistin in obese/diabetic individuals has been demonstrated [16]. However, a lowered protein expression of resistin was measured in white adipose tissues and in sera of different animal models of obesity (*ob*/*ob*, *+db*/*+db*, KKAy and high-fat-diet-induced obese mice) [17–20]. The reason for the apparent discrepancy in the resistin levels measured between different diabetic/obese animal models is unknown. In addition, medical/surgical weight loss improved endothelial functions in humans without a correlation to plasma resistin levels [21]. Nonetheless, acute administration of resistin (10 ng/ml) reduced bradykinin-induced dilation of isolated dog coronary artery [22]. In healthy human subjects, antioxidant supplementation (2 g/day vitamin C for 2 weeks) significantly reduced the plasma resistin level, with an increased level of reduced glutathione (GSH) [23]. However, the effects of folic acid, which also possesses antioxidant properties as reported in previous studies [24,25], on plasma resistin and GSH levels in *+db*/*+db* mice remain to be determined.

So far, most studies on the evaluation of the role(s) of endothelium dysfunction in diabetes-related cardiovascular problems have been performed using male animals. The effects of folic acid consumption on the cardiovascular functions of female species under diabetic conditions are largely unknown. In this study, we hypothesized that oral folic acid supplementation may provide beneficial effects on endothelial functions via modulation of Akt/heat shock protein 90 (HSP90)/eNOS cascade with a concomitant change in PTEN (phosphatase and tensin homolog deleted on chromosome 10) expression in endothelial cells and plasma levels of resistin and GSH in obese/diabetic (female; *+db*/*+db*) mice, which serve as an animal model of human type 2 diabetes mellitus.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents used in this study were obtained from Sigma-Aldrich (USA), unless otherwise stated. Resistin, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY 294002), geldanamycin and triciribine were purchased from Calbiochem-Novabiochem (USA).

2.2. Animals

Six-month-old female C57BL/KsJ mice [lean/nondiabetic (*+db*/*+m*) mice: 16.5±2.0 g; obese/diabetic (*+db*/*+db*) mice: 58.2±3.4 g], imported from Australia and subsequently bred in the Laboratory Animals House facilities of our university, were housed under a 12:12-h light–dark cycle (0700 h, on; 1900 h, off) and given standard rodent chow (folic acid, 7.9 ppm; Prolab RMH 2500, Australia) and water *ad libitum* before they were sacrificed. The diabetic (*+db*/*+db*) mice displayed typical phenotypes of obesity, hyperinsulinemia and hyperglycemia, as previously reported by our group [2]. All experiments performed in this study were approved by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong (approval nos. 04/054/MIS and 07/003/MIS). The use of experimental animals adhered to the recommendations of the Declaration of Helsinki and internationally accepted principles. Every effort was made to limit animal suffering and the number of animals used in these experiments. Mice were divided randomly into three groups: controls (fed an equal volume of distilled water), 5.7 μg/kg folic acid (equivalent to ~400 μg/70 kg, the daily intake for adults recommended by the US Food and Drug Administration) and 71 μg/kg folic acid (equivalent to a human dose of ~5 mg/70 kg). They were fed by oral gavage (using a feeding needle) daily for four consecutive weeks before they were sacrificed. There was no casualty or obvious sign of toxicity throughout the course of the experiments, and all mice involved survived.

2.3. Measurement of the isometric tension of isolated thoracic aorta

Thoracic aortas were dissected from animals immediately after sacrifice, and the isometric tension change of isolated aorta in response to drug challenge was measured, as previously described [2,26,27]. To exclude the involvement of cyclooxygenase and acetylcholinesterase, we included indomethacin (1 μM, a nonselective cyclooxygenase inhibitor) and neostigmine (10 μM, an acetylcholinesterase inhibitor) in the physiological salt solution throughout the experiments. Inhibitors [N^G-nitro-L-arginine methyl ester (L-NAME), resistin, LY 294002, geldanamycin and triciribine] were added to the organ bath 30 min before the administration of phenylephrine for active tone induction for the construction of the cumulative concentration–response curve by acetylcholine. The contractile force generated by phenylephrine was normalized to high K^+ (40 mM, which elicited similar magnitudes of aortic contraction in *+db*/*+m* and *+db*/*+db* mice) so as to adjust the concentration of phenylephrine (0.4–1 μM) used to elicit a comparable degree of contraction, as we have previously reported [2,27], under different conditions/manipulations (e.g., endothelium removal and addition of L-NAME, LY 294002, geldanamycin and triciribine) in both strains.

2.4. Plasma glucose and lipid profile measurements

During the course of oral folic acid supplementation, blood samples were collected from the mouse's tail vein once a week. Glucose and insulin levels were determined using commercially available kits, as reported previously [2]. At the end of the treatment period (i.e., 4 weeks), blood samples of individual mice were collected by heart puncture after sacrifice. The samples were immediately immersed in ice and centrifuged at 4°C. The serum collected was stored at −80°C before analysis. The serum levels of high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, total cholesterol and triglyceride were measured using the ALYCON system with Roche reagents.

2.5. Determination of plasma resistin and folate levels

Plasma resistin level was measured in triplicate using a commercially available enzyme-linked immunosorbent assay (ELISA) kit specific for mouse resistin (BioVendor Research and Diagnostic Products, Brno, Czech Republic), in accordance with the manufacturer's instructions. Plasma folate levels were measured in triplicate using a commercial assay (Beckman, USA).

2.6. Measurement of GSH and oxidized glutathione blood levels

An equal volume of chilled 2 M perchloric acid containing 4 mM EDTA was mixed with blood (0.2 ml) collected immediately after heart puncture. The collected mixture was centrifuged at 10,000×*g* for 15 min at 4°C, and acidic supernatant was collected for glutathione determination, as described previously [28]. Briefly, 0.2 ml of the acidic supernatant was neutralized with 0.1 ml of 2 M potassium hydroxide containing 3 M morpholinopropane sulfonic acid. The levels of GSH, oxidized glutathione (GSSG) and

Table 1
Effects of oral folic acid supplementation (5.7 and 71 μg/kg) on body weight, daily chow consumption and weight of the abdominal fat tissues of *+db*/*+m* and *+db*/*+db* mice

	+db/*+m* mice			*+db*/*+db* mice		
	Control	5.7 μg/kg folic acid	71 μg/kg folic acid	Control	5.7 μg/kg folic acid	71 μg/kg folic acid
Body weight (g)	16.5±2.0	14.3±1.7	17.2±2.2	58.2±3.4 *	51.4±1.2 **	49.2±2.0 **
Chow consumption (g)	1.1±0.1	1.0±0.1	1.1±0.2	1.1±0.1	1.2±0.1	1.1±0.1
Weight of abdominal fat tissues (g)	0.4±0.2	0.4±0.1	0.5±0.2	5.5±0.1 *	5.0±0.1 **	5.0±0.1 **

Data are expressed as mean±S.E.M.
* $P<.05$, *+db*/*+m* mice (control) versus *+db*/*+db* mice (control).
** $P<.05$, *+db*/*+db* mice: controls versus folic-acid-treated mice.

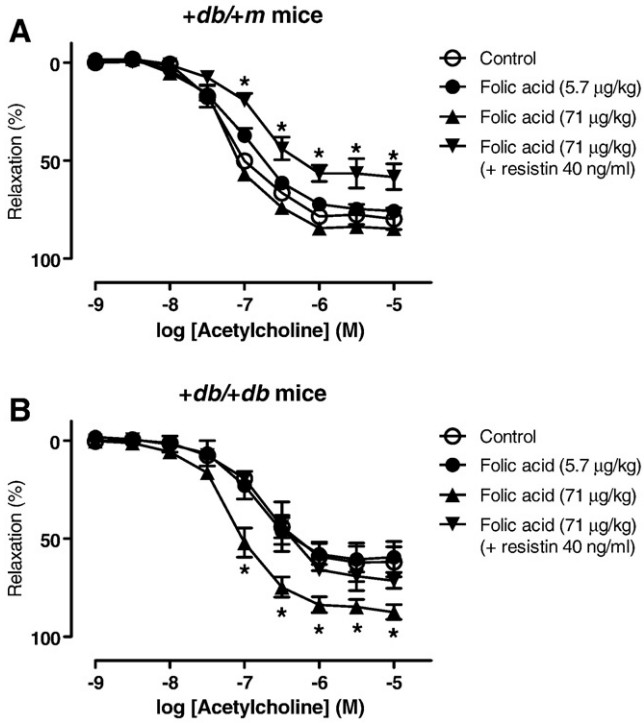

Fig. 1. Effects of oral folic acid supplementation (4 weeks) on the vasodilation response of isolated thoracic aorta (endothelium-intact). Acetylcholine-induced relaxation (in the presence of 10 μM neostigmine and 10 μM indomethacin) of the phenylephrine (1 μM)-precontracted isolated thoracic aorta of (A) *+db/+m* mice and (B) *+db/+db* mice. Control (○); folic acid [5.7 μg/kg (●); 71 μg/kg (▲)] (n=5–6); and folic acid (71 μg/kg) plus resistin (40 ng/ml) (▼) (n=4). (A) *P<.05, *+db/+db* mice treated with folic acid (71 μg/kg) (▲) versus controls (●, *+db/+db* mice). (B) *P<.05, *+db/+m* mice treated with folic acid (71 μg/kg) plus resistin (40 ng/ml) (▼) versus controls (○, *+db/+m* mice).

total glutathione (GSH plus GSSG) were determined using the glutathione reductase enzymatic assay and glyoxalase I method, as described previously [29].

2.7. Western blot analysis

Thoracic aortas (endothelium-intact) were homogenized in the presence of protease inhibitors to obtain protein extracts. Protein concentrations were determined using the BCA™ protein assay kit (Pierce, USA). Samples (25 μg of protein per lane) were loaded onto a 10% SDS polyacrylamide gel electrophoresis gel. After electrophoresis (90 V, 150 min), separated proteins were transferred (12 mA, 45 min) to polyvinylidene difluoride membrane (Bio-Rad, USA). Nonspecific binding sites were blocked with 5% nonfat dry milk for 120 min, and blots were then incubated with individual antibody type: anti-PTEN (1:1000; Cell Signalling, USA), anti-Akt (1:1000; Santa Cruz Biotechnology, USA), anti-phospho-Akt (Ser473) (1:1000; Santa Cruz Biotechnology), anti-eNOS (Cell Signalling), anti-caveolin-1(1:1000; Santa Cruz Biotechnology) and anti-phospho-eNOS (Ser1177) (1:1000; Cell Signalling), overnight at 4°C. Anti-goat horseradish-peroxidase-conjugated IgG (1:1000; Bio-Rad) or anti-rabbit horseradish-peroxidase-conjugated IgG (1:1000; Bio-Rad) was used to detect the binding of its correspondent antibody. In some experiments, endothelium was removed mechanically from the aorta before proteins were extracted. Western blot experiments were repeated for comparison. Membranes were stripped and reblotted with anti-β-actin antibody (1:8000; Sigma-Aldrich) to verify an equal loading of protein in each lane for a comparison of the expressions of proteins of interest. The protein expressions were detected with Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, USA) and quantified using the Scion Image (v. 6.03) analysis program. The optical density of each band was normalized to that of β-actin.

2.8. Immunoprecipitation of eNOS and HSP90

Three hundred micrograms of total protein was immunoprecipitated with 2 μg of anti-eNOS antibody (Cell Signalling) at 4°C for 6 h, followed by protein A-agarose beads (Sigma-Aldrich) at 4°C overnight. The immunoprecipitate was washed three times using lysis buffer containing protease inhibitors. The protein complex was then released from the agarose beads by adding 150 μl of Laemmli buffer and then boiled for 10 min. Forty micrograms of the protein complex was subjected to Western blot analysis using anti-HSP90 antibody (Santa Cruz Biotechnology), as described above. HSP90 recruitment was calculated as the densitometric value of HSP90 divided by the densitometric value of total eNOS.

2.9. Statistical analysis

Data are expressed as mean±S.E.M. n refers to the number of mice used for the experiments. Statistical comparisons were performed using analysis of variance (one-way or two-way, followed by *post hoc* Bonferroni's correction) or Student's t test, where appropriate. Difference was considered to be statistically significant at P<.05.

3. Results

A progressive decrease in the body weight of obese/diabetic (*+db/+db*) mice receiving folic acid treatment (5.7 μg/kg) was observed, resulting in a 10% reduction in body weight after 4 weeks. No further decrease in body weight was observed when the *+db/+db* mice received a higher dose of folic acid (i.e., 71 μg/kg) (n=24–26 for each group) (Table 1). In contrast, there was no change in the body weight of lean/nondiabetic (*+db/+m*) mice after folic acid treatments (5.7 and 71 μg/kg) (n=24–26 for each group) (Table 1).

Despite a decrease in the body weight of *+db/+db* mice after folic acid treatment, there was no apparent change in daily chow consumption throughout the course of folic acid treatment (Table 1). Folic acid consumption (5.7 and 71 μg/kg) caused no apparent change in the weight of abdominal (omental) fat tissues (a metabolically active fat depot) of *+db/+m* mice (Table 1). In contrast, the weight of abdominal fat tissues was reduced by about 9% in *+db/+db* mice after folic acid treatment (5.7 and 71 μg/kg) (Table 1).

Lower plasma folate levels were detected in control (drug-free) *+db/+db* mice (29.62±4.08 μg/L) compared to *+db/+m* mice (45.22±6.13 μg/L) (P<.01). The plasma levels of folate in mice were elevated after folic acid consumption [*+db/+m* mice: 5.7 μg/kg folic acid: 98.73±5.07 μg/L, 71 μg/kg folic acid:

Table 2
Effects of oral folic acid supplementation (5.7 and 71 μg/kg) on the blood biochemistry of *+db/+m* and *+db/+db* mice

	+db/+m mice			*+db/+db* mice		
	Control	5.7 μg/kg folic acid	71 μg/kg folic acid	Control	5.7 μg/kg folic acid	71 μg/kg folic acid
Fasting insulin (pmol/L)	32±3	29±1	31±2	767±40***	754±66	737±51
Fasting glucose (mmol/L)	5.0±0.3	5.3±0.7	5.1±0.3	16.1±1.7***	15.9±2.0	15.2±1.5
Total cholesterol (mmol/L)	2.02±0.11	1.98±0.05	2.01±0.09	3.38±0.15*	3.44±0.18	3.24±0.09
HDL cholesterol (mmol/L)	1.98±0.05	1.76±0.12	2.01±0.13	1.12±0.11*	0.93±0.07	0.98±0.12
LDL cholesterol (mmol/L)	0.68±0.12	0.72±0.08	0.75±0.16	1.26±0.18*	1.14±0.13	1.31±0.23
Triglyceride (mmol/L)	1.86±0.21	1.76±0.11	1.65±0.23	4.46±0.21*	4.11±0.27	3.21±0.28**

Data are expressed as mean±S.E.M.
* P<.05, *+db/+m* mice (control) versus *+db/+db* mice (control).
** P<.05, *+db/+db* mice: control versus folic-acid-treated (71 μg/kg) mice.
*** P<.001, *+db/+m* mice (control) versus *+db/+db* mice (control).

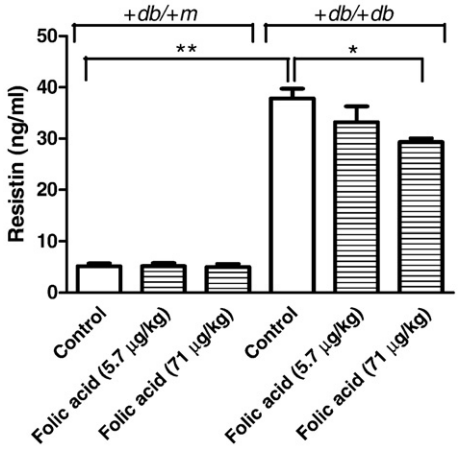

Fig. 2. Effects of oral folic acid supplementation (4 weeks) on plasma resistin levels. Mice (*+db/+m* and *+db/+db*) (control, □) were orally fed folic acid (5.7 μg/kg and 71 μg/kg, ▤), and plasma resistin level was measured using an ELISA kit specific for mouse resistin. **P<.01, *+db/+m* mice (control) versus *+db/+db* mice (control). *P<.05 versus controls (n=5–6).

181.46±7.12 μg/L; *+db/+db* mice: 5.7 μg/kg folic acid: 56.32±6.03 μg/L, 71 μg/kg folic acid: 122.37±9.54 μg/L; P<.01 compared to the respective controls].

In phenylephrine (1 μM)-preconstricted aortic preparation, cumulative administration of acetylcholine [with 10 μM neostigmine (an anti-cholinesterase) and 10 μM indomethacin] caused a concentration (10 nM–10 μM)-dependent relaxation in *+db/+m* and *+db/+db* mice, with maximum relaxation observed at 10 μM (Fig. 1A and B) (n=5–6). In contrast, no significant change (<5%) in phenylephrine-mediated precontractile tone was observed in time-matched controls of both strains (data not shown). When compared with *+db/+m* mice, a smaller magnitude of acetylcholine-elicited relaxation plus a decrease in sensitivity to acetylcholine were observed in *+db/+db* mice (IC_{50}: *+db/+m* vs. *+db/+db* mice: 70.61±2.23 vs. 175.8± 3.60 nM) (Fig. 1B). Interestingly, in *+db/+db* mice receiving high-dose (71 μg/kg) but not low-dose (5.7 μg/kg) folic acid, blunted acetylcholine-induced aortic relaxation could be restored (Fig. 1B), and the magnitude of relaxation was similar to that observed in controls (*+db/+m* mice) (Fig. 1B). In contrast to *+db/+db* mice, folic acid consumption (5.7 and 71 μg/kg) did not modify acetylcholine-induced relaxation in *+db/+m* mice (Fig. 1A).

The acetylcholine-induced relaxation observed in *+db/+m* and *+db/+db* mice was suppressed by resistin (40 ng/ml, 30-min incubation) (n=4 for each group) (Fig. 1A and B). In addition, the acetylcholine-induced relaxation observed in *+db/+m* and *+db/+db* mice was abolished (<6% relaxation was observed at 10 μM acetylcholine) by L-NAME (20 μM) (n=5). In mice fed folic acid (5.7 and 71 μg/kg), the acetylcholine-elicited aortic relaxation of both

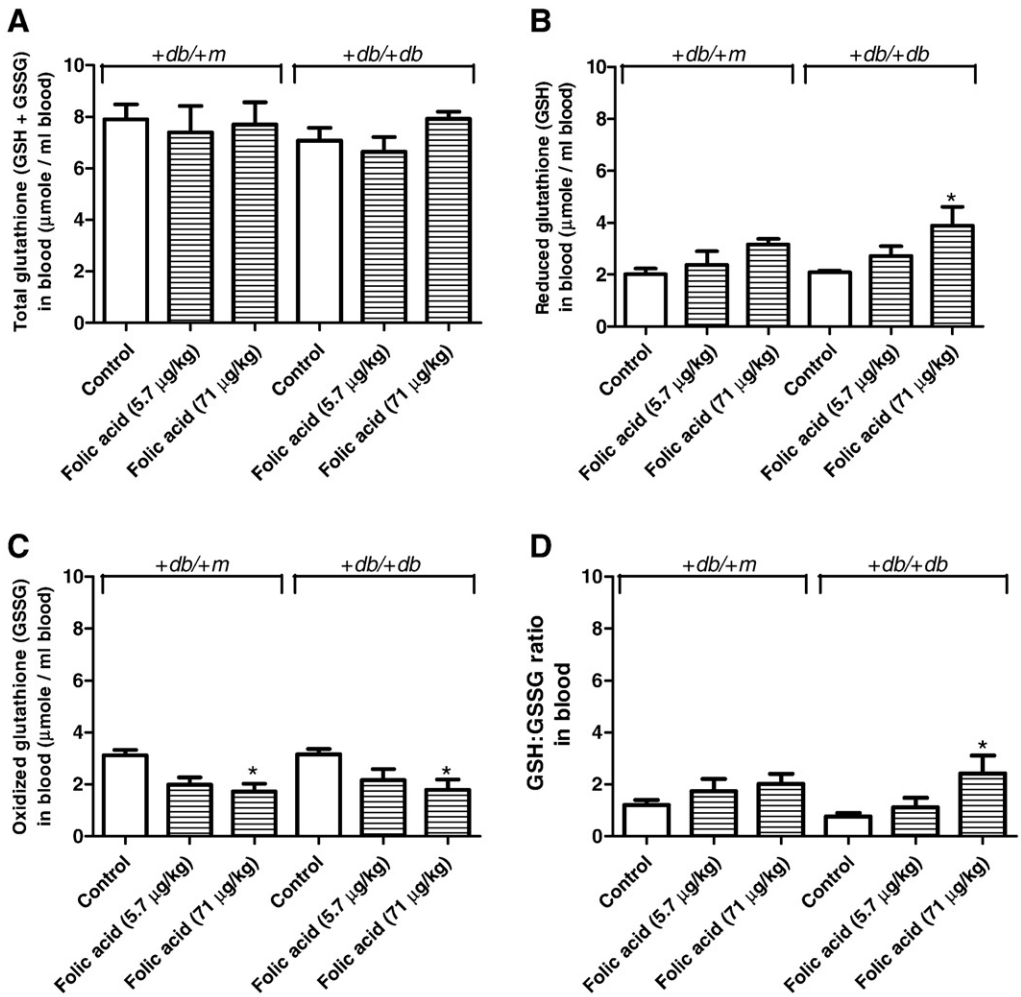

Fig. 3. Effects of oral folic acid supplementation (4 weeks) on (A) total glutathione level, (B) GSH level, (C) GSSG level and (D) GSH/GSSG ratio in the blood. Mice (*+db/+m* and *+db/+db*) (control, □) were orally fed folic acid (5.7 and 71 μg/kg, ▤). Results are expressed as mean±S.E.M. *P<.05 versus controls (n=4–6).

strains was equally sensitive to L-NAME, and there was <5% relaxation elicited by 10 μM acetylcholine (data not shown).

The acetylcholine-elicited relaxation in *+db/+db* and *+db/+m* mice was abolished by LY 294002 (1 μM, a PI3K inhibitor), geldanamycin (1 μg/ml, a specific inhibitor of HSP90) and triciribine (1 μM, an Akt inhibitor) (n=4; data not shown). All blockers used in this study, applied alone, did not alter the baseline tension of the preparations.

The plasma glucose and insulin levels measured in *+db/+db* mice were higher than those recorded in *+db/+m* mice (Table 2). However, folic acid supplementation (5.7 and 71 μg/kg) did not alter the plasma glucose and insulin levels (n=5–6 for each group) (Table 2).

In controls (i.e., without folic acid supplementation), the levels of total cholesterol and LDL cholesterol measured in *+db/+db* mice were higher than those measured in *+db/+m* mice (P<.05) (Table 2). In contrast, the HDL cholesterol level measured in *+db/+db* was lower than that measured in *+db/+m* mice (P<.01) (Table 2). There was no significant change in total cholesterol, HDL cholesterol and LDL cholesterol in both strains after folic acid treatment (Table 2).

The triglyceride level measured in *+db/+db* mice was higher than that measured in *+db/+m* mice (Table 2). A significant decrease in triglyceride level was observed in *+db/+db* mice receiving 71 μg/kg folic acid (P<.05 vs. controls) (Table 2). There was a trend of reduction in triglyceride levels in *+db/+m* mice after folic acid supplementation, but the change was statistically insignificant (Table 2) compared to controls.

The plasma resistin level measured in *+db/+db* mice was approximately sevenfold higher than that detected in *+db/+m* mice (37.82±2.17 and 5.12±0.87 ng/ml for *+db/+db* and *+db/+m* mice, respectively; P<.01). Interestingly, high-dose folic acid (71 μg/kg) caused a significant reduction in resistin level measured in *+db/+db* mice from 37.82±2.17 to 29.31±0.91 ng/ml (P<.05). However, the plasma resistin level measured in *+db/+m* mice remained at a relatively constant level after folic acid consumption (Fig. 2).

The total (reduced and oxidized) glutathione levels (i.e., GSH and GSSG) measured in the blood of control *+db/+db* and *+db/+m* mice were similar, and folic acid consumption did not alter the GSH and GSSG levels (Fig. 3A). GSH and GSSG levels were estimated from the total glutathione content (Fig. 3B and C), and folic acid (5.7 μg/kg) did not alter the GSH and GSSG levels in both strains. In addition, folic acid (71 μg/kg) did not alter GSH levels, but decreased GSSG levels measured in *+db/+m* mice. Nevertheless, the change in GSH/GSSG ratio was statistically insignificant (Fig. 3D). In contrast, folic acid (71 μg/kg) increased GSH levels and decreased GSSG levels in *+db/+db* mice, with a GSH/GSSG ratio higher than that recorded in controls (P<.05) (Fig. 3D).

The protein expression level of caveolin-1 in aortic homogenates of *+db/+db* mice was higher than that detected in *+db/+m* mice (P<.001) (n=6) (Fig. 4). However, folic acid consumption (5.7 and 71 μg/kg) caused no apparent change in the protein expression level of caveolin-1 measured in both strains (n=6) (Fig. 4).

In contrast to caveolin-1, the basal protein expression levels of PTEN in aortic homogenates were similar in both strains (Fig. 5A). Folic acid consumption caused a dose-dependent reduction in the protein expression of PTEN measured in both *+db/+db* and *+db/+m* mice (Fig. 5A). However, the effect of folic acid on the reduction in PTEN protein expression was greater in *+db/+db* mice than in *+db/+m* mice (41% vs. 21%) (Fig. 5A). No PTEN was detected in the endothelium-denuded aorta lysates of both strains (n=4; data not shown).

To evaluate the involvement(s) of Akt/eNOS cascade and the possible activation of individual component, we measured the protein expressions of Akt, p-Akt, eNOS and p-eNOS of aortic homogenates. Activation of Akt and eNOS was represented by the amount of

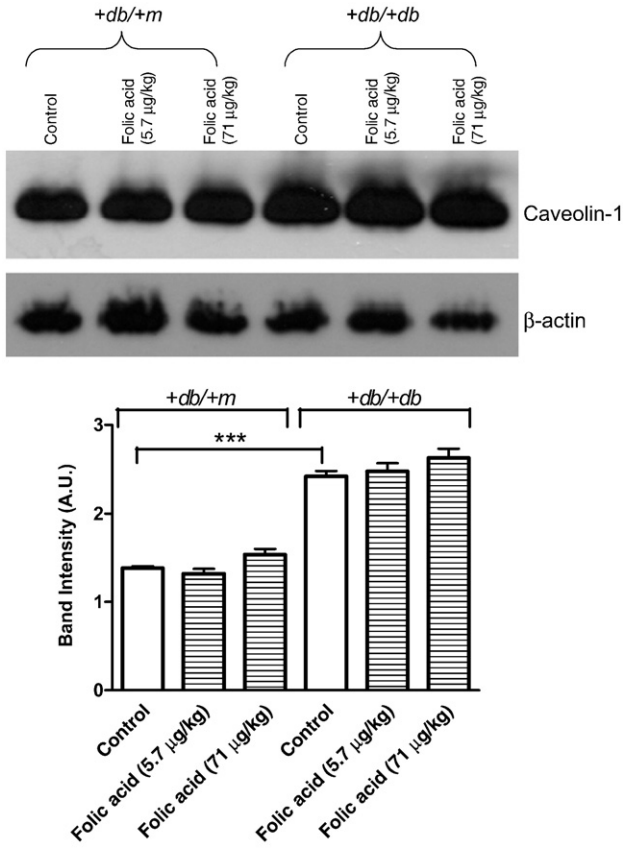

Fig. 4. Effects of oral folic acid supplementation (4 weeks) on the protein expression of caveolin-1. Mice (*+db/+m* and *+db/+db* mice) (control, □) were orally fed folic acid (5.7 and 71 μg/kg, ▭). Individual protein expression of the thoracic aorta (endothelium-intact) was determined using Western blot analysis. Results are expressed as the mean (in arbitrary units; normalized to β-actin) ±S.E.M of four independent experiments. ***P<.01, *+db/+m* mice (control) versus *+db/+db* mice (control).

phosphorylated forms of Akt and eNOS (i.e., p-Akt and p-eNOS). Folic acid consumption caused a dose-dependent increase in p-eNOS and p-Akt expressions in both strains (Fig. 5B and C), with no apparent changes in eNOS and Akt expression (Fig. 5B and C). However, folic acid consumption caused a greater increase in p-eNOS and p-Akt expressions in *+db/+db* mice (eNOS: 56% increase in *+db/+m* mice and 107% increase in *+db/+db* mice; p-Akt: 49% increase in *+db/+m* mice and 79% increase in *+db/+db* mice) (Fig. 5B and C). No eNOS and Akt were detected in the endothelium-denuded aorta lysates of both strains (n=4; data not shown).

The effect of oral folic acid supplementation on eNOS/HSP90 interaction was evaluated using immunoprecipitation methods. Our results revealed that folic acid consumption caused a dose-dependent increase in eNOS/HSP90 interaction in the aortic homogenates of both strains (Fig. 6). However, folic acid (71 μg/kg) caused a greater increase in eNOS/HSP90 interaction in *+db/+db* mice (51% increase in *+db/+m* mice and 94% increase in *+db/+db* mice) (Fig. 6).

4. Discussion

Herein we illustrate that folic acid consumption [71 μg/kg/day, which is equivalent to ~5 mg/day adult (70 kg) consumption, for 4 weeks] improved the blunted acetylcholine-induced aortic relaxation in female obese/diabetic (*+db/+db*) mice. Our results are consistent with a previous study [10] in which folic acid (i.e., 5 mg/70 kg/day) provided a more significant effect, compared to a low dose of folic acid, on reversing the endothelial dysfunction of the mesenteric

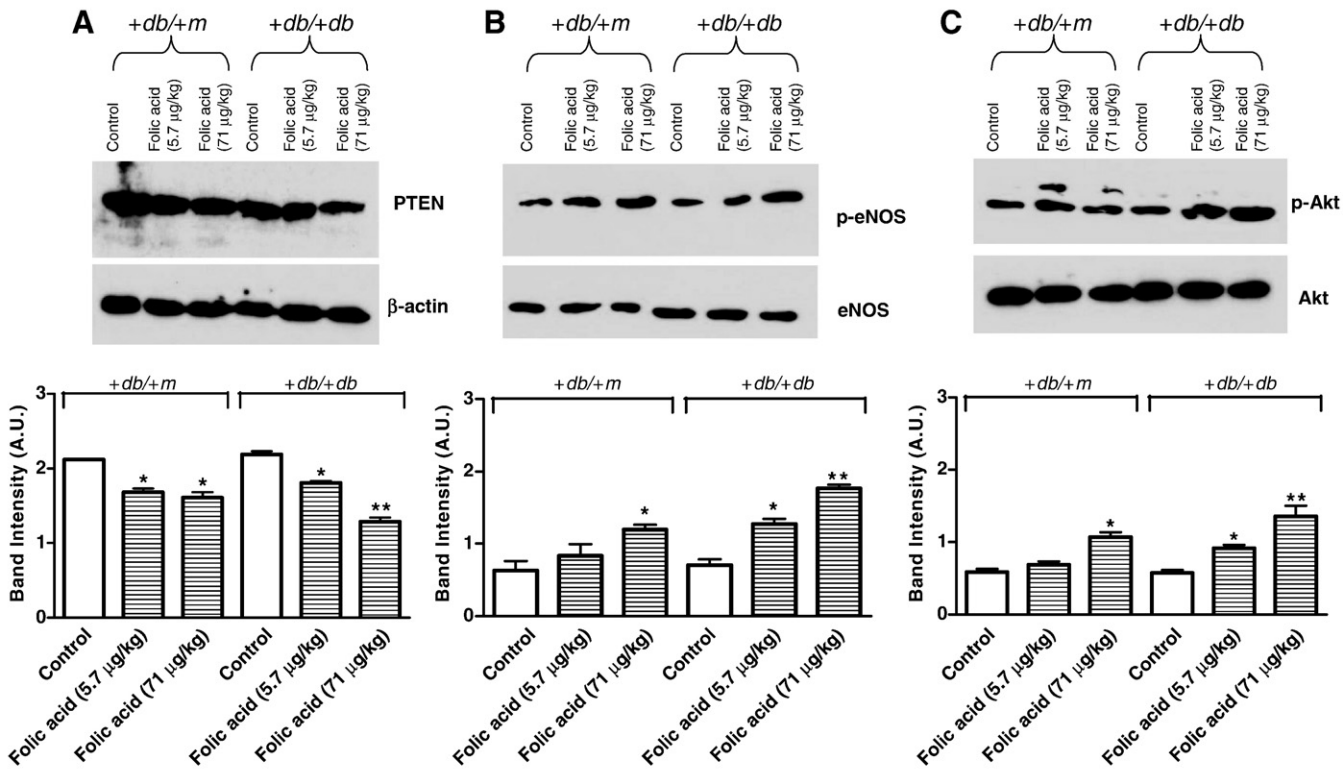

Fig. 5. Effects of oral folic acid supplementation (4 weeks) on the protein expression of (A) PTEN, (B) p-eNOS and eNOS, and (C) p-Akt and Akt. Mice (*+db/+m* and *+db/+db* mice) (control, □) were orally fed folic acid (5.7 and 71 μg/kg, ▤). The individual protein expression of the thoracic aorta (endothelium-intact) was determined using Western blot analysis. Results are expressed as the mean (in arbitrary units; normalized to β-actin) ±S.E.M of four independent experiments. (A) *P<.05 and **P<.01 versus controls. (B) *P<.05 and **P<.01 versus controls. (C) *P<.05 and **P<.01 versus controls. (D) *P<.05 and **P<.01 versus controls. (E) *P<.05 versus controls.

artery of heterozygous cystathionine-β-synthase-deficient mice. More importantly, in our study, the blunted relaxation observed in *+db/+db* mice after the administration of 71 μg/kg/day (for 4 weeks) folic acid was restored to a magnitude similar to that observed in *+db/+m* mice. It has been reported that folic acid supplementation (5 mg/day for 6 weeks) improves the flow-mediated dilation of the brachial artery of patients with coronary artery disease [12]. In contrast to Clark et al. [10], eradication of the acetylcholine-elicited relaxation of both strains by L-NAME (an eNOS inhibitor) suggests that NO released from endothelial cells is solely responsible for the acetylcholine-induced relaxation observed in our study.

PTEN is a lipid/protein phosphatase that influences NO release by serving as a negative regulator of the PI3K/Akt signaling pathway [30,31]. In human aortic endothelial cells, palmitic acid causes an up-regulation of PTEN activity and transcription, which leads to inactivation of eNOS via the p38/Akt signaling pathway [32]. In addition, Akt-mediated eNOS activation of human aortic endothelial cells was inhibited by human cytomegalovirus via up-regulation of PTEN [33]. The expression of PTEN in the tibialis anterior muscle was up-regulated in mice under conditions of chronic diabetic/insulin resistance, suggesting the participation of PTEN [34]. Consistent with a previous report in which a similar PTEN protein expression was detected in the isolated aorta of deoxycorticosterone acetate salt hypertensive and sham rats [35], our study clearly illustrates that the protein expression levels of PTEN detected in *+db/+m* and *+db/+db* mice were similar. Nonetheless, folic acid supplementation resulted in a significant reduction in PTEN expression in the isolated aorta, and a greater magnitude of suppression was observed in *+db/+db* mice. As mentioned above, the down-regulation of PTEN protein expression observed is probably correlated with, or responsible for, enhanced Akt and eNOS activation after folic acid treatment.

The activities of eNOS in the endothelial cells of rat and dog were markedly suppressed under diabetic/high-glucose conditions, which are related to the impairment of HSP90/eNOS interaction [36,37]. In our study, folic acid consumption increased eNOS activity (as reflected by an increased level of p-eNOS), probably via enhanced Akt phosphorylation (i.e., p-Akt) and greater magnitude of the HSP90/eNOS interaction. The eradication of acetylcholine-induced relaxation in both *+db/+db* and *+db/+m* mice by LY 294002 (a selective PI3K inhibitor), geldanamycin (an HSP90 inhibitor) and triciribine (a selective Akt inhibitor) further strengthened our conclusions on the obligatory role of PI3K/HSP90/eNOS/Akt cascade. We have previously reported that the impaired acetylcholine-induced aortic relaxation observed in *+db/+db* mice is probably related to the enhanced protein expression of caveolin-1 [2]. In addition, atorvastatin increases eNOS activation and NO production in bovine aortic endothelial cells via down-regulation of caveolin-1 and enhanced HSP90/eNOS interaction [38]. However, in our study, folic acid consumption did not alter the caveolin-1 protein expression of both strains.

Impaired NO-mediated relaxation is related to an increased generation of reactive oxygen species, which reduce the bioavailability of NO [39]. In this study, folic acid consumption (71 μg/kg) slightly increased the blood levels of GSH (a strong physiological antioxidant) and the GSH/GSSG ratio estimated in *+db/+db* mice. Such a small increase in antioxidant activity may contribute minimally to normalization (caused by folic acid consumption) of the blunted acetylcholine-induced relaxation observed in *+db/+db* mice.

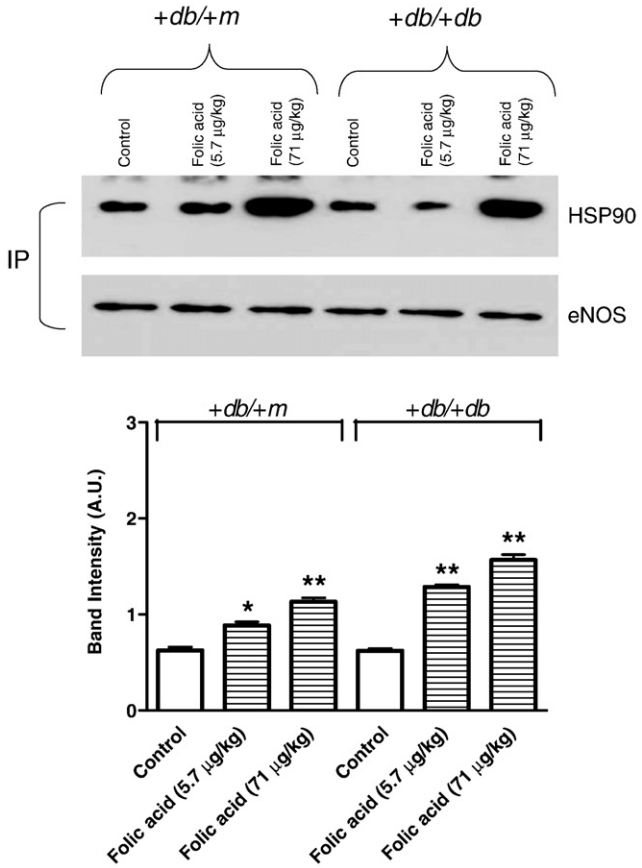

Fig. 6. Effects of oral folic acid supplementation (4 weeks) on HSP90/eNOS interaction. Mice (*+db/+m* and *+db/+db*) (control, □) were orally fed folic acid (5.7 and 71 μg/kg, ▭), and HSP90/eNOS protein interaction in the thoracic aorta (endothelium-intact) was determined by immunoprecipitation (IP) methods (n=4). Results are expressed as the mean (in arbitrary units, normalized to β-actin) ±S.E.M of four independent experiments. *P<.05 and **P<.01 versus controls.

Interestingly, folic acid does not have a good antioxidant effect (as measured by cell-free 1,1-diphenyl-2- picrylhydrazyl assay; unpublished data). In addition, plasma folate levels detected in *+db/+db* mice were significantly lower than those observed in *+m/+db* mice before folic acid consumption. Folic acid supplementation managed to elevate plasma folate levels in both species. However, the reduced folate levels detected in *+db/+db* mice failed to reach levels the same as or similar to those observed in *+m/+db* mice after folic acid supplementation. Thus, it is tempting to speculate that the antioxidant effects of folic acid treatment are probably related to folate formed *in vivo*. This notion is supported by a previous observation that 5-MTHF (the active form/metabolite of folic acid) enhanced eNOS activity and NO production in immortalized mouse brain endothelial cells [40].

Previous studies have demonstrated that the expression of resistin, a polypeptide secreted from adipose tissues [41,42], is enhanced/up-regulated in obese/diabetic patients and animal models [16]. Consistent with previous studies [43,44], the plasma resistin level measured in *+db/+db* mice was ~7-fold higher than that detected in *+db/+m* mice. It has been reported that an elevated resistin level inhibited basal and insulin-stimulated Akt phosphorylation/eNOS activation via an up-regulation of PTEN expression in cultured human aortic endothelial cells [45]. There was also an increase in insulin signaling cascade in the liver and a reduction in serum resistin level in PTEN-deleted mutant mice [46]. However, our study revealed that, although the resistin level measured in *+db/+db* mice was higher than that detected in *+db/+m* mice, the basal expressions of PTEN, p-eNOS and p-Akt estimated were similar in both strains. It implies that, at least in our system, resistin level may not be significantly correlated to PTEN expression and NOS/Akt activation. Nonetheless, our study clearly demonstrates that resistin can influence vascular response because, when applied acutely, it suppressed acetylcholine-induced relaxation in both *+db/+m* and *+db/+db* mice. Our results are in line with a report indicating that the bradykinin-induced *in vitro* dilation of canine coronary artery is inhibited by resistin applied acutely [22].

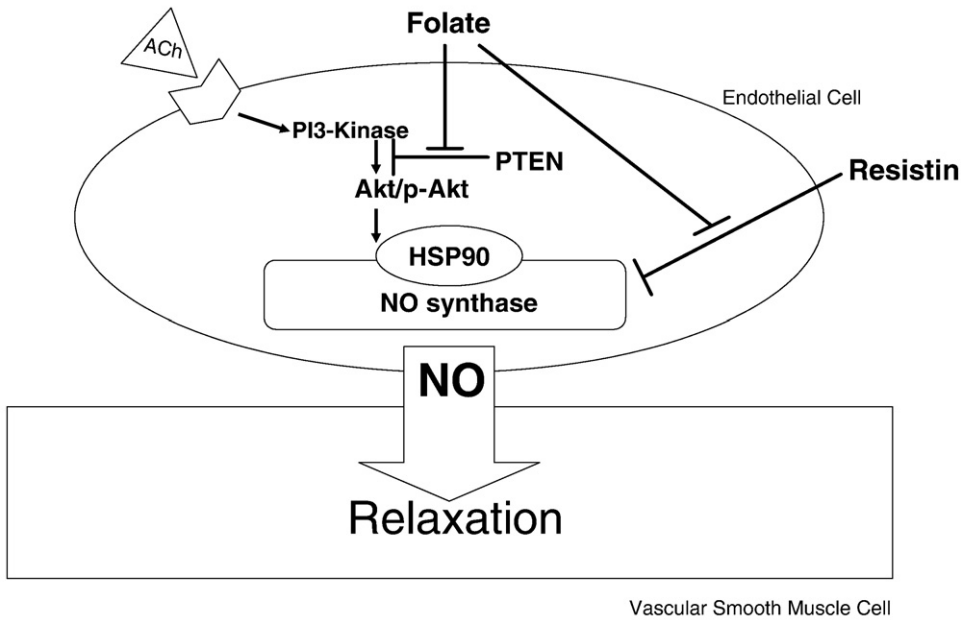

Fig. 7. A schematic illustration showing the major events occurring in the endothelial cells and vascular smooth muscle cells of the aorta of mice fed folic acid.

In conclusion, oral folic acid supplementation restored the blunted acetylcholine-induced aortic relaxation observed in *+db/+db* mice, probably via enhancement of the activity of PI3K/HSP90/eNOS/Akt cascade. In addition, folic acid consumption caused a greater down-regulation of the PTEN protein expression of the endothelium, as well as a greater reduction in plasma resistin level (Fig. 7) plus a slight improvement of antioxidative status in *+db/+db* mice. All these effects offered by folic acid consumption may probably make it a beneficial addition to the treatment of vascular disorders/complications associated with diabetes mellitus.

Acknowledgements

We are grateful to the Li Ka Shing Institute of Health Sciences and the Institute of Vascular Medicine (Faculty of Medicine, The Chinese University of Hong Kong) for financial support (to Y.W. Kwan). This project was financially supported by the RGC Earmarked Grants of Hong Kong SAR, People's Republic of China (reference nos. 4107/01M and 4166/02M, project code 2140565), and Direct Grants for Research (The Chinese University of Hong Kong) (reference no. 2401149; project codes 2041231 and 2401296). Mr. S.W. Seto, Ms. T.Y. Lam, Ms. Alice L.S. Au and Mr. Wayne Y.W. Lee are recipients of postgraduate studentships from the Department of Pharmacology (The Chinese University of Hong Kong). We thank Ms. Jian Hong Wu (State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University) for technical assistance in measuring cholesterol and lipoproteins levels. Provision of the Student Campus Work Scheme by the Chou's Foundation Fund and the Student Campus Work Scheme (Shaw College, The Chinese University of Hong Kong) is also appreciated. Proofreading of the manuscript by Mr. Ho Yeung Lam is also acknowledged.

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