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The administration of folic acid reduces intravascular oxidative stress in diabetic rabbits

Nilima Shukla, Gianni D. Angelini, Jamie Y. Jeremy*
Department of Cardiac Surgery, Bristol Royal Infirmary, Bristol, UK
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Abstract

There is evidence that plasma homocysteine augments angiopathy in patients with diabetes mellitus. Although lowering homocysteine with folic acid improves endothelial function, the precise mechanisms underlying this effect are unknown. To study this area further, the effect of administration of folic acid to diabetic rabbits on intraaortic oxidative stress was studied by assessing the formation of superoxide (O_2^-), 8-isoprostane $F_{2\alpha}$ (8-$IPF_{2\alpha}$), and prostacyclin (as 6-keto-$PGF_{1\alpha}$) as well as acetylcholine-stimulated relaxation and $gp47^{phox}$ content. Nonketotic diabetes mellitus was induced in New Zealand rabbits with alloxan, and low- and high-dose folic acid was administered daily for 1 month. Rabbits were killed, aortae were excised, and rings were prepared. Rings were mounted in an organ bath, and relaxation was elicited with acetylcholine. The O_2^- release was measured spectrophotometrically; the $gp47^{phox}$ expression, by Western blotting; and the 8-$IPF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ formation, by enzyme-linked immunosorbent assay. Blood was collected for measurement of homocysteine, red blood cell folate, and glucose. In aortae from the diabetic rabbits, acetylcholine-induced relaxation was significantly impaired compared with that in untreated controls. The O_2^- release, $p47^{phox}$ expression, and 8-$IPF_{2\alpha}$ formation were all enhanced and 6-keto-$PGF_{1\alpha}$ formation was reduced compared with controls. All these effects were reversed by both low- and high-dose folic acid. Plasma total homocysteine was reduced by high-dose, but not low-dose, folic acid. Red blood cell folate was elevated in both groups. The improvement of endothelial function in patients receiving folic acid may be due to inhibition of nicotinamide adenine nucleotide phosphate oxidase (NADPH) oxidase expression and therefore conservation of nitric oxide and prostacyclin bioavailability, 2 vasculoprotective factors.

1. Introduction

Although there is evidence that elevated levels of plasma homocysteine augment diabetic angiopathy [1], the etiology and indeed importance of this association to diabetic angiopathy are unclear. However, the intravascular formation of superoxide (O_2^-) has been implicated in the etiology of angiopathy associated with both diabetes mellitus (DM) [2] and hyperhomocysteinemia (HHC) [3,4]. Among its many vasculopathic effects, O_2^- reacts with nitric oxide ($NO^\cdot$) to form reactive nitrogen species that reduces $NO^\cdot$ bioavailability [5-7]. Because $NO^\cdot$ provides vasculoprotection [8], this interaction has been deemed important in the etiology of diabetic angiopathy [7]. Increased O_2^- formation and a concomitant decrease in $NO^\cdot$ formation by vascular tissue from animal models of both DM [9,10] and HHC [11,12], as well as in patients with DM [13,14] and HHC [15,16], are well documented. The overproduction of O_2^- in both DM and HHC has been ascribed, in part, to the intravascular overexpression of nicotinamide adenine nucleotide phosphate oxidase (NADPH) oxidase [2,11,12].

The administration of folic acid, which lowers plasma homocysteine [17], improves endothelial function in patients with both DM and HHC [18-20]. The therapeutic action of folic acid has been interpreted, in part, as being due to lowering of plasma homocysteine [17]. Indeed, a reduction of plasma total homocysteine by 3 μmol (ie, 20%-30%) by folic acid is associated with a significant reduction in cardiovascular episodes including myocardial infarction and stroke [17], although this has been contested by other reports [21,22]. Folic acid and its active metabolite, 5-methyltetrahydrofolate, also elicit effects that are independent of homocysteine [23-25]. These include protection against

* Corresponding author. Department of Cardiac Surgery, Bristol Heart Institute, Bristol Royal Infirmary, BS2 8HW Bristol, UK. Tel.: +44 0117 928 2699.
E-mail address: j.y.jeremy@bris.ac.uk (J.Y. Jeremy).

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endothelial NO synthase (eNOS) uncoupling, conservation of tetrahydrobiopterin, and inhibition of O_2^- formation [23-25]. Apart from effects on forearm blood flow, however, little is known of the in vivo mechanisms underlying the therapeutic effects of folic acid.

To clarify this area, therefore, the effect of oral administration of folic acid to rabbits rendered diabetic with alloxan on intravascular oxidative stress was studied. Both low and high doses of folic acid were studied in an attempt to dissect out homocysteine-lowering effects from direct actions of folic acid. Indices of oxidative stress were acetylcholine-elicited relaxation (NO-dependent), superoxide formation, and expression of $p47^{phox}$, a key subunit of the NADPH oxidase complex. 8-Isoprostane (8-IP) formation was also measured because 8-IP is formed by reactive oxygen species and as such is a marker of oxidative stress and is an independent risk factor for vascular disease [26]. 8-Isoprostane also promotes intravascular oxidative stress through an up-regulation of NAPDH oxidase expression [27]. By contrast, prostacyclin (PGI_2) has diametrically opposite effects to 8-IP; in particular, it is a potent inhibitor of NADPH oxidase expression [27]. Furthermore, arterial PGI_2 formation is reduced by oxidative stress [27]; and reduced vascular PGI_2 formation has long been associated with DM [28]. Thus, relative formation of PGI_2 and 8-IP was also investigated. The roles of the endothelium and the media of the aorta in mediating effects were determined by comparing denuded with intact aortic tissue.

2. Methods

2.1. Induction of diabetes, dosing, and sample collection

The principles of laboratory care were adhered to according to National Institutes of Health and UK Home Office Animals Care regulations. Ethical approval for the study had been obtained from the local Committee at the University of Bristol. Male New Zealand white rabbits (3 kg) were injected intravenously with alloxan (Sigma Chemical, Poole Dorset, United Kingdom) via the lateral ear vein at a stat dose of 65 mg/kg [11,29]; and age-matched controls, with saline alone. The diabetic rabbits were fed ad libitum with SDS standard rabbit chow (SDS, Whitham, United Kingdom) and allowed free access to water. Blood was sampled at sacrifice for plasma glucose. Urine was also monitored over the duration of diabetes for glucose, ketone bodies, and proteins with Multistix (Ames Division, Miles Laboratories, Stoke Poges, Buckinghamshire, United Kingdom) as previously described [11,29]. Any animals displaying ketonuria or proteinuria were excluded from the study. Folic acid was administered by dissolving the appropriate amount in drinking water. Diabetic rabbits are markedly polyuric and polydypsic [29]. To adjust the folic acid content of water, the rabbits' intake of water was measured over 1 week before dosing with folic acid. This stabilized within 1 week and did not increase thereafter. Water was changed daily. Doses of folic acid were based on what would be considered to be high and low dose in man, that is, 0.5 mg/3 kg per day and 0.1 mg/3 kg per day. Blood samples were anticoagulated with EDTA (final concentration, 5 mmol/L) and centrifuged at 2000*g* for 10 minutes at 4°C. Whole blood was collected for red blood cell (RBC) folate. Plasma was aspirated; and total homocysteine content was analyzed using reverse-phase high-pressure liquid chromatography with fluorescence detection, and RBC folate concentrations were measured by immunoassay by the Department of Chemical Pathology at the Bristol Royal Infirmary [30].

2.2. Organ bath experiments

After 4 weeks, rabbits were killed by intravenous injection of pentobarbitone (Euthatal 100 mg/kg, Merial Health Ltd., Marlow, Essex, UK) via the lateral ear vein. Thoracic aortae were removed and placed in cold Dulbecco modified Eagle medium (GIBCO BRL Life Technologies, Paisley, Scotland, United Kingdom). Adventitia was removed, and 2-mm aortic rings were prepared and mounted in organ baths for isometric tension studies [11,29]. The rings were mounted vertically in 20-mL chambers, equipped with 2 parallel platinum electrodes, containing Kreb Ringer bicarbonate buffer (KRB) with the following composition (millimoles per liter in distilled water): 119 NaCl, 4.7 KCl, 1.17 $MgSO_4 \cdot 7H_2O$, 1.18 KH_2PO_4, 2.5 $NaHCO_3$, 2.5 $CaCl_2$, 5 glucose, maintained at 37°C by a thermoregulated circuit. Rings were suspended between 2 tissue bearers, one in fixed position and the other attached to a force-displacement transducer; and data were recorded using a MacLab (AD Instruments, Chalgrove, Oxfordshire, UK) [11]. The KRB was gassed with a mixture of 95% O_2–5% CO_2 maintained at pH 7.4. An initial tension of 2 g was applied to the suspended tissue strips and rings equilibrated for 1 hour with frequent changes of KRB. After equilibration, tissues were precontracted with phenylephrine (100 μmol/L) and then relaxed with cumulative doses of acetylcholine (0.01-10 μmol/L). To determine the possible effects on systems downstream of NO release at the medial levels, the endothelium-independent vasodilator, sodium nitroprusside (0.01-10 μmol/L), was also studied [11,12,29]. Relaxation responses were expressed as a percentage inhibition of phenylephrine-induced contraction.

2.3. Superoxide formation

The measurement of superoxide release by aortic rings was performed by detection of ferricytochrome *c* reduction [31]. Aortic rings (with and without endothelium; removed by rubbing with a cotton bud) were equilibrated in Dulbecco modified Eagle medium without phenol red for 10 minutes at 37°C in a 95% air–5% CO_2 incubator (Heraeus, Hera Cell; Kandro Laboratory Products, Langenselbold, Germany). Twenty micromoles per liter of horseradish cytochrome *c* (Sigma Chemical) with or without 500 U/mL copper-zinc superoxide dismutase (Sigma Chemical) was added to the segments and incubated at 37°C in a 95% air–5% CO_2 incubator for 1 hour. The final volume of the reaction mixture was 0.5 mL per well. The reaction medium was then removed,

and optical density was measured by spectrophotometry at 550 nm and converted to nanomoles of cytochrome *c* reduced superoxide using the molar extinction $\Delta E_{550\ nm} = 21.1$ (mmol/L)$^{-1}$·cm^{-1}. The reduction of cytochrome *c* that was inhibitable with superoxide dismutase reflected actual superoxide release. Segments were blotted, dried, and weighed, data being expressed as nanomoles of superoxide per milligram tissue per hour. To determine the source of the O_2^-, aortic rings were incubated with 100μmol/L apocynin (Sigma), 100 μmol/L diphenylene iodonium chloride (DPI) (Sigma; NADPH oxidase inhibitor), 100 μmol/L rotenone (Sigma; an inhibitor of mitochondrial respiration), or 100 μmol/L allopurinol (Sigma; an inhibitor of xanthine oxidase), and 500 μmol/L L-nitroarginine methyl ester (inhibitor of NOS) for 1 hour before measurement of O_2^- [31]. A previous study had demonstrated that these concentrations of inhibitors were adequate in reducing the activity of the target systems [31].

2.4. Western analysis of $p47^{phox}$

Aortic segments were frozen in liquid nitrogen, crushed in a pestle and mortar, and suspended in 50 mmol/L Tris buffer (pH 7.4) containing 1% vol/vol Triton X-100, EDTA (10 mmol/L), phenylmethylsulfonyl fluoride (1 mmol/L), pepstatin (0.05 mmol/L), and leupeptin (0.2 mmol/L). Samples were initially centrifuged at 3000 rpm for 30 minutes, and supernatants were recentrifuged at 20 000*g* for 2 hours at 4°C. Supernatants were then boiled at a 1:1 ratio with 50 mmol/L Tris, pH 6.8, containing 4% wt/vol sodium dodecyl sulfate, 10% vol/vol glycerol, 4% vol/vol 2-mercaptoethanol, and 2 mg/mL bromophenol blue. Samples of equal protein (100 μg) were loaded onto 12% Tris-glycine sodium dodecyl sulfate gels and separated by electrophoresis. After transfer to nitrocellulose, the blots were primed with monoclonal antibody 48 at 2.5 μg/mL final concentration and, as a control for loading, antiactin mouse monoclonal antibody at 1:250 (Cedarlanem, Ontario, Canada). The blots were then incubated with goat anti–mouse antibody conjugated to horseradish peroxidase (1:2000 dilution) and developed by enhanced chemiluminescence (Amersham International, Oxfordshire, UK). Rainbow markers (14-220 kd, Amersham) were used for molecular weight determination. Bands were quantified with a BioRad GS-690 densitometer and Molecular Analyst Software (BioRad, Hemel Hempstead, Herts, UK) [11,12,31].

2.5. Eicosanoid formation

Aortic rings were prepared using a scalpel blade and incubated at 37°C for 6 hours to allow preparative release of eicosanoids to subside [32,33]. One ring in triplicate for each animal was then incubated with A23187, which elicits formation though activation of phospholipase A_2. In one group, the endothelium was removed; and in another, measurements were made with intact endothelium. Rings were then incubated at 37°C for 1 hour, the supernatants were removed, and PGI_2 (as 6-keto-$PGF_{1\alpha}$) and 8-isoprostane $F_{2\alpha}$ (8-$IPF_{2\alpha}$) were measured using enzyme-linked immunoassay kits (Alexis, Bingham, Notts, UK) [31]. Data were related to amount of eicosanoid generated per milligram tissue per hour.

2.6. Data analysis and statistics

Data were collated and analyzed using Microsoft Excel (Microsoft, Redmond, WA), and nonparametric statistical analysis was carried out using an Intercooled Stata 8 statistics package (Stata, College Station, TX). Using the Kruskal-Wallis test, it was established that the data on aortic functions were all normally distributed; and as such, parametric statistics was deemed applicable. All measurements represent the mean of means. Quadruplicate measurements were made for each parameter per animal, and the mean was calculated. Each data point is expressed as mean ± SEM (n = 6). Paired comparisons between 2 groups was performed using paired Student *t* test where analysis of variance indicated significance for the multiple comparison. Statistical significance was accepted when $P < .05$. For plasma and serum measurements, the Bartlett test for equality of variance (a necessary assumption for a 1-way analysis of variance) was significant, indicating that nonparametric methods of analysis were required. Thus, values are expressed as median and ranges; and Mann-Whitney *U* tests were applied to test the statistical significance between control and treated groups.

3. Results

The starting weights in both the control and diabetic rabbit groups were similar (control: median, 3.0 kg; range, 2.9-3.1; n = 18; diabetic: median, 3.1; range, 2.9-3.2; n = 18). At 4 weeks after commencement of the study, there were no significant alterations of weight between any of the groups. Plasma glucose concentrations (nonfasting) were increased in the 1-month diabetic group compared with the control group (Table 1). Plasma homocysteine concentrations were not statistically different between controls and diabetic rabbits after 1 month (Table 1). After administration of folic

Table 1
Blood levels of glucose (millimoles per liter), total homocysteine (micromoles per liter), and RBC folate (milligrams per deciliter) 4 weeks after onset of study

	Plasma glucose	Plasma total homocysteine	RBC folate
Untreated control	6.9 (6.3-7.4)	12.5 (9.7-13.3)	39 (32-54)
Untreated diabetic	27.9 (19.4-44.2)	10.9 (6.7-12.3)	33 (23-49)
Control low folic	6.3 (6.3-7.4)	10.2 (6.4-13.8)	78 (36-95)*
Control high folic	6.6 (5.8-7.2)	9.1 (7.1-11.3)*	210 (110-340)*
Diabetic low folic	27.9 (23.6-39.2)	9.7 (8.7-11.3)	88 (52-109)*
Diabetic high folate	27.9 (19.4-36.2)	8.4 (6.7-13.3)*	189 (121-306)*

Data are expressed as median (range); n = 6 animals.

* $P < .05$, comparing treated controls with untreated controls or untreated diabetic with treated diabetics.

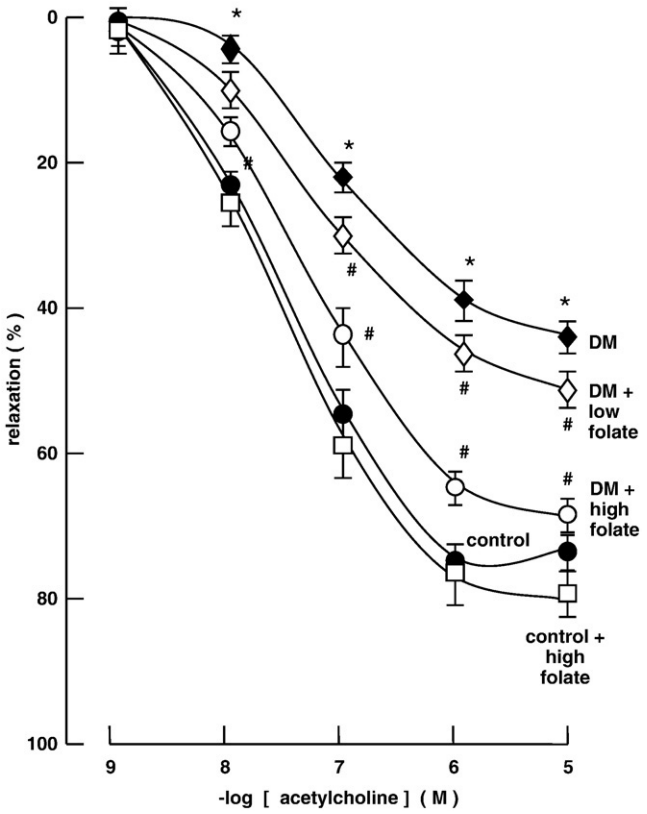

Fig. 1. Effect of folic acid administration to diabetic rabbits on acetylcholine-stimulated aortic relaxation: controls (●); diabetic (♦); diabetic + low-dose folic acid (◇); diabetic + high-dose folic acid (○); control + high-dose folic acid (□). Each point = mean ± SEM, n = 6 rabbits. *$P < .05$, untreated controls vs untreated diabetics. #$P < .05$, treated diabetics vs untreated diabetics.

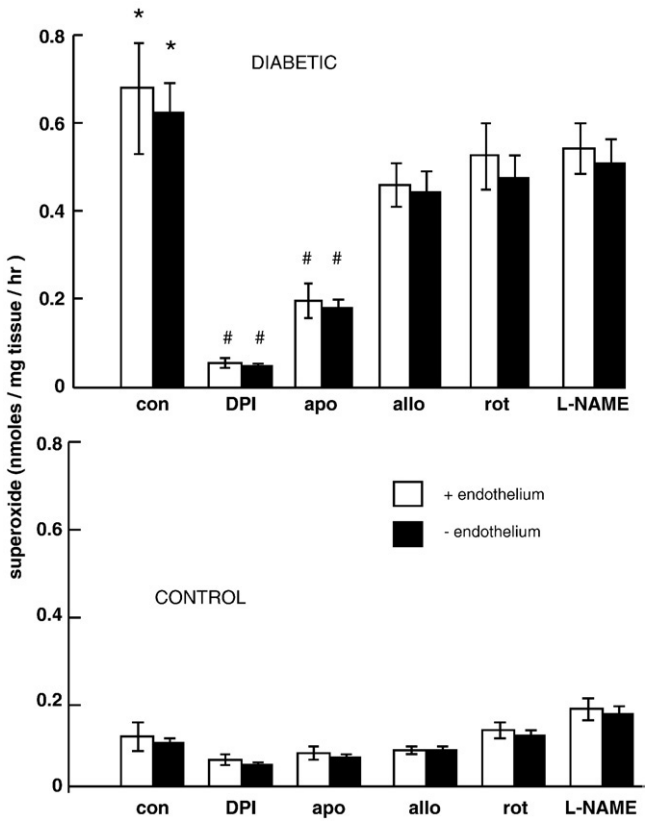

Fig. 2. Superoxide formation by isolated aorta (with and without endothelium) from diabetic and control rabbits and effect of 100 μmol/L apocynin and 100 μmol/L DPI (NADPH oxidase inhibitors), 100 μmol/L allopurinol (xanthine oxidase inhibitor), 100 μmol/L rotenone (inhibitor of mitochondrial electron transport chain), and 500 μmol/L L-nitroarginine methyl ester (NOS inhibitor). Each point = mean ± SEM, n = 6 animals. *$P < .05$, comparing untreated controls to untreated diabetics. #$P < .05$, significantly reduced compared with untreated diabetic controls. Apo indicates apocynin; allo, allopurinol; rot, rotenone; L-NAME; L-nitroarginine methyl ester; con, control.

acid to both control and diabetic groups, plasma total homocysteine was significantly reduced in the high-dose folic acid group but not the low-dose group (Table 1). Folic acid was significantly elevated in all animals whose diet was supplemented with folic acid, whereas plasma glucose was unaffected (Table 1). The RBC folate was increased in rabbits receiving both low-dose and high-dose folic acid (Table 1).

After 1 month, in aortae from the DM group, Ach-induced relaxation was significantly impaired in the untreated diabetic group compared with that in controls (Fig. 1), which was significantly reversed by folic acid administration and to a greater degree by high-dose as compared with low-dose folic acid (Fig. 1). By contrast, there were no significant differences in relaxation elicited by sodium nitroprusside between aortae from any of the groups (Tables 1 and 2), indicating that alterations downstream of NOS (eg, guanylyl cyclase) were unaffected by DM.

Table 2
Effect of folic acid administration to diabetic rabbits on sodium nitroprusside–stimulated aortic relaxation (percentage)

	Sodium nitroprusside (mol/L)					
	1×10^{-8}	3×10^{-8}	1×10^{-7}	3×10^{-7}	1×10^{-6}	3×10^{-6}
Untreated control	2 ± 0.3	6 ± 0.3	15 ± 1.5	50 ± 5	82 ± 18	95 ± 17
Untreated diabetic	0.5 ± 0.02	3 ± 0.1	11 ± 1.2	44 ± 4	78 ± 15	87 ± 16
Control low folic	3 ± 0.2	5 ± 0.1	14 ± 1.5	53 ± 7	88 ± 18	98 ± 16
Control high folic	4 ± 0.4	6 ± 0.2	18 ± 1.3	49 ± 5	89 ± 18	90 ± 7
Diabetic low folic	1 ± 0.2	5 ± 0.1	14 ± 1.4	48 ± 8	77 ± 14	88 ± 6
Diabetic high folate	3 ± 0.3	4 ± 0.1	16 ± 1.4	50 ± 4	78 ± 15	91 ± 9

Each point = mean ± SEM, n = 6 rabbits. No significant changes were determined.

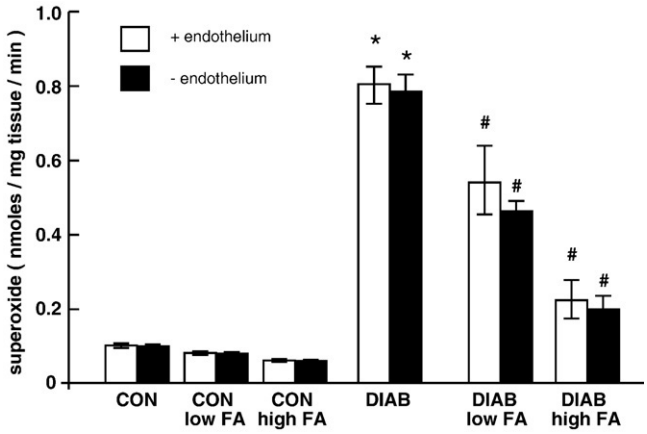

Fig. 3. Effect of folic acid administration on superoxide formation by isolated aorta (with and without endothelium) from diabetic and control rabbits. Each point = mean ± SEM, n = 6 rabbits. *$P < .05$, untreated controls vs untreated diabetics. #$P < .05$, treated diabetics vs untreated diabetics. FA indicates folic acid; DIAB, diabetic.

The O_2^- formation was significantly enhanced in aortic rings from diabetic rabbits compared with controls (Fig. 2). In both cases, removal of the endothelium had no significant effect, indicating that the principal source of O_2^- is the medial region of the artery (Fig. 2). In turn, O_2^- release was significantly inhibited by apocynin and DPI (both NADPH oxidase inhibitors) but not by rotenone, allopurinol, or NOS,

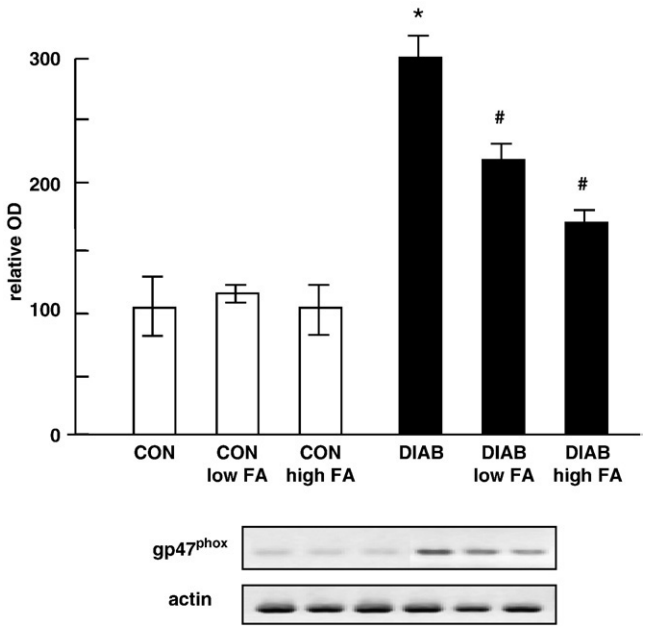

Fig. 4. Effect of folic acid administration on aortic gp47^{phox} expression in control and diabetic rabbits. The lower panel shows the representative blots including actin (loading control); and the upper panel shows the results of the densitometric analyses of 6 blots, expressed as relative optical per square millimeter. Each point = mean ± SEM, n = 6 rabbits. *$P < .05$, untreated controls vs untreated diabetics. #$P < .05$, treated diabetics vs untreated diabetics. OD indicates optical density.

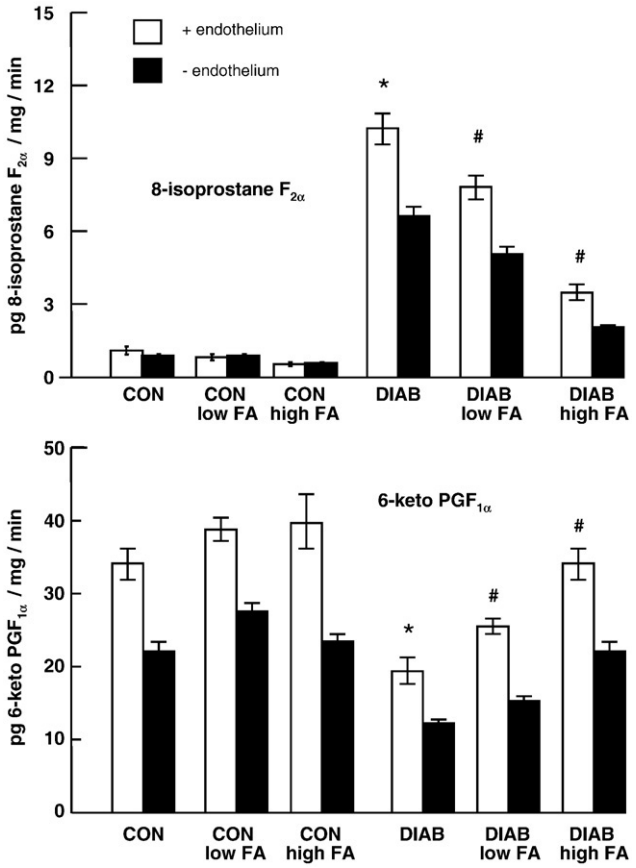

Fig. 5. Effect of folic acid administration on the formation of 8-IPF$_{2\alpha}$ and PGI$_2$ (as 6-keto-PGF$_{1\alpha}$) by isolated aorta (with and without endothelium) from diabetic and control rabbits. Each point = mean ± SEM, n = 6 rabbits. *$P < .05$, untreated controls vs untreated diabetics. #$P < .05$, treated diabetics vs untreated diabetics.

consolidating that DM promotes induction of NADPH oxidase and that this is the principal source of O_2^-. The administration of both low- and high-dose folic acid reduced aortic O_2^- release from both diabetic and control rabbits (Fig. 3). Aortic gp47^{phox} protein content was significantly increased in untreated diabetic group compared with untreated controls (Fig. 4), an effect reduced by the administration of both low- and high-dose folic acid (Fig. 4). Aortic 8-IP formation was decreased in untreated diabetic group compared with untreated controls (Fig. 5), an effect reduced by the administration of both low -and high-dose folic acid (Fig. 5). In contrast, aortic PGI$_2$ formation (as 6-keto-PGF$_{1\alpha}$) was markedly enhanced in untreated diabetic group compared with untreated controls, an effect reversed by the administration of both low- and high-dose folic acid (Fig. 5).

4. Discussion

The present study firstly confirms that experimental DM in the rabbit induces the intraarterial overproduction of O_2^- and reduces acetylcholine-stimulated (NO-dependent)

relaxation, which is consistent with previous reports [34-36]. Because O_2^- formation was blocked by NADPH oxidase inhibitors and the level of aortic NADPH oxidase subunit 47^{phox} was augmented, it is concluded that the principal source of O_2^- in this diabetic model is NADPH oxidase, which is again consistent with previous reports [34-37]. The media, rather than the endothelium, is the main source of the O_2^-. Thus, in this model of type 2 DM, O_2^- derived from NADPH oxidase located in the media reduces NO bioavailability at the endothelial level and therefore reduces relaxation of aortae from diabetic rabbits. Because NO derived from eNOS also inhibits NADPH oxidase expression and activity in the media of arteries [38], it follows that the reduction of NO may further augment NADPH oxidase expression. In turn, because NO is vasculoprotective by inhibiting events such as vascular smooth muscle cell proliferation, adhesion molecule expression, and thrombosis, the overexpression of NADPH oxidase may contribute to diabetic angiopathy.

In the present study, aortic 8-$IPF_{2\alpha}$ formation was also increased by DM. 8-Isoprostane is also formed principally by the medial region of the artery because removal of endothelium had little effect on overall release of the eicosanoid. 8-Isoprostane is formed by reactions of reactive oxygen species with arachidonic acid [26]. 8-Isoprostane has similar biological effects as TXA_2 in that it activates TXA_2 receptors, augments platelet aggregation, and constricts arteries [26]. Furthermore, 8-IP up-regulates the expression of NADPH oxidase and promotes O_2^- formation in vascular cells [27]. Thus, 8-IP formation may augment oxidative stress through a self-perpetuating positive feedback loop. By contrast, PGI_2 formation, which has diametrically opposite effects to both TXA_2 and 8-IP [27], was markedly reduced in aortae from diabetic rabbits, a well-established consequence of DM [28]. Furthermore, O_2^- derived from NADPH oxidase reduces PGI_2 formation but promotes both TXA_2 and 8-IP formation [27], possibly by negation of PGI_2 synthase activity by nitration [28]. Iloprost (an analogue of PGI_2), in direct contrast to 8-IP and TXA_2, is a potent inhibitor of NADPH oxidase expression [27]. Thus, a reduction of PGI_2 and concomitant up-regulation of 8-IP may further augment the expression of NADPH oxidase.

The present study demonstrates that the administration of folic acid at doses that would be considered therapeutically effective in man [17] reduces intravascular oxidative stress in the diabetic rabbit. Both low- and high-dose folic acid reduced O_2^- formation, reduced 47^{phox} protein levels, reversed impairment of NO-mediated relaxation, reduced 8-$IPF_{2\alpha}$ formation, and prevented the reduction of PGI_2 formation in aortae from diabetic rabbits. Folic acid reduced plasma homocysteine levels, but only significantly at the higher dose of folic acid, indicating that folic acid independently improves endothelial function in this model rather than a reduction of homocysteine per se.

The therapeutic action of folic acid has largely been interpreted as being mediated by a lowering of plasma homocysteine, indicating that homocysteine is independently vasculopathic [17]. However, several in vitro studies have now demonstrated that millimolar homocysteine is required to elicit direct vascular effects, indicating that homocysteine alone is not independently angiopathic [39-41]. It is important to note that in the present study plasma homocysteine was not elevated in diabetic animals and in fact was reduced. This is in consistent with some reports that plasma homocysteine is reduced in the diabetic patient [42]. This indicates further that the effects of folic acid is not mediated by a lowering of homocysteine alone. It is also notable that a previous study demonstrated that homocysteine, at physiological levels, augmented the impairment of endothelial function in aortae from diabetic rabbits, indicating that the plasma levels of homocysteine need not be elevated for them to have a vasculopathic effect in DM [11].

By contrast, the active metabolite of folic acid, 3-methyltetrahydrofolate, has potent biological effects that are independent of homocysteine [23-25]. In particular, 3-methyltetrahydrofolate protects against eNOS uncoupling, conserves tetrahydrobiopterin bioactivity, and inhibits superoxide formation [43]. Low-dose and high-dose folate elicits beneficial effects independently of homocysteine in both hyperhomocysteinemic mouse [23] and in cardiovascular disease patients [24]. Our data are consistent with these studies and confirm that folic acid and/or its active metabolite elicits direct antioxidative effects, including the suppression of NADPH oxidase expression.

Controversy continues unabated as to whether folic acid is therapeutically useful in preventing cardiovascular disease per se [20-22]. In a key study, it has been demonstrated that folic acid administration to type 2 DM patients reduces homocysteine levels but has no effect on other markers of low-grade inflammation [44] This latter study is of relevance because de Jager et al [45] reported that endothelial dysfunction and low-grade inflammation together, but not independently, explain the increased cardiovascular mortality in type 2 DM patients. Several diverse strategies have been shown to reduce low-grade inflammation. These include conventional pharmacological agents, such as nonsteroidal anti-inflammatory drugs, glitazones, statins, and angiotensin-converting enzyme inhibitors, as well as diet and exercise [46-49]. Thus, given the evidence that folic acid improves endothelial health, then adjunct therapy with other drugs that reduce low-grade inflammation may prove more effective than folic acid alone.

In conclusion, the present study demonstrates that folic acid administration has an emphatic inhibitory effect on intravascular oxidative stress associated with DM and conserves NO bioactivity. These effects may not be due to a lowering of homocysteine but to a primary effect of folic acid and its active metabolite(s) on the preservation of eNOS integrity. This has a secondary effect of preventing the increased up-regulation of NADPH oxidase and therefore the generation of superoxide and increased isoprostane

formation coupled with decreased PGI_2 formation. These effects may be of relevance to the future use of folic acid to treat diabetic angiopathies.

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