Homocysteine enhances impairment of endothelium-dependent relaxation and guanosine cyclic monophosphate formation in aortae from diabetic rabbits

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Abstract

Aims/hypothesis. Both diabetes mellitus and hyperhomocysteinaemia are risk factors for cardiovascular disease and are associated with impaired endothelial nitric oxide and with excess superoxide formation. To explore potential vasculopathic interactions between these risk factors, the effect of homocysteine on endothelium-dependent relaxation and cyclic GMP formation was investigated in aortae from diabetic rabbits.

Methods. Rabbits were rendered diabetic by intravenous injection of alloxan. Six months later, the aortae were excised, cut into rings and mounted in an organ bath for isometric measurement of acetylcholine-evoked relaxation in rings pre-contracted with phenylephrine. Cyclic GMP formation by aortic rings after stimulation with acetylcholine, calcium ionophore A23187 and sodium nitroprusside was assessed using radioimmunoassay. The effect of homocysteine on these parameters was then studied.

Results. Ach-evoked relaxation and cyclic GMP formation induced with acetylcholine and calcium ionophore A23187 were impaired in aortae from diabetic rabbits compared with the control rabbits, effects that were reversed with superoxide dismutase (SOD) and augmented by 10–100 $\mu\text{mol/l}$ homocysteine, an effect again reversed by SOD.

Conclusion/interpretation. These data show that the bioavailability of nitric oxide is reduced in aortae from diabetic rabbits due to excess production of superoxide, an effect augmented by homocysteine. These results indicate that patients with diabetes mellitus could be susceptible to homocysteine-mediated angiopathy at lower concentrations than those that promote vasculopathy in non-diabetic patients. [Diabetologia (2002) 45:1325–1331]

Keywords Diabetes, homocysteine, superoxide, nitric oxide, angiopathy.

Both diabetes mellitus and hyperhomocysteinaemia (HH) are risk factors for cardiovascular disease (CVD) [1, 2] and are associated with impaired endothelial nitric oxide and with excess superoxide (O_2^-)

formation [3, 4, 5, 6, 7, 8, 9, 10, 11]. Clinically, there are now a number of studies indicating that homocysteine (Hcy) potentiates CVD in diabetic patients [2]. The underlying mechanisms that assess the vasculopathic impact of HH and diabetes mellitus, both alone or interactively, are not completely understood. It is now established, however, that Hcy promotes oxidative stress, including superoxide formation which could be mediated by Hcy-mediated vasculopathy [3, 4, 5]. Superoxide elicits a number of pro-atherogenic effects including endothelial dysfunction, oxidation of lipids and the promotion of vascular smooth muscle cell (VSMC) proliferation [5]. Superoxide also reacts readily with nitric oxide to produce peroxynitrite

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Abbreviations: HH, Hyperhomocysteinaemia; O_2^- , superoxide; SOD, superoxide dismutase; VSMC, vascular smooth muscle cell; Hcy, homocysteine; ONOO, peroxynitrite

(ONOO), thereby reducing the bioavailability of nitric oxide [12]. In turn, impaired endothelium nitric oxide dependent relaxation of arteries in patients with HH has been reported [13, 14]. Diminished NO formation by Hcy in laboratory animals and in vitro systems is well documented [3, 4, 5, 15, 16]. Since reduced nitric oxide formation is associated with vascular disease [5, 6], the pathogenic impact of Hcy could be mediated, in part, through an O_2^- -mediated reduction of NO bioavailability [4, 5]. Similarly, diabetes mellitus is also associated with impaired endothelial function and reduced nitric oxide formation as well as increased O_2^- generation by the vasculature [7, 8, 9, 10, 11].

It is reasonable to propose, therefore, that the potentiation of diabetic angiopathy by Hcy could be mediated by a common denominator mechanism: i.e. the impairment of nitric oxide formation by the overproduction of O_2^- . In order to test this proposal we studied the effect of exogenous Hcy on endothelium-dependent relaxation and cyclic GMP formation (index of endothelial nitric oxide bioactivity) in aortae from hyperglycaemic, non-ketotic, diabetic rabbits.

Materials and methods

Drugs and materials. Acetylcholine, alloxan, calcium ionophore A23187, catalase, DL-homocysteine, indomethacin, isobutylmethylxanthine, phenylephrine, sodium nitroprusside, CuZn superoxide dismutase and all buffer components were purchased from Sigma Chemical (Poole, Dorset, UK). Dual range [^{125}I] cGMP kits were purchased from Amersham Radiochemicals (Amersham, UK). All animals received humane care according to the Home Office Animal Care regulations (UK), for which ethics committee approval and licensing had been obtained.

Induction of diabetes. Male New Zealand white rabbits (3 kg) matched for age were injected intravenously with alloxan (Sigma Chemical, Poole Dorset) via the lateral ear vein at a stat dose of 65 mg/kg [17]. The diabetic rabbits were fed ad libitum with SDS standard rabbit chow (SDS, Whitham, UK) and allowed free access to water. Blood was sampled at monthly intervals for serum urea and electrolytes, cholesterol, triglycerides and glucose by the routine section of the Department of Clinical Chemistry, Royal Free Hospital, London. Urine was also monitored over the duration of diabetes for glucose, ketone bodies and proteins with Multistix (Ames Division, Miles Laboratories, Stoke Poges, Buckinghamshire, UK).

Organ bath studies. The rabbits were killed by cervical dislocation 6 months after the induction of diabetes mellitus and the aortae were rapidly excised and placed in cold oxygenated Krebs solution (KRB). Adventitial tissue was removed and the aortae were cut into 2 mm rings and mounted vertically in 15 ml capacity organ attached to Grass FTO3 force displacement transducer and data recorded on disc using MacLab. In each experiment, four rings were set up simultaneously. An initial tension of 2 g was applied to the suspended tissue strips, which were then equilibrated for 30 min, after which time tension was reset. The experiment commenced after a further equilibration time of 30 min. DL-Hcy ($\pm$ SOD) were then added to the organ bath chamber at different concentrations and

left to equilibrate for 30 min. Contraction was then elicited with phenylephrine (PE; 1–3 μ mol/l), the concentration of PE being adjusted to give the same level of tension (2 g) in all rings. The rings were then relaxed with a cumulative concentrations of the endothelium vasodilator, acetylcholine (ACh; 0.01–10 μ mol/l) or the endothelium-independent vasodilator, sodium nitroprusside (SNP; 0.001–10 μ mol/l).

Cyclic GMP formation. Aortae from the control rabbits and diabetic rabbits were cut into 2 mm segments as described. Rings were then placed in polypropylene tubes containing 250 μ mol/l of the phosphodiesterase (PDE) inhibitor, isobutylmethylxanthine (IBMX) in KRB and various concentrations of Hcy and/or CuZnSOD or catalase. After 30 min incubation at 37°C, cGMP formation was stimulated with: (i) acetylcholine (generates nitric oxide through receptor mediated increase in Ca^{2+} and NOS activation), (ii) calcium ionophore A23187 (activates nitric oxide synthase through an increase of cytosolic Ca^{2+}) and (iii) sodium nitroprusside (SNP: activates guanylyl cyclase directly). Tubes were incubated for a further 20 min at 37°C. Reactions were stopped by the addition of 1 mol/l perchloric acid and the tissues sonicated (3 $\times$ 30 s; Soniprep, MSE, Bucks, UK). After centrifugation at 1000 g for 15 min, supernatants were taken and neutralised with 1 mol/l K_3PO_4 . Aliquots were then taken and acetylated with triethylamine/acetic anhydride (1/2, v/v) and diluted with PBS (pH 7.4). To these cGMP standards (0–256 fmoles) 200 μ l diluted antisera against cGMP antisera containing [^{125}I] cGMP was added. After overnight incubation at 4°C, antisera against rabbit globulins in phosphate buffer was added to each tube and incubated for 15 min on melting ice. Tubes were then centrifuged at 2500 rpm for 10 min. Supernatants were decanted into vials and scintillation fluid added and counted in a gamma particle counter. Standard curves were compiled and unknown values calculated.

Data analysis and statistics. Comparisons of weights, plasma glucose and plasma lipids between the 6-month diabetic groups and the age-matched controls were carried out using the Wilcoxon test (paired values). For the cGMP measurements data are related to mg tissue per min (wet weight). Each data point is expressed as mean $\pm$ standard error of the mean (SEM). Responses of isolated aortic rings to ACh and SNP are expressed as the percentage of relaxation of PE-induced contraction, with maximal relaxation being arbitrarily set at basal tension (i.e. 100%). Organ bath and cGMP data were analysed with a paired Student's *t* test for the multiple comparison using ANOVA subjected to a Bonferroni adjustment. Statistical significance was accepted when the *p* value was less than 0.05.

Results

Animal weights, serum glucose and cholesterol concentrations. The starting weights in both the control and diabetic rabbit groups were similar (control: median, 3.0 kg; range, 2.7–3.5, *n*=6; diabetic: median, 3.1; range, 2.8–3.6, *n*=6). The diabetic rabbits were (*p*<0.03) lighter than the control group (control: median, 4.1 kg; range, 3.5–4.35, *n*=6; diabetic: median, 3.6; range, 3.0–3.9, *n*=6). Serum glucose concentrations (non-fasting) were increased (*p*<0.009) in the 6-month diabetic group (32.2; 18.3–41.1, *n*=6) compared with the control group (median, 6.4 mmol/l; range, 6.1–7.5 mmol/l, *n*=6). Serum cholesterol con-

Table 1. Comparison of phenylephrine-induced contractions (% maximal) in aortae from diabetic rabbits and age-matched control rabbits (6 months duration). Data = mean $\pm$ SEM, $n=6$

Concentration of phenylephrine (mol/l)						
	1×10^{-8}	3×10^{-8}	1×10^{-7}	3×10^{-7}	1×10^{-6}	3×10^{-6}
Control rabbits	5 ± 0.5	15 ± 1.2	43 ± 3.6	64 ± 5.2	83 ± 8.3	94 ± 9
Diabetic rabbits	6 ± 0.4	16 ± 1.2	45 ± 4.3	67 ± 5.4	88 ± 6.2	95 ± 7.6

Table 2. Comparison of sodium nitroprusside (SNP)-induced relaxation (% maximal) in aortae from diabetic (6 months duration) and age-matched control rabbits. Data = mean $\pm$ SEM, $n=6$

Concentration of SNP (mol/l)						
	1×10^{-8}	3×10^{-8}	1×10^{-7}	3×10^{-7}	1×10^{-6}	3×10^{-6}
Control rabbits	2 ± 0.2	3 ± 0.3	12 ± 1.3	50 ± 5.9	85 ± 8.8	99 ± 7.4
Diabetic rabbits	3 ± 0.2	2 ± 0.1	14 ± 1.4	53 ± 5.3	88 ± 8.1	99 ± 6.9

Fig. 1. Acetylcholine-evoked relaxation of aortic rings from diabetic (solid circles) rabbits and control (open circles) rabbits, and diabetic+300 U/ml SOD (open squares) 6 months after the induction of diabetes. N.B. 300 U/ml SOD had no effect on relaxation in aortae from control animals. Each point = mean $\pm$ SEM, $n=6$ animals. * $p < 0.001$ control vs diabetic **Fig. 2.** Effect of homocysteine (Hcy) on acetylcholine-evoked relaxation of aortic rings from diabetic (6 months duration) and control rabbits. Each point = mean $\pm$ SEM, $n=6$. * $p < 0.001$ control vs diabetic; ** $p < 0.01$ diabetic control vs homocysteine additions. Diabetic without additions (solid circles); control without additions (open circles); diabetic plus 10 $\mu\text{mol/l}$ Hcy (open squares); diabetic plus 100 $\mu\text{mol/l}$ Hcy (solid triangles); diabetic 100 $\mu\text{mol/l}$ Hcy + 300 U/ml SOD (open triangles)

centrations (non-fasting) were not significantly different between the 6-month control group (median = 1.2 mmol/l; range, 0.6–2.3; $n=6$) and the diabetic group (median = 0.9 mmol/l; range, 0.5–1.3; $n=6$). Serum triglycerides were not different between the control and diabetic groups [17].

Organ bath studies and cGMP concentrations. PE-induced contractions were not different in aortae from the diabetic rabbits compared with the control rabbits (Table 1). However, Ach-stimulated relaxation was impaired in aortae from diabetic rabbits compared to the control rabbits (Fig. 1). The impairment of Ach-stimulated relaxation in diabetic animals was reversed

with SOD (300 U/ml) in aortae from diabetic rabbits but SOD had no effect on the control rabbits (Fig. 1). In contrast, there were no differences in sodium nitroprusside (SNP)-stimulated relaxation of aortae from diabetic rabbits compared to the control rabbits (Table 2).

Following pre-incubation for 30 min with Hcy (10 $\mu\text{mol/l}$ and 100 $\mu\text{mol/l}$), there was a further inhibition of Ach-stimulated relaxation in aortae from diabetic rabbits, an effect reversed with 300 U/ml SOD (Fig. 2). In contrast, incubation for 30 min with Hcy (10 $\mu\text{mol/l}$ and 100 $\mu\text{mol/l}$) had no effect on Ach-stimulated relaxation in aortae from the control rabbits.

Fig. 3 Effect of homocysteine (Hcy) on acetylcholine-evoked relaxation of aortic rings from control (non-diabetic) rabbits. Control without additions (open circles); plus 10 µmol/l Hcy (open squares); plus 100 µmol/l Hcy (open triangles); 100 µmol Hcy+300 U/ml SOD. Each point = mean $\pm$ SEM, $n=6$

Fig. 4 Effect of homocysteine (Hcy) (with and without SOD) on acetylcholine-evoked A23187-evoked cGMP formation by aortic rings from diabetic rabbits (6 months duration) and control rabbits. Each bar = mean $\pm$ SEM, $n=6$. ** $p<0.001$ diabetic vs control rabbits * $p<0.01$ diabetic vs homocysteine additions

bits (Fig. 3). Furthermore, Hcy (at 10 µmol/l and 100 µmol/l), had no effect on SNP (nitric oxide donor)-mediated relaxation (Table 3).

Both Ach-stimulated and A23187-stimulated cGMP formation was impaired in aortae from diabetic

Fig. 5 Effect of homocysteine (Hcy) (with and without 300 U/ml SOD) on calcium ionophore A23187-evoked cGMP formation by aortic rings from diabetic rabbits (6 months duration) and control rabbits. Each bar = means $\pm$ SEM, $n=6$. ** $p<0.001$ diabetic vs control rabbits * $p<0.01$ diabetic vs homocysteine additions

Table 4 Effect of homocysteine (Hcy) on sodium nitroprusside [SNP (1 µmol/l)]-stimulated cGMP formation (fmol·mg tissue⁻¹·min⁻¹) [means $\pm$ SEM, $n=6$] in aortae from diabetic and control rabbits

	Zero	10 µmol/l Hcy	100 µmol/l Hcy
Control rabbits	880 $\pm$ 76	870 $\pm$ 83	890 $\pm$ 67
Diabetic rabbits	875 $\pm$ 56	860 $\pm$ 56	856 $\pm$ 54

rabbits compared to control rabbits (Figs. 4 and 5). The similarity of the impairment of cGMP formation to that of relaxation in response to Ach consolidates that the reduction of relaxation in diabetic aortae is due to an impairment of the nitric oxide-cGMP axis rather than other pathways (e.g. EDHF or PGI₂). In contrast, there were no differences in sodium nitroprusside (SNP)-stimulated cGMP formation by aortae from diabetic rabbits compared to control rabbits (Table 4). Following pre-incubation for 30 min with Hcy (10 µmol/l and 100 µmol/l), there was further inhibition of Ach-stimulated and A23187-stimulated cGMP formation in aortae from diabetic rabbits, an effect reversed with 300 U/ml SOD, but no significant changes in the control rabbits (Figs. 4, 5).

Table 3 The effect of 30-min preincubation of homocysteine (Hcy) at 10 µmol/l (+10) and 100 µmol/l (+100) on sodium nitroprusside (SNP)-evoked relaxation (% maximal) in aortae

Concentration of SNP (mol/l)	1 $\times$ 10 ⁻⁸	3 $\times$ 10 ⁻⁸	1 $\times$ 10 ⁻⁷	3 $\times$ 10 ⁻⁷	1 $\times$ 10 ⁻⁶	3 $\times$ 10 ⁻⁶
C+zero	2 $\pm$ 0.2	3 $\pm$ 0.3	12 $\pm$ 1.3	50 $\pm$ 5	83 $\pm$ 8	99 $\pm$ 7
C+10 Hcy	2 $\pm$ 0.2	4 $\pm$ 0.1	11 $\pm$ 1.8	48 $\pm$ 4	84 $\pm$ 5	93 $\pm$ 6
C+100Hcy	1 $\pm$ 0.2	4 $\pm$ 0.1	9 $\pm$ 1.5	46 $\pm$ 6	88 $\pm$ 8	90 $\pm$ 6
D+zero	2 $\pm$ 0.2	3 $\pm$ 0.3	12 $\pm$ 1.3	49 $\pm$ 5	85 $\pm$ 8	99 $\pm$ 7
D+10 Hcy	3 $\pm$ 0.2	2 $\pm$ 0.1	14 $\pm$ 1.4	53 $\pm$ 5	88 $\pm$ 8	98 $\pm$ 6
D+100 Hcy	3 $\pm$ 0.2	2 $\pm$ 0.1	14 $\pm$ 1.4	53 $\pm$ 5	88 $\pm$ 8	94 $\pm$ 6

from control (C) or diabetic rabbits (D) of 6 months duration. Each point = mean $\pm$ SEM, $n=6$

Discussion

The similarity of the impairment of cGMP formation to that of relaxation in response to Ach consolidates that the reduction of relaxation in diabetic aortae is caused by an impairment of the nitric oxide-cGMP axis rather than other pathways (e.g. EDHF or PGI₂). Nitric oxide release following endothelial nitric oxide synthase (eNOS) activation is mediated by an increase in cytosolic calcium, which in the case of Ach is mediated by a receptor-linked mechanism and in the case of calcium ionophore A23187 through non-specific calcium influx [6]. Since SNP-stimulated relaxation and cGMP formation were not affected (indicating no effect on guanylyl cyclase), the impairment of Ach-mediated relaxation, as well as Ach- and A23187-stimulated cGMP formation in the aortae from diabetic animals, indicates that the impairment is not due to an inhibition of Ach receptors or associated signal transduction systems (e.g. G proteins, phospholipase C and inositol trisphosphate formation) but to a reduction in nitric oxide bioavailability. In contrast, there were no differences in sodium nitroprusside (SNP)-stimulated cGMP formation by aortae from diabetic rabbits compared to control rabbits. Taken together, these data indicate that diabetes mellitus reduces NOS activity and/or the bioavailability of nitric oxide but does not reduce guanylyl cyclase activity in aortae from diabetic rabbits. The impairment of both Ach-stimulated and A23187-stimulated cGMP formation was reversed by SOD, indicating that this impairment was caused by increased superoxide formation. These data are in keeping with previous reports of impaired endothelium dependent relaxation and nitric oxide formation in arteries from diabetic animals [8, 9, 17].

The reversal of the reduced relaxation and cGMP formation in response to Ach and A23187 by SOD in aortae from diabetic rabbits also indicates that this impairment is due to excess O₂⁻. Since O₂⁻ reacts with nitric oxide to form peroxynitrite (ONOO), effectively reducing local nitric oxide availability [5, 6, 12], this explains the present effects of diabetes mellitus on endothelium-dependent relaxation. Although ONOO itself can promote relaxation, it is 1000 times less potent than nitric oxide [19]. ONOO also rapidly breaks down to produce other toxic free radicals [12]. Several studies have now shown a reduction of nitric oxide and an increase in O₂⁻ formation by the vasculature of diabetic laboratory animals [8, 9, 20, 21, 22, 23]. In support of these observations, the adenoviral gene transfer of both eNOS and SOD have been shown to reverse the reduction of endothelium-dependent relaxation in the diabetic rabbit [24, 25].

The over-production of O₂⁻ in diabetic animals has been ascribed, in part, to a reduction in endogenous vascular SOD activity [26]. In humans, Type I (insulin-dependent) diabetes mellitus is also associated

with reduced SOD activity due to increased glycation of the enzyme [27]. The excess production of O₂⁻ by the vasculature of diabetic animals has also been attributed to the over-expression of endogenous O₂⁻ generating enzymes, including NADPH oxidase, xanthine oxidase, cyclooxygenase and lipoxygenase as well as the auto-oxidation of glucose and mitochondrial respiration [8, 7, 20].

In our study, pre-incubation with 10 µmol/l and 100 µmol/l Hcy for 30 min caused a further impairment of both Ach-evoked relaxation and cGMP formation in aortae from diabetic rabbits but had no effect in aortae from age-matched control animals. This marked potentiating effect was also reversed by SOD, indicating that Hcy augments an already established increase in O₂⁻ formation by vascular tissue from diabetic animals, possibly by acting at a common denominator site. In previous studies in which the effect of Hcy on endothelium-dependent relaxation was investigated, exposure to 1 mmol/l Hcy for 3 h was required to elicit an inhibitory effect in isolated rat aortic rings [18]. Since Hcy autoxidises to form O₂⁻ [3] it is reasonable to suggest that arterial tissue from diabetic animals could accelerate the auto-oxidation of Hcy. Thus, the inhibitory effects of Hcy obtained at 10 µmol/l and 100 µmol/l constitutes a striking reduction in the inhibitory capacity of the amino acid on nitric oxide formation and actions (i.e. cGMP formation). As discussed above, there are other junctures at which Hcy could be exerting this effect: an increase in pro-oxidant enzyme activity (e.g. NADPH oxidase), an inhibition of antioxidant systems (e.g. SOD) or both.

Clinically, the relation between diabetes mellitus, Hcy and angiopathy is not clear. A number of studies have indicated a potentiating association between diabetes mellitus, Hcy and CVD [2, 28, 29, 30] and increased plasma Hcy concentrations in patients with Type I diabetes mellitus [31, 32, 33, 34]. In Type II (non-insulin-dependent) diabetic patients, fasting and post-methionine load Hcy concentrations are also above normal [35, 36]. There are other reports, however, that Hcy concentrations are not increased in diabetes mellitus and that homocysteinaemia is not correlated with angiopathy in diabetic patients [2, 35, 38, 39]. Our studies indicate that increased plasma concentrations of Hcy might not necessarily be a prerequisite for the promotion of angiopathy in diabetes mellitus, which in turn could explain the lack of correlation between increased Hcy and angiopathy in diabetes mellitus found in some studies.

Although the concentration at which Hcy was inhibitory in this study (10 µmol/l) "normophysiological", in vivo, circulating Hcy exists in several forms. Approximately 80% of Hcy is bound to proteins in blood [40, 41]. The remaining unbound Hcy can form a dimer with itself, can exist as a homocysteine-cysteine complex as well as in a reduced or oxidised form

[40, 41], attributes that could be central to its pathological influence in diabetes mellitus. An examination of all these different forms of Hcy was beyond the scope of our study.

In conclusion, our study shows that concentrations of Hcy that have no effect on aorta from normal rabbits markedly impair endothelium-dependent relaxation and nitric oxide-dependent cGMP formation in aortae from diabetic rabbits. Since these effects were reversed by SOD, we conclude that the effect is mediated by an augmentation of O_2^- formation perhaps due to reduced SOD activity, increase pro-oxidant enzymes or an acceleration of Hcy oxidation. This, in turn, indicates that concentrations of Hcy that have no effect in normal tissue can exert a deleterious impact on nitric oxide-dependent systems when diabetes mellitus is present. For intervention, supplementation of the diet with folate or the B_6 and B_{12} vitamins have been used to reduce plasma Hcy [1, 2, 3]. Given the evidence that diabetes mellitus and Hcy could interact to promote angiopathy through the generation of superoxide [4, 5] the administration of SOD mimetics or antioxidants, possibly as adjuvants to vitamin supplements, could be an alternative strategy in this particular clinical scenario.

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