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Abnormal metabolic fate of nitric oxide in Type I diabetes mellitus

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Abstract

Aims/hypothesis. Reduced bioavailability of endothelium-derived nitric oxide is implicated in diabetic macrovascular and microvascular disease. In patients with diabetes, we hypothesised that protein glycosylation can alter nitric oxide binding affinity of haemoglobin and plasma proteins, hence reducing nitric oxide availability and causing an alteration in nitric oxide metabolism.

Methods. Binding of nitric oxide to haemoglobin was studied across a range of glycosylation levels in vitro (HbA_{1c} 5.9 to 9.8%). In clinical studies nitrate, nitrite, nitrosyl haemoglobin and plasma nitrosothiols were measured in venous blood from 23 patients with uncomplicated Type I (insulin-dependent) diabetes mellitus and 17 non-diabetic control subjects. Samples were analysed at baseline and after nitric oxide was added ex vivo.

Results. Nitric oxide-haemoglobin binding was increased at a HbA_{1c} greater than 8.5% compared with 5.9% ($p<0.01$). Basal nitrosyl haemoglobin was higher in diabetic patients compared with the control subjects (0.59±0.12 μmol/l vs 0.24±0.12 μmol/l, $p<0.05$). Plasma nitrosothiols, and nitrite and nitrate (NOx) concentrations were similar in diabetic patients compared with the control subjects (7.64±0.79 μmol/l vs 5.93±0.75 μmol/l, 13.98±2.44 μmol/l vs 12.44± 2.15 μmol/l, respectively). In blood from diabetic patients, added nitric oxide was metabolised preferentially to nitrosyl haemoglobin and plasma nitrosothiols, with a twofold increase in nitrosyl haemoglobin observed across all concentrations of nitric oxide ($p<0.05$). These preferential increases correlated positively with HbA_{1c}.

Conclusion/interpretation. Nitrosyl haemoglobin is increased in patients with Type I diabetes. Preferential metabolism to nitrosyl haemoglobin and nitrosothiols occurs after increases in nitric oxide. Our results show an accentuated association between nitric oxide and glycosylated proteins, especially deoxygenated haem. An altered metabolic fate of nitric oxide could influence microvascular regulation and tissue perfusion. [Diabetologia (2002) 45:1515–1522]

Keywords Haemoglobin, glycosylation, nitric oxide, diabetes mellitus, bioavailability, metabolism, nitrosyl haemoglobin.

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Abbreviations: NOx, Total nitrate and nitrite; RSNO, plasma nitrosothiols; HbNO, nitrosyl haemoglobin; HbSNO, S-Nitrosohaemoglobin.

Atherosclerosis and microvascular disease are the main causes of mortality and morbidity in patients with Type I (insulin-dependent) diabetes mellitus [1, 2]. Endothelial dysfunction with reduced bioavailability of nitric oxide has been linked to atherogenesis and non-diabetic glomerulosclerosis [3, 4] and is likely to play a role in the development of diabetic vasculopathy [5, 6]. The underlying mechanisms of endothelial dysfunction are not clear, in particular how diabetes affects the balance between nitric oxide production and inactivation. Little attention has been given to

the possibility that diabetes might directly affect the metabolism and vascular availability of nitric oxide.

In healthy subjects, nitric oxide released intraluminally by endothelial cells is rapidly metabolised. The main pathways for nitric oxide metabolism are: (i) oxidation of oxyhaemoglobin to form methaemoglobin and nitrate ion, (ii) combination with deoxyhaemoglobin (or deoxygenated haem groups on partially oxygenated haemoglobin) to form nitrosyl haemoglobin (HbNO), and (iii) nitrosylation of thiol groups on haemoglobin (HbSNO) and other proteins to form s-nitrosothiols (RSNO). In addition, pathological processes such as diabetes are associated with increased levels of superoxide radicals (O_2^-), which combine quickly with nitric oxide to produce damaging peroxynitrite.

The formation of relatively stable nitric oxide metabolites seems to provide a protected transport mechanism, allowing nitric oxide to be transported through regions of high production (i.e. conduit arteries) to be released downstream in the microcirculation, and thus influence microvascular tone. Nitric oxide release within the systemic microcirculation is suggested by an arterio-venous gradient of nitrite and HbNO after nitric oxide inhalation in normal subjects [7], of nitrite and high-molecular weight RSNOs following exercise in normal subjects [8] and of nitrate and nitrite during exercise training in hypercholesterolaemic patients [9]. Nitric oxide can also be transferred from high-molecular weight to low-molecular weight thiols [10], possibly allowing nitric oxide to be delivered to the vascular bed to elicit vasodilatation. It has been suggested that nitric oxide bound as HbNO is moved to a 'back-pocket' on haemoglobin to form HbSNO during re-oxygenation of the haemoglobin molecule in the pulmonary circulation. Nitric oxide can subsequently be released from HbSNO (along with oxygen) in hypoxic tissue, or passed on to small molecular weight plasma proteins to form RSNO [11, 12].

A reanalysis of nitric oxide-haemoglobin reactions in vitro, under physiological conditions showed the formation of HbNO to compete with the oxidation reaction, even at high oxygen saturation. It was proposed that haemoglobin, rather than serving to eliminate nitric oxide, acts to preserve its bioactive state [13]. Nitric oxide bound to the haem sites of the alpha-subunits of haemoglobin might act as a negative allosteric effector of haemoglobin [14]. HbNO was also shown to have an enhanced Bohr effect, promoting oxygen release with increased sensitivity to tissue acidosis [15]. To clarify the role of nitric-oxide-haem interactions in vivo, arterial and venous concentrations of nitric oxide metabolites were measured at baseline and after nitric oxide inhalation. The authors concluded that nitric-oxide-haem reaction pathways predominate in vivo [7]. These results suggest that nitric-oxide-haem interactions can act to regulate vascular tone and influence oxygen supply to the peripheral microvascular circulation, possibly triggering the release of nitric oxide in regions of low pO_2.

Hyperglycaemia accelerates the formation of AGE, including glycosylated haemoglobin, albumin and other plasma proteins. We proposed that glycosylation of blood proteins changes their binding affinity for nitric oxide, thereby altering the metabolic fate of nitric oxide in Type I diabetic patients. To test this hypothesis, we incubated nitric oxide with haemoglobin at different levels of glycosylation and measured the nitric-oxide-protein products formed. To confirm our results in vivo, we measured nitric oxide metabolites in venous blood from diabetic patients and control subjects under basal conditions and again after exposing the blood to increasing concentrations of exogenous nitric oxide ex vivo.

Materials and methods

Laboratory study. Haemoglobin standards of specific HbA_{1c} levels (5.9, 7.2, 8.5 or 9.8%) were obtained (Euro-trol, Wageningen, Netherlands). The nitric oxide donor, NOC-9 (Alexis, UK) was added to the haemoglobin standards and incubated for 30 min at 37 °C in air. NOC-9 was selected because 50% of the nitric oxide is released within 2 min at 37 °C, pH 7.4, providing a transient burst of free nitric oxide [16]. We confirmed this using a nitric oxide electrode (700 μm; Harvard apparatus). The sample was then separated from free nitric oxide, NOx and NOC-9 by passing through a Sephadex G25 column. Fractions containing haemoglobin were collected and assessed for HbNO and HbSNO using a nitric oxide electrode system. Haemoglobin concentrations were measured using the haemoglobin cyanide (cyanmethaemoglobin) method [17].

Clinical study. Venous blood samples were drawn from 23 patients with Type I diabetes who were attending the outpatient clinic as part of their routine clinical management. Blood samples were non-fasting and without dietary restriction, providing a mid-afternoon blood sample under "normal" conditions (insulin, diet) for each subject. Patients had no overt diabetic complications and were compared with 17 control subjects matched for age and gender. Control blood samples were also non-fasting and without dietary restriction. This study was approved by the local ethics board (Bro Taf, Wales, UK) and all subjects gave their informed consent.

The diabetic patients did not differ from the control subjects in terms of age (30.8±1.4 vs 28.8±1.4 years, NS), haemoglobin concentration (13.0±0.6 vs 13.8±0.5 g/dl, NS), haematocrit (0.39±0.01 vs 0.40±0.01, NS) and red blood cell count (4.44±0.13 vs 4.64±0.15×10^{12}/l, NS). Lipid peroxidation (measured as F2-isoprostane levels) was used as a marker of oxidative stress and was similar in diabetic and control plasma samples (761±61 pg/ml vs 651±40 pg/ml, NS). Mean HbA_{1c} values were higher in the diabetic patients than in the control subjects (9.24±0.41 vs 5.46±0.07, $p<0.05$).

Venous blood samples were drawn into each of four EDTA-containing Vacutainers. The nitric oxide donor, NOC-9 was added immediately to the samples to provide final added concentrations of 0, 5, 25 and 50 μmol/l nitric oxide. All four samples were incubated for 3 min at 37°C before being transferred to ice and centrifuged at 2000 rpm for 10 min at 4°C. Plasma was separated and stored at –80 °C for analysis of nitrate and

nitrite and RSNO. The red blood cell slurry was snap frozen in liquid nitrogen and stored at –80 °C for HbNO measurement by electron paramagnetic resonance (EPR) at liquid nitrogen temperature.

Electrode detection of HbNO and HbSNO from haemoglobin standards. This method was adapted from a chemiluminescence method described previously [18]. Briefly, the fresh haemoglobin samples containing HbNO and HbSNO were split in two. Cyanide (potassium ferricyanide (0.2 mmol/l) and potassium cyanide (0.2 mmol/l) in 0.5 mmol/l EDTA) was added to one – this served to stabilise HbSNO and oxidise HbNO. EDTA (0.5 mmol/l) was added only to the second sample. After 30 min of incubation on ice, the samples were separated on a G25 column. A 200 µl aliquot of the haemoglobin fraction was added to a reaction chamber containing glacial acetic acid (70%), I_2 and 50 mg potassium iodide under a stream of N_2 gas. Nitric oxide released from the sample was carried by the gas via a NaOH (1 mol/l) trap before detection by a nitric oxide electrode (700 µm; Harvard) in the gas phase. HbNO was calculated from the current detected in the sample without CN (HbNO + HbSNO) minus the current detected in the sample with CN added (HbSNO). The system was calibrated using NOC-9. Effluent from the column was used as a blank.

Patient HbNO. HbNO measurement was made by EPR using a Varian spectrometer operating at a frequency of 9.24 GHz and 100 kHz field modulation. A microwave power of 10 mW and magnetic field sweep of 200 Gauss was used. The frozen red blood cell pellet (200 µl) was placed in a finger sample dewar filled with liquid nitrogen. Typically, 4 min spectra were recorded using a modulation amplitude of 4 Gauss and 0.5 s time constant. The HbNO signals were measured using a spectral simulation package (EW Voight software). This EPR method was calibrated using the above nitric oxide electrode method using red blood cell samples having known amounts of nitric oxide added. We found no appreciable difference between the two techniques, with the advantage that EPR afforded batch analysis of patient samples that remained frozen and had no appreciable loss of HbNO over time.

Plasma nitrate and nitrite (NOx). Plasma NOx was measured using a fluorometric method [19]. Plasma samples were prefiltered to remove NOC-9 and contaminating plasma proteins. NADPH (10 µmol/l) was added to each 50 µl plasma sample. Nitrite was measured from the fluorometric signal formed by the reaction between nitrite and naphthylethylene diamine (DAN; 0.62 M). Nitrate was measured by its conversion to nitrite by nitrate reductase and further reaction with DAN. A total of 50 mmol/l glucose-6-phosphate, 1.6 U/ml glucose-6-phosphate dehydrogenase and 0.8 U/ml nitrate reductase in sodium phosphate buffer of 14 mmol/l was added. For nitrite measurement, a similar volume of sodium phosphate buffer was added. Samples were incubated for 1–1.5 h at 37°C. After incubation, DAN in 0.62 mol/l HCl was added with further incubation for 10 min at room temperature protected from light. The reaction was halted by adding 2.8 N NaOH and the fluorescence measured after 10 min using a Perkin Elmer Fluorescent Spectrophotometer (emission = 450 nm, excitation = 365 nm).

Plasma RSNO. S-nitrosylation of plasma proteins was quantified by the Saville reaction [20]. 1.0% sulfanilamide in 0.5 mol/l HCl was added to 500 µl plasma sample to measure background nitrite. RSNO was measured by adding 500 µl 0.5 mol/l HCL containing 0.2% $HgCl_2$ and 1.0% sulfanilamide to 500 µl plasma sample. 500 µl of 0.5 mol/l HCl containing 0.02% N-1-naphthyl-ethylenediamine dihydrochloride was added to both samples. After light-protected incubation for 10 min, the concentration of RSNO was measured spectrophotometrically at 540 nm using S-nitrosoglutathione, G-SNO (Sigma-Aldrich, UK) as a standard. Background absorbance of nitrite was subtracted from the RSNO signal. No appreciable decomposition of G-SNO occurred over the time course of the assay.

Statistical analysis. All data are presented as means ± SEM. For baseline values from both laboratory and clinical studies, differences between means were compared by unpaired Student's *t* test. Multiple means were compared by ANOVA, followed by paired or unpaired Student's *t* test, as appropriate. Correlations were carried out using Pearson's correlation coefficient. A p of less than 0.05 was considered statistically significant.

Results

Laboratory study. In vitro, nitric oxide binding to haemoglobin was not increased significantly between HbA_{1c} 5.9% and 8.5% (0.046±0.003% vs 0.053± 0.008% moles of nitric oxide bound to haemoglobin per mole of haem subunit, NS). However, a twofold increase in the total amount of nitric oxide bound to haemoglobin was observed with HbA_{1c} of 9.8% (0.046±0.003% vs 0.083±0.022%, $p<0.01$) (Fig. 1). A similar trend occurred in experiments where the ratio of nitric oxide to haemoglobin was decreased ($p<0.01$).

Clinical study. HbNO concentrations were higher in blood drawn from patients with diabetes than from control subjects (0.59±0.12 µmol/l vs 0.24± 0.12 µmol/l, $p<0.05$). Baseline RSNO was similar in the diabetic and control group (7.64±0.79 µmol/l vs 5.93±0.75 µmol/l, NS) as was NOx (13.98± 2.44 µmol/l vs 12.44±2.15 µmol/l, NS).

Incubation of blood with exogenous nitric oxide to assess metabolic capacity resulted in dose-dependent increases in HbNO and NOx in both groups. In the diabetic group, increasing concentrations of nitric

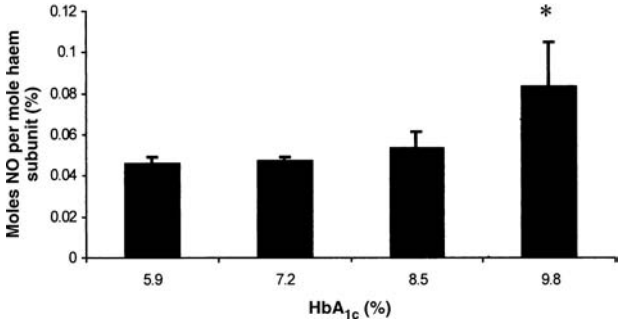


Fig. 1. Formation of total nitrosylated haemoglobin (HbNO and Hb-SNO) after incubation of nitric oxide and haemoglobin at different levels of glycosylation, $n=4$ for each group. *Denotes when means were significantly different from 5.9% ($p<0.01$)

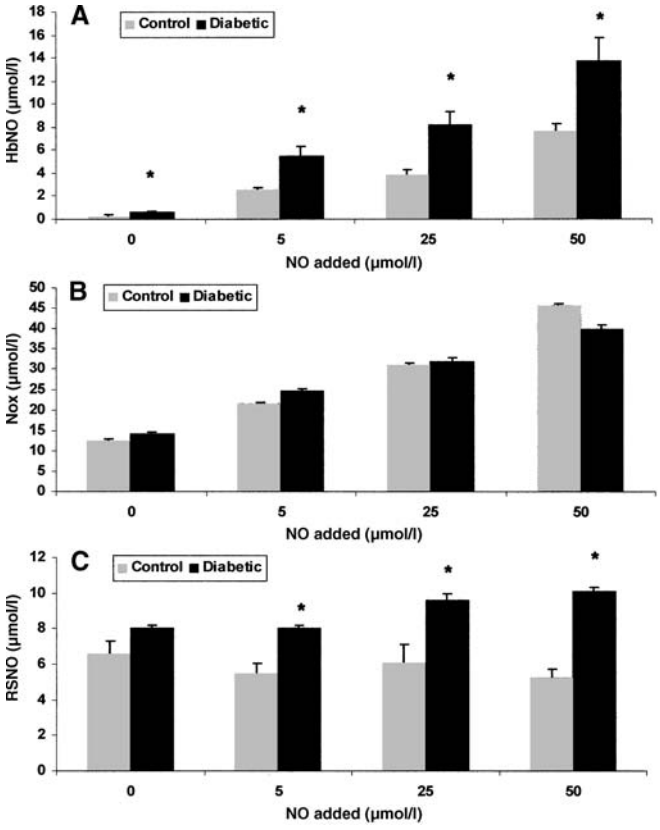


Fig. 2A–C. Comparison of HbNO (**A**), NOx (**B**) and plasma RSNO (**C**) between venous blood of diabetic (*black*) and control (*grey*) subjects at baseline and after 5, 25 and 50 µmol/l of exogenous nitric oxide was added. A significant difference between the diabetic and control groups is indicated by a *($p<0.05$)

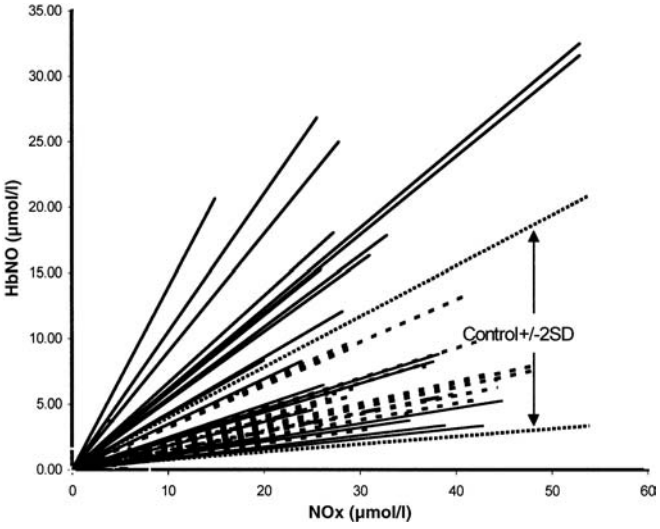


Fig. 3. Formation of HbNO vs NOx in venous blood isolated from diabetic (*solid black lines*) and control (*dashed black lines*) subjects after ex vivo addition of 5, 25 and 50 µmol/l nitric oxide. All values were corrected for baseline. Control distribution (±2SD) is shown by the *two dotted lines*

oxide also resulted in a dose-dependent increase in plasma RSNO. Little change was observed in the control group ($p<0.05$, Fig. 2). For all concentrations of nitric oxide added, HbNO and plasma RSNO formation was greater in blood isolated from the diabetic patients than in blood isolated from the control subjects ($p<0.05$, Fig. 2A, C). No significant difference in NOx was observed between the diabetic and control groups (Fig. 2B).

Lineweaver-Burke analysis of patient and control HbNO data showed that the Km values (concentration of nitric oxide at half maximal velocity) for nitric oxide binding by haemoglobin in control and patient groups were similar (about 5 µmol/l nitric oxide). The Vmax was higher for the patient group ($p<0.05$).

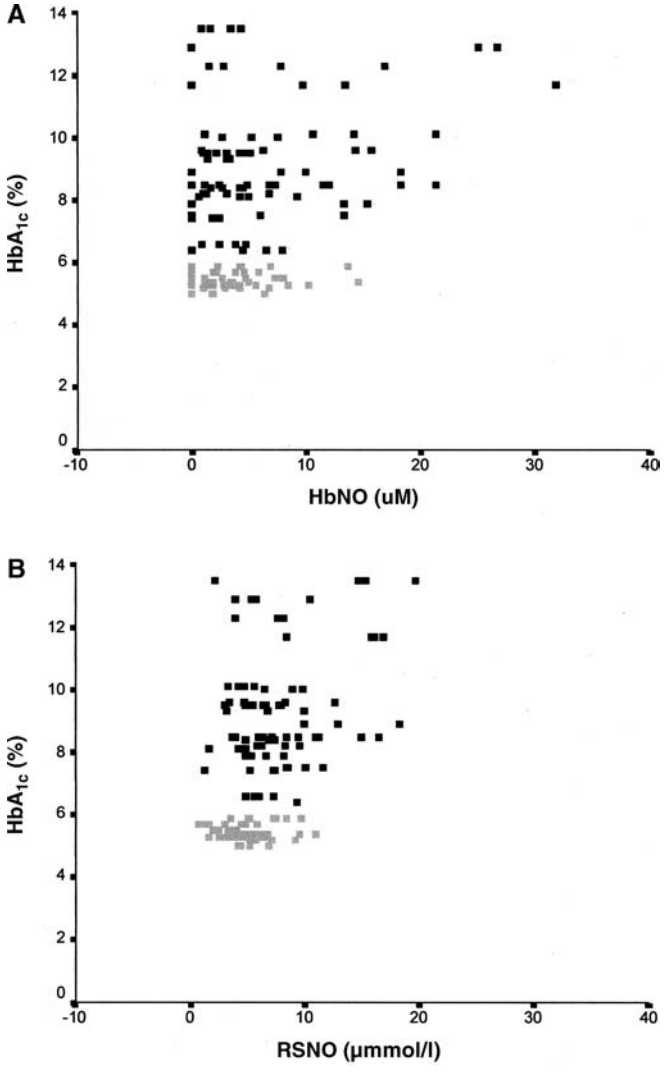
Apportion of NO to the three pathways of metabolism. Nitric oxide metabolites were plotted against each other at each concentration of exogenous nitric oxide (Fig. 3). As expected, a positive correlation was observed between HbNO and NOx in both groups ($r=0.56$ and $r=0.84$, both $p<0.01$). The gradient of this relation indicates the ratio of HbNO to NOx formation. A higher rate of HbNO formation in the diabetic group is reflected by the higher average line gradient compared with the average line gradient of the control group (0.44±0.07 vs 0.19±0.02, $p<0.01$). There was no correlation between HbNO and RSNO or NOx and RSNO. Line gradients for these correlations were not different between diabetic and control groups.

Approximately half of the diabetic subjects fell within the 'control range', but an apparently separate group had higher gradients reflecting increased HbNO relative to NOx formation. Consequently, the diabetic data was divisible into two sub-groups (those within and those outside the average control line gradient ± 2SD). Of the diabetic patients 44% had gradients within the control range (i.e. between 0.054 and 0.338) and 56% had gradients that were higher than the control subjects. Average line gradients for the "abnormal" versus "normal" diabetic sub-groups were 0.65±0.08 and 0.159±0.02, respectively ($p<0.01$). The clinical characteristics of these diabetic patients (those within and those outside the control range) are presented in Table 1. The two sub-groups did not differ in terms of triglyceride, cholesterol, or blood glucose. Of interest, a higher incidence of retinopathy and microalbuminuria was observed in those diabetic patients having an abnormal nitric oxide metabolism.

Relation between nitric oxide metabolites and HbA_{1c}. All together, HbA_{1c} correlated positively with both an increased formation of HbNO ($r=0.30$, $p<0.01$) and RSNO ($r=0.39$, $p<0.01$) but not with NOx (Fig. 4). Analysis of the data from diabetic patients alone also showed HbA_{1c} to correlate positively with HbNO and RSNO ($p<0.05$). We found no correlation between nitric oxide metabolites and other clinical phenotypic characters. No correlation was observed between

Table 1. Clinical characteristics of diabetic patient group

Parameter	Means ± SEM	
Age (years)	30.78±1.39	
Duration (since onset) (years)	13.20±1.75	
HbA_{1c} (%)	9.24±0.41	
Cholesterol (mmol/l)	5.01±0.21	
Triglyceride (mmol/l)	2.06±0.42	
Blood glucose (mmol/l)	9.07±2.32	
	Diabetics within control range (%)	Diabetics outside control range (%)
Albuminuria absent	100	85
Present	0	15
Retinopathy absent	75	50
Background	12.5	40
Proliferative	12.5	10
Neuropathy absent	100	100
Present	0	0
Peripheral vascular disease	0	0
Insulin basal bolus	41.7	45.5
Actrapid/mono	8.3	18.2
Mix	16.7	27.3
act/insulin	8.3	0
Other	25	9.1



glycaemia and increased NO metabolites based on patient blood glucose concentrations on the day of the experiment.

When the HbA_{1c} of the two sub-groups described above were compared, the subgroup showing an increased HbNO formation had a higher HbA_{1c} compared to those with 'normal' HbNO formation (9.63±0.52 vs 8.05±0.51, $p<0.05$).

Discussion

The principal findings from these studies are firstly, that venous blood isolated from Type I diabetic patients has increased HbNO compared with blood isolated from control subjects ($p<0.05$). Secondly, nitric oxide added to blood isolated from patients with Type I diabetes ex vivo leads to preferentially increased HbNO and RSNO compared with the control subjects. Thirdly, a preferential increase in HbNO in blood from patients with diabetes correlates positively with HbA_{1c} in vitro and in vivo. Our results are consistent with preferential binding of nitric oxide to glycosylated blood proteins, especially deoxygenated haem subunits, in patients with Type I diabetes mellitus.

Our results show that, in both patient and control groups, nitric oxide-haemoglobin reactions are the predominant metabolic pathways for free nitric oxide.

Fig. 4. Relation between HbA_{1c} and HbNO formation (**A**) and RSNO formation (**B**) for control (*light grey*) and diabetic (*black*) subjects

In the control group, little change was observed in plasma RSNO concentration after nitric oxide was added to blood. This is consistent with the observation that low-molecular weight RSNOs do not increase after nitric oxide inhalation in healthy subjects [7]. In health, RSNO can act as a relatively unchanging pool of nitric oxide, with haemoglobin remaining available to metabolise 'spare' nitric oxide via the oxidation pathway (to form NOx) or via the addition pathway to form an nitric oxide store (HbNO). In the diabetic group, increasing nitric oxide concentration resulted in increased RSNO. This suggests glycosylation of proteins (other than haemoglobin) affects nitric oxide binding, at least at higher nitric oxide concentrations. There could also be exchange of nitric oxide between haemoglobin and thiol-containing proteins (RSH) such that increased binding of nitric oxide by glycosylated haemoglobin could consequently result in increased RSNO under certain conditions.

The positive correlation observed between HbNO and RSNO formation and HbA_{1c} in these studies strongly suggests that preferential nitric oxide-binding to blood proteins could be attributable to glycosylation. Hyperglycaemia accelerates the formation of AGE, including glycosylated haemoglobin (HbA_{1c}) and plasma proteins. The increased tendency to HbNO production in patients with diabetes points to abnormal interaction between nitric oxide and deoxyhaemoglobin. It could be relevant that deoxyhaemoglobin is more susceptible to glycosylation than oxyhaemoglobin [21] and that oxygen and ligand binding properties of haemoglobin are altered by glycosylation [22]. An analysis of s-nitrosylation of human glycosylated haemoglobin compared to non-glycosylated haemoglobin has previously shown that glycosylation leads to increased formation and decreased decomposition of s-nitroso-haemoglobin [23]. It seems likely that these hyperglycaemia-induced structural changes in the haemoglobin molecule will also influence the interaction with nitric oxide, increasing the binding association and/or decreasing the dissociation rate of nitric oxide and thus cause an accumulation of HbNO in vivo. This theory is supported by the observation that the increased concentration of HbNO observed at baseline in blood isolated from diabetic patients was not matched by a decrease in RSNO or NOx.

The significance of our finding of two apparently distinct sub-groups within the larger diabetic patient group remains unclear. The sub-group exhibiting an abnormal nitric oxide metabolism had an average HbA_{1c} of 9.63%. Our in vitro results show increased nitric oxide binding to haemoglobin only at a HbA_{1c} of above 8.5%. These findings indicate that an abnormal nitric oxide metabolism could be a direct consequence of poor glycaemic control, and that this occurs only above a critical level of glycosylation.

The UKPDS [24] and DCCT [25] studies showed that intensive treatment and reduction in HbA_{1c} to 7.0% decreased the frequency and severity of long-term microvascular and neurological complications. Conventional therapy resulted in relatively higher HbA_{1c} (7.9% and 9.0% respectively) and was associated with increased incidence of diabetic complications. Our in vitro studies show "normal" binding of nitric oxide by haemoglobin at a HbA_{1c} of 7.2% and an abnormal binding at 9.5%. It is difficult to compare the DCCT or UKPDS end points (diabetic complications) with our HbNO results as neither study measured clinical outcomes across a range of HbA_{1c}. We found normal binding at 8.5% in vitro, and the average HbA_{1c} of the diabetic patients that fell within the control range was 8.1%. Diabetic patients with abnormal nitric oxide metabolism had an average HbA_{1c} of 9.6%.

Our findings might have direct bearing on the bioactivity of nitric oxide in the microcirculation. The bioavailability of nitric oxide produced by vascular endothelium might depend on its inactivation by glycosylated haemoglobin and other proteins, as well as inactivation by reactive oxygen intermediates [25]. In addition recent work has indicated that some nitric oxide metabolites could provide a protected transport and delivery mechanism for nitric oxide. The arteriovenous gradients (with the exception of nitrite) were observed during times of exercise or increased concentrations of nitric oxide, suggesting nitric oxide metabolites can act as a compensatory mechanism to provide vasodilation and therefore oxygen to hypoxic tissue. Altered nitric oxide activity in people with diabetes mellitus is not satisfactorily explicable simply in terms of altered nitric oxide production or inactivation. The confounding factor could be that nitric oxide can be transported and released in the microvasculature. Such a carrier action has been attributed to RSNOs, including nitroso-haemoglobin (Hb-SNO), but other metabolites including HbNO and nitrite might also be involved [7, 8, 10, 11]. Our ex vivo studies show that, in blood isolated from diabetic patients, nitric oxide is metabolised preferentially to HbNO and RSNO. This hyperglycaemic-induced alteration in nitric oxide metabolism could affect nitric oxide bioavailability and limit peripheral nitric oxide release despite low pO_2, thereby contributing to microvascular disease and impaired tissue perfusion.

Although only a small proportion of total circulating haemoglobin is nitrosylated (<1%), our data indicate this stores approximately 260 nmol nitric oxide per litre in the form of HbNO in control subjects. It has recently been shown [26] that an infusion of 36 μmol/min nitric oxide or 5 nmol/min s-nitrosoglutathione into the brachial artery of healthy subjects resulted in large increases in downstream radial artery diameter and forearm blood flow that were compara-

ble to those after stimulation of endogenous nitric oxide formation with acetylcholine or bradykinin. These results provide evidence that this concentration of transported nitric oxide is bioactive in human blood and the increased concentrations reported in our study represent a physiologically significant pool of nitric oxide.

In summary, we show preferential binding of endogenous and exogenous nitric oxide to glycosylated deoxy-haemoglobin and consequently an altered metabolic fate of nitric oxide in patients with Type I diabetes mellitus. We suggest that an altered profile of circulating metabolites of nitric oxide will be matched by altered nitric oxide regeneration and bioactivity in the systemic microcirculation.

Currently, glycosylation of haemoglobin is regarded simply as a useful means of assessing diabetic control. It has not been considered a potential mediator of disease. The present findings do not confirm this to be the case, but they point to a need to re-consider the significance of glycosylation. There are several potential reasons for targeting protein glycation to prevent complications in diabetes mellitus, including deleterious effects on membrane fluidity [27], structural defects [28], or oxidative stress as a direct result of production of reactive oxygen intermediates [29]. If an altered metabolic fate of nitric oxide caused by abnormal deoxyhaemoglobin binding proves harmful, our work highlights a new and important reason for targeting glycosylation of proteins as a therapeutic intervention in Type I diabetes.

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