Nitric Oxide Synthesis and Isoprostane Production in Subjects With Type 1 Diabetes and Normal Urinary Albumin Excretion

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The role of nitric oxide (NO) and free radicals in the development of microvascular disease in type 1 diabetes remains unclear. We have measured NO and isoprostane (a stable marker of in vivo lipid peroxidation) production in 13 type 1 diabetic subjects with normal urinary albumin excretion and 13 healthy volunteers. Whole-body NO synthesis was quantified by measuring the urinary excretion of 15N-nitrate after the intravenous administration of L-[15N]2-arginine. The urinary excretion of the major urinary metabolite of 15-F2t-isoprostane (8-iso-prostaglandin-F2α), 15-F2t-IsOP, was quantified as a marker of in vivo lipid peroxidation. Whole-body NO synthesis was significantly higher in diabetic subjects compared with control subjects (342 ± 216 nmol 15N-nitrate/mmol creatinine [95% CI of the difference 45–207], P = 0.005). This increase was not explained by a difference in renal function between the 2 groups. There was no difference in 2,3-dinor-5,6-dihydro-F2t-isoP excretion between diabetic subjects and control subjects (44.8 ± 7.8 vs. 41.4 ± 10.0 ng/mmol creatinine, mean ± 95% CI). However, there was an inverse correlation between NO synthesis and free radical activity in subjects with diabetes (r = -0.62, P = 0.012) that was not observed in control subjects (r = 0.37, P = 0.107). We conclude that whole-body NO synthesis is higher in type 1 diabetic subjects with normal urinary albumin excretion than in control subjects. The inverse correlation between isoprostane production and NO synthesis in diabetic subjects is consistent with the hypothesis that NO is being inactivated by reactive oxygen species. Diabetes 49:857–862, 2000

The Diabetes Control and Complications Trial has confirmed that tight blood glucose control significantly delays the onset and slows the progression of microvascular complications in type 1 diabetes (1). However, despite optimum blood glucose control, a substantial proportion of diabetic subjects in this study developed serious microvascular complications. In contrast, in the Wisconsin Epidemiological Study, a significant proportion (30%) of subjects with type 1 diabetes did not manifest severe microvascular complications after 10 years of follow-up, although they had chronic poorly controlled diabetes (2). It would be useful if we could understand this discrepancy underlying the development of microvascular disease so that those at risk could be identified. The benefit/risk ratio of maintaining tight blood glucose control could be increased if targeted toward individuals at greatest risk of complications due to hyperglycemia.

The complex issues involved in both the pathogenesis of and individual susceptibility to the angiopathy associated with type 1 diabetes have been well documented (3–5). The endothelium and, in particular, the production of nitric oxide (NO) have been the subject of considerable research in recent years. NO maintains basal vasodilator tone, is a potent platelet anti-aggregant, reduces the ability of monocytes to adhere to endothelial cells and to oxidize LDL cholesterol, and inhibits the proliferation of vascular smooth muscle cells (6,7), thus serving an important physiological role in the normal microvasculature.

Functional studies of the action of NO in type 1 diabetes have produced conflicting results, especially when the effects of muscarinic agonist–mediated release of NO (e.g., acetylcholine, carbachol, and methacholine) in the vascular bed in the forearm are measured (8–14). N⁴-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of endothelial nitric oxide synthase, provides a more specific measurement of basal vasodilatation due to NO. Two studies have shown impaired forearm vasoconstriction to L-NMMA, implying a reduced contribution of NO to vascular tone (9,10). However, this defect may only be present in type 1 diabetic subjects with microalbuminuria (urinary albumin excretion rate [UAER] 20–200 µg/min) (10).

Until recently, the methodology to accurately quantify free radical production in vivo has suffered from limitations that have made interpretation difficult. The discovery of F2-isoprostanes, a group of biologically active compounds pro-
duced principally by the nonenzymatic free radical metabolism of arachidonic acid (15), appear to provide a reliable measure of oxidant injury in vivo (16–18) and may also participate as mediators of oxidant injury (19). The action of NO is closely linked to the balance between its production and that of superoxide (O$_2^-$), a reactive oxygen species also produced by the endothelium. O$_2^-$ interacts with NO to produce the highly reactive oxygen species peroxynitrite (ONOO$^-$) (20). ONOO$^-$ catalyzes the formation of isoprostane in LDL in vivo (21). Furthermore, in vitro studies have demonstrated an increase in production of isoprostanes in the presence of hyperglycemia (22). This can induce mitogenesis in vascular smooth muscle and mesangial cells (22,23) and therefore may play a significant role in the development of the vascular complications observed in type 1 diabetic patients.

We have developed a sensitive and accurate stable isotope method to quantify NO synthesis in humans (24). Using this method, we explored the relationship between NO and free radical production in a group of type 1 diabetic subjects with normal UAERs and a group of well-matched nondiabetic healthy volunteers.

**RESEARCH DESIGN AND METHODS**

Subjects with type 1 diabetes attended outpatient clinics in the Royal London and St Bartholomew’s Hospital Trust, where approval for the study was obtained from the Research Ethics Committee. Matching healthy volunteers were recruited from local staff in the hospita.
The increase in NO production observed in the diabetic group was not explained by a difference in blood pressure control, lipid profile, height, or weight between the diabetic subjects and healthy volunteers; both groups were well matched for all variables. In addition, all subjects were asked not to take part in any physical training or exercise during the 3 days before and during the study.

There was no difference in creatinine clearance between the 2 groups. Similarly, there was no difference in the elimination rate constants for $^{15}$N-nitrate. Therefore, we are confident that the renal handling of $^{15}$N-nitrate was not different for diabetic subjects compared with control subjects. However, when we correlated the amount of $^{15}$N-nitrate excreted with creatinine clearance, we obtained a weak positive relationship in the diabetic subjects ($r = 0.49$, $P < 0.05$) with no correlation in the control subjects ($r = -0.26$, NS). This finding may support an association between NO production and hyperfiltration in type 1 diabetes.

We assume that the available metabolic pool of L-arginine is the same in both groups because there is evidence that the effect of L-arginine on the NO pathway in diabetic subjects is not different from that in healthy volunteers (29). Furthermore, when L-arginine was infused directly into the forearm at 50 times the dose used in this study, there was no effect on the acetylcholine-induced vascular response in type 1 diabetic subjects or healthy volunteers (30). Therefore, any difference in the intracellular enrichment of L-[15N]-arginine would not account for the increased production of $^{15}$N-nitrate in the diabetic study group.

We found no relationship between the daily amount of insulin used in the diabetic subjects and the urinary excretion of $^{15}$N-nitrate, nor was there a relationship between NO synthesis or isoprostane production and HbA$_1C$ levels. Although it has been shown that hyperinsulinemia increases the synthesis/release of NO (31,32), we cannot say whether any of our subjects had hyperinsulinemia. To quantify the amount of insulin present would have required measurement of an insulin profile over the 36-h period during which we measured NO synthesis, and this was not performed in our study subjects.

Functional studies of NO action in the forearm vasculature of type 1 diabetic subjects have given inconsistent results. Differences in patient characteristics and the length of disease

TABLE 2
Urinary excretion of total nitrate and $^{15}$N-nitrate at each 12-h period after intravenous administration of L-[15N]-arginine

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Type 1 diabetes</th>
<th>Healthy volunteers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total nitrate (µmol/l)</td>
<td>Creatinine (µmol/l)</td>
</tr>
<tr>
<td>0–12 h</td>
<td>1,450 ± 246</td>
<td>10.0 ± 1.3</td>
</tr>
<tr>
<td>12–24 h</td>
<td>1,306 ± 149</td>
<td>11.3 ± 1.9</td>
</tr>
<tr>
<td>24–36 h</td>
<td>1,197 ± 194</td>
<td>7.0 ± 1.2</td>
</tr>
<tr>
<td>Total (0–36 h)</td>
<td>3,937 ± 634</td>
<td></td>
</tr>
<tr>
<td>Healthy volunteers</td>
<td>0–12 h</td>
<td>795 ± 130</td>
</tr>
<tr>
<td></td>
<td>12–24 h</td>
<td>960 ± 122</td>
</tr>
<tr>
<td></td>
<td>24–36 h</td>
<td>887 ± 107</td>
</tr>
<tr>
<td>Total (0–36 h)</td>
<td>2,642 ± 241</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SE ($n = 13$). Difference from healthy volunteers: *$P = 0.004$; †$P = 0.005$; ‡$P = 0.002$. 

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duration, in addition to fluctuations in ambient blood glucose and insulin concentrations during these studies, may explain some of the variability in vasodilatory responses observed. However, notwithstanding the fact that there are differences in the characteristics of the type 1 diabetic patients studied, most studies have found no difference between diabetic and control subjects when the vascular responses to carbachol (8,10), acetylcholine (9), and methacholine (11) were measured in the forearm.

Although a reduced response to methacholine was reported by Johnstone et al. (12), blood glucose does not appear to have been controlled during the forearm blood flow measurements, and volunteers received high-dose aspirin for 3 days before the study day. Furthermore, it is now clear that methacholine acts largely through mechanisms other than the L-arginine/NO pathway in the forearm (33); therefore, changes in forearm blood flow in the diabetic subjects may have been mediated through some mechanism other than NO. O’Driscoll et al. (13), using historical control subjects, reported a diminished response to acetylcholine in normoalbuminuric type 1 diabetic patients. However, vasodilatory responses to acetylcholine in the forearm can be significantly affected by differences in forearm length due to the rapid metabolism of acetylcholine by cholinesterase (34).

Using L-NMMA, a defect in basal NO action was demonstrated in type 1 diabetic subjects with chronic disease when measurements were performed under euglycemic conditions. This defect was only significantly different from control subjects in type 1 diabetic subjects with microalbuminuria (10). In support of this finding, Mäkimatilla et al. (14) demonstrated no difference in stimulated release of NO using acetylcholine or in basal NO action using L-NMMA in the forearm vasculature of type 1 diabetic subjects with normal urinary albumin excretion under euglycemic conditions. In contrast, type 1 diabetic subjects with minimal urinary albumin loss showed a diminished vascular response to the systemic administration of L-NMMA (29). Although L-NMMA has the advantage of providing a specific measurement of basal vasodilation due to NO, it will not distinguish between a reduced production of NO with increased sensitivity or an increased production of NO with a decreased sensitivity to NO in the vasculature. We have not performed a functional study in our group of type 1 diabetic subjects with normal urinary albumin excretion nor are we inferring that the increase in NO production observed in the diabetic subjects is a direct eval-

![FIG. 2. The rate of elimination of \( ^{15}N \)-nitrate was calculated by plotting the amount of \( ^{15}N \)-nitrate to be excreted on a logarithmic scale versus time. The elimination rate constant for subjects with type 1 diabetes \((\cdot;-0.087)\) was not different from that for control subjects \((\cdot; -0.091)\) \((P = 0.344)\).](image)

![FIG. 3. Urinary \( ^{15}N \)-nitrate and 2,3-dinor-5,6-dihydro-F\(_2\)t-IsoP excretion in male \((n = 8)\) and female \((n = 5)\) type 1 diabetic subjects and control subjects. Horizontal bars represent median values. The highest \( ^{15}N \)-nitrate excretion was observed in female diabetic subjects. \(^*P = 0.037\) female vs. male diabetic subjects, \(^{**}P = 0.017\) female vs. male control subjects. The excretion of \( ^{15}N \)-nitrate remained significantly higher in male diabetic subjects compared with control subjects, independent of the outlier in the diabetic group. There was no difference in urinary excretion of 2,3-dinor-5,6-dihydro-F\(_2\)t-IsoP between male and female diabetic subjects or control subjects. \(\cdot\), Male type 1 diabetic patient; \(\bigcirc\), female type 1 diabetic patient; \(\bigtriangleup\), male control subject; \(\triangle\), female control subject.](image)

![FIG. 4. Spearman rank correlation of urinary excretion of \( ^{15}N \)-nitrate and 2,3-dinor-5,6-dihydro-F\(_2\)t-IsoP in subjects with type 1 diabetes \((A)\) and matched control subjects \((B)\). There is a significant inverse correlation in diabetic subjects \((r = -0.62, P = 0.012)\) that is not present in control subjects \((r = 0.37, P = 0.107)\).](image)
vation of endothelial production. The source and the functional assessment of NO production needs to be assessed in a future study.

Mäkimatilla et al. (14) also found no difference in basal NO action and an increase in stimulated NO release in type 1 diabetic subjects with macroalbuminuria (UAER 1,210 ± 434 µg/min [mean ± SE]) compared with normoalbuminuric diabetic subjects and control subjects. However, 7 of the 10 diabetic subjects in this group had been treated with ACE inhibitors (ACE-I). ACE inhibitors have been shown to reverse the defect in basal action of NO in essential hypertension (35) and to produce a significant sympatholytic effect in the forearm circulation, which is mediated at a postsynaptic level (36). In addition, the reversal of endothelial dysfunction induced by ACE inhibitors was sustained 3 days after discontinuing ACE inhibition in subjects with coronary artery disease (37). ACE inhibitors have also been shown to increase the response to acetylcholine-mediated NO release in type 1 diabetes (13). Therefore, the role of ACE inhibition in the reversal of endothelial dysfunction, in addition to any sympatholytic action that may be taking place, makes it difficult to interpret the forearm blood flow results in the macroalbuminuric group of type 1 diabetic subjects in the Finnish study (14).

The observation that F₂-isoprostanes, the most reliable measure of oxidative stress in vivo (38), are not elevated in the group of diabetic subjects in this study compared with nondiabetic healthy control subjects is important. The origin of the NO production we measured is unclear; however, the finding that an increase in NO synthesis is inversely correlated with a measurement of oxidative stress is consistent with the hypothesis that NO is being inactivated by enhanced oxidative species. In 2 preliminary reports of isoprostane production in diabetes, plasma concentrations and urinary excretion of F₂-isoprostanes were increased in diabetic subjects compared with control subjects (39,40). However, it is unclear whether the subjects studied in either of these studies had type 1 or type 2 diabetes. Although there is concern that urinary 8-iso-prostaglandin-F₂α may reflect local renal production rather than systemic production of 8-iso-prostaglandin-F₂α (41), there was a large variance in urinary excretion of 8-iso-prostaglandin-F₂α among the diabetic subjects reported by Catella-Lawson et al. (40). This variance could possibly be explained by inclusion of diabetic subjects with different patient characteristics, such as differences in level of diabetic control, urinary albumin excretion, or duration of diabetes. In a third study of 23 poorly controlled type 1 diabetic subjects, levels of urinary 8-iso-prostaglandin-F₂α, measured by immunoreactivity, were found to be elevated compared with age-matched healthy control subjects (42).

In the presence of an isoprostane concentration similar to that in control subjects, the unexpectedly high synthesis of NO found in the diabetic group in this study may indicate a protective role for NO against reactive oxygen species. This finding may be particularly relevant in this group of diabetic subjects, all of whom had normal urinary albumin excretion, despite the majority who had type 1 diabetes for >20 years. In vitro studies have demonstrated an ability of NO to significantly inhibit both iron and ONOO⁻-induced lipid peroxidation (43). Although we cannot say in absolute figures how much NO is being produced in vivo in the diabetic subjects, the percentage of ¹⁵N-arginine converted to ¹⁵N-nitrate is significantly higher in the diabetic subjects compared with control subjects, reflecting a significantly higher NO production. The antioxidant effects of NO are related to its rate of production in proportion to that of O₂⁻, with high concentrations of NO producing inhibition of chain-propagating lipid peroxidation (44,45). It is therefore possible that some diabetic individuals are able to produce large amounts of NO, which may act as an antioxidant to reactive oxygen species produced during the hyperglycemic state of diabetes. It is conceivable that the high production of NO in the diabetic subjects in this study may have protected them from developing microalbuminuria. However, this will need to be confirmed by quantifying NO production in diabetic subjects who have developed microalbuminuria.

The increase in NO production documented in female diabetic subjects is consistent with our previous report in non-diabetic subjects, in which a sex difference was observed in whole-body NO synthesis (46). This may be related to estrogen production and could be an important aspect of management of type 1 diabetes in female subjects at the postmenopausal stage.

We have shown that patients with long-standing type 1 diabetes and normal urinary albumin excretion have increased NO synthesis compared with healthy control subjects. We did not demonstrate an increase in F₂-isoprostane production (an index of oxidative stress) in diabetic subjects compared with control subjects, but identified an inverse relationship between NO synthesis and F₂-isoprostane production in diabetic subjects that was not present in control subjects.

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