The influence of improved glycaemic control with insulin and sulphonylureas on acute phase and endothelial markers in Type II Diabetic subjects

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Abstract

Aims/hypothesis. Improved glycaemic control might reduce both microvascular and macrovascular complications of Type II diabetes (non-insulin-dependent) mellitus. To explore such possible mechanisms, we investigated the effects of intensive treatment on markers of endothelial dysfunction and of acute phase activation, using both sulphonylureas and insulin.

Methods. In a randomised cross-over study we gave sulphonylureas or insulin each for a period of 16 weeks to 22 poorly controlled Type II diabetic subjects who were being treated by diet. There was a 4 week washout period between each treatment. Subjects were studied at baseline and at the end of each treatment.

Results. Treatment with sulphonylureas and insulin resulted in similar improvements in glycaemic control (glycated haemoglobin, baseline: 11.8 [(SD 2.2)%; after sulphonylureas: 8.6 (1.2)%, $p < 0.001$; after insulin: 8.6 (1.2)%, $p < 0.001$] and in insulin sensitivity {metabolic clearance rate of glucose, baseline: median 1.75 [interquartile (IQ) range 1.41, 2.27] ml · kg⁻¹ · min⁻¹; after sulphonylureas: 2.41 (1.82, 3.01) ml · kg⁻¹ · min⁻¹, $p = 0.001$; after insulin: 2.23 (1.92, 2.75) ml · kg⁻¹ · min⁻¹, $p = 0.027$ }. There were no significant changes in concentrations of endothe-

lial markers von Willebrand factor, cellular fibronectin, thrombomodulin, tissue plasminogen activator, soluble E-selectin or soluble intercellular adhesion molecule-1 or in urinary albumin excretion rate after either treatment period. Concentrations of C-reactive protein were not significantly influenced by sulphonylureas but fell after insulin [baseline: median 4.50 (IQ range 1.37, 6.44) µg · ml⁻¹; sulphonylureas: 2.69 (0.88, 9.65) µg · ml⁻¹ ($p = 0.53$); insulin: 2.07 (1.16, 5.24) µg · ml⁻¹ ($p = 0.017$)]. There were, however, no significant effects of either treatment on circulating concentrations of fibrinogen ($p = 0.28$ – 0.34) or of the proinflammatory cytokines interleukin-6 or tumour necrosis factor- α ($p = 0.65$ – 0.79).

Conclusion/interpretation. Markers of endothelial dysfunction and concentrations of proinflammatory cytokines in Type II diabetes are not influenced by improved glycaemic control over 16 weeks. Improved metabolic control with insulin could, however, be associated with reduced concentrations of the acute phase marker C-reactive protein. [Diabetologia (2000) 43: 1099–1106]

Keywords Glycaemic control, insulin, sulphonylureas, insulin resistance, endothelial dysfunction, tumour necrosis factor- α , interleukin-6, C-reactive protein.

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Abbreviations: CHD, Coronary heart disease; CRP, C-reactive protein; AGE, advanced glycation end products; SSPG, steady state plasma glucose; MCR, metabolic clearance rate; sE-sel, Soluble E-selectin; sICAM-1, soluble intercellular adhesion molecule; tPA, tissue plasminogen activator; vWF, von Willebrand factor; AER, albumin excretion rate.

Improved glycaemic control in Type I (insulin-dependent) diabetes mellitus has been shown to reduce the incidence of microvascular complications [1]. The UK Prospective Diabetes Study has recently shown statistically significantly lower risks of microvascular disease with intensified glycaemic control [2]. The mechanism of the relation between glycaemia and both microvascular and macrovascular disease is controversial. Microalbuminuria in patients with Type I diabetes and also those with Type II (non-insulin-dependent) diabetes mellitus clusters with a pronounced predisposition to severe microvascular (proliferative retinopathy) and macrovascular [coronary heart disease (CHD)] complications and it has been postulated that endothelial dysfunction underlies these associations [3,4].

Insulin resistance has been linked to cardiovascular risk in diabetic and non-diabetic subjects [5, 6]. Insulin resistance in itself clusters with microalbuminuria and with other markers of endothelial dysfunction [7]. We have recently suggested that these associations are the consequence of the common antecedence of adipose tissue-derived proinflammatory cytokines [8]. These molecules are expressed and released by adipocytes and endothelial cells as well as by inflammatory cells [9–11], and have important roles in insulin signalling [12] and in endothelial activation [13]. In addition, the circulating cytokine interleukin-6 (IL-6) is the major determinant of hepatic production of the acute phase proteins C-reactive protein (CRP) and fibrinogen [14]. There is growing evidence that increased concentrations of CRP predict the incidence of CHD over as long as 15 years [15–17] and it has been proposed that CRP itself has some role in the atherothrombotic process [18,19]. Circulating concentrations of the cytokines IL-6 and tumour necrosis factor- α (TNF- α) are increased in diabetic patients [20,21], perhaps as a consequence of the effects of advanced glycation end-products (AGE) on the expression of these cytokines by endothelial cells and macrophages [22,23]. Moreover, hyperglycaemia could have direct effects on endothelial function, either through mechanisms associated with 'pseudohypoxia' [24] or activation of protein kinase C [25] or through receptor-mediated activation by AGE [26].

We used stored blood samples from a cross-over study investigating the effects of improved glycaemic control on fibrinolysis, using sulphonylureas and insulin for 16 weeks each, in 22 poorly controlled Type II diabetic patients to assess the influences of reducing glycaemia on markers of endothelial activation, on hepatic acute phase proteins and on insulin sensitivity. We have previously shown that these two modes of therapy have different effects on fibrinolytic activity [27] which we believe results from divergent effects on endogenous production of insulin precursor molecules. Thus the study design also enabled

us to compare the effects of these two different modes of therapy on endothelial and acute phase variables. The hypotheses we wished to test were that:

1. Improved glycaemic control, using either insulin or sulphonylureas, is associated with reduced concentrations of circulating markers of endothelial dysfunction.
2. Improved glycaemic control, using either insulin or sulphonylureas, is associated with reduced concentrations of acute phase markers and of cytokines.
3. Improvements in insulin action consequent upon improved glycaemic control are related to reduced endothelial and acute phase activation.

Subjects and methods

Subjects. Poorly controlled Type II diabetic patients ($n = 22$) referred to the outpatient department of the Whittington Hospital were recruited for a cross-over study to investigate the effects of different modes of glycaemic control on fibrinolytic activity [27]. In this study we used frozen samples from subjects investigated with the earlier protocol and whose characteristics are shown in Table 1. Diabetes was diagnosed using World Health Organisation (WHO) criteria [28]. The subjects were on diet alone and were investigated before the instigation of additional treatment. Patients with a history of ischaemic heart disease, renal or hepatic disease were excluded, as well as those on any other medication including antihypertensive agents and aspirin. The study was approved by the Islington Health Authority Ethical Committee and all subjects gave informed consent.

Study design. The study design has been described previously [27]. Briefly subjects were treated with insulin [premixed human soluble and isophane 30:70, (Humulin M3, Lilly, Indianapolis, Ind., USA)] or sulphonylurea (glibenclamide) once or twice daily each for 16 weeks in a randomised cross-over design, with a washout period of 4 weeks. Although this period of washout is inadequate to allow glycated haemoglobin values to return to baseline, it was not felt justifiable to withdraw drug treatment for longer in these poorly controlled patients. Dosage adjustment was made by telephone twice weekly on the basis of home blood glucose monitoring and weekly (first 4 weeks) followed by monthly visits, aiming for a fasting plasma glucose concentration of 6.0 mmol/l or less. The mean doses were 27.3 ± 11.7 units a day for insulin and 8.8 ± 5.6 milligrams a day for glibenclamide. Subjects were seen at baseline, after 16 weeks of treatment 1, after a 4 week washout period and after 16 weeks of treatment 2, having been told to omit their treatment on the evening before and the morning of study.

Subjects attended the Department of Medicine after an overnight fast. Weight, height, waist-to-hip ratio and skinfold measurements were done as described previously [27] and after a 10-min rest blood pressure was recorded in duplicate with a random zero sphygmomanometer (Hawksley, Lancing, Sussex). Fasting venous blood samples were taken without venous stasis for measurement of concentrations of plasma glucose (fluoride), fructosamine, insulin, endothelial and hepatic acute phase markers, proinflammatory cytokines (lithium heparin), fibrinogen (acid citrate dextrose) and values of glycated

Table 1. Characteristics of subjects, glycaemia and insulin sensitivity at baseline and after interventions

	Baseline	After sulphonylurea	After insulin	<i>p</i> (sulphonylurea vs insulin)
Age	56.3 (8.0)			
Sex (female, male)	8 f, 14 m			
Duration (years)	4.0 ± 6.2			
BMI (kg/m ²)	28.9 ± 3.8	30.5 ± 4.2 ^c	30.9 ± 4.2 (<i>n</i> = 20) ^c	0.31
Waist:hip ratio	0.98 ± 0.11	0.93 ± 0.08 ^a	0.93 ± 0.09 (<i>n</i> = 20) ^a	0.92
Subscapular:triceps ratio	1.72 ± 0.67	1.92 ± 0.76 (<i>n</i> = 21)	2.23 ± 1.01 (<i>n</i> = 19) ^b	0.03
Fasting plasma glucose (mmol/l)	12.7 ± 3.0 (<i>n</i> = 21)	9.3 ± 2.2 (<i>n</i> = 21) ^c	9.9 ± 1.9 (<i>n</i> = 19) ^b	0.16
Glycated haemoglobin (%)	11.8 ± 2.2	8.6 ± 1.2 ^c	8.6 ± 1.2 (<i>n</i> = 20) ^b	0.63
Fructosamine (μmol/l)	2.53 ± 0.67	1.96 ± 0.46 ^c	1.98 ± 0.31 (<i>n</i> = 19) ^c	0.77
Metabolic clearance rate of glucose (ml · kg ⁻¹ · min ⁻¹)	1.75 (1.41–2.27) (<i>n</i> = 20)	2.41 (1.82–3.01) ^c	2.23 (1.92–2.75) (<i>n</i> = 19) ^a	0.56
Fasting insulin (pmol/l)	26.4 (12.6–42.7)	37.8 (20.5–54.1) ^b	43.8 (23.9–76.8) (<i>n</i> = 20) ^c	0.39

Data are shown as means ± SD or as median (range) for skewed data. The number (*n*) of subjects is 22 except where indicated otherwise. Significance of differences were analysed using paired Student's *t*-tests on untransformed or logarithmi-

cally transformed data. Significance of differences after sulphonylurea or insulin compared with baseline are shown in the corresponding column.

^a *p* < 0.05 ^b *p* < 0.01 ^c *p* < 0.001

haemoglobin (EDTA). Blood was centrifuged at 3000 *g* for 10 min at 4°C and stored immediately at –70°C.

An insulin sensitivity test was done using the modified Harano technique [29] as described previously [27]. This comprises a continuous infusion of insulin [50 mU · kg⁻¹ · h⁻¹ Actrapid (Novo Nordisk, Basingstoke, UK)] and glucose (6 mg · kg⁻¹ · min⁻¹) for 150 min, with arterialised blood being drawn from the contralateral arm every 5 min from 120 to 150 min for calculation of steady state plasma glucose concentration (SSPG). Metabolic clearance rate of glucose (MCR-glucose) was calculated as

MCR-glucose = (glucose infusion rate /SSPG) (ml · kg⁻¹ · min⁻¹).

Biochemical methods. Plasma glucose was measured using a glucose oxidase method (Glucose II analyser, Beckman, Brea, Calif., USA). Fructosamine was measured using a commercial kit (Roche Diagnostics, Hatfield, UK) with a Cobas Fara centrifugal analyser (intra-assay CV < 3%, normal range 1.55–2.10 μmol/l) and haemoglobin A_{1C} by electroendosmosis (Corning, Palo Alto, Calif., USA) (normal range 6.5–8.5%). Insulin was assayed using a specific in-house 2-site immunoenzymometric assay [30] and fibrinogen using the von Claus method (BCL, Lewes, Sussex). Plasma concentrations of thrombomodulin, von Willebrand factor, cellular fibronectin [31], soluble E-selectin (sE-sel), soluble intercellular adhesion molecule-1 (sICAM-1), tissue plasminogen activator (tPA) antigen and C-reactive protein (CRP) were measured at the Gaubius Laboratory, TNO-PG, Leiden, Netherlands. Thrombomodulin was assayed using an ELISA kit (Diagnostic Stago, Asnières, France) [32], von Willebrand factor (vWF) antigen by an ELISA essentially as described [33] using polyclonal antibodies from Dako (Glostrup, Denmark) and cellular fibronectin using a sandwich ELISA with a monoclonal antibody IST-9 (Sera-Lab, Crawley Down, UK) against the ED-A domain for capture and a peroxidase-conjugated polyclonal fibronectin antibody (Dako) for detection. Soluble E-selectin was measured with the Biotrak h-sELAM-1 ELISA kit (Amersham Life Science, Little Chalfont, UK) and sICAM-1 with a BioSource Europe (Nivelles, Belgium) ELISA kit. Tissue plasminogen activator antigen was measured by ELISA (Organon

Teknika, Turnhout, Belgium) which recognise both the free form of the factor and complexes of tPA with plasminogen activator inhibitor-1. The C-reactive protein was measured with a highly sensitive ELISA procedure [34], that has a range of 0.25–10.25 μg/ml and an inter-assay CV of 8%. Tumour necrosis factor-α and IL-6 were measured by enzyme-linked immunosorbent assays (ELISA) (R and D Systems, Oxford, UK). Each subject collected three separate timed-overnight urine samples in the week before the study and albumin concentration was measured by an in-house adaptation of a previously described immunoassay [27,35]. Mean albumin excretion rate (AER) was expressed as μg · min.

Statistical methods. The data were analysed with the Statistical Package for the Social Sciences (SPSS-PC) and are expressed as mean SD for normally distributed variables and median [interquartile (IQ) range] for skewed variables. As reported previously [27], concentrations of plasma glucose, fructosamine and haemoglobin A_{1C} values increased during the 4-week washout phase but did not return to baseline values between treatments. For this reason, as in our previous analyses, the variables after improved glycaemic control with either sulphonylureas or insulin were compared with the pretreatment baseline, the validity of this approach being confirmed by a lack of treatment order effect. The data were analysed by paired Student's *t*-tests for normally distributed data (before or after logarithmic transformation) or by Wilcoxon's rank-sum test for those data which remained skewed after logarithmic transformation (AER). Pearson or Spearman's rank correlation was used to explore the relations between changes in the variables. Statistical significance was taken as a *p* value of less than 0.05. We estimated that the number of subjects studied provided 80% power to detect, at the 5% level, a treatment difference of 11% in vWF and fibrinogen, of 13% in tissue plasminogen activator, of 20% in AER, of 38% in CRP, of 15% in IL-6, and of 9% in TNF-α. The same power existed to detect treatment differences of between 11% and 17% in other variables.

Table 2. Markers of endothelial function at baseline and after interventions

	Baseline	After sulphonylurea	After insulin	<i>p</i> (sulphonylurea vs insulin)
von Willebrand factor (%)	142 (116–209)	136 (108–183)	140 (110–205)	0.77
Cellular fibronectin (%)	304 ± 162	308 ± 184	311 ± 191	0.89
Thrombomodulin (ng/ml)	126 ± 71	119 ± 67	125 ± 64	0.13
Tissue plasminogen activator antigen (ng/ml)	25.0 ± 8.5	26.5 ± 6.9	27.9 ± 6.9	0.46
Soluble E-selectin (ng/ml)	31.9 (23.4–43.9) (<i>n</i> = 16)	30.4 (23.1–60.0) (<i>n</i> = 15)	30.7 (24.5–42.9)	0.11
Soluble intercellular adhesion molecule ⁻¹ (ng/ml)	498.6 (445.2–698.9)	545.7 (423.9–730.2) (<i>n</i> = 16)	580.2 (484.8–671.6)	0.60
Albumin excretion rate (µg/min)	7.9 (5.9–22.4) (<i>n</i> = 20)	6.9 (5.0–24.0) (<i>n</i> = 20)	6.8 (4.0–14.6)	0.76

Data are shown as means ± SD or as median (range) for skewed data. The number (*n*) of subjects is 17 except whose indicated otherwise. Significance of differences were analysed using paired Student's *t* tests on untransformed or logarithmically transformed data, except for albumin excretion rate,

which remained skewed after transformation, where Wilcoxon's test was used. Significance of differences after sulphonylurea or insulin compared with baseline are shown in the corresponding column

Table 3. Acute phase proteins and proinflammatory cytokines at baseline and after interventions

	Baseline	After sulphonylurea	After insulin	<i>p</i> (sulphonylurea vs insulin)
Fibrinogen (mg/dl)	251 ± 46 (<i>n</i> = 22)	239 ± 30 (<i>n</i> = 22)	243 ± 47 (<i>n</i> = 19)	0.67
C-Reactive protein (µg/ml)	4.50 (1.37–6.44)	2.69 (0.88–9.65)	2.07 (1.16–5.24) ^a	0.12
Interleukin-6 (pg/ml)	2.30 (1.55–5.35)	1.80 (1.37–4.19)	1.85 (1.42–4.30)	0.82
Tumour necrosis factor-α (pg/ml)	3.00 (2.57–4.20)	3.21 (2.64–4.50)	3.06 (2.58–4.60)	0.83

Data are shown as means ± SD or as median (range) for skewed data. The number (*n*) of subjects is 17 except where indicated otherwise. Significance of differences were analysed using paired Student's *t* tests on untransformed or logarithmi-

cally transformed data. Significance of differences after sulphonylurea or insulin compared with baseline are shown in the corresponding column.

^a *p* < 0.05

Results

We studied 22 subjects (14 men and 8 women) at baseline and after 16 weeks of treatment with sulphonylureas and 20 completed both limbs of the study. One subject declined insulin therapy and one developed renal calculi during the insulin phase. Analyses of all the variables presented showed no evidence of effects of treatment order. Both treatments resulted in an statistically significant increase in body mass index, treatment with insulin, but not with sulphonylureas, resulted in a statistically significant increase in subscapular-to-triceps ratio, implying truncal fat deposition. The treatments produced parallel falls in glycaemia as indicated by the concentrations of fasting plasma glucose, fructosamine and glycated haemoglobin values, and parallel increases in serum insulin concentration. Values of glycated haemoglobin and concentrations of fructosamine were reduced to close to and within the normal range respectively, although fasting plasma glucose concentrations were marginally increased because treatment omitted before the study protocol. As reported previously [27], both treatments produced an increase in MCR-glucose, which was not statistically significantly different between the two treatments.

The effects of these interventions on concentrations of plasminogen activator inhibitor-1 and of proinsulin-like molecules have also been reported previously [27].

We estimated seven markers of endothelial activation/dysfunction, six in plasma and AER (Table 2). For none of these variables was there a significant difference either between concentrations at baseline and after either treatment phase or after the two treatment periods.

Fibrinogen concentrations were not significantly influenced by sulphonylurea or insulin treatment (Table 3). Concentrations of CRP were significantly lower after insulin treatment (*p* = 0.017) but not after sulphonylurea treatment (*p* = 0.53) (Fig. 1). Concentrations of IL-6 and TNF-α were not influenced by improved glycaemic control and were not different between treatments (Table 3).

We sought correlations between changes in the three measures of glycaemia and in those of insulin sensitivity, with changes in the endothelial and acute phase markers. Because we found no statistically significant between-treatment differences for any variable except CRP and in view of the the small numbers of subjects and the additional risk of spurious associations with increasing numbers of comparisons,

Fig. 1. Concentrations of C-reactive protein at baseline and after interventions

S = after sulphonylurea, I = after insulin, * $p = 0.017$, ** $p = 0.53$, *** $p = 0.12$

we combined the changes from baseline to the end of treatment on both interventions. Despite there being no significant effect of either treatment on concentrations of vWF, changes in these concentrations with treatment correlated with each measure of change in glycaemia ($r = 0.40$ – 0.49). There were no other consistent correlations of changes in measures of glycaemia or insulin sensitivity with those in other endothelial or acute phase markers.

Discussion

We have investigated 22 subjects before and after 16 weeks of intensive treatment with a sulphonylurea and with insulin, which resulted in virtual normalisation of concentrations of fructosamine and glycated haemoglobin values with the assays used for this study. We found a parallel improvement in insulin sensitivity with both treatments. Neither treatment resulted, however, in any change in any of the circulating endothelial markers or in AER. Although there was no statistically significant effect on the concentrations of fibrinogen or the proinflammatory cytokines IL-6 and TNF- α , those of CRP fell significantly with insulin but not with sulphonylurea treatment.

We used the seven measures shown in Table 2 as markers of endothelial activation. It is recognised that several of the circulating markers are synthesised by several cell types, and could thus lack specificity. Moreover, a number of endothelial markers respond to acute phase stimuli [8,36,37] so that the delineation between endothelial marker and acute phase protein is not distinct. For this reason we have referred to

CRP and fibrinogen as ‘hepatic acute phase proteins’ [38].

Some studies have suggested hyperglycaemia influences a variety of markers of endothelial dysfunction, either directly mediated through glucose [24,25,39–41] or through the influence of AGEs [22,23,26,42,43] or Amadori-modified proteins [44]. There are few studies which have assessed the influence of improved glycaemic control on such markers. Changes in glycaemia have been associated with parallel alterations in endothelium-dependent vasodilatation [45–47] and reduced concentrations of endothelial markers [48], although a decrease in values of glycated haemoglobin is not universally associated with a decrease in vWF concentrations [49–51]. In our study, despite there being no significant change in concentrations of vWF, there were positive associations between changes in each measure of glycaemia and those in vWF, although multiple comparisons increase the risks of Type I error. This might suggest that a potentially beneficial effect of improved glycaemic control is neutralised by adverse changes in vWF concentrations mediated by treatment-related mechanisms. We found no associations between changes in concentrations of sE-sel and the degree of glycaemia and insulin sensitivity, associations which have been described previously [48,52]. The lack of influence of improved glycaemic control on AER contrasts with previous reports in newly diagnosed [53,54] and established [55] diabetic patients.

There are few data on the influence of glycaemic control, treatment or insulin resistance on circulating concentrations of cytokines and hepatic acute phase proteins [56,57]. Both the oxidative stress associated with hyperglycaemia [58] and the influence of AGEs on endothelial cells and macrophages [22, 23] could link poor glycaemic control with increased expression and secretion of proinflammatory cytokines. Our finding of reductions in CRP concentrations with insulin but not with sulphonylurea treatment, unassociated with changes in concentrations of cytokines,

could be a spurious finding consequent upon multiple comparisons. It does, however, raise the possibility of actions of insulin on hepatic expression of acute phase proteins. Insulin has been shown to be a powerful inhibitor of cytokine-induced transcription of acute phase proteins in rat hepatoma cells [59]. Our observation that changes in concentrations of CRP were not associated with changes in concentrations of cytokines might support such an interpretation. The smaller increase in insulin concentrations on sulphonylurea treatment did not produce a fall in concentrations of CRP ($p = 0.53$). We found no parallel changes in fibrinogen concentrations, possibly because fibrinogen concentrations in diabetes seems to be related to changes in half-life in diabetic patients [60], in contrast to those of CRP, which are virtually dependent on synthetic rate [61]. Insulin treatment could be associated with increases in fibrinogen concentrations [62,63].

In non-diabetic subjects, we have found statistically significant relations between some markers of hepatic acute phase activation and several components of the insulin-resistance syndrome [8]. In this study, despite statistically significant improvements in insulin sensitivity, this was not accompanied by reductions in circulating concentrations of proinflammatory cytokines, which argues against a role for such molecules in the insulin resistance associated with hyperglycaemia.

Thus, in a crossover study, we have found no influence of improved glycaemic control on markers of endothelial dysfunction, although insulin treatment was associated with a fall in the concentration of CRP. These observations could imply that other approaches to improving endothelial function, such as treatment with angiotensin converting enzyme inhibitors or antioxidants, could have greater advantages than glycaemic control in the prevention of CHD in people with Type II diabetes.

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