Total Antioxidant Status and Coronary Artery Calcification in Type 1 Diabetes

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OBJECTIVE — Type 1 diabetes is associated with a high risk of coronary heart disease (CHD), despite the absence of dyslipidemia. Oxidative modification may render LDLs more atherogenic. We aimed to assess antioxidant status in type 1 diabetes and its association with coronary artery calcification (CAC).

RESEARCH DESIGN AND METHODS — Total antioxidant status (TAS) of serum was measured using the Trolox equivalent antioxidant capacity assay in 48 type 1 diabetic and 25 nondiabetic subjects. The presence of CAC was assessed in the diabetic subjects using electron beam computed tomography.

RESULTS — TAS was reduced in type 1 diabetic subjects compared with nondiabetic subjects (Mann-Whitney U test, P < 0.0001). There were associations between TAS and HbA1c (r = −0.43, P = 0.0026) and duration of diabetes (r = −0.35; P = 0.0157). Significant CAC was considered present if the Agatston score was >10. The diabetic subjects with significant CAC were older (P < 0.0001); had longer duration of diabetes (P = 0.0002); were more likely to have high blood pressure (P = 0.040); had higher total cholesterol concentration (P = 0.039); serum creatinine concentration (P = 0.003), and urinary albumin–to–creatinine ratio (P = 0.022); and had lower serum TAS (P = 0.018) compared with those without significant calcification. In logistic regression with CAC as the dependent variable, TAS was entered as a predictor, and the effects on its predictive value of adding other explanatory variables in bivariate analyses were assessed. The power of TAS to predict CAC was independent of many of the traditional CHD risk factors. Whereas TAS as a predictor was no longer statistically significant when age or duration of diabetes were entered into the model, the odds ratio for a TAS concentration above the median value predicting significant CAC only increased from 0.19 to 0.26 and 0.32, respectively.

CONCLUSIONS — TAS is reduced in type 1 diabetes and is associated with the presence of CAC.

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Type 1 diabetes is associated with a high risk of coronary heart disease (CHD) (1), despite the absence of dyslipidemia. Total and LDL cholesterol concentrations are normal or decreased, and HDL cholesterol concentrations are normal or increased (2). There is evidence to suggest that oxidative damage is increased in diabetes (3). Whereas hyperglycemia can result in the generation of free radicals through several biochemical pathways (nonenzymatic glycation, the polyol pathway, and glucose autoxidation), the mechanisms underlying the oxidative damage in diabetes are not understood. Free radicals can result in consumption of antioxidant defenses and enhanced susceptibility to lipid peroxidation. Oxidative modification of LDL may be an important step in the atherosclerotic process (4).

Coronary artery calcification (CAC) detected by electron beam computed tomography (EBCT) has been shown to strongly correlate with prevalent CHD in type 1 diabetic subjects (5). The amount of CAC correlates with the histological coronary atherosclerotic burden (6).

We hypothesized that the total antioxidant status (TAS) of serum is reduced in type 1 diabetes and that the TAS of serum in type 1 diabetic subjects is inversely associated with CAC detected by EBCT.

RESEARCH DESIGN AND METHODS — Type 1 diabetic subjects were recruited consecutively from the diabetic clinics at St Mary’s and St Charles Hospitals in London, U.K. Subjects had required insulin treatment within 1 year of diagnosis of diabetes and had undetectable fasting C-peptide concentrations (<100 pmol/l). Subjects were free from other significant medical conditions and had creatinine concentrations within the normal range. Healthy nondiabetic subjects were recruited from hospital staff. To investigate the effects of age and sex on TAS and CAC, recruitment of subjects was not limited according to age or sex, so that groups were not age- and sex-matched. Subjects taking nutritional supplements that contained substances commonly assumed to be antioxidants were not included. All subjects attended the Metabolic Day Ward at St Mary’s Hospital for venesection after a 10-h overnight fast and for clinical assessment by the same investigator (J.V.). In the diabetic subjects, clinical assessment involved assessing the presence of retinopathy, peripheral neuropathy, and peripheral vascular disease (PVD). The presence of retinopathy was defined as the detection of at least
one microaneurysm by direct ophthalmoscopy. The presence of peripheral neuropathy was defined as the absence of both ankle jerks and/or the bilateral absence of vibration sensation at the medial malleolus using a 128-Hz tuning fork. The presence of peripheral vascular disease was defined as the absence to palpation of all four foot pulses or a previous digital (or more proximal limb) amputation performed for ischemia. A single early morning urine sample was collected for measurement of albumin–to–creatinine ratio, and a ratio of >2.0 was taken to indicate the presence of microalbuminuria. Type 1 diabetic subjects also attended the Royal Brompton Hospital for imaging. We obtained local ethical committee approval and informed consent from all subjects.

**Biochemical methodology**

Clotted blood was centrifuged at 1,500 rpm for 30 min, and the serum was separated and frozen at −70°C. Samples were thawed and analyzed in batches. TAS was measured using the Trolox equivalent antioxidant capacity (TEAC) assay (Randox Laboratories, San Francisco, CA), a method developed by Miller et al. (7). Both water- and lipid-soluble antioxidants contained in the biological sample under investigation inhibit production of the radical cation of 2,2'-azinobis(3-ethylbenzothiazoline 6-sulfonate). Production of the radical cation was measured spectrophotometrically. The system was calibrated with Trolox, a water-soluble vitamin E analog. The assay was performed on a Cobas-Mira analyzer (Hoffmann-La Roche). The intra- and interassay coefficients of variation were 1.7 and 3.5%, respectively.

Blood glucose, HbA1c, total cholesterol, triglycerides, HDL cholesterol, creatinine, and urinary albumin–to–creatinine ratio were measured using standard laboratory techniques.

**EBCT to detect CAC**

Type 1 diabetic subjects underwent EBCT of the heart to detect CAC. High-resolution noncontrast enhanced EBCT examinations (Imatron C-100 scanner; Imatron, South San Francisco, CA) were performed. We obtained 40 contiguous 3 mm–thick transaxial images for each subject, commencing at the root of the aorta and proceeding caudally through the apex of the heart. The scans, acquired during one or two separate breathholding sequences, were triggered by the electrocardiographic signal at 80% of the RR interval, near the end of diastole and before atrial contraction, to minimize the effect of cardiac motion. The rapid image acquisition time virtually eliminates motion artifact related to cardiac contraction. For each study, a CAC score was determined using the method of Agatston et al. (8). The CAC score is the product of the area of CAC (at least two contiguous pixels, corresponding to an area of 0.51 mm², with a computed tomography [CT] density ≥130 Hounsfield units) and a factor rated 1–4 dictated by the maximum CT density within that lesion. A CAC score was calculated for each of the major coronary arteries (the left main, the left anterior descending, the circumflex, and the right coronary arteries), the sum of which gave the total CAC score. The radiation exposure was <1 mSv. All scans were scored by the same radiologist. A small repeatability study (n = 20), involving the same radiologist and EBCT scanner and involving type 1 diabetic subjects, demonstrated that the within-observer agreement for classification of CAC to ≤10 or >10 was perfect (κ = 1.00) (H.M. Colhoun, personal communication).

**Statistical analysis**

For comparison of a continuous variable between two groups, the Mann-Whitney U test was used; for comparison of a continuous variable among three groups, the Kruskal-Wallis test was used. Continuous variables are expressed as medians with interquartile ranges. For comparison of categorical variables between two groups, Fisher’s exact test was used. The Spearman rank correlation test was used to assess the relationship between two continuous variables in a group. Multiple regression was used to assess the relationship between two or more continuous or categorical variables and a dependent variable, and residual analyses were performed. If residual analysis failed to establish homoscedasticity, or if the residuals were not normally distributed, then variables were either transformed or, if appropriate transformations were not possible, treated as categorical variables.

CAC required analysis as a categorical variable. A CAC score ≤10 was taken to indicate no significant coronary calcification, and a CAC score >10 was taken to indicate significant coronary calcification. Recent guidelines for the interpretation of CAC detected by EBCT have suggested that a score of ≤10 represents a <10% probability of significant CHD (9). Categorical divides of any calcification and of CAC scores of 400 have been shown to correlate with prevalent CHD in type 1 diabetic subjects (5). The association between TAS and CAC was therefore further explored by dividing CAC into three categories (CAC score of 0, 1–400, and >400).

Smoking status was treated categorically as ever or never smoked. Systolic and diastolic blood pressures were treated both as continuous variables and, to take account of subjects treated with antihypertensive agents, as categorical variables: subjects were classified as having low or high values, and those treated with antihypertensive agents were assigned to the high-value category. The categorical divides were 130 and 80 mmHg for systolic and diastolic blood pressure, respectively. For regression models involving parameters measured in the type 1 diabetic group, it was necessary to treat TAS as a categorical variable, with the median value for the diabetic group as the categorical divide.

For all statistical evaluations, a two-tailed P value <0.05 was considered significant. The Arcus Quickstat Biomedical package was used for the analyses (Longman Software Publishing).

**RESULTS** — A total of 48 type 1 diabetic and 25 nondiabetic subjects were recruited. Table 1 illustrates the demographic, clinical, and biochemical characteristics of both groups. In the diabetic group, HbA1c was 7.65% (interquartile [IQ] range 6.90–8.85), the duration of diabetes was 19 years (13–31); 25 (52%) had retinopathy, 12 (25%) had neuropathy, and 17 (35%) had microalbuminuria. Four (8%) of the diabetic subjects were known to have CHD (>50% stenoses on coronary angiography); one of the four subjects was also known to have cerebrovascular disease (CBVD), having had a previous transient ischemic attack. Five (10%) of the diabetic subjects (including the four subjects with CHD) had PVD: one had had a below-knee amputation, one had had digital amputations, and three had absent foot pulses; none had had peripheral revascularization procedures performed, and none reported
intermittent claudication at the time of study.

TAS was significantly lower in the diabetic than in the control subjects (1.26 [IQ range 1.21–1.33] vs. 1.43 [1.33–1.50] mmol/l; \( P < 0.0001 \); this held true for both male (1.29 [1.24–1.36] vs. 1.46 [1.40–1.52]; \( P < 0.0001 \)) and female subjects (1.24 [1.20–1.32] vs. 1.32 [1.28–1.47]; \( P = 0.0079 \)). In the diabetic group, TAS was similar in male and female subjects (1.29 [1.24–1.36] vs. 1.24 [1.20–1.32] mmol/l; \( P = 0.1543 \)); in the control group, TAS was significantly greater in male than in female subjects (1.46 [1.40–1.52] vs. 1.32 [1.28–1.47] mmol/l; \( P = 0.0155 \)). TAS decreased with age in male subjects (\( r = 0.34; P = 0.0283 \)), particularly in diabetic male subjects (\( r = 0.49; P = 0.0088 \)); however, there was no significant association between TAS and age in female subjects (\( r = 0.12; P = 0.5141 \)).

In the diabetic group, there were significant associations between TAS and HbA1c (\( r = 0.43; P = 0.0026 \)) and between TAS and duration of diabetes (\( r = 0.35; P = 0.0157 \)). There was insufficient power to demonstrate these associations in diabetic male and female subjects separately. In the diabetic group, there were no significant associations between TAS and BMI, smoking status, systolic or diastolic blood pressure, fasting glucose concentration, cholesterol concentration, triglyceride concentration, HDL cholesterol concentration, creatinine concentration, or the urinary albumin-to-creatinine ratio. TAS was significantly lower in those diabetic subjects with retinopathy compared with those without retinopathy (1.25 [IQ range 1.20–1.31] vs. 1.30 [1.24–1.37] mmol/l; \( P = 0.04 \)). This association was not independent of HbA1c; when either TAS or retinopathy were treated as the dependent variable in bivariate analyses, the association between TAS and retinopathy was no longer significant when HbA1c was entered into either model. There were no associations between TAS and the presence of nephropathy or peripheral neuropathy. In the control group, aside from the association with sex, there were no other significant associations with TAS.

The 48 diabetic subjects were divided into those without significant CAC (CAC score \( \leq 10; n = 28 \)) and those with significant CAC (>10; \( n = 20 \)). Table 2 illustrates the demographic, clinical, and biochemical characteristics of the two groups of type 1 diabetic subjects, divided according to the presence of significant CAC. All subjects with clinical evidence of CHD, CBVD, or PVD had significant CAC detected by EBCT.

Age and duration of diabetes were greater in those with coronary calcification (\( P < 0.0001 \) and \( P = 0.0002 \), respectively). Systolic blood pressure, treated as a continuous variable, was higher in those with coronary calcification (\( P = 0.012 \)); furthermore, the proportion of diabetic subjects categorized as having high systolic blood pressure was greater in those with coronary calcification (75 vs. 43%, \( P = 0.040 \)). Total cholesterol concentration was greater in those with coronary calcification (\( P = 0.039 \)), whereas triglyceride and HDL cholesterol concentrations were not significantly different. Although HbA1c tended to be higher in those with coronary calcification, this was of borderline statistical significance (\( P = 0.053 \)).

TAS was lower in those with coronary calcification (\( P = 0.018 \)), and their plasma creatinine concentrations and urinary albumin-to-creatinine ratios were higher (\( P = 0.0027 \) and \( P = 0.022 \), respectively). The proportion of subjects who had ever smoked (50 vs. 36%, \( P = 0.382 \)) and the proportions of subjects with retinopathy, microalbuminuria, and peripheral neuropathy were not significantly greater in those with coronary calcification.

The predictive value of TAS in determining the presence of significant CAC in the type 1 diabetic subjects and the extent to which its predictive value was affected by other variables was assessed using a multiple logistic regression model with CAC as the categorical dependent variable. TAS, as a categorical variable, was entered first as a predictor in the model, and the presence of diabetes, age, and duration of diabetes were entered subsequently as predictors.
and the effects on its predictive value of adding other explanatory variables in bivariate analyses were assessed. Table 3 demonstrates the degree to which the odds ratio, 95% confidence intervals, and P value for TAS as a predictor are affected by adjusting for other explanatory variables in bivariate analyses. The power of TAS to predict CAC was independent of many of the traditional CHD risk factors. Only age and duration of diabetes reduced the predictive power. In a young-onset disease such as type 1 diabetes, it is difficult to separate out the effects of age from the effects of duration of disease. Adjusting for age and duration of diabetes only increased the odds ratio for TAS from 0.19 to 0.26 and 0.32, respectively, suggesting that only part of the association between TAS and CAC was accounted for by these covariates. To the best of our knowledge, no other studies have demonstrated an association between a measure of oxidative stress and CAC in subjects with type 1 diabetes. Therefore, if oxidative stress does contribute to the higher CHD risk in type 1 diabetes, its effect is not mediated solely by hyperglycemia.

CONCLUSIONS — This study has demonstrated a negative association between TAS and CAC in subjects with type 1 diabetes. The power of TAS to predict CAC was independent of many of the traditional CHD risk factors. Only age and duration of diabetes reduced the predictive power. In a young-onset disease such as type 1 diabetes, it is difficult to separate out the effects of age from the effects of duration of disease. Adjusting for age and duration of diabetes only increased the odds ratio for TAS from 0.19 to 0.26 and 0.32, respectively, suggesting that only part of the association between TAS and CAC was accounted for by these covariates. To the best of our knowledge, no other studies have demonstrated an association between a measure of oxidative stress and CAC in diabetes.

We have demonstrated reduced TAS in type 1 diabetic compared with nondiabetic control subjects. The reduction in TAS in type 1 diabetic subjects was associated with increasing HbA1c and duration of diabetes. A reduction in TAS was also associated with increasing age, at least in men. Others have demonstrated reduced antioxidant capacity in type 1 diabetics. Significant reductions in total radical trapping parameter (TRAP) were found in the plasma of poorly controlled type 1 diabetic subjects, together with inverse associations between TRAP values and indexes of glycemic control (10). Reduced TRAP was also found in well-controlled type 1 diabetic subjects without evident complications (11). Although HbA1c was associated with some of the variation in TAS in the current study, and may have contributed to reduced TAS in type 1 diabetic compared with control subjects, HbA1c did not affect the association between TAS and CAC. Therefore, if oxidative stress does contribute to the higher CHD risk in type 1 diabetes, its effect is not mediated solely by hyperglycemia.

Several assays have been developed to assess the TAS of a biological sample (rev. in [12]). We have previously used the TEAC assay to demonstrate that higher preoperative TAS concentrations result in lower postoperative lipid peroxide concentrations and smaller postoperative increases in cardiac troponin T concentrations after coronary artery bypass graft-
Table 3—Risk of type 1 diabetic subjects having significant CAC (>10) if the serum total antioxidant status is greater than the median value (1.26 mmol/l Trolox equivalent), with adjustment for covarates

<table>
<thead>
<tr>
<th>Model</th>
<th>Odds ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univariate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>0.19 (0.05–0.66)</td>
<td>0.0095</td>
</tr>
<tr>
<td>Bivariate-adjusted for:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.26 (0.05–1.45)</td>
<td>0.1245</td>
</tr>
<tr>
<td>Sex</td>
<td>0.14 (0.03–0.57)</td>
<td>0.0064</td>
</tr>
<tr>
<td>Duration of diabetes</td>
<td>0.32 (0.07–1.36)</td>
<td>0.1224</td>
</tr>
<tr>
<td>Smoking status (ever or never)</td>
<td>0.18 (0.05–0.67)</td>
<td>0.0100</td>
</tr>
<tr>
<td>Systolic BP (continuous variable)</td>
<td>0.13 (0.03–0.38)</td>
<td>0.0077</td>
</tr>
<tr>
<td>Systolic BP (low or high)</td>
<td>0.14 (0.03–0.59)</td>
<td>0.0070</td>
</tr>
<tr>
<td>Diastolic BP (continuous variable)</td>
<td>0.19 (0.05–0.70)</td>
<td>0.0125</td>
</tr>
<tr>
<td>Diastolic BP (low or high)</td>
<td>0.18 (0.05–0.66)</td>
<td>0.0093</td>
</tr>
<tr>
<td>HbA1c</td>
<td>0.22 (0.05–0.98)</td>
<td>0.0469</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>0.20 (0.05–0.78)</td>
<td>0.0201</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.19 (0.05–0.69)</td>
<td>0.0121</td>
</tr>
<tr>
<td>Log(triglyceride)</td>
<td>0.14 (0.03–0.57)</td>
<td>0.0062</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.15 (0.04–0.60)</td>
<td>0.0071</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.17 (0.04–0.64)</td>
<td>0.0089</td>
</tr>
<tr>
<td>Urinary albumin-to-creatinine ratio</td>
<td>0.19 (0.05–0.71)</td>
<td>0.0130</td>
</tr>
</tbody>
</table>

BP, blood pressure.

It has been suggested that increases in oxidative stress in type 1 diabetic women compared with men could account for the loss of sex difference in CHD incidence in type 1 diabetes (14). However, we were unable to demonstrate an effect of sex on TAS in type 1 diabetic subjects. In nondiabetic control subjects, men had significantly higher values than women. Whereas this is contrary to what might have been expected based on sex differences in CHD incidence in nondiabetic subjects, the number of control subjects was probably too small to allow reliable subgroup analysis.

EBCT-detected CAC has been strongly correlated with clinical CHD in type 1 diabetes (5). The odds ratio of clinical CHD increased from 1.0 in type 1 diabetic men without calcification to 4.3 and 50.9 in men with CAC scores of 1–399 and of ≥400, respectively. In type 1 diabetic women, the odds ratio increased from 2.2 to 3.8 and 28.8 in the same CAC score categories (5). We have demonstrated a progressive decrease in TAS in the same CAC score categories. All subjects with clinical evidence of macrovascular disease had significant coronary calcification detected by EBCT in the current study. Type 1 diabetes is associated with a loss of the sex differences in cardiovascular mortality (1) and CAC (15) seen in the nondiabetic population. We were unable to demonstrate sex differences in CAC in the type 1 diabetic subjects studied.

Associations have been demonstrated between CHD incidence in North American type 1 diabetic subjects followed prospectively for 6 years and age, duration of diabetes, systolic blood pressure, and total cholesterol (16). Associations between CAC and these variables were demonstrated in the current study. A strong association between nephropathy and CHD in type 1 diabetes has been suggested (17), although the extent to which such an association is independent of blood pressure and lipid parameters remains unclear (18). Associations between CAC and plasma creatinine concentration and between CAC and urinary albumin-to-creatinine ratio were demonstrated in the current study. No association between CHD incidence and HbA1c was demonstrated in North American type 1 diabetic subjects (16), and cross-sectional associations in both European and North American type 1 diabetic populations between prevalent CHD and hyperglycemia have been inconsistent (18). The association between CAC and HbA1c in the current study was of only borderline statistical significance. Whereas an association between CHD incidence in type 1 diabetic subjects and smoking has been demonstrated (16), there was no cross-sectional association between prevalent CHD and smoking in the European and North American type 1 diabetic populations (18). Whereas there was a higher proportion of subjects who had ever smoked in those with significant CAC in the current study, this failed to achieve statistical significance.

A limitation of this study was that our numbers were relatively small, particularly for the comparison between those type 1 diabetic subjects without and those with significant CAC. However, a post hoc power calculation based on the one standard deviation difference in loge TAS between those type 1 diabetic subjects without and those with significant CAC suggested that a sample of ~20 subjects in each group was sufficient to detect such a difference with 90% power and 5% significance. Other comparisons were performed on larger numbers, with differences as large as, or even larger than, the 1 SD difference in loge TAS.

Another limitation of the study was the imprecise measurement of clinical parameters, such as assessments for the presence of microvascular complications. True associations between these complications and CAC may have therefore been missed. Furthermore, the imprecision of CAC scores on the basis of single, rather than duplicate, EBCT scans has been acknowledged (19). Nevertheless, we have demonstrated an association between TAS and CAC. Although there may be some participation bias (in that not all type 1 diabetic subjects invited to participate in the study agreed to do so), it is highly unlikely that such bias would affect the relation between TAS and CAC score specifically.

Although the study was cross-sectional, it is possible that reduced antioxidant status resulted in coronary calcification, representing CHD. It is also possible, however, that ischemia resulting from CHD was causing the reduction in antioxidant status. A prospective study would be required to establish causality. An association between oxidative stress and CHD in type 1 diabetes raises the possibility that interventions directed toward manipulating antioxidant status may reduce the increased risk of CHD in type 1 diabetes.
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