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Unaltered Antioxidant Activity of Plasma in Subjects at Increased Risk for IDDM

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Evidence suggests that free oxygen radicals are involved in the destruction of islet β-cells in insulin-dependent diabetes mellitus (IDDM). Therefore, we determined the plasma antioxidant activity in 51 healthy unaffected children and adolescents randomly chosen from a study of β-cell autoimmunity in school-children in northern Finland. Twenty-two subjects tested positive for one or more IDDM-associated autoantibodies and 9 subjects had at least two of the three antibodies tested (antibodies against islet cells, ICA; glutamic acid decarboxylase, GADA; insulin, IAA). There was no significant association of total plasma antioxidant potential, plasma concentrations of α-tocopherol, ascorbic acid, protein thiols, or uric acid with the presence of ICA, GADA, or IAA. A reduced first-phase insulin response to intravenous glucose was also not associated with reduced plasma antioxidant activity. These results indicate that the plasma antioxidant activity is not decreased in subjects at increased risk for IDDM. Furthermore, the results suggest that the clinical onset of IDDM is not preceded by signs of increased systemic oxidative stress in plasma.

Keywords: Insulin-dependent diabetes mellitus, antioxidants, oxidative stress

Abbreviations: IDDM, insulin-dependent diabetes mellitus; TRAP, total peroxyl radical-trapping potential; TAS, total antioxidant status; IAA, insulin autoantibody; ICA, islet cell autoantibody; GADA, glutamic acid decarboxylase autoantibody; IVGTT, intravenous glucose tolerance test; FPIR, first-phase insulin response

INTRODUCTION

Evidence suggests that free oxygen radicals mediate cytokine-induced islet β-cell destruction[1,2] and that β-cells are susceptible to oxidative insult.[3] Low serum α-tocopherol may be associated with an increased incidence of insulin-dependent diabetes mellitus (IDDM),[4] and the plasma concentration and activity of antioxidants

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is decreased in patients with overt IDDM.[5–7] It is not known, however, whether the plasma antioxidant activity decreases before the clinical onset of IDDM. Therefore, we determined the total antioxidant activity and the concentrations of four chain-breaking antioxidants in plasma from 51 unaffected children and adolescents, 22 of whom were at increased risk for clinical onset of the disease, having one or more autoantibody specificities associated with IDDM.[8]

SUBJECTS AND METHODS

Subjects

The study population, derived from a study on β-cell autoimmunity in schoolchildren in northern Finland,[9] was comprised of 51 healthy children and adolescents (25 males, mean age 13.8 years, range 9.5–18.1 years). In 1994, 3661 unaffected schoolchildren from the original study population were screened for islet cell antibodies (ICA) and for antibodies to the 65 kD isoform of glutamic acid decarboxylase (GADA). All children who initially tested positive for ICA and/or GADA ($n = 109$) were invited for retesting in 1996, and for each individual an initially-autoantibody-negative control was matched for sex, age, and school. Out of 218 invited subjects, 210 were given a clinical examination combined with blood sampling for autoantibody analyses and a short intravenous glucose tolerance test (IVGTT). Fifty-one individuals were studied at random for plasma antioxidant activity. The blood samples were obtained after overnight fasting. The samples for the analyses of antioxidant activity were drawn into tubes containing EDTA, and plasma was separated immediately. Sera for the quantification of IDDM-associated autoantibodies and insulin were stored at −20°C until analyzed. The research design was approved by the ethics committees of the Medical School, University of Oulu, and Tampere University Hospital, and informed consent was obtained from the participating subjects and/or their parents.

Methods

IDDM-Associated Autoantibodies

ICA levels were determined by a standard immunofluorescence method using sections of frozen human group O pancreas.[10] End point dilution titers were examined for positive samples and the results were expressed in Juvenile Diabetes Foundation (JDF) units relative to an international reference standard.[11] The detection limit was 2.5 JDF units.

GADA levels were measured with a radioligand assay, as previously described.[12,13] The cut-off limit for antibody positivity was set at the 99th percentile in 372 non-diabetic children and adolescents, i.e., 6.6 RU.

Insulin autoantibody (IAA) levels were analyzed with a radiobinding assay modified from that described by Palmer *et al.*[14] A subject was considered to be positive for IAA when the specific binding exceeded 68 nU/ml (99th percentile in 105 non-diabetic subjects).

Intravenous Glucose Tolerance Tests

IVGTTs were performed following a fast of 10–16 h according to an international standardized protocol.[15] Blood samples were taken twice before the infusion of glucose (−5 and 0 min), and 1, 3, 6, and 10 min after glucose infusion had been completed. Serum insulin concentrations were determined with an enzyme-linked immunosorbent assay.[16] The first-phase insulin response (FPIR) was defined as the sum of the 1- and 3-min serum insulin concentrations. FPIR levels < 46 mU/l, which correspond to the fifth percentile of FPIR levels measured in 100 healthy subjects of similar age, were considered as abnormal.

Total Plasma Antioxidant Activity

The total plasma antioxidant activity was assessed by two separate methods, i.e., the total peroxyl radical-trapping potential (TRAP) and the total antioxidant status (TAS). TRAP was

measured using a chemiluminescence-enhanced modification[17] of the method described by Wayner *et al.*[18] Thermal decomposition of 2,2′-azobis[2-amidinopropane]hydrochloride (ABAP, Polysciences, Warrington, PA) generated peroxyl radicals at a constant rate at 37°C. Peroxyl radicals were detected using luminol (Sigma Chemical Co., St. Louis, MO) -enhanced chemiluminescence with a luminometer. Plasma samples inhibited chemiluminescence for a time that was directly proportional to the TRAP values. The extinction time of each sample was compared to that of 1 mol of the water soluble tocopherol analog Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a gift from F. Hoffman–La Roche Ltd, Basel, Switzerland), capable of trapping 2 mol of peroxyl radicals. TRAP was expressed as μmol of peroxyl radicals trapped by one liter of the sample (μM). A theoretical TRAP ($TRAP_{Calc}$) was derived from concentrations of individual antioxidants based on their stoichiometric peroxyl radical-trapping factors as follows: $TRAP_{Calc}$ = [uric acid] * 2.0 + [α-tocopherol] * 2.0 + [ascorbic acid] * 0.7 + [sulfhydryl groups, –SH] * 0.4.[19] Provision was made for an unidentified fraction of measured TRAP ($TRAP_{Unid}$), which was the difference between the measured TRAP and $TRAP_{Calc}$ ($TRAP_{Unid} = TRAP - TRAP_{Calc}$). The intra-assay coefficient of variation for TRAP was 2%. Plasma TAS[20] was measured using a commercial reagent kit (Total Antioxidant Status, NX 2332, Randox Laboratories, Cromwell, UK) according to the manufacturer's application instructions for the Hitachi 704 automated analyzer. Briefly, a peroxidase/H_2O_2 system was used for oxidation of ABTS (2,2′-azinobis[3-ethylbenzthiazoline-6-sulfonic acid]) chromogen to produce colored $ABTS^+$ radical cations. Antioxidants suppressed the formation of $ABTS^+$ to an extent that was proportional to the total plasma antioxidant potential. The intra-assay coefficient of variation was 2.7% for TAS ($n = 7$).

The plasma concentrations of uric acid and ascorbic acid were measured by high-performance liquid chromatography (HPLC) with an electrochemical detector.[21] The concentration of α-tocopherol was measured by the modified HPLC method,[22] in which an ultraviolet detector is replaced with an LC-amperometric detector (Bioanalytical Systems Inc., West Lafayette, IN). The protein thiol groups were determined as previously described.[23]

Statistics

Student's *t*-test and analysis of variance were used to compare continuous variables between groups. Pearson's correlation matrix was used for the correlation analysis. Calculations were performed using the Statistica/Win 5.0 software (Statsoft Inc., Tulsa, OK). Data are expressed as mean ± SD.

RESULTS

Among the 51 children and adolescents studied, 9 tested positive for at least two of the three IDDM-associated autoantibody specificities out of the three analyzed, whereas 13 had only one detectable autoantibody and 29 tested negative for all three autoantibodies. There were eight subjects with a decreased FPIR. Subjects with at least one autoantibody specificity or with a decreased FPIR were considered at increased risk for IDDM.

There were no significant differences in plasma TRAP, TAS, or in the concentrations of antioxidants between subjects with IDDM-associated autoantibodies and the controls (Table I). Subjects with a reduced FPIR had a higher plasma TAS than subjects with a normal insulin response, but there were no other significant differences between these two groups (Table II). There was no correlation between plasma TRAP, TAS, or concentrations of antioxidants and the levels of IDDM-associated antibodies or FPIR. There was, however, a significant correlation between age and plasma uric acid ($r = 0.41$, $p = 0.003$), and also consequently between age and TRAP ($r = 0.31$, $p = 0.03$) and TAS ($r = 0.33$, $p = 0.02$). There was

TABLE I Plasma TRAP, TAS, and antioxidants in subjects with at least two IDDM-associated autoantibodies, in those with one antibody specificity, and in antibody negative control subjects. There were no significant differences between the groups

	≥2 AB ($n=9$)	1 AB ($n=13$)	Control subjects ($n=29$)	p
Age (years)	13.4 ± 2.7	13.8 ± 2.4	13.9 ± 2.4	0.84
TRAP (μM)	952 ± 83	998 ± 114	975 ± 140	0.70
$TRAP_{Calc}$ (μM)	711 ± 86	717 ± 92	682 ± 123	0.59
$TRAP_{Unid}$ (μM)	241 ± 21	281 ± 103	293 ± 118	0.50
TAS (mM)	1.43 ± 0.09	1.43 ± 0.06	1.47 ± 0.11	0.41
α-Tocopherol (μM)	18.9 ± 6.2	18.2 ± 3.4	20.1 ± 5.4	0.51
Ascorbic acid (μM)	59.3 ± 15.2	46.5 ± 14.0	50.8 ± 18.1	0.21
Protein thiols (μM)	666 ± 57.7	667 ± 161	641 ± 66.5	0.65
Uric acid (μM)	183 ± 40.8	191 ± 60.8	175 ± 58.1	0.71

p-Values by analysis of variance.

TABLE II Plasma TRAP, TAS, and concentrations of antioxidants in unaffected children with a reduced first-phase insulin response (FPIR) to intravenous glucose and in those with normal FPIR

	Reduced FPIR ($n=8$)	Normal FPIR ($n=39$)	p
TRAP (μM)	996 ± 137	964 ± 117	0.51
$TRAP_{Calc}$ (μM)	705 ± 97	685 ± 108	0.63
$TRAP_{Unid}$ (μM)	290 ± 116	279 ± 118	0.81
TAS (mM)	1.52 ± 0.09	1.44 ± 0.10	0.02
α-Tocopherol (μM)	20.2 ± 4.9	19.1 ± 5.4	0.62
Ascorbic acid (μM)	48.1 ± 14.7	51.8 ± 18.1	0.59
Protein thiols (μM)	625 ± 82.6	662 ± 102	0.35
Uric acid (μM)	191 ± 57.5	173 ± 51.6	0.39

p-Values by Student's t-test.

also a fairly strong correlation between plasma TRAP and TAS values ($r=0.43$, $p=0.001$).

DISCUSSION

There is one earlier report that plasma TAS is reduced in ICA-positive first-degree relatives of IDDM patients.[24] A first-degree relative testing positive for ICA has an approximately 35–40% risk of progressing to clinical IDDM over the next five years,[25,26] whereas the risk has been estimated to be approximately 6–10% in ICA-positive subjects from the general population.[8] On the other hand, the risk increases substantially in the general population (up to approximately 30%) if an individual tests positive for at least two IDDM-associated autoantibodies. The highest risk is conferred by a reduced FPIR in combination with one or more autoantibodies.[27]

In the present study, we used a combination of three autoantibodies and FPIR as indices of the risk for IDDM, and multiple parameters to assess plasma antioxidant activity. No difference could be observed, however, in any of the indicators of plasma antioxidant activity between the children with two or more disease-associated antibodies and those with no detectable autoantibodies. The subjects with a decreased FPIR had an even higher TAS level than those with a normal insulin response. The number of autoantibody-positive children with a reduced FPIR was so low in this study ($n=4$) that comparison with the remaining subjects was not meaningful.

Some antioxidants, e.g., flavonoids, beta-carotene, and bilirubin, might account for some of the $TRAP_{Unid}$. There were, however, no differences between our study groups in $TRAP_{Unid}$. It must be emphasized that the major antioxidant defense is probably based on the actions of

transition metal binding proteins, such as hemoglobin and ceruloplasmin,[28] not measured by the assays used for the present study. On the other hand, if increased free radical generation and oxidative stress occur only locally around the pancreatic islet cells, this would probably not be reflected in the antioxidant activity in the peripheral circulation. Our study provides no indication of reduced plasma antioxidant activity in individuals at increased risk for IDDM, however, and the benefit of antioxidant supplementation for prevention of IDDM is thus called into question.

In conclusion, we observed no major changes in the plasma antioxidant activity in unaffected children and adolescents with IDDM-associated autoantibodies and/or a reduced insulin response to intravenous glucose. These data suggest that there is no increased systemic oxidative stress in subjects at increased risk for IDDM.

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