The relationship between chronic glycaemic control and oxidative stress in type 2 diabetes mellitus

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Accepted: 8 April 2008

ABSTRACT

This study compares the lipid peroxidation marker urinary thiobarbituric acid reactive substances (TBARS) and antioxidants including plasma α-tocopherol (vitamin E), plasma (P-GSH-Px) and erythrocyte glutathione peroxidase (E-GSH-Px) activities, and plasma selenium levels in two groups of type 2 diabetic subjects (both $n=20$) with a disease duration of ≤2 (GP1) and 4–6 years (GP2), and non-diabetic age and gender-matched control subjects (CG, $n=20$). The mean (standard deviation [SD]) age of the groups was similar at 41(10) years. Fasting blood and midstream urine samples were obtained from diabetic and non-diabetic subjects attending the diabetic clinic and HbA1c, fructosamine, urine TBARS, total antioxidant (TAS) levels, P-GSH-Px, E-GSH-Px and plasma selenium and vitamin E concentrations were measured. HbA1c (%) and fructosamine levels in the GP1 and GP2 diabetic subjects and the controls were 5.75 (0.67), 11.43 (2.01) and 4.33 (0.47), and 3.09 (0.57), 6.09 (1.15) and 1.67 (0.31), respectively (GP1 *vs*. GP2, GP1 *vs*. GC and GP2 *vs*. CG, all $P<0.001$). Elevated urinary TBARS (μmol/mmol urinary creatinine) in the GP1, GP2 and GC groups were 2.47 (0.37), 3.73 (0.93) and 1.18 (0.24), respectively (GP1 *vs*. GP2, GP1 *vs*. GC and GP2 *vs*. CG, all $P<0.001$). A significant correlation between HbA1c and TBARS was also noted ($r^2=0.894$, $P<0.001$) but only in the GP2 subjects. TAS levels were only decreased in the GP2 group compared to control values (0.59 [0.18] *vs*. 1.74 [0.21], $P<0.001$). Plasma vitamin E concentrations (μmol/L) of 34.11 (3.31), 9.57 (2.20) and 39.01 (2.91) were observed in the GP1, GP2 and GC groups, respectively (GP1 vs. CG, $P<0.05$ and GP1 *vs*. GP2 and GP *vs*. CG, both $P<0.001$). E-GSH-Px (U/g Hb) and P-GSH-Px (U/L) activities in GP1, GP2 and CG groups were also decreased at 57.04 (4.31), 24.0 (8.94) and 67.6 (4.29), and 6.16 (1.56), 2.67 (0.47) and 8.72 (0.31), respectively (E-GSH-Px: CG *vs*. GP1, $P<0.01$, CG *vs*. GP2 and GP1 *vs*. GP2, both $P<0.001$; P-GSH-Px: CG *vs*. GP1, CG *vs*. GP2 and GP1 *vs*. GP2, all $P<0.001$). Plasma selenium levels (μmol/L) were only significantly decreased in GP2 compared to both GP1 and CG values (0.49 [0.29] *vs*. 1.67 [0.80] *vs*. 1.79 [0.26], both $P<0.001$). These observations support the suggestion that chronic hyperglycaemia can influence the generation of free radicals, which may lead ultimately to increased lipid peroxidation and depletion of antioxidants, and thereby enhanced oxidative stress in subjects with type 2 diabetes mellitus.

KEY WORDS: Biochemical markers.
Diabetes mellitus.
Oxidative stress.

Introduction

The development of diabetic complications is a complex pathophysiological process involving a combination of protein glycation, polyol accumulation and free radical-mediated oxidative stress.[1,2] The last process is probably mediated by activation of protein kinase C (PKC)[3] resulting from poor long-term glycaemic control and glucose toxicity.[4–6] Recent studies[1,2] suggest that the above pathophysiological mechanisms all interact, resulting in an amplification of their own individual consequences. Furthermore, diabetes-associated oxidative stress is probably a result of an increased production of plasma free-radical concentrations and a significant reduction in antioxidant defence mechanisms,[7,8] and may involve activation of the c-jun N-terminal kinase (JNK) signalling pathway as a result of glucose toxicity.[9]

Although oxidative stress, implicated in impaired insulin secretion in type 2 diabetes[5,10] and activated by acute glucose fluctuations,[6] has been defined as a disturbance in the balance between the production of free radicals or reactive oxygen species (ROS) and antioxidant defences,[11] which may then lead to tissue injury,[12] suggesting that it should more correctly be defined as disrupted redox signalling and control. It can produce major interrelated derangements of cell metabolism, including DNA damage, protein damage and lipid peroxidation.[13–16] If oxidative stress persists, eventually it will lead to molecular damage and tissue injury.[17]

Antioxidants are defined as any substance that, when present at low concentration compared with those of oxidative substrates, considerably delays or inhibits oxidation of the substrate. Antioxidants inhibit the rate of oxidation but cannot be distinguished from radicals that play a pathophysiological role in the development of tissue damage. Furthermore, a variety of antioxidant defence systems operate, including enzymatic and non-enzymatic antioxidants.[17,18]

The aim of the present study is to investigate the

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relationship between long-term glycaemic control assessed by HbA1c measurements, lipid peroxidation and antioxidant defence mechanisms in non-insulin-dependent diabetes mellitus.

Materials and methods

Type 2 diabetics subjects (20 males, 20 females) attending the diabetic clinic were selected randomly. The diabetic subjects were separated into two groups according to duration of disease, with group 1 (GP1) and group 2 (GP2) having a disease duration of ≤2 years and 6–8 years, respectively. Duration of diabetes was defined as the time between diagnosis and initial visit for the present study. The characteristics of both the diabetic groups and the control group (CG) are given in Table 1. The CG consisted of healthy non-diabetic individuals who were gender- and age-matched to allow appropriate comparison.

Fasting blood and midstream urine (MSSU) samples were obtained from subjects in each of the groups. Serum HbA1c and fructosamine were measured using commercially available kits (Roche Diagnostics, Germany), with methods based on a turbidometric inhibition immunoassay and a colorimetric assay, respectively, in which the enol form of fructosamine reduces nitrotetrazolium blue to a formazan compound, monitored at 546 nm.

Urine thiobarbituric acid reactive substances (TBARS) were quantitated by the fluorimetric technique developed by Yagi,[19] based on the reaction between thiobarbituric acid (TBA) and malondialdehyde (MDA) producing a MDA-TBA complex, while plasma vitamin E (α-tocopherol) was measured using reverse-phase high-performance liquid chromatography (HPLC) using a C18 column and methanol as a mobile phase.[20]

Total antioxidant status was determined using a commercially available kit[21] (Randox Laboratories, Antrim, Northern Ireland) with a method based on the suppression of production of a free-radical cation species (ABTS; 2,2′-azin-di-[3-ethylbenzthiazoline sulphonate]) and plasma selenium by atomic absorption spectrometry.[22] Plasma glutathione peroxide (P-GSH-Px) and erythrocyte glutathione peroxidase (E-GSH-Px) were measured using commercial kits supplied by Randox Laboratories.[23] In the latter determinations, glutathione peroxidase catalyses the oxidation of glutathione (GSH) in the presence of cumene hydroperoxide to produce oxidised glutathione (GSSG), which, in the presence of glutathione reductase and NADPH, produced GSH and a concomitant increase in the oxidation of NADPH to $NADP^+$ and a decrease in absorbance at 340 nm.

Results, expressed as mean (SD), were analysed using one-way analysis of variance (one-way ANOVA) followed by Bonferroni's multiple comparison test. P values <0.05 were considered significant.

Table 1. Clinical characteristics of the study groups.

	Control group (CG, n=20)	Group 1 (GP1, n=20)	Group 2 (GP2, n=2)
Duration (years)	–	≤ 2	6–8
Age (years)	41[17]	40[18]	40[18]
Male/female	10/10	10/10	10/10
Insulin therapy	–	8	9
Metformin	–	8	9
Glibenclamide	–	6	4
Metformin and glibenclamide	–	3	3

Results (mean [SD]) were compared using a one-way analysis of variance test (one-way ANOVA) followed by Bonferroni's multiple comparison test. No significant difference was noted when subject age or group gender mix was analysed.

Results

Compared to non-diabetic values (reference range: 4.4–5.7) significantly elevated levels of HbA1c were noted in both diabetic groups (both P< 0.001, Table 2). Furthermore, GP2 HbA1c values were significantly higher than those in GP1 (P<0.001). A two- and three/four-fold increase in fructosamine levels were observed in GP1 and GP2 values, respectively (both P<0.001), compared to CG values. In addition, mean GP2 fructosamine levels were approximately double those observed in GP1 (P<0.001). Similarly, urine TBARS, a non-specific indicator of lipid peroxidation, were also elevated two-fold and three-fold in GP1 and GP2, respectively, compared to CG group values (both P<0.001). Once again, disease duration also produced significantly different results, with values in GP2 being 44% higher than those in GP1 (P<0.001). In addition, a significant and positive apparent correlation between HbA1c and urinary TBARS was noted, but only in the diabetic group with chronic hyperglycaemia (GP2; r^2=0.894, P<0.001).

Mean total antioxidant levels were only slightly (not significantly) decreased in the GP1 group. In contrast, a three-fold decrease in values was noted in the chronic hyperglycaemic group (GP2), compared to both GP1 and CG group values (both P<0.001). A similar pattern was also observed in plasma α-tocopherol (vitamin E) values. However, while a significant 15% decrease was observed between CG and GP1 group values (P<0.001), the values observed in GP2 were three- to four-fold decreased compared to CG and GP1 values (both P<0.001). In addition, significant but negative apparent correlations were noted between HbA1c and plasma α-tocopherol (vitamin E) (r^2=0.3678, P<0.05), and between urine TBARS and plasma α-tocopherol (vitamin E) (r^2=0.896, P<0.01), but only in the GP2 group. Mean plasma selenium concentrations were similar in the CG and GP1 group subjects at 1.6–1.8 μmol/L, but decreased significantly by three- to four-fold in GP2 group subjects compared to the other groups (both P< 0.001).

Decreased E-GSH-Px levels (U/g Hb) were observed in both diabetic groups compared to control group values (both P<0.001). In the chronic hyperglycaemia group (GP2), E-GSH-Px levels were significantly decreased when compared to values from CG and GP1 subjects by three-fold and 2.5-fold, respectively (both P<0.001). A similar results pattern was noted for P-GSH-Px values, with values reduced in comparison to CG values by 30% and 300%, respectively (both P<0.001), in the GP1 and GP2 groups. In addition, GP2 values for P-GSH-Px were 40% of those observed in GP1 individuals (P<0.001). An apparent negative significant correlation between P-GSH-Px values and plasma selenium concentrations was also noted, but only in GP2 diabetic subjects (r^2 = 0.633, P< 0.001).

Table 2. Biochemical markers of oxidative stress in type 2 diabetes.

	CG (n=20) (1)	GP1 (n=20) (2)	GP2 (n=20) (3)	*P* values 1*vs*2	1*vs*3	2*vs*3
Disease duration (years)	–	2	6–8			
HbA1c (%)	4.33 [0.47]	5.75 [0.67]	11.43 [2.01]	***	***	***
Fructosamine (mmol/L)	1.67 [0.31]	3.09 [0.57]	6.09 [1.15]	***	***	***
TBARS (µmol/mmol creatinine)	1.18 [0.24]	2.47 [0.37]	3.73 [0.93]	***	***	***
TAS (mmol/L)	1.74 [0.21]	1.56 [0.58]	0.59 [0.18]	NS	***	***
Plasma Vit. E (µmolL)	39.01 [2.19]	34.11 [3.31]	9.57 [2.20]	**	***	***
E-GSH-Px (U/g Hb)	67.6 [4.29]	57.04 [4.31]	24.0 [8.94]	**	***	***
P-GSH-Px (U/L x 10^{-3})	8.72 [0.31]	6.16 [1.56]	2.67 [0.47]	***	***	***
Plasma selenium (µmol/L)	1.79 [0.26]	1.67 [0.80]	0.49 [0.29]	NS	***	***

Results, expressed as mean [SD], were analysed using one-way analysis of variance (one-way ANOVA) followed by Bonferroni's multiple comparison test. $^{**}P<0.01$; $^{***}P<0.001$; NS not significant).

TBARS: thiobarbituric acid, TAS: total antioxidants, Vit. E: vitamin E, E-GSH-Px: erythrocyte glutathione peroxidase, P-GSH-Px: plasma erythrocyte glutathione peroxidase.

Discussion

This study investigates the relationship between chronic glycaemic control, as assessed by HbA1c and fructosamine measurements, and markers of lipid peroxidation and oxidative stress in two groups of adult Caucasian type 2 diabetic patients differing in disease duration by approximately five years. The results clearly show that levels of antioxidants and antioxidant mechanisms decreased with disease duration and are associated with worsening long-term glycaemic control. Furthermore, these data are consistent with the involvement of protein glycation, polyol accumulation and free radical-mediated oxidative stress[1,2] in the pathogenesis of diabetic complications, the development of which are driven ultimately by poor long-term glycaemic control and glucose toxicity.[4–6] The results of the present study also suggest that, in addition to effective glycaemic surveillance in diabetic patients, dietary antioxidant supplementation also should be considered.

The *in vivo* measurement of reactive free radicals is difficult because of their reactivity, short half-life, low concentration and the complexity of the products formed.[24] Consequently, measurement of TBARS has been used to reflect free-radical generation and peroxidative damage. While spectrophotometric, fluorimetric and HPLC methods have all been used to quantitate MDA formation in various body fluids and tissues, the fluorimetric method used in the current study to estimate urine TBARS has been shown to measure the TBA-MDA adduct rather than the lipid peroxides as previously thought. However, this method has been shown to be analytically robust and to provide a reliable assessment of MDA levels, an end product of lipid peroxidation.[25,26]

However, while serum or plasma samples are usually analysed to quantitate MDA formation, urine excretion of TBARS was assessed in the present study and the results clearly demonstrate that increased MDA formation and lipid peroxidation, as measured by the urine TBARS-MDA assay,[27,28] significantly correlated with prolonged hyperglycaemia in type 2 diabetics. Consequently, the accumulation of glycosylation products (i.e., HbA1c) may have predictive value for the presence of an enhanced rate of lipid peroxidation in subjects with poor glycaemic control. Consequently, the above observations are consistent with the suggestion that glycation products can be associated with the generation of free radicals, and subsequently enhanced lipid peroxidation.[17,29] In essence, the longer the duration of diabetes, the poorer the diabetic control and the more glycation occurs, and, as a consequence, the greater the oxidative stress.

In order to assess antioxidant defences, vitamin E, P-GSH-Px, E-GSH-Px and selenium were measured. Many studies have demonstrated significant depletion or impaired antioxidants such as plasma vitamin E,[30,31] both erythrocyte and plasma glutathione peroxidase[32,33] activities, and plasma selenium content[34] and increased lipid peroxidation in the diabetic state. Furthermore, increased HbA1c and decreased glycaemic control have been linked to both the rate of lipid peroxidation and impaired antioxidant scavengers and the presence of enhanced oxidative stress in subjects with diabetes[29,35] – results that are similar to those found in the present study. However, although antioxidant biochemical markers measured in type 2 diabetics in the present study were within laboratory reference intervals, they were significantly reduced compared to non-diabetic control values, and they tended to be at the low end of the reference range.

In the non-diabetic state, antioxidants play a protective role in reducing free radical-mediated oxidative stress, but the threshold of protection can vary dramatically as a function of their activity and balance. In non-diseased conditions, antioxidant defence mechanisms are sufficient to counteract the effects of free-radical formation and activity, but in chronic hyperglycaemia associated with the diabetic state they may be overwhelmed because of increased oxidative stress. Consequently, the results of the present study are consistent with increased free-radical production, as a result of poor metabolic control, which leads to consumption of chain-braking antioxidants, increased lipid peroxidation and enhanced oxidative stress.

Indeed, recent studies have now demonstrated the therapeutic potential of manipulating diabetes-induced

oxidative stress. For example, the potential of α-tocopherol and mixed tocopherol supplementation to reduce systemic oxidative stress,[36] improved peripheral insulin sensitivity following short-term oral α-lipoic acid administration,[37] and decreased intracellular oxidative stress following insulin therapy[38] all have been demonstrated recently in type 2 diabetics. □

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