Increased Plasma Oxidizability and Decreased Erythrocyte and Plasma Antioxidative Capacity in Patients with NIDDM

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Abstract

Background: Atherosclerosis and microvascular complications in patients with non-insulin-dependent diabetes have been linked to increased oxidative stress. The glutathione redox cycle is a major determinant of the antioxidative capacity of plasma and its constituents.

Methods: We attempted to investigate plasma oxidation and plasma and erythrocyte glutathione and glutathione enzymes in 20 patients with NIDDM, compared with euglycemic matched controls. Plasma oxidation was analyzed both basally (without) and as induced by 2,2'-azobis,2-amidopropane hydrochloride measured by the generation of thiobarbituric acid reactive substances and lipid peroxides.

Results: There was a significant increase in oxidation both basally (without) and as induced by AAPH. Plasma glutathione was lowered by 50% ($P<0.01$) and erythrocyte glutathione peroxidase, glutathione s-transferase and glutathione reductase activities were lower by 30%, 27% and 46%, respectively ($P<0.01$) in the patients with NIDDM.

Conclusions: Confronted by increased oxidation, patients with NIDDM show an abnormal plasma and erythrocyte antioxidative capacity, which may result in an accelerated rate of complications.

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Cardiovascular diseases are two to four times more prevalent in patients with non-insulin-dependent diabetes mellitus [1,2], which is explained only partially by the traditional coronary risk factors. Recent attention has been given to the possibility that diabetic patients are exposed to increased oxidative stress [3,4]. Indeed, there have been reports on increased lipid peroxides in human plasma and in exprimental diabetes [5–7]. A central role is played by advanced protein glycosylation, which generates advanced glycation end products that may participate in the pathogenesis of vascular, neural, renal, connective tissue and lens complications in NIDDM [8].

NIDDM = non-insulin-dependent diabetes mellitus

Oxidative modification of low density lipoprotein is required for atherogenesis and is another link between enhanced oxidative stress, NIDDM and accelerated atherosclerosis. A variety of antioxidants — including natural nutrients, plasma and erythrocyte enzymes — operate in order to scavenge free radicals and protect the LDL molecule against oxidation [9,10]. Glutathione and its related enzymes play an important role in antioxidative reactions [11–13]. However, in addition to increased oxidative stress, decreased antioxidant capacity has been described in NIDDM, which may result from consumption of antioxidants [14,15] or other mechanisms.

In this study we measured plasma oxidation and plasma and erythrocyte antioxidative capacity in patients with NIDDM and compared the results with a normal control group. Plasma and erythrocyte glutathione, glutathione peroxidase, erythrocyte glutathione reductase and glutathione s-transferase activities were also determined.

Patients and Methods

We investigated 20 NIDDM patients, 4 women and 16 men aged 25–60 years (average 52±8), who were non-smokers and consuming an American Diabetes Association diet. None had cardiac or renal complications as confirmed by history, physical examination, ECG and renal function tests. The characteristics of the patients are shown in Table 1. The patients were compared to a healthy control group matched

Table 1. Characteristics of patients with NIDDM

	Patients (n=20)	Control (n=20)
Age (yr)	52±8 (30–68)	48±9 (28–64)
M/F	16/4	16/4
BMI (kg/m^2)	29±4** (23–31)	26±3 (21–30)
Glucose (mg/dl)	200±60** (108–305)	92±12 (72–110)
Cholesterol (mg/dl)	221±32* (166–267)	188±39 (141–274)
Triglycerides (mg/dl)	203±92** (104–405)	130±58 (64–243)

M/F = male to female ratio; BMI = weight to height^2 bone mass index. All results are means±SD. *$P<0.05$; ** $P<0.01$ (comparing patients with normoglycemic control subjects)

by age and sex, who were thinner and had lower blood cholesterol and triglyceride concentrations [Table 1].

Blood was taken into Na_2 EDTA (1 mmol/L) tubes after a 14 hour fast, and plasma was separated after centrifugation at 2,000 rpm for 10 min at room temperature. Plasma 4 ml was dialyzed against phosphate-buffered saline overnight at 4°C. Then 2 ml plasma was incubated for 2 h at 37°C without (control) or with (100 mmol/L) AAPH.

AAPH generates peroxy radicals at a constant rate [16], and under the above conditions plasma lipid peroxidation was maximal. The samples were then kept at 4°C and analyzed for their oxidation state within 2 h by using the TBARS assay that measured malondialdehyde equivalents [17]. In addition, plasma peroxides were measured at 365 nm by using extinction coefficient of $2.46 \times 10^4\ M^{-1}cm^{-7}$[18]. For erythrocyte enzymes, heparinized blood was kept in ice. Erythrocytes were separated using phycol buffer and centrifuged at 4°C for 20 min at 1,700 rpm. Erythrocytes were rewashed by normal saline for measurement of the enzymes.

Erythrocyte glutathione reductase activity was measured according to Beutler [19], with and without the addition of FAD. Plasma and erythrocyte glutathione peroxidase activities were measured by the Kakatnur and Jelling method [20], erythrocyte glutathione transferase by the Habig method [21], and plasma and erythrocyte glutathione by the DTNB glutathione reductase method [22].

Statistical analysis

All measurements were performed in triplicate and presented as mean±SEM. Student's *t* test was used for all analyses. The computer software program STATEASE (version 1.00, Data Plus Systems Inc., New York) was used for computations.

Results

Plasma oxidation increased both at baseline and following incubation with AAPH. Diabetic plasma generated a fourfold PD [Figure 1A and B] and a 1.7-fold malondialdehyde at baseline, compared with the control. Upon AAPH incubation, there was a 1.4-fold increase in PD generation and a 1.7-fold increase in MDA generation ($P<0.01$) in the patients with NIDDM compared with the controls [Figure 1C and D]

When erythrocyte and plasma antioxidant enzymes were compared with control values, erythrocyte glutathione peroxidase activity was 30% lower in NIDDM ($P<0.01$) [Figure 2A]. Erythrocyte glutathione transferase was decreased by 27% ($P<0.05$) in NIDDM [Figure 2B]. Glutathione reductase was decreased by 46% without, and by 39% with FAD ($P<0.01$) in the diabetic patients [Figure 2C]. There was a 50% decrease in plasma glutathione in the diabetic patients ($P<0.01$), and a nonsignificant decrease in erythrocyte glutathione concentration in the NIDDM patients [Figure 3A and B].

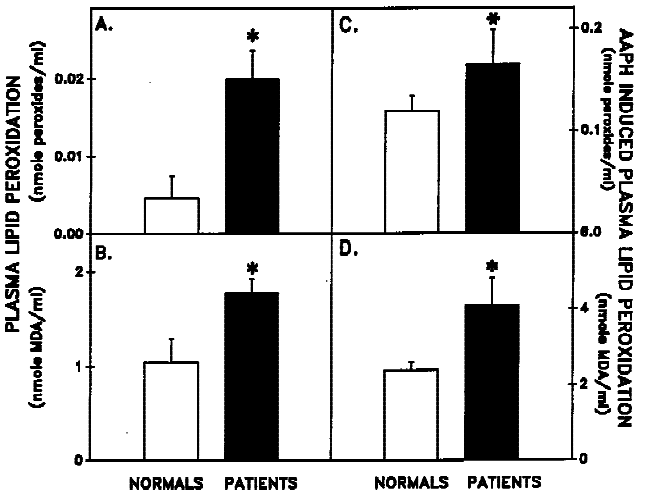

Figure 1. Plasma lipid peroxidation in 20 patients with NIDDM (black bars) and 20 normoglycemic control subjects (open bars). Plasma was dialyzed against PBS at 4°C for 24 h. **A** and **B** refer to plasma lipid peroxidation at the basal state. **C** and **D** refer to plasma susceptibility to oxidation (upon AAPH). Plasma oxidation products, PD and malondialdehyde (MDA) were measured at 37°C before and after incubation with 100 mM AAPH for 2 h. *$P<0.01$. All results are means±SEM.

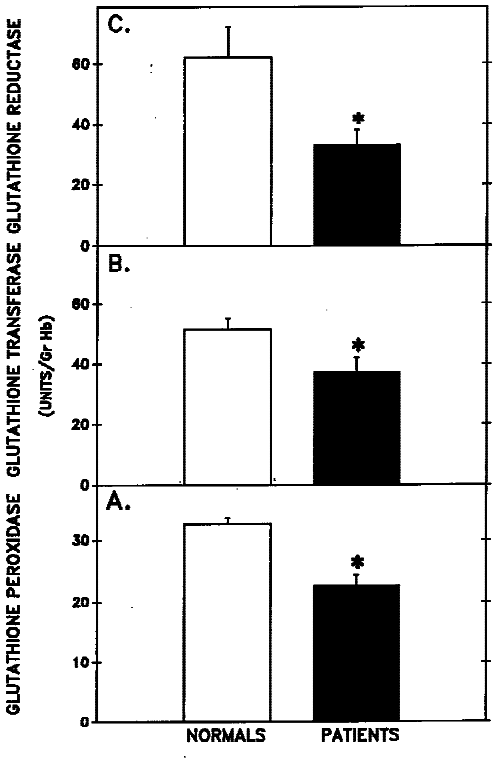

Figure 2. Glutathione cycle enzymes, glutathione peroxidase (**A**), glutathione transferase (**B**) and glutathione reductase (**C**) were measured in erythrocytes derived from 20 patients with NIDDM (black bars) and compared to 20 normoglycemic control subjects.

AAPH = 2,2'-azobis,2-amidopropane hydrochloride
TBARS = thiobarbituric acid reactive substances
PD = plasma peroxide

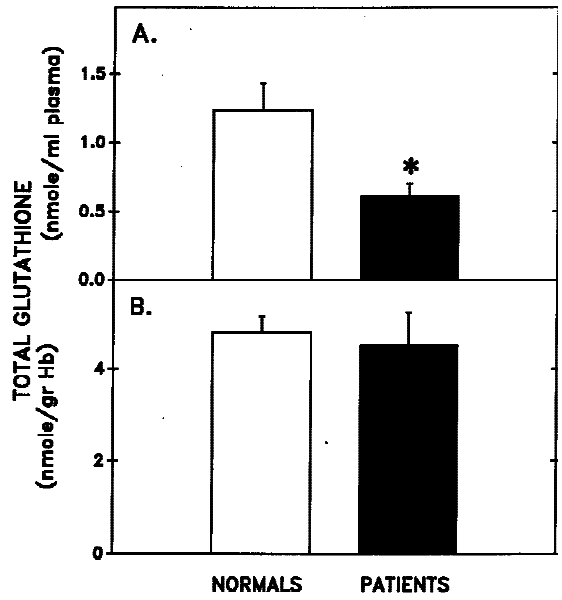

Figure 3. Plasma (**A**) and erythrocyte (**B**) glutathione in 20 patients with NIDDM (black bars) compared to 20 normoglycemic patients (open bars). * P <0.01. All results are means±SEM.

Discussion

Our results show a significant increase in the extent of plasma oxidation both at the basal state and with induced oxidation in diabetic plasma. Using different assays to measure plasma oxidation products, it has been repeatedly demonstrated that patients with NIDDM generate increased amounts of peroxides [5,6]. Moreover, some correlation has been shown between the degree of glycemic control and the existence of vascular complications in NIDDM patients [23]. Since we eliminated subjects with vascular or other complications, the small number of patients did not enable the correlation between diabetic metabolic control and the oxidative state. We are aware of only one study that utilized the same methodology as ours (AAPH-induced plasma lipid peroxidation) and exhibited increased plasma oxidizability in NIDDM [24].

Regarding plasma and erythrocyte antioxidative enzyme activities, they were all reduced to a significant extent in the patients with NIDDM compared to normal controls. The reasons for altered antioxidant defense systems in diabetes are variable. Hyperglycemia and autoglycoxidation of sugars involve the lipoproteins, proteins and other plasma constituents, all of which result in free radical generation [8,23]. The same oxidative processes may affect the glutathione protein and its enzymes, thus the glutathione redox cycle will be affected. Hokkaido [25] observed a significant decrease in the activity of the enzyme γ-glutamyl-cysteine-synthatase, which generated glutathione (due to glycation of this enzyme) in patients with diabetes. Moreover, incubation of this enzyme *in vitro* with increasing amounts of glucose inhibited its activity. Another potential mechanism related to abnormal glutathione enzymes involves the polyol pathway in tissue exposed to hyperglycemia [26]. This pathway results in a decrease in the ratio of NADPH/NADP (due to increased aldose reductase activity). Consequently, there is a decrease in glutathione and its redox cycle enzymes upon interference with its regeneration. Low activity of glucose-6-phosphate dehydrogenase was shown in the liver of diabetic rats [27] and may also result in low glutathione concentration.

Apparently normal mechanisms against oxidation that require the regeneration of vitamin C by glutathione and its enzymes are defective in diabetes. It has been repeatedly demonstrated that ascorbic acid is oxidized and consumed with consequent low plasma and tissue levels in diabetes [27,28]. It is suggested that glutathione is consumed in these reactions to regenerate vitamin C. As the normal activity of glutathione and its redox enzymes is dependent on provision of amino acids and nutrients in the diet (such as riboflavin and selenium), dieting and food restriction may affect the enzymes. Our patients were generally in good health and maintained a stable weight and nutrient balance, which eliminates the possibility of nutrient deficiency.

Our results showing both increased plasma oxidizability and abnormal protective activity against oxidation in plasma and erythrocytes extend the growing body of evidence emphasizing the importance of oxidative stress in diabetes and its complications (macro- and microvascular). The potential of introducing antioxidants to NIDDM patients to combat these alterations has to be seriously discussed and investigated.

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Capsule

Alzheimer's amyloid

Cerebral deposition of amyloid beta-peptide (A-beta) is an early and critical feature of Alzheimer's disease. A-beta generation depends on proteolytic cleavage of the amyloid precursor protein (APP) by two unknown proteases: beta-secretase and gamma-secretase. These proteases are prime therapeutic targets. A transmembrane aspartic protease with all the known characteristics of beta-secretase was cloned and characterized by Vassar et al. Overexpression of this protease, termed BACE (for beta-site APP-cleaving enzyme), increased the amount of beta-secretase cleavage products, and these were cleaved exactly and only at known beta-secretase positions. Antisense inhibition of endogenous BACE messenger RNA decreased the amount of 13-secretase cleavage products, and purified BACE protein cleaved APP-derived substrates with the same sequence specificity as beta-secretase. Finally, the expression pattern and subcellular localization of BACE were consistent with that expected for beta-secretase. Future development of BACE inhibitors may prove beneficial for the treatment of Alzheimer's disease.

Capsule

Fibrocytes as hearing aids

X-linked non-syndromic mixed deafness (DFN3) is caused by mutations in the gene encoding transcription factor Brn-4. To investigate possible pathogenetic mechanisms, Minowa et al. from Japan established a mouse model of the disorder by targeted mutagenesis. The Brn4-deficient mice were profoundly deaf and showed a substantial reduction in endocochlear potential. There were no gross defects in the middle ear or cochlear structures, but severe pathological anomalies were seen in fibrocytes, a population of cells implicated in the regulation of cochlear potassium homeostasis. Thus, DFN3 differs from many other forms of deafness in that it appears to arise from malfunction of a non-sensory cell type.

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