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Age-independent oxidative stress in elderly patients with non-insulin-dependent diabetes mellitus

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Summary

Impaired antioxidant defence is implicated in the development of cardiovascular complications in non-insulin-dependent diabetes (NIDDM). However, as many of these patients are elderly, observed changes in antioxidant status may be due to the patient's age rather than their disease. We sampled blood from 47 elderly NIDDM patients (21 male and 26 female; mean age ± SD, 75.62 ± 7.97 years), 66 young (30 male and 36 female; 24.52 ± 4.72 years) and 58 healthy elderly volunteers (17 male and 41 female; 70.74 ± 4.85 years), and measured the antioxidant glutathione, the marker for free-radical-damage lipid hydroperoxide products (LHP), vitamin E and total antioxidant capacity (TAC). There was a significant increase in LHP in the healthy elderly group compared with the young volunteers (3.14 ± 1.5 vs. 2.14 ± 1.38 μmol/l, $p < 0.01$). The values were much higher in NIDDM patients (7.02 ± 2.29 μmol/l, $p < 0.0001$ vs. healthy elderly). There was a reduction in TAC in healthy elderly compared with the young (359.99 ± 54.82 vs. 471.47 ± 94.29 μmol/l trolox equivalents, $p < 0.0001$), but there was no further reduction in NIDDM patients. Similarly, glutathione was reduced to the same degree in healthy elderly and NIDDM patients (0.29 ± 0.09, 0.30 ± 0.11 vs. 0.54 ± 0.19 μmol/l in young volunteers, $p < 0.0001$). Vitamin E concentrations were comparable in all groups (26.34 ± 5.39 young volunteers, 31.50 ± 8.23 healthy elderly and 30.98 ± 9.03 μmol/l NIDDM patients), but after correction for serum cholesterol there was a significant reduction in the diabetic group compared with the young, but not with the elderly (5.54 ± 1.55 vs. 6.67 ± 1.86 vs. 6.31 ± 1.85 (μmol/l)/(mmol/l), $p < 0.01$). We have demonstrated an age-dependent reduction in total antioxidant capacity and glutathione defence and an age-independent increase in LHP in elderly patients with NIDDM. Reduced concentrations of vitamin E were demonstrated in NIDDM patients compared with young, but not elderly, volunteers. Increased oxidative damage occurs independently of age in NIDDM patients despite comparable antioxidant defences in this age group.

Introduction

Free radicals are continuously produced during aerobic metabolism.[1] These unstable species may cause oxidative damage to DNA, carbohydrates, proteins and lipids that are normally counteracted by protective antioxidants. Oxidative defence is provided by a number of enzymes and vitamins, including the chain-breaking scavengers vitamin E, vitamin C and glutathione.[2–4] In times of increased free radical production, individuals may become deficient in these antioxidants. Incomplete scavenging of reactive radicals leads to oxidation of cellular lipids, proteins, nucleic acids and glycoconjugates which results in fragmentation and cross-linking. This may ultimately lead to cell death with widespread pathological

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consequences.[5] The imbalance between protective antioxidants (antioxidant defence) and increased free radical production, leading to oxidative damage, is known as oxidative stress. An example of this process is oxidation of membrane-bound lipids and lipoproteins known as lipid peroxidation.[6] Oxidation of circulating low-density lipoprotein (LDL) has been linked to the initiation and progression of atherosclerosis and ultimately to the pathogenesis of cardiovascular disease.[7]

Oxidative stress has been implicated in many diseases associated with ageing[8] and in the ageing process itself.[9] We have recently demonstrated that advanced age, in the absence of disease, is associated with decreased antioxidant defence and increased oxidative damage.[10] The degree of oxidative stress was accentuated in chronic illness, particularly if increasing severity or an acute event was serious enough to require hospital admission.

Morbidity and mortality are increased in patients with NIDDM when compared with the general population, particularly in relation to coronary artery disease. One possible explanation for this is that oxidative stress may be a contributory factor. It is well established that there is an increased production of damaging free radicals in NIDDM patients that may be due to auto-oxidation of glucose and glycosylated proteins.[5,11–13]

However, most patients with NIDDM are elderly and if poor antioxidant defence is to be implicated in the development of vascular complications, then age-related perturbations in those parameters must be taken into account. This study was designed to determine whether observed changes in oxidative stress in elderly NIDDM patients were a manifestation of the illness or simply a reflection of the ageing process itself.

Methods

Patients

We studied 66 young healthy volunteers (30 male and 36 female, mean age $\pm$ SD 24.5 $\pm$ 4.7 years) and 58 healthy elderly subjects (17 male and 41 female, 70.7 $\pm$ 4.8 years) recruited from a sample of community-based individuals. Health was defined as an absence of major medical or surgical illness in the previous 5 years, no hospital admissions, no current medication, and a subjective perception of good health as determined by health questionnaire. These individuals were compared with 47 elderly patients (21 male and 26 female, 75.6 $\pm$ 8.0 years) with non-insulin-dependent diabetes (NIDDM) attending the Diabetes Resource Centre at a University Teaching Hospital. Patients were receiving either diet only (8), diet plus oral hypoglycaemic agents (30) or diet and insulin (9) for glycaemic control. The majority of patients studied had evidence of vascular complications including hypertension, coronary artery disease (CAD), peripheral vascular disease (PVD) and cerebrovascular disease (CVD), but none had been hospitalized in the previous 6 months. In addition to their diabetic medication, the majority of patients were also receiving antihypertensive and antianginal agents. Diabetes control was assessed by glycated haemoglobin (HbA_1C) measurements. The study was approved by the Local Medical Ethical Committee, and all volunteers and patients gave informed written consent.

Sample collection

Venous blood (non-fasting) was sampled into plain tubes for measurements of serum cholesterol, TAC and vitamin E and into EDTA and lithium-heparin tubes for measurement of total glutathione and LHP, respectively. Samples were placed on ice and centrifuged within an hour at 3500 rpm, 4°C for 15 min. To ensure stability of glutathione, plasma was treated with 30% perchloric acid (PCA). After mixing thoroughly, samples were centrifuged at 11000 rpm for 5 min and stored at -80°C until analysis. Samples from the healthy elderly group were collected in the community.

Assays

Total plasma glutathione was measured by enzymerate assay using the colorimetric dye 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB).[14] This method relies on the recycling of the oxidized and reduced forms of glutathione by the enzyme glutathione reductase, determined by spectrophotometry at a wavelength of 412 nm. Lipid hydroperoxides (LHP) were determined by the ferrous-oxidation of the colorimetric dye xylenol orange at a wavelength of 560 nm in conjunction with the specific hydroperoxide discriminant triphenylphosphine as described by Nourooz-Zadeh *et al.*[15] TAC was determined by enhanced chemiluminescence.[16] Vitamin E concentrations were determined by reverse-phase high-performance liquid chromatography (HPLC) with UV-detection at 292 nm using tocopherol acetate as internal standard.[17] Serum cholesterol was measured by standard enzymic assay.

Statistics

A minimum sample size of 47 was needed to detect a 20% change in measures of oxidative stress having 80% power at the 95% confidence limit. Results are expressed as mean $\pm$ SD. Statistical difference between groups was determined by analysis of

variance (ANOVA). The level of significance was taken as $p<0.05$. To determine correlation between variables, a Pearson linear regression model was used.

Results

Oxidative defence

Plasma glutathione concentrations were significantly reduced in healthy elderly volunteers compared with young controls (mean ± SD 0.29 ± 0.09 and 0.54 ± 0.19 μmol/l, $p<0.0001$) (Figure 1, Table 1). Patients with NIDDM had comparable glutathione concentrations to the healthy elderly. Similarly, TAC measurements were significantly reduced in both healthy elderly volunteers and diabetic patients compared with the young (360.0 ± 55 μmol/l, 386.4 ± 99 vs. 471.5 ± 94, $p<0.01$). Despite this, there were no significant changes in vitamin E in any of the groups studied (Table 1). However, after correction for serum cholesterol, there was a significant reduction in vitamin E in the diabetic group compared to the young volunteers (5.54 ± 1.55 vs. 6.67 ± 1.86 (μmol/l)/(mmol/l), $p<0.01$). Although the diabetic patients also had lower cholesterol-corrected vitamin E concentrations than the healthy elderly, the differences were not significant.

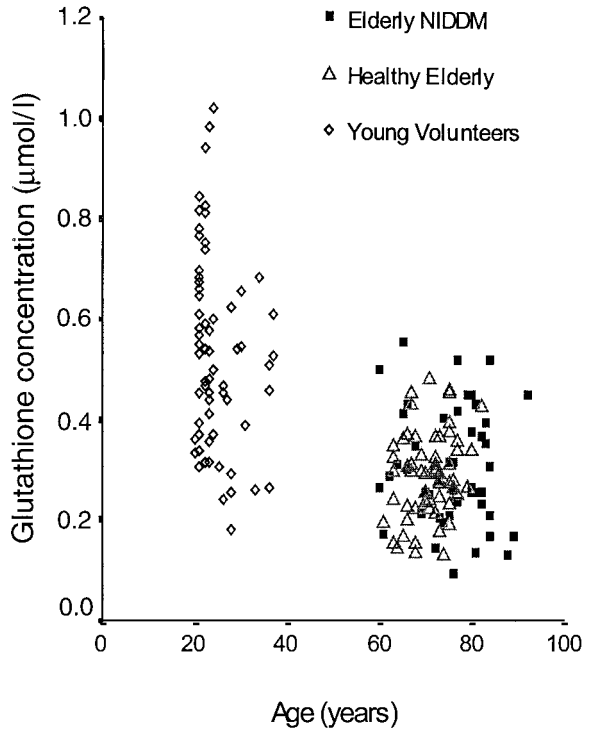

Figure 1. Plasma glutathione concentrations in healthy individuals and elderly patients with NIDDM.

Oxidative damage

There was evidence of increased oxidative damage with age. A modest, but significant increase in LHP occurred in the healthy elderly population compared with young volunteers (2.14 ± 1.4 and 3.14 ± 1.5 μmol/l, $p<0.01$). In patients with diabetes, the increase in LHP was striking at 7.0 ± 2.3 μmol/l, $p<0.0001$ (Figure 2).

All NIDDM patients had reasonable glycaemic control (HbA_1C 8.7 ± 1.2%), and there was no correlation between HbA_1C and lipid peroxidation levels ($r^2=0.002$). Similarly, there was no correlation between BMI or smoking history and markers of oxidative stress in the diabetic patients.

Discussion

Patients with NIDDM have an increased mortality and morbidity compared with non-diabetics and are more likely to develop CAD, cerebrovascular and peripheral vascular disease.[18] This excess risk for vascular disease can only partly be explained by the traditional risk factors for the general population such as smoking, hypertension and raised cholesterol.[19] It has been proposed that oxidative stress may be associated with the pathogenesis of the complications of non-insulin-dependent diabetes (NIDDM), particularly vascular disease.[5,12]

Previous studies are consistent with our own findings of increased oxidative stress in NIDDM. Sundaram *et al.* studied patients with NIDDM in their fifth and sixth decades, and showed an increase

Table 1 Oxidative stress measurements in healthy elderly individuals and elderly individuals with NIDDM compared with young volunteers

	LHP (μmol/l)	TAC (μmol/l trolox)	GSH (μmol/l)	Vitamin E (μmol/l)	Vitamin E/Cholesterol (μmol/l)/(mmol/l)
Young volunteers	2.14 ± 1.38	471.5 ± 94.3	0.54 ± 0.19	26.3 ± 5.4	6.67 ± 1.86
Healthy elderly	3.14 ± 1.50*	360.0 ± 54.8*	0.29 ± 0.09**	31.5 ± 8.2	6.31 ± 1.85
Patients with NIDDM	7.02 ± 2.29***	386.4 ± 99.8*	0.30 ± 0.11**	31.0 ± 9.0	5.54 ± 21.4*

Results are means ± SD. LHP, lipid hydroperoxide products; TAC, total antioxidant capacity; GSH, glutathione. *$P<0.01$ **$P<0.0001$ compared with young volunteers. ***$P<0.0001$ compared with healthy elderly.

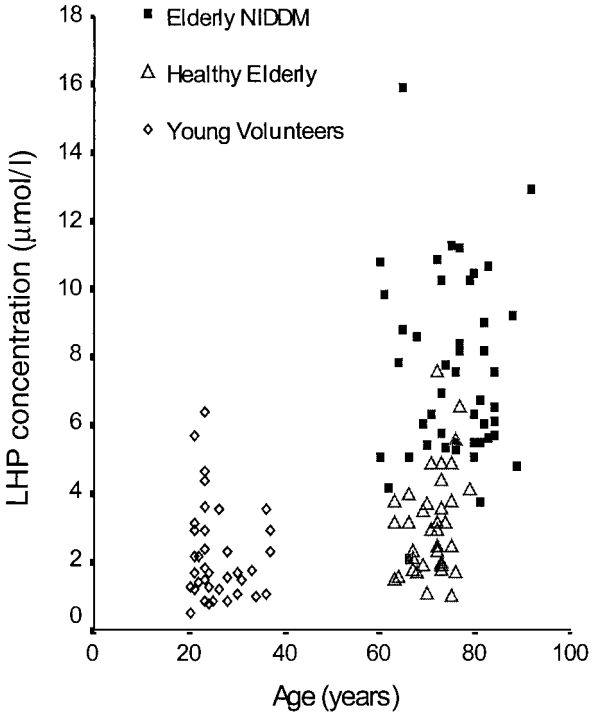

Figure 2. Plasma LHP concentrations in healthy individuals and elderly patients with NIDDM.

in lipid peroxidation from the onset of disease.[20] This became progressively worse with time and with the development of complications. The method used has been criticized, however (i.e. the simple TBARS assay), because it measures many parameters in addition to lipid peroxidation, and is affected by the lipid content of the sample.[21] Using the ferrous-oxidation of xylenol orange assay to determine lipid hydroperoxides, Nourooz-Zadeh *et al.* suggested that changes in oxidative stress were related to the underlying metabolic abnormalities in NIDDM rather than to the onset of complications.[21] This is supported by the finding of raised lipid peroxidation products in patients with impaired glucose tolerance (IGT), even before the onset of frank diabetes.[22]

Studies have consistently demonstrated deficiency in individual antioxidants in NIDDM patients[20–23] with reduced concentrations of glutathione, vitamin C and the antioxidant enzymes superoxide dismutase (SOD) and catalase as well as a decrease in total radical trapping antioxidant parameter (TRAP), suggestive of reduced total antioxidant defence.[23] Some,[20,21] but not all, authors found a decrease in vitamin E in patients with NIDDM even when corrected for serum cholesterol. Surprisingly, Ceriello *et al.* demonstrated a cholesterol-adjusted increase in vitamin E despite reduced TRAP in diabetic patients.[23] Overall, these studies suggest that oxidative stress and impaired antioxidant defence is a feature of NIDDM that is present early in the disease and may contribute to its progression and even to the development of complications.

One issue that has not previously been addressed is the impact of age on antioxidant status in NIDDM. Many patients who develop diabetic complications are elderly, so perturbations in oxidative stress parameters may simply reflect the ageing process. In the present study, we have clearly demonstrated that NIDDM patients developed oxidative damage in excess of that expected from age-matched controls. This was apparent despite similar reductions in antioxidant defence including TAC, glutathione and vitamin E in the healthy elderly and diabetic groups. Elderly NIDDM patients are therefore more susceptible to free-radical-induced damage than can be accounted for by normal ageing. Possible explanations for this include poor functioning of protective antioxidants in elderly diabetics, or greatly increased amounts of free radicals that overwhelm the defence system in this condition.

Oxidation of low-density lipoprotein (LDL) cholesterol is a key step in the formation of atheroma and is thought to be a major factor in the development of cardiovascular disease.[7,24] This may be of particular importance in patients with NIDDM, because in addition to raised total LDL cholesterol levels, they tend to have a pro-atherogenic lipid profile composed of small dense LDL particles, raised triglycerides and moderately raised cholesterol.[19,25–28] Small, dense LDL particles are known to be more readily oxidized than larger particles, partly because they have less protective vitamin E.[29–31] Once modified, they are taken up by macrophages in the arterial intima via the scavenger receptor, eventually forming foam cells, which predispose to the formation of atherosclerotic plaques.[32]

The increased oxidative damage that we have demonstrated in elderly patients with NIDDM may therefore predispose to the development of atherosclerosis. This is apparent despite comparable antioxidant defence in age-matched controls. We propose that these patients may have supernormal requirements for antioxidants. Supplementation, with known free radical scavengers such as vitamins E and C, have a potential role in boosting antioxidant defence[33,34] and may be important in this group.

It has been established that consumption of fruit and vegetables rich in antioxidant vitamins is associated with a lower risk of death from coronary disease.[35–37] Vitamin supplementation, particularly with vitamin E, may yet prove to have an additional protective role, but intervention studies have not clarified what the appropriate dose is or for how long supplementary vitamins should be taken.[38–42] In addition, scavenging antioxidants act synergistically,[43,44] so supplementation with two or more may enhance their individual effects. Following the

demonstration of increased oxidative damage in elderly patients with NIDDM highlighted in this study, further studies are now needed to assess the role of antioxidant supplementation in those patients with and without clinically apparent cardiovascular disease.

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