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Altered antioxidant enzyme defences in insulin-dependent diabetic men with increased resting and exercise-induced oxidative stress

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ABSTRACT

Impaired antioxidant defences may predispose to the increased resting and exercise-induced oxidative stress found in patients with insulin-dependent diabetes mellitus (IDDM). We investigated major erythrocyte antioxidant enzyme activities at rest and in response to sustained, moderate intensity physical exercise in young diabetic men (n = 9) previously reported to have markedly elevated plasma lipid peroxidation and blood glutathione levels compared with control men (n = 13) (Laaksonen *et al.* 1996). At rest, erythrocyte glutathione reductase activity was 15% higher in the diabetic group (P = 0.049). Se-glutathione peroxidase and glutathione-S-transferase activities were similar in both groups. Red cell Cu, Zn-superoxide dismutase and catalase activities were lower in the IDDM group (P = 0.033 and P = 0.023, respectively). After 40 min of exercise at 60% of the subjects' peak oxygen consumption, Se-glutathione peroxidase activity rose by about 14% in the control group (P = 0.003), but not in the IDDM group (P = 0.47). Exercise did not cause significant changes in other enzyme activities in either group. To conclude, lower erythrocyte Cu, Zn-superoxide dismutase and catalase activity in young men with IDDM at rest may contribute to increased oxidative stress. On the other hand, increased glutathione reductase activity may represent a compensatory upregulation of glutathione homeostasis in response to increased oxidative stress. Upregulation of Se-glutathione peroxidase activity in response to physical activity appeared to be impaired in men with IDDM.

Keywords antioxidant, blood, erythrocyte, free radical, glutathione, lipid peroxidation, oxidative stress, reactive species, redox.

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Impaired antioxidant defences may play a key role in the increased oxidative stress found in patients with insulin-dependent diabetes mellitus (IDDM) at rest (Yaqoob *et al.* 1994, Griesmacher *et al.* 1995, Laaksonen *et al.* 1996) and in response to physical exercise (Laaksonen *et al.* 1996). Oxidative stress has been increasingly implicated in the accelerated atherosclerosis and microvascular complications of diabetes mellitus (Wolff *et al.* 1991, Lyons 1993, Tesfamariam 1994). Impairments in antioxidant defences reported in IDDM include altered glutathione metabolism (Godin *et al.* 1988, Jain & McVie 1994, Yaqoob *et al.* 1994, Di Simplicio *et al.* 1995) and decreased superoxide dismutase (SOD) activity (Nath *et al.* 1984, Kawamura *et al.* 1992, Yaqoob *et al.* 1994). In diabetes, production of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Ceriello *et al.* 1991, Wolff *et al.* 1991) is increased through glucose auto-oxidation, protein glycation and formation of advanced glycation end-products (Wolff *et al.* 1991, Lyons 1993, Schmidt *et al.* 1994) and impaired nitric oxide and prostaglandin metabolism (Tesfamariam 1994). Coupled with increased ROS production, compromised antioxidant defences could predispose diabetic patients to increased oxidative stress.

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Decreased blood total glutathione has been found in diabetic patients, generally either in older, mainly non-insulin-dependent diabetic patients (Murakami *et al.* 1989) or poorly defined populations (Jain & McVie 1994). We recently reported increased blood total glutathione and plasma thiobarbituric acid reactive substance (TBARS, an index of lipid peroxidation) levels in young men with IDDM in moderate control and without major diabetic complications (Laaksonen *et al.* 1996). Thus in young otherwise healthy IDDM patients, increased blood total glutathione may represent a compensatory response to increased oxidative stress.

Tissue glutathione plays a central role in antioxidant defence (Sen & Hänninen 1994). Reduced glutathione detoxifies reactive oxygen species such as hydrogen peroxide and lipid peroxides directly or in a glutathione peroxidase (GPX) catalysed mechanism. Glutathione reductase (GRD) catalyses the NADPH-dependent reduction of oxidized glutathione, serving to maintain intracellular glutathione stores and a favourable redox status. Glutathione-S-transferase (GST) catalyses the reaction between the -SH group and potential alkylating agents, rendering them more water-soluble and suitable for transport out of the cell. GST can also use peroxides as a substrate (Alin *et al.* 1985).

Cu, Zn-superoxide dismutase (Cu, Zn-SOD) and catalase are also major antioxidant enzymes. Cu, Zn-SOD is mostly in the cytosol and dismutates superoxide to hydrogen peroxide. Catalase is a hydrogen peroxide decomposing enzyme mainly localized to peroxisomes or microperoxisomes.

Even moderate exercise may increase free radical production beyond the capacity of antioxidant defences, resulting in oxidative stress (Alessio 1993, Ji 1993, Sen *et al.* 1994a, b, Sen 1995). Because patients with IDDM have impaired antioxidant defenses and increased oxidative stress at rest, they could potentially be more susceptible to exercise-induced oxidative stress. In our earlier study which found increased resting and exercise-induced plasma TBARS in young otherwise healthy men with IDDM, plasma TBARS had a strong negative correlation with peak oxygen consumption ($\dot{V}O_{2\ peak}$) in diabetic men (Laaksonen *et al.* 1996). Thus, although exercise induces oxidative stress acutely, physical fitness may have a protective effect against lipid peroxidation in diabetes. Regular physical exercise can strengthen antioxidant defences and may decrease resting and acute exercise-induced oxidative stress (Alessio & Goldfarb 1988, Sen *et al.* 1992, Atalay *et al.* 1996). Exercise also acutely induces complex changes in antioxidant enzymes, which can either be protective or predispose to oxidative stress (Alessio 1993, Ji 1993, Sen & Hanninen 1994, Sen 1995). To our knowledge, no studies have been published regarding acute exercise-induced changes in antioxidant enzyme defences and their relationship to fitness and oxidative stress in diabetes mellitus.

The purpose of this study was to extend our knowledge of resting and exercise-induced oxidative stress and antioxidant defences and the relationship to physical fitness in IDDM by assessing selected major erythrocyte antioxidant enzyme activities at rest and in response to sustained, moderate intensity physical exercise in young diabetic men who showed markedly elevated plasma TBARS and blood glutathione levels.

MATERIALS AND METHODS

Young otherwise healthy men with IDDM (n = 9) and healthy male control subjects (n = 14) were recruited into the study after obtaining informed consent (Laaksonen *et al.* 1996). Briefly, diabetic subjects were chosen from patients followed at the Kuopio University Hospital Diabetes Clinic. Control subjects were volunteers from the local university student population. Clinical and biochemical data are displayed in Table 1. Diabetic and control subjects were well matched for age, body mass index (BMI) and physical fitness (as

Table 1 Characteristics of diabetic and control men

	Diabetic group (n = 9)	Control group (n = 13)
Age (years)	23 ± 1.7	23 ± 2.9
Body mass index (kg m^{-2})	23.5 ± 2.5	23.3 ± 1.7
$\dot{V}O_{2\ peak}$ (mL kg^{-1} min^{-1})	46 ± 6.9	45 ± 6.0
Exercise frequency (times $week^{-1}$)	2.6 ± 1.6	1.2 ± 1.6
Duration of diabetes (years)	9 ± 5.8	N/A
Haemoglobin	149 ± 3.9	152 ± 8.8
Haemoglobin A_{1c} (%)	7.3 ± 1.7	N/A
Daily insulin dose (IU kg^{-1})	0.6 ± 0.3	N/A
Serum total cholesterol (mmol L^{-1})	4.3 ± 0.5	4.9 ± 1.0
HDL cholesterol (mmol L^{-1})	1.2 ± 0.3	1.5 ± 0.5
Serum triglycerides (mmol L^{-1})	1.5 ± 1.2	1.1 ± 0.4

Data are means ± SD.
N/A, not applicable; HDL, high-density lipoprotein.

measured by $\dot{V}O_{2\ peak}$). Reasons for exclusion included any cardiovascular or pulmonary disease, vitamin supplementation, chronic medication other than insulin and regular participation in organized athletic events or highly intense physical activity. None of the diabetic subjects had clinically evident atherosclerotic disease, nephropathy (overnight urinary albumin excretion was $< 10\ \mu g\ min^{-1}$ and serum creatinine was normal), or neuropathy, and only two of them had mild background retinopathy, not requiring laser treatment. Diabetic subjects were in fair glycaemic control (the mean HbA_{1c} of the last two measurements over the preceding 6–8 months was 7.3 ± 1.7%). The study was approved by the ethics committee of the Kuopio University Hospital, Finland.

Exercise testing

All subjects underwent an exercise test to determine $\dot{V}O_{2\ peak}$, using an electrically braked bicycle ergometer (Tunturi EL 400, Turku, Finland), breath by breath gas monitoring (Medikro 919, Kuopio, Finland) and continuous ECG. One to two weeks later, subjects exercised for 40 min at 60% of their $\dot{V}O_{2\ peak}$ after a 5 min warm up at 60 W. On the day of exercise, the subjects ate a light, carbohydrate-rich breakfast 2–4 h before testing.

Blood sample collection and preparation

Routine screening laboratory tests and lipoprotein and HbA_{1c} determinations were carried out on blood samples drawn in a fasting state in the morning separately from the exercise tests. Samples for erythrocyte glutathione-dependent enzymes and Cu, Zn-SOD and catalase assays were taken from an antecubital vein 5 min before and within 2 min after exercising at 60% $\dot{V}O_{2\ peak}$ for 40 min. Treated samples were frozen at −80 °C until later determination. Fingerstick blood glucose determinations were carried out immediately before and after exercise at 60% $\dot{V}O_{2\ peak}$ in the diabetic group only.

Plasma TBARS and blood total glutathione (TGSH) levels were determined as described before (Sen *et al.* 1994b). Plasma TBARS results were missing for one control subject.

Determination of erythrocyte Se-GPX, GRD and GST activity was carried out using washed red cell haemolysates as described before (Sen *et al.* 1992), with the following modifications: H_2O_2 was used as a substrate instead of cumene peroxide to detect selenium dependent GPX activity; enzyme activity was expressed as $\mu mol\ min^{-1}\ g^{-1}$ haemoglobin.

Determination of erythrocyte Cu, Zn-SOD activity was based on the reduction of nitroblue tetrazolium in a xanthine-xanthine oxidase-dependent superoxide generating system (Beauchamp & Fridovich 1971). The activity of SOD that could cause a 50% inhibition of superoxide produced by the reduction of nitroblue tetrazolium was defined as 1 unit (U).

Red cell catalase activity was determined by monitoring the decrease in absorbance at 240 nm in the presence of 10 mM hydrogen peroxide (Aebi 1984). One U of catalase activity was defined as the decomposition of 1 M hydrogen peroxide min^{-1} at 25 °C.

Serum cholesterol and triglyceride were measured enzymatically using a Hitachi 717 analyser (Tokyo, Japan). The same method was also used for high-density lipoproteins (HDL) after removal of LDL and very low-density lipoproteins (VLDL) by dextran sulphate/$MgCl_2$ (Penttilä *et al.* 1981).

Blood HbA_{1c} was measured in diabetic subjects using liquid cation exchange chromatography (normal range 4.0–6.0%).

The SPSS/PC+ software (SPSS, Chicago, IL) was used for statistical analyses. Results for the groups are expressed as means ± SD. Differences between the group means were analysed for significance using the unpaired Student's *t*-test. Differences within the same group before and after exercise were tested with repeated measures ANOVA. Pearson's correlation and univariate regression analysis was used to assess the associations between selected variables. Statistical significance was defined as $P < 0.05$.

RESULTS

Peak oxygen consumption

The mean $\dot{V}O_{2\ peak}$ values in both groups were similar (Table 1).

Fingerstick blood glucose levels in the diabetic group before and after exercise

Pre- and post-exercise blood glucose levels were 10 ± 5 and 8 ± 5 mmol L^{-1}, respectively.

Plasma TBARS levels before and after exercise

Plasma TBARS levels were increased in the IDDM group both before and after exercise (Laaksonen *et al.* 1996, Table 2). Plasma TBARS levels rose markedly in both groups in response to exercise.

Blood total glutathione levels before and after exercise

Blood TGSH levels were increased in the IDDM group both before and after exercise (Laaksonen *et al.* 1996, Table 2). Mean blood TGSH remained unchanged in response to exercise in both groups.

Table 2 Plasma thiobarbituric acid reacting substance (TBARS) and blood total glutathione (TGSH) levels before and after exercise in diabetic and control men

	Before exercise		After exercise	
	Diabetic group	Control group	Diabetic group	Control group
n	9	13	9	13
Plasma TBARS (μmol L^{-1})	2.2 ± 0.72*	0.86 ± 0.38	3.3 ± 0.63†‡	1.4 ± 0.54§
Blood TGSH (mmol L^{-1})	1.2 ± 0.22*	0.94 ± 0.15	1.2 ± 0.25‡	0.9 ± 0.12

Data are means ± SD.
*, $P \leq 0.005$, diabetic vs. control group, Student's unpaired *t* test.
†, $P = 0.001$, diabetic group, before vs. after exercise, ANOVA repeated measures.
‡, $P = 0.001$, diabetic vs. control subjects, Mann-Whitney *U* test.
§, $P = 0.012$, control group, before vs. after exercise, ANOVA repeated measures.

Erythrocyte glutathione-dependent enzymes at rest

Red cell GRD activity at rest was 15% higher in the diabetic group ($P = 0.049$, Fig. 1a.). Neither Se-GPX nor GST activity differed between the two groups (Fig. 1b & c).

Erythrocyte Cu, Zn-SOD and catalase activities at rest

Erythrocyte Cu, Zn-SOD and catalase activities at rest were significantly lower in the diabetic group ($P = 0.007$ and $P = 0.023$, Fig. 2).

Erythrocyte glutathione-dependent enzyme activities after exercise

Post-exercise red cell Se-GPX activity rose modestly with exercise in the control group ($P = 0.003$, Fig. 1b) but not in the IDDM group. Post-exercise Se-GPX activity was higher in the control group than in the IDDM group ($P = 0.046$). Post-exercise GRD activity was also higher in the IDDM group than in the control group ($P = 0.032$). Exercise did not significantly affect GRD or GST activity in either group.

Erythrocyte Cu, Zn-SOD and catalase activities after exercise

There were no significant exercise-induced changes in either Cu,Zn-SOD or catalase activity in either group (Fig. 2).

Correlations and univariate regression analysis

At rest, red cell GST activity correlated inversely with blood HbA_{1c} activity ($r = -0.79$, $P = 0.012$). There were no other significant correlations between the antioxidant enzymes and serum lipid levels, prevailing glucose levels, HbA_{1c} or $\dot{V}O_{2\ peak}$ in the IDDM group. Moreover, plasma TBARS levels (Laaksonen *et al.* 1996) did not correlate with any of the erythrocyte antioxidant enzyme activities.

DISCUSSION

Otherwise healthy young diabetic men had lower erythrocyte Cu, Zn-SOD and catalase activity, but higher GRD activity. Coupled with increased plasma TBARS and blood total glutathione levels in the diabetic men (Laaksonen *et al.* 1996), these changes may reflect increased susceptibility to oxidative stress and compensatory adaptations of glutathione metabolism in response to increased oxidative stress.

Erythrocyte glutathione-dependent enzymes at rest

Elevated erythrocyte GRD activity in the diabetic group may partially explain increased total blood glutathione stores found in the IDDM group (Laaksonen *et al.* 1996) by increasing conversion of GSSG to GSH, since excess GSSG is exported out of the cell (Srivastava & Beutler 1969). Increased GRD activity in IDDM is consistent with some (Godin *et al.* 1988, DiSimplicio *et al.* 1995), but not all (Ståhlberg & Hietanen 1991), previous reports. In contrast to our study, most other human studies in which decreased blood TGSH levels have been found have been in older, mainly non-insulin-dependent subjects (Murakami *et al.* 1989) or poorly defined populations (Jain & McVie 1994). In a recent study investigating platelet glutathione levels in IDDM patients only slightly older than those in our study and in moderate glycaemic control, TGSH content was similar and GRD activity elevated in IDDM (DiSimplicio *et al.* 1995). In the IDDM patients without nephropathy, TGSH levels were nearly 20% higher than in control subjects, although no statistical comparison was reported (DiSimplicio *et al.* 1995).

Induction of the polyol pathway by hyperglycaemia has been proposed as a major mechanism leading to the depletion of glutathione reported in some other studies (DeMattia *et al.* 1994, Roy *et al.* 1997). Godin *et al.* (1988), however, reported that red cells from diabetic rats and human red cells incubated in glucose were markedly resistant to hydrogen peroxide-induced de-

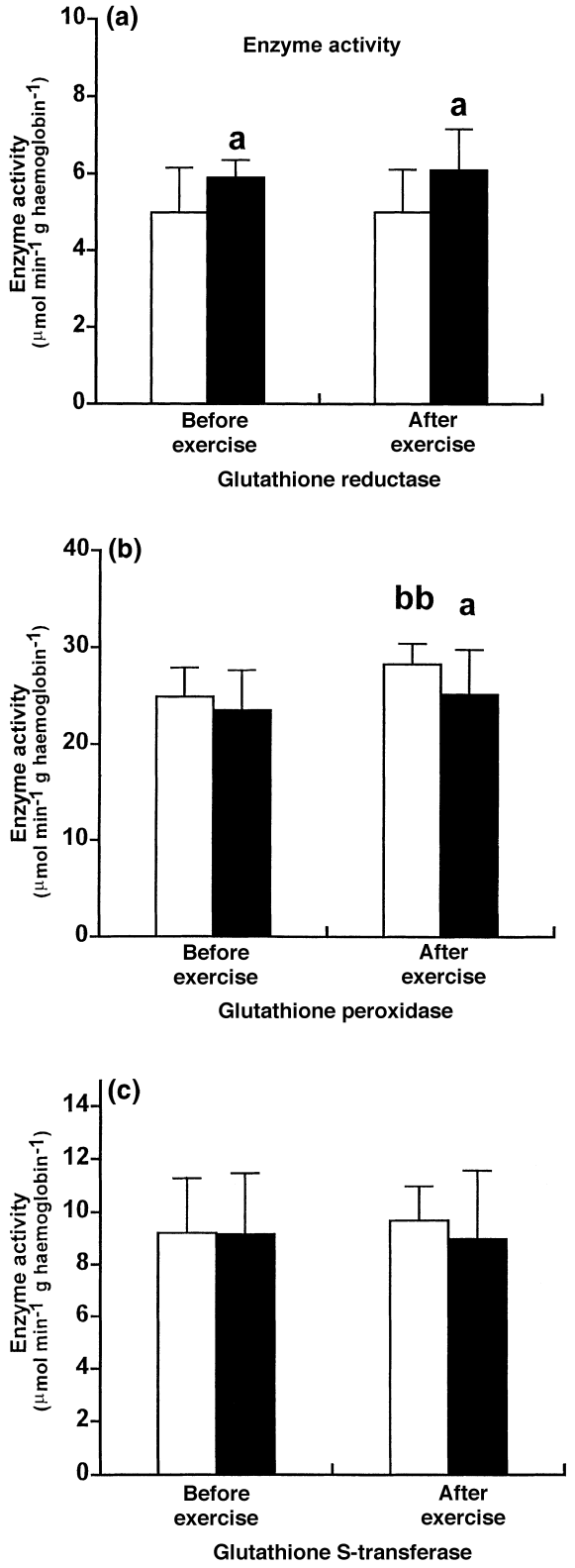

Figure 1 Red cell glutathione reductase (a). glutathione peroxidase (b) and glutathione -S-transferase (c) activity before and after exercise in diabetic (■) and control (□) men. a, diabetic vs. control groups; b, before vs. after exercise. a, $P < 0.05$; bb, $P < 0.01$. Data shown are mean ± SD.

pletion of GSH, possibly through induction of the pentose phosphate pathway. Our study and those of others (Godin *et al.* 1988, DiSimplicio *et al.* 1995) suggest that, at least in some diabetic populations, polyol pathway-induced decreases in the NADPH/$NADP^+$ ratio or other abnormalities in thiol metabolism do not occur to such an extent to prevent upregulation of blood TGSH levels and red cell GRD activity. Young otherwise healthy men with IDDM may possibly compensate partially for increased oxidative stress by upregulation of the glutathione system.

No differences at rest between diabetic and control men were found in erythrocyte Se-GPX and GST activities. Decreased (Yaqoob *et al.* 1994) and unchanged (Godin *et al.* 1988) Se-GPX activity in IDDM has previously been reported. Interestingly, GST was the only enzyme which was inversely correlated with HbA_{1c} levels. *In vitro* studies have shown decreased liver GST activity in a hyperglycaemic medium (Yadav *et al.* 1994). Human studies assessing erythrocyte GST activity in IDDM are limited. However, Ståhlberg & Hietanen (1991) also found no difference in GST activity between IDDM and control subjects.

Erythrocyte Cu, Zn-SOD and catalase activities at rest

Our finding of decreased red cell Cu, Zn-SOD activity in IDDM at rest agree with some (Nath *et al.* 1984, Kawamura *et al.* 1992, Yaqoob *et al.* 1994), but not all (Godin *et al.* 1988), previous studies. Kawamura *et al.* (1992) have found decreased activity of glycated red cell Cu, Zn-SOD fractions, elevated glycated Cu, Zn-SOD levels and decreased overall Cu, Zn-SOD activity in IDDM. Decreased Cu, Zn-SOD activity coupled with increased superoxide production (Ceriello *et al.* 1991, Wolf *et al.* 1991) could predispose to increased oxidative stress, especially if not compensated with increased catalase or Se-GPX activity. Superoxide may react with other reactive oxygen species such as nitric oxide to form highly toxic species such as peroxynitrite, in addition to direct toxic effects (Tesfamariam 1994). Alternatively, superoxide can be dismutated to much more reactive hydrogen peroxide, which through the Fenton reaction can then lead to highly toxic hydroxyl radical formation (Wolff *et al.* 1991). Thus decreased catalase activity could also contribute to the increased oxidative stress found in the IDDM subjects. Elevated glucose (Yadav *et al.* 1994) and hydrogen peroxide levels (Ou & Wolff 1994) have also been shown to inactivate catalase.

Effect of exercise on red cell antioxidant enzyme activities

Se-GPX response to exercise seemed to be impaired in the IDDM group. Se-GPX activity rose significantly with exercise only in the control group, and unlike at

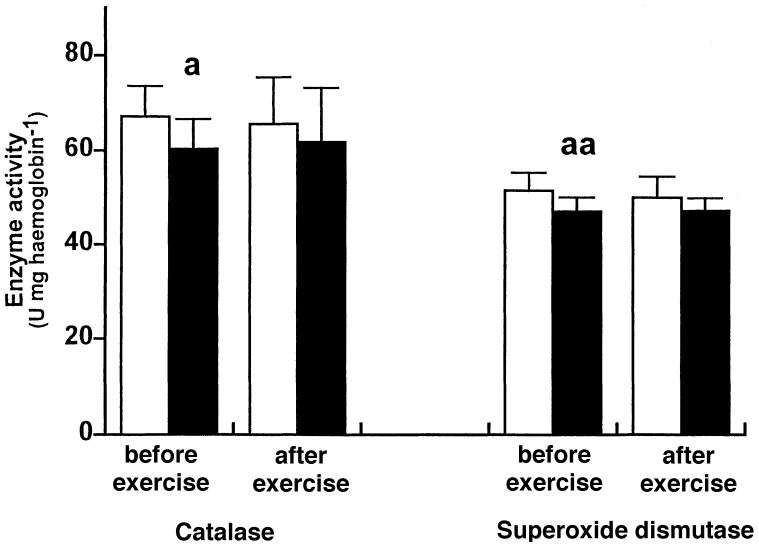

Figure 2 Red cell catalase superoxide dismutase activity before and after exercise in diabetic and control men. a, diabetic (■) vs. control (□) groups. a, $P < 0.05$; aa, $P < 0.01$. Data shown are mean ± SD.

rest, Se-GPX activity was higher in the control men than in the diabetic men. The explanation for lack of significant upregulation of Se-GPX in the diabetic group is unclear. Upregulation of GPX in response to acute exercise has been found in skeletal muscle in animal experiments (Ji 1993), although in some human studies no changes in erythrocyte GPX activity were reported (Ohno *et al.* 1986, Duthie *et al.* 1990, Rokitzki *et al.* 1994).

Red cell GRD, GST, catalase and Cu, Zn-SOD activity were unaffected by exercise. The response of erythrocyte catalase activity to acute exercise in our study agrees with some previous studies also showing no change after exercise (Ohno *et al.* 1986, Duthie *et al.* 1990, Rokitzki *et al.* 1994). Because catalase is localized to peroxisomes or microperoxisomes (Michiels *et al.* 1994), it would be expected that GPX, located in the cytosol and mitochondria, would be more sensitive to exercise-induced peroxide and peroxyl radical formation. The response of erythrocyte Cu, Zn-SOD activity to exercise has been very inconsistent in the literature (Ohno *et al.* 1994)

The strong inverse correlation between $\dot{V}O_{2\ peak}$ and plasma TBARS found in the diabetic group only (Laaksonen *et al.* 1996) was not clearly explained by the antioxidant enzymes measured. The lack of correlation between $\dot{V}O_{2\ peak}$ and any of the red cell antioxidant enzyme activities measured may be because of the complex interrelationships between different antioxidants in the antioxidant defence system, or because the apparent protective effect of physical fitness could conceivably be mediated through effects on different antioxidants or pro-oxidants.

Decreased red cell Cu, Zn-SOD and catalase activity at rest may predispose to the increased oxidative stress found in young men with IDDM in fair glycaemic control. On the other hand, erythrocyte GRD upregulation may reflect a compensatory adaptation to increased oxidative stress and could contribute to the increased blood total glutathione levels in the diabetic men. Exercise-induced red cell Se-GPX upregulation appeared to be impaired in the diabetic men.

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