

Inflammation and nitric oxide production in skeletal muscle of type 2 diabetic patients

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Abstract

An inflammatory process may be involved in nitric oxide production in skeletal muscle of type 2 diabetic patients. Nitric oxide generation in skeletal muscle was assessed in 14 non-complicated type 2 diabetic patients and in 12 healthy subjects. In samples of quadriceps femoris muscle, endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), nitrite, nitrate and nitrotyrosine were determined. The macrophage-specific antigen CD163, the T-cell membrane factor CD154 and tumour necrosis factor- α (TNF- α) were also assayed. In six patients, ultrastructural analysis of muscle was performed. Nitrites and nitrates were increased in patients as compared to controls (22.7 ± 4.5 and 32.7 ± 7.0 vs 16.0 ± 2.9 and 22.8 ± 4.0 $\mu\text{mol/mg}$ protein; $P < 0.001$, Mann-Whitney U test). Endothelial NOS was similar in diabetic and control subjects (36.4 ± 13.8 vs 36.3 ± 6.8 ng/mg

protein), contrasting with the significant increase of iNOS recorded in patients (34.3 ± 13.0 vs 8.5 ± 2.8 ng/mg protein, $P < 0.00002$). Nitrotyrosine levels were higher in the patient than in the control group (42.1 ± 24.4 vs 10.3 ± 2.5 ng/mg protein, $P < 0.00002$), as were CD163 (10-fold) and TNF- α (fourfold) levels. Furthermore, CD154 levels were detectable only in the patient samples (10.2 ± 5.3 ng/mg protein). By multiple-regression analysis, changes in glycated haemoglobin values could predict 96% variation in nitrotyrosine. Macrophages were present in all muscle samples analysed by electromicroscopy. The increased levels of CD163, CD154 and TNF- α indicate that an inflammatory process occurs in skeletal muscle of type 2 diabetic patients. This may contribute to iNOS induction, muscle damage and insulin resistance.

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Introduction

There is growing evidence that chronic inflammation has a role in the pathogenesis of type 2 diabetes (Pickup *et al.* 2000, Marette 2002), but it has been shown that nitric oxide (NO) has a regulatory effect in the immune system (van der Veen 2001). However, excessive NO formation mediates non-specific tissue damage and has been found to be involved in the development of muscle insulin resistance in diet-induced obesity (Perreault & Marette 2001).

The synthesis of NO is catalysed by at least three isoforms of nitric oxide synthase (NOS). Two of the isoforms are constitutively expressed, one in endothelial cells (eNOS) and the other in the brain (nNOS); the third one, inducible (i) NOS, is induced by inflammatory cytokines such as interleukin-1 (IL-1) or tumour necrosis factor alpha (TNF- α) in macrophages and many other cell types (Moncada *et al.* 1991, Beck *et al.* 1999). At low concentration, NO stimulates guanylate cyclase activity,

which mediates physiological vasodilatation, inhibits platelet aggregation and leukocyte adhesion, protects against cytokine-induced damage and is a major derived immunosuppressive factor for T-cell immunity (Moncada *et al.* 1991, van der Veen 2001). However, the activation of the inducible isoform (iNOS) through inflammatory stimulants produces NO in thousand-fold larger quantities, which mediate pathological processes (Beck *et al.* 1999). NO can be transformed in reaction with another free radical superoxide (O_2^-) to form peroxynitrite (ONOO^-). This reaction between NO and O_2^- is extremely strong and is three times faster than the rate at which superoxide dismutase scavenges O_2^- . Peroxynitrite can nitrate lipids and proteins. Nitration can cause protein dysfunction, but few proteins that appear nitrated *in vivo* have been identified; one of them is nitrotyrosine, which is a marker of peroxynitrite production (Beckman & Koppenol 1996).

Many biochemical pathways associated with hyperglycaemia (such as glucose auto-oxidation, polyol pathways,

eicosanoid synthesis and protein glycation) increase the production of O_2^- (Di Mario & Pugliese 2001, Zou *et al.* 2002). There are also indications that O_2^- can be produced by eNOS (Schmidt *et al.* 1996, Wang *et al.* 2000). Therefore, the simultaneous production of NO and O_2^- is possible.

The generation of NO in type 2 diabetes, mainly investigated in vascular endothelium, has been a controversial issue. The endothelial dysfunction of the disease has been associated with decrement in NO production, impaired NO-mediated, endothelium-dependent relaxation, or increased NO production accompanied by augmented neutralisation of NO by O_2^- (Pieper 1998). Nitrotyrosine detection in plasma of diabetic patients (Ceriello *et al.* 2001), together with the increased expression of NOS and O_2^- production in human aortic endothelial cells exposed to high levels of glucose (Cosentino *et al.* 1997), supports the hypothesis that there is an increased production of NO and peroxide in diabetic patients.

The isoform nNOS has been detected in various normal human skeletal muscles at the surface membrane of the fibres and at the neuromuscular junctions. Low levels of iNOS have also been reported (Stamler & Meissner 2001). In addition, NOS activity similar to eNOS has been demonstrated in skeletal muscle, as well as mRNA expression of iNOS and eNOS (Wang *et al.* 2001). In pathological conditions such as heart failure and autoimmune myopathies, the expression of iNOS in skeletal muscle is highly increased (Stamler & Meissner 2001). iNOS expression in muscle cells was induced by the combination of IL-1 α - and interferon gamma (IFN- γ), both *in vitro* and *in vivo* (Adams *et al.* 2002), and by the combination of TNF- α and IFN- γ in cultured skeletal muscle cells (Bedard *et al.* 1998).

NO is generated by macrophages during the process of antigen presentation to T cells (van der Veen 2001). The activated T helper cell (Th) expresses co-stimulatory molecules that, together with several cytokines, including IFN- γ , induce NO production in the macrophage. One of the T-cell co-stimulatory molecules is CD40L (CD154), which is part of receptor-ligand families, including the TNF-receptor and TNF family (van der Veen 2001). CD154 is expressed on activated mature T cells, but not in resting T cells, and is involved in the amplification and regulation of inflammatory responses (van Kooten & Banchereau 2000). Chronic activation of T cells, as reported in autoimmunity and organ rejection, is dependent upon overexpression of CD154. The elevated levels of CD154 in individuals with type 1 or 2 diabetes were considered as evidence of a proinflammatory state (Varo *et al.* 2003).

The aim of the present study was to analyse the production of NO in skeletal muscle of type 2 diabetic patients and to find out whether this process is related to inflammatory factors. Therefore, the enzymes eNOS and

iNOS, and the products of NO formation, NO_2^- , NO_3^- and nitrotyrosine, were determined in quadriceps femoris muscle of these patients, as well as CD163, a specific antigen of human macrophages (Malm *et al.* 2000). The inflammatory cytokine TNF- α , and the indicator of activated $CD4^+$ cells, CD154 (van Kooten & Banchereau 2000), were also determined in the muscle samples. To confirm the presence of macrophages, some muscle sections were observed with the electron microscope.

Materials and Methods

Subjects

Fourteen type 2 diabetic patients (eight men and six women), aged 46.6 ± 7.5 years, were treated with oral hypoglycaemic agents (glibenclamide, gliclazide or metformin) until blood glucose was under 13.1 mmol/l. Diagnosis of type 2 diabetes was made 2 months to 13 years before the study. The studied subjects were descendants of a mixture of white Europeans, black Africans and native South American Indians. None of the subjects were hypertensive or had signs of renal or retinal complications. The control group was formed by 12 healthy volunteers (nine men and three women) of similar age (45.6 ± 12.6 years). The body-mass index (BMI) of patients was 27.2 ± 5.1 and of control subjects 24.3 ± 3.9 . The characteristics of the subjects are shown in Table 1.

Muscle biopsy

A muscle biopsy was taken from the vastus lateralis part of quadriceps muscle with a Bergström needle, under local anaesthetic and antiseptic conditions. Consent of all patients and control subjects was obtained after the muscle biopsy procedure was explained to them. The study was approved by the hospital ethics committee. Muscle samples were frozen in isopentane cooled in liquid nitrogen and kept at -80°C until processing. In six patients, part of the muscle sample was fixed in 3% glutaraldehyde in phosphate buffer, at pH 7.4 and 320 mOsmol, for ultrastructural study.

Ultrastructure

The muscle sample was postfixed in 1% OsO_4 and embedded in epon; sections were cut with a diamond knife in a Porter-Blum MT2 ultramicrotome, stained with uranyl acetate and lead citrate, and observed in a Hitachi H-500 transmission electron microscope, at an acceleration voltage of 100 kV.

Assessment of NO products in muscle

Frozen muscle samples were weighed, and the tissue was minced in a glass homogeniser with 1 ml of buffer,

Table 1 Type 2 diabetic patients and control subjects characteristics

	Patients		Control subjects		P
	Means \pm s.d.	Range	Means \pm s.d.	Range	
Sex (M/F)	8/6		9/3		
Age (years)	46.6 \pm 7.5	32–59	45.6 \pm 12.6	27–67	NS
BMI	27.2 \pm 5.1	19.11–35.69	24.30 \pm 3.9	19.80–26.64	NS
Glycaemia (mmol/l)	8.00 \pm 2.76	5.06–13.11			
Glycated haemoglobin (mmol/l)	11.2 \pm 5.7	5.6–23.3			
Diagnosis time of the disease (months)	37 \pm 43	2–156			

BMI: body mass index.

Tris-HCl 10 mmol/l, pH 7.5; NaCl, 150 mmol/l; EDTA, 5 mmol/l; triton X-100, 1% (v/v); 10 μ g/ml leupeptin; 10 μ g/ml aprotinin; and 2.5 g/ml pepstatin A. The homogenised tissue was placed in an Eppendorf tube and centrifuged at 90 g. The supernatant was used for the rest of the assays. Protein concentration was assessed by the BCA protein assay kit (Pierce Biochemicals, Rockford, IL, USA).

NO levels were determined indirectly by quantification of their oxidised products of degradation, nitrates and nitrites, using nitrate reductase and the Greiss reagent (Green *et al.* 1982) by the method of Moshage *et al.* (1995). Briefly, the supernatants of homogenised tissue were diluted fourfold with distilled water and incubated with nitrate reductase from *Aspergillus* spp. in order to quantify the total amount of NO products (nitrites plus nitrates). In the absence of the enzyme, only nitrite concentrations are determined. After 30-min incubation at 37 °C in the presence of the enzyme and its cofactors NADPH and FADH₂, and further incubation for 10 min with sodium pyruvate and lactic dehydrogenase to degrade excess NADPH, the samples were deproteinised with zinc sulphate, and 100 μ l of the supernatant were mixed with 100 μ l of Greiss reagent (a solution containing equimolar amounts of solution A (2.5% phosphoric acid and 10% sulphamide) and solution B (0.1% ethylenediamine)). A standard curve was obtained with sodium nitrate dissolved in water or in a pool of normal human serums. Nitrite concentration was determined at 540 nm by an ELISA plate reader (Labsystems Multiscan MCC/340, Pulttitte 8, Helsinki, Finland). The interassay and intra-assay coefficients of variation for the Greiss reaction were 5.6% and 3.6% respectively.

The total amount of nitrotyrosine was determined by a standard sandwich ELISA, as described by Ye *et al.* (1996). All antibodies, the mouse immunoglobulin (Ig) G monoclonal antibody, for capturing the modified amino acid, the polyclonal against nitrotyrosine, and the polyclonal goat antirabbit IgG-peroxidase, were purchased from Upstate Biotechnology (Lake Placid, NY, USA). The nitrotyrosine was quantified by a standard curve with known concentrations of nitrotyrosine from chemically

modified bovine serum albumin. The interassay and intra-assay coefficient of variations were 8% and 11% respectively. The sensitivity of the assay was 50 pg/ml.

Determination of NOS in muscle

Determination of constitutive eNOS A quantikine kit purchased from R & D Systems (Minneapolis MN, USA) was used to ascertain the levels of eNOS in muscle tissue according to the manufacturer's instructions. The sensitivity was 25 pg/ml under the conditions tested, using recombinant eNOS. The inter- and intra-assay variations were less than 8%.

Determination of iNOS A standard sandwich ELISA was used to determine iNOS. Briefly, the monoclonal antihuman iNOS (Serotec Corporation, Kidlington, Oxford, UK) was adsorbed to the plate at a concentration of 1 μ g/well with PBS. The plate was then blocked with 1% bovine serum and 0.05% Tween 20 in PBS for 1 h at 37 °C. Then, 100 μ l pure or 1:2,1:4 diluted (in PBS) supernatant of homogenised tissue were incubated overnight at room temperature. The plates were washed, and the secondary antibody, a polyclonal rabbit antihuman iNOS (Serotec), was incubated at a concentration of 1 μ g/ml for 1 h at 37 °C. The plates were washed again and incubated with goat antirabbit IgG-peroxidase for 1 h at 37 °C. Finally, the plates were washed and incubated with tetramethyl benzidine and read at 492 nm in an ELISA reader system (Labsystem, MA, USA). Purified iNOS (by affinity column) was used to perform a standard curve. The sensitivity of the assay was 40 pg/ml. No cross-reaction was observed when recombinant eNOS was used in the assay. The inter- and intra-assay variation was 12%.

Assessment of CD163 in muscle

A sandwich ELISA was performed as described by Sulahian *et al.* (2001), with minor modifications. Briefly, monoclonal anti-CD163 (clone ED-Hu1, Serotec) was used to capture CD163, and the biotinylated

clone RM3/1 was used to detect the molecule. The homogenised muscle samples were diluted 1:10 in distilled water prior to being added to the plates and incubated overnight. The rest of the procedure was performed as described by Sulahian *et al.* (2001), except that CD163 was purified from the supernatant of phormol myristate acetate (PMA)-activated monocytes by affinity chromatography using the monoclonal clone ED-Hu1, and the purified protein was used to perform the standard curve. The detection limit was 100–10 000 pg of purified CD163. The specificity was confirmed by immunoprecipitation of CD163 in homogenised muscle tissue prior to the assay.

Assessment of TNF- α in muscle

TNF- α was assessed by a third-generation TNF- α quantikine assay (R & D Systems) according to the manufacturer's instructions except that samples were diluted as suggested for serum samples and incubated for 18 instead of 3 h. The sensitivity of the assay was 0.5–3 pg/ml.

Assessment of CD154 in muscle

CD154 levels were detected by a commercial sandwich immunoenzymatic assay purchased from Chemicon Corporation (Temecula, CA, USA). The ELISA plates were already coated with the capturing monoclonal. The samples were diluted as specified by the manufacturer in the assay buffer, and the only modification was that the samples were incubated for 18 h at 4 °C. Quantitative analysis was performed with a standard curve. The detection limit was 1 ng CD154 per mg of protein.

Blood samples

Glucose was assayed by an enzymatic method (Qualitest). Glycated HbA was determined by resin column separation (Sigma kit).

Statistical analysis

Results are expressed as mean \pm S.D. of the mean. Comparison between patients and controls was made by the Mann–Whitney U test. Correlations were made with the Pearson and Spearman tests. A stepwise multiple-regression analysis (Statistica, StatSoft Inc., Tulsa, OK, USA) was performed. A value of $P < 0.05$ was considered statistically significant.

Results

At the time of the muscle biopsy, blood glucose was 8.00 ± 2.76 mmol/l. In nine patients, the values of glycated haemoglobin were available ($11.19 \pm 5.71\%$). In

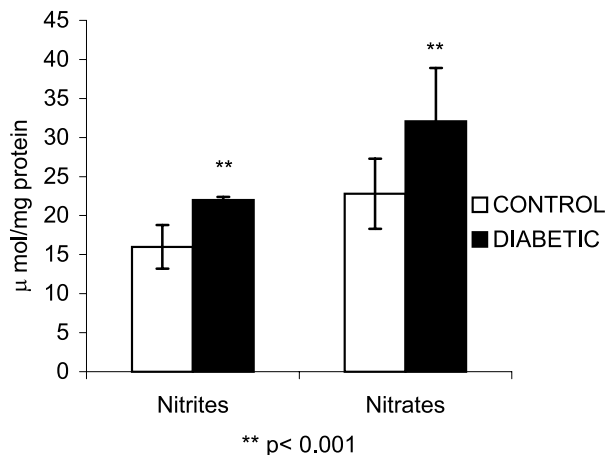


Figure 1 Levels of nitrites and nitrates in quadriceps muscle of type 2 diabetic patients (black columns) and control subjects (white columns). Mean values \pm S.D. in $\mu\text{mol/mg}$ protein.

diabetic patients, nitrites and nitrates in skeletal muscle were 40% higher than in control subjects (NO_2^- 16.0 ± 2.9 $\mu\text{mol/mg}$ protein in controls vs 22.7 ± 4.5 $\mu\text{mol/mg}$ protein in patients ($P < 0.0009$); NO_3^- 22.8 ± 4.0 $\mu\text{mol/mg}$ protein in controls vs 32.7 ± 7.0 $\mu\text{mol/mg}$ protein in patients ($P < 0.0007$) (Fig. 1)). The ratio $\text{NO}_3^-/\text{NO}_2^-$ did not differ between control and diabetic subjects (1.43 ± 0.19 and 1.50 ± 0.39).

The eNOS level was similar in diabetic and control subjects (36.3 ± 6.8 ng/mg protein in controls; 36.4 ± 13.8 ng/mg protein in patients), but iNOS was fourfold higher in the skeletal muscle of the diabetic group (8.5 ± 2.8 ng/mg protein in controls; 34.3 ± 13.0 ng/mg protein in patients ($P < 0.00002$) (Fig. 2)). Nitrotyrosine showed a similar difference, around 300% higher in

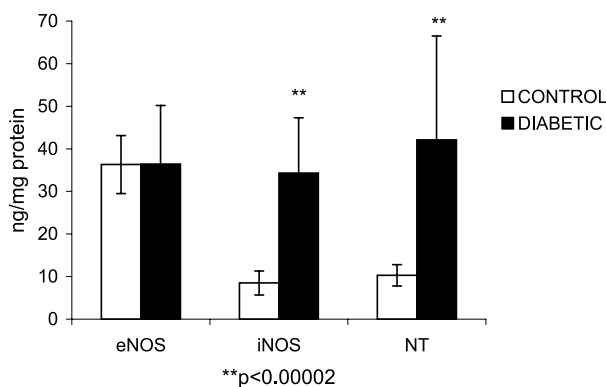


Figure 2 Levels of constitutive endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and nitrotyrosine (NT) in quadriceps muscle of type 2 diabetic patients (black columns) and control subjects (white columns). Mean values \pm S.D. in ng/mg protein.

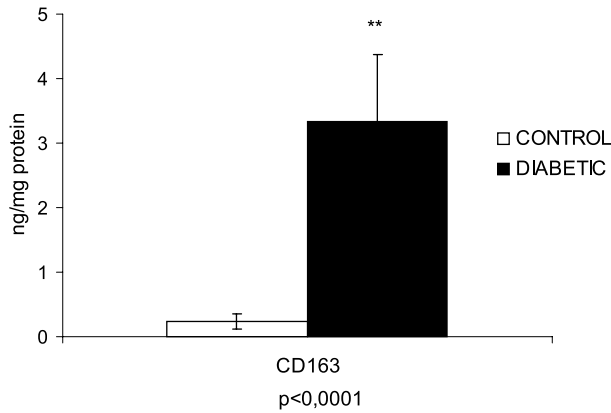


Figure 3 CD163 in quadriceps muscle of type 2 diabetic patients (black column) and control subjects (white column). Mean values \pm s.d. in ng/mg protein.

diabetic patients (control subjects 10.3 ± 2.5 ng/mg protein; diabetic patients 42.1 ± 24.4 ng/mg protein ($P < 0.00002$) (Fig. 2)).

CD163 was analysed in all patients and in nine control subjects. Patients showed over 10 times more CD163 than the control subjects (0.24 ± 0.12 ng/mg protein in controls; 3.33 ± 1.05 ng/mg protein in patients ($P < 0.00008$) (Fig. 3)). TNF- α was 44.8 ± 15.8 pg/mg protein in control subjects and 177.6 ± 147.0 pg/mg protein in the patient group ($P < 0.01$) (Fig. 4)).

CD154 was found only in homogenates of diabetic patients and was undetectable in those of the control subjects (10.2 ± 5.3 ng/mg protein as compared to undetectable; limit of the method 1 ng/mg protein), implying the presence of activated IFN- γ -producing CD4⁺ cells.

The level of iNOS and of CD163 correlated to the levels of nitrotyrosine (Fig. 5). Plasma glycated haemoglobin values showed a direct correlation to TNF- α (Fig. 6), nitrotyrosine and CD163 (Fig. 7).

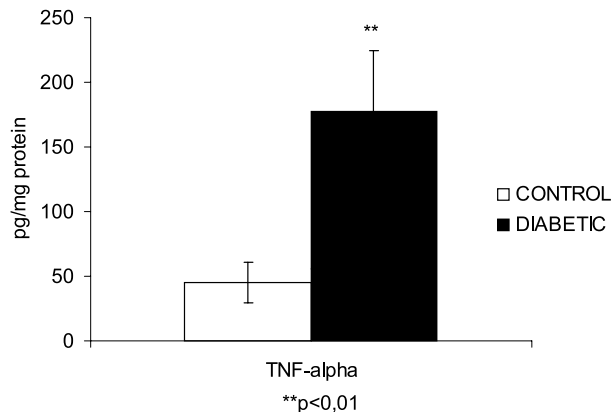


Figure 4 Tumour necrosis factor alpha (TNF-alpha) in quadriceps muscle of type 2 diabetic patients (black column) and control subjects (white column). Mean values \pm s.e.m. in pg/mg protein.

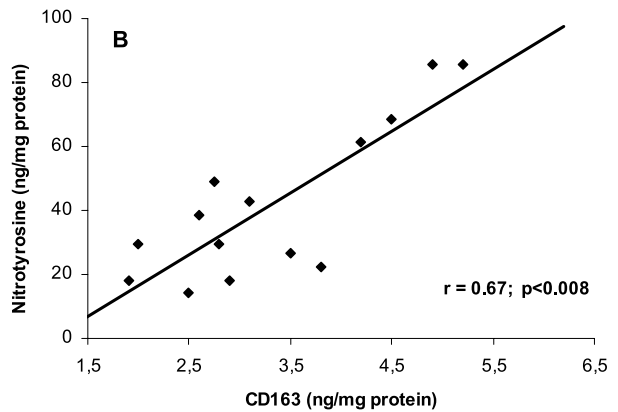
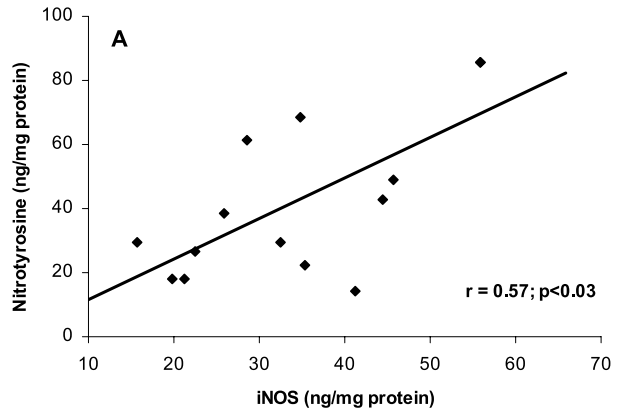


Figure 5 Correlations (Spearman) of inducible nitric oxide synthase (iNOS) (A) and CD163 (B) to nitrotyrosine.

Multiple-regression analysis showed that the variation in nitrotyrosine could be predicted from changes in glycated haemoglobin, the percentage of explained variance being 96%; after adding the next step of the multivariate regression analysis, the age of the patients, the fraction of

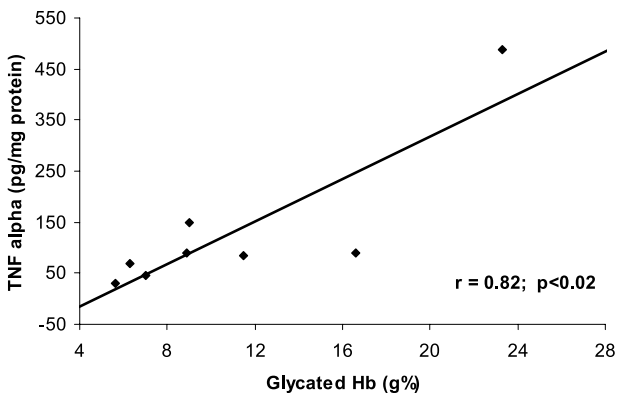


Figure 6 Correlation (Spearman) of glycated haemoglobin (Hb) to tumour necrosis factor alpha (TNF- α).

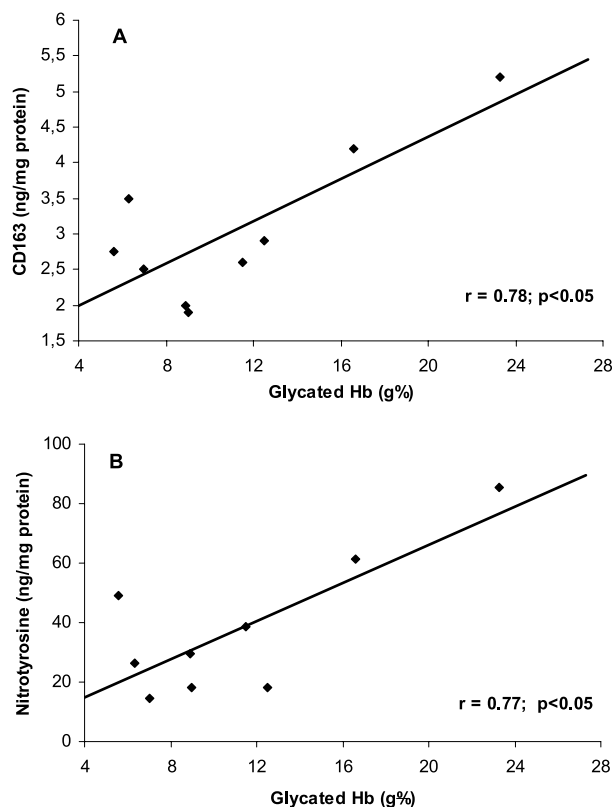


Figure 7 Correlations (Pearson) of glycated haemoglobin (Hb) to DC163 (A) and nitrotyrosine (B).

explained variance increased to 99%. The variation in the sum of NO_2^- and NO_3^- was explained primarily by the variation in CD163 (96%); with the addition of TNF- α , it was a total of 99%.

At ultrastructural observation, macrophages were present in the interstitial space close to capillaries in the muscle sections of the six patients that were examined for this purpose. An example is shown in Fig. 8.

Discussion

In the present report, increased levels of iNOS and nitrotyrosine were found in skeletal muscle of type 2 diabetic patients, indicating a high production of NO. NO_2^- and NO_3^- were also increased, but not to the same extent as nitrotyrosine and iNOS. This suggests that a high proportion of NO reacted with superoxide to produce peroxynitrite, finally resulting in nitrotyrosine formation.

In a skeletal muscle sample, there are muscle fibres, connective tissue, microvessels and nerve fibres. eNOS and iNOS have been demonstrated to be present in endothelial cells and muscle fibres; therefore, we cannot rule out that iNOS may be activated in muscle and/or endothelial cells. However, the presence of macrophages shown by the increased levels of CD163, which is a good

marker for these cells (Malm *et al.* 2000), together with the finding of macrophages in the biopsies of all six patients that were examined, provides evidence for the involvement of macrophages in muscle alteration of type 2 diabetic subjects. Furthermore, the increased levels of CD154 and TNF- α indicate that an inflammatory process is involved in iNOS activation and NO production (van der Veen 2001).

Pickup *et al.* (2000) found increased circulating levels of IL-6 and TNF- α in type 2 diabetic subjects, but these cytokines were decreased in cultured diabetic blood. It is possible that the migration of white cells to tissues such as skeletal muscle is important for cytokine production.

In the present results, the variation in levels of the NO products NO_2^- and NO_3^- was explained in the multiple-regression analysis by the variation in CD163 (96%), and adding TNF- α explained 99% of the variation. This strongly suggests a role for macrophages in activation of NO production. CD163 is a highly expressed macrophage membrane protein belonging to the scavenger receptor cysteine-rich (SRCR) domain family (Graversen *et al.* 2002). It has been demonstrated that iNOS expression in skeletal muscle *in vivo* and *in vitro* requires IL-1 β and IFN- γ to induce NF κ B activation (crucial for iNOS transcription) (Adams *et al.* 2002). The increase in iNOS expression required more than two signals related to an immune inflammatory reaction. Activated T cells produce IFN- γ , which in turn increases macrophage IL-1 β , IL-6 and, as a consequence, TNF- α (Adams *et al.* 2002). We detected that CD154 was present in the muscle homogenates of the type 2 diabetic patients, compared to undetectable levels in control subjects. Other investigators reported that soluble CD154 (CD40L) is elevated in plasma of type 1 and type 2 diabetic patients (Varo *et al.* 2003) and in serum of type 2 diabetic patients with coronary artery disease (Marx *et al.* 2003). Thus, one may assume that an inflammatory reaction observed in the diabetic muscle involves T-cell and macrophage activation, and specifically inflammatory cytokines that enhance NO, superoxide, peroxynitrite and consequently nitrotyrosine generation.

It has been recently reported that obese tissue is characterised by macrophage infiltration (Weisberg *et al.* 2003, Xu *et al.* 2003). In the present work, muscle samples were macroscopically free of fat, and no adipocytes were seen at ultrastructural analysis. Although four patients and one control were over 27, neither TNF- α nor CD163 muscle levels were related to BMI; however, this does not exclude that macrophage presence in muscle may have some relationship to intramuscular lipids.

The possible link between hyperglycaemia and inflammation

CD163 has been identified as the endocytic receptor binding haemoglobin in complex with the plasma protein haptoglobin (Graversen *et al.* 2002). It is conceivable that

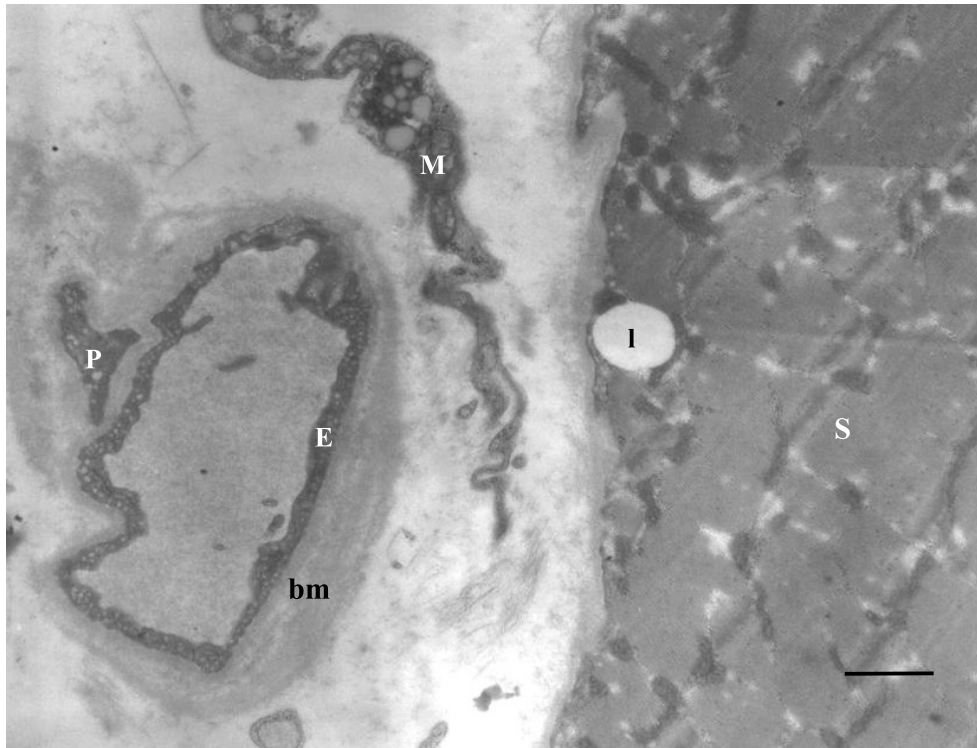


Figure 8 Transmission electron microscope oblique section of vastus lateralis part of quadriceps muscle of a type 2 diabetic patient. Bar=1 μm . Section of a muscle fibre (right): Sarcomere (S). Empty space of a lipid droplet (I). Cell in the centre of the section is a macrophage (M). Capillary (left): endothelial cell (E), thick basement membrane (bm) and pericyte (P).

glycated haemoglobin, and maybe other advanced glycation end products (AGEs), would trigger macrophage attraction into tissues. In the present work, a correlation was found between glycated haemoglobin and CD163, supporting this possibility.

Origin of superoxide

AGEs may cause pathological changes by generation of reactive oxygen species, probably acting through receptors for AGE (Yan *et al.* 1994, Wautier *et al.* 2001); therefore, activation of iNOS will coincide with production of O_2^- , leading to peroxynitrite production. The addition of glycated human oxyhaemoglobin to normal omental microvessels impaired NO-mediated responses by a mechanism involving superoxide anions (Vallejo *et al.* 2000). In addition, reaction products of iNOS, AGEs and NF κ B have been found in aortic endothelial and smooth muscle cells of streptozotocin diabetic Mongolian gerbils (Nishigaki *et al.* 1999), and normalising levels of mitochondrial reactive oxygen species, which were increased by hyperglycaemia in cultured bovine aortic endothelial cells, prevented glucose-induced activation of protein kinase C, formation of AGEs, sorbitol accumulation and NF κ B activation (Nishikawa *et al.* 2000).

In the present results, the variation in nitrotyrosine could be predicted mainly by the changes in glycated haemoglobin, indicating that superoxide formed by the action of AGEs would eventually lead to the formation of peroxynitrite and tyrosine nitration. It is not known whether NO-derived nitrogen species can nitrate tyrosine residues of the insulin receptor and give rise to insulin resistance, although this is theoretically possible (Perreault & Marette 2001). If this did occur, a vicious circle would perpetuate hyperglycaemia and insulin resistance.

Nitrotyrosine has been found in plasma of diabetic patients, although it was undetectable in plasma of control subjects (Ceriello *et al.* 2001). However, in the present study, nitrotyrosine was found in skeletal muscle of normal subjects but in a quantity fourfold lower than in diabetic patients.

In conclusion, iNOS is increased in skeletal muscle samples of type 2 diabetic patients. Although this enzyme may be induced in muscle fibres and endothelial cells, the presence of macrophages and the increased levels of TNF- α and CD154 are evidence of an inflammatory process with macrophage and T-cell activation. The moderate increase of the NO products NO_2^- and NO_3^- , contrasting with the markedly augmented levels of nitrotyrosine, indicates that NO reacted with O_2^- to produce

ONOO⁻, which finally nitrated tyrosine residues. The superoxide ion necessary to react with NO for the production of ONOO⁻ may have been generated in relation to AGE formation, as suggested by the correlation between glycated haemoglobin and nitrotyrosine.

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