Nitric oxide synthase in skeletal muscle fibres of patients with type 2 diabetes

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Muscle-derived nitric oxide (NO) mediates fundamental physiological actions on skeletal muscle [1-4] including glucose uptake into muscle cells [5]. Recently, we have shown that the altered glucose metabolism in skeletal muscle of patients with type 2 diabetes (T2D) is associated with changes in the metabolic profile of individual muscle fibres [6], but fibre-type specific changes in NO synthase (NOS) expression in skeletal muscle of T2D patients remain to be elucidated. Here we investigated fibre-type related NOS expression in vastus lateralis muscle of T2D patients compared with healthy individuals with normal glucose tolerance (NGT). Cytophotometrical assay and Western blotting did not reveal any quantitative differences between NOS expression in muscles from NGT and T2D subjects. Positive NOS immunoreactivity in vastus lateralis of T2D patients was found to be associated with fast-oxidative glycolytic (FOG) muscle phenotype. This indicates that NOS expression in T2D patients correlates both with skeletal muscle fibre type distribution and the activity of oxidative and glycolytic enzymes.

There is increasing interest in the role of nitric oxide (NO) in common metabolic disorders such as type 2 diabetes (T2D). The underlying mechanisms by which theses complications arise and manifest remain poorly understood. Investigators have demonstrated that some of the changes that occur as a result of the diabetic environment include reduced bioavailability of NO [7]. NO originates via the oxidative L-arginine pathway catalyzed by a NO-synthase (NOS) family constituted by three distinct NOS isoforms (EC. 1.14.13.39). Neuronal and endothelial NOS (also designated NOS1 and NOS3) were identified in and cloned from neuronal and endothelial cells, respectively, whereas inducible NOS was isolated originally from activated macrophages and was therefore called macrophage NOS (also designated NOS2). Muscle fibre-type specific NOS expression in healthy skeletal muscles is profoundly described [1, 8]. We have earlier reported that NOS immunoreactivity was found to be associated primarily with fast-oxidative glycolytic (FOG) muscle fibres [8, 9]. However, studies about fibre related NOS expression in skeletal muscles from T2D patients are missing.

Aim of the present study was to investigate the NOS1-3 expression in the vastus lateralis muscles of T2D patients in comparison to healthy individuals with NGT and to show a relationship of NOS1-3 to defined fibre types. The muscle fibres were typed according to three classification systems as slow and fast (physiological fibre types), as I, IIA, IIX (myosin

based ATPase fibre types) and as SO (slow oxidative), FOG (fast oxidative glycolytic) and FG (fast glycolytic; physiological-metabolic fibre types). We measured NOS expression in defined muscle fibres *in situ* cytophotometrically and in the muscle homogenate by Western blotting.

Anthropometric and metabolic characteristics of subjects are summarized in Table 1. Age, sex distribution and anthropometric parameters were not significantly different between the NGT and T2D groups. In parallel with the altered parameters of glucose metabolism and insulin sensitivity, maximal aerobic capacity (VO_{2max}) was significantly lower in the T2D group. The analysis of vastus lateralis muscle biopsies revealed that all three NOS isoforms were co-localized in the same fibres as evidenced from examination of adjacent immunolabelled sections, illustrated in Fig. 1. To classify the NOS-positive fibres, we examined serial sections of vastus lateralis muscle subjected to enzyme histochemical reactions for demonstration of ATPase, GPDH and SDH activities and immunolabelled with antibodies against fast myosin, NOS1 (on behalf of all NOS isoforms) (Fig. 2). Western blotting (Fig. 3) confirmed the expression of all three NOS isoforms in vastus lateralis muscles of NGT and T2D patients. 13 fibres were identified and classified. The NOS-positive fibres were FOG fibres including IIA, IIAX and IIX fibres. After further subtyping of FOG fibres in FOGI (more glycolytic) and FOGII (more oxidative), IIA fibres correlated to FOGI and IIX and IIAX fibres correlated to FOGII.

In this study, we found a co-expression of NOS 1-3 in the metabolic defined fibre subtypes of FOG. This agrees with our earlier findings in skeletal muscles of healthy subjects [8], patients with type 1 diabetes (T1D) [10] and patients with Duchenne muscular dystrophy [9, 11]. The correlation of NOS expression to the myosin-based ATPase fibre types was not obvious due to their metabolic heterogeneity. However, NOS1-3-positive fibres were clearly defined as FOG fibres. We have previously demonstrated that fast fibres without or very low oxidative metabolism fast glycolytic (FG) fibres were NOS-negative [8]. This implies that NO as modulator of muscle functions is involved in oxidative metabolism in connection with fast force development, which only occurs in FOG fibres. Therefore, we confirm here the distinct role of FOG fibres in muscle function already suggested in our earlier studies [12].

Cytophotometrical assessment of NOS1-3 immunoreactivity showed no significant differences between T2D and NGT subjects (Fig. 4). Our findings that the expression of all three NOS isoforms in skeletal muscles does not significantly differs between NGT and T2D individuals are in accordance with the report of Kashyap et al. [13, 14] who did not found any differences in NOS protein content in muscle homogenates of healthy and T2D individuals.

In contrast to T2D patients, we measured reduced expression of NOS 1-3 expression in skeletal muscle of T1D patients, compared to NGT subjects [10]. These alterations could be linked to the reduction of FOG fibres [10] and the lack of C-peptid [15]. In muscles of T2D patients, frequency of FOG fibres and C-peptid level were not significantly changed, in contrast to T1D [6, 15]. In both T1D and T2D, chronic hyperglycemia occurs, the insulin sensitivity is preserved in T1D and impaired in T2D. This suggests that hyperglycemia and impaired insulin sensitivity do not effect the NOS expression in skeletal muscle fibres.

Notably, the NO levels in quadriceps muscle of T2D patients were earlier reported to be significantly higher than in healthy controls[16]. In this study, we show that the NOS expression in FOG fibres does not significantly differ between NGT and T2D individuals. This poses a question; why total NO plasma levels in T2D patients are nevertheless significantly lower compared with healthy controls [17]. The apparent inconsistency can be explained by an increased oxidative stress in diabetes [16, 18] resulting in withdrawing NO from its regular physiological course via the scavenging actions of superoxides. Consequently, the NO bioavailability gets drastically reduced [19]. As a result, the cell is getting short of bioactive NO and responses with up-regulation of NOS and other enzymes engaged in detoxification of ROS [20]. This is in line with our earlier hypothesis that NO deficiency with consequent muscle cell degeneration in muscular dystrophies may result from NO scavenging by superoxides rather than from reduced NOS expression [11]. Our findings suggest that antioxidants and pharmacological activators of the L-arginine-NO-cGMP pathway may appear to be useful adjuvants in the treatment of T2D patients. A better understanding of the NO generation in muscles of T2D patients may provide further insight into fundamental mechanisms underlying the development of a clinical phenotype in diabetes.

Acknowledgements

This study was supported by grants from the Deutsche Forschungsgemeinschaft (BL 580/3-1) and the Bundesministerium für Bildung und Forschung, Interdisciplinary Centre for Clinical Research at the University of Leipzig (01KS9504/1, Project NO3) (to M.B.).

Author contributions

K.P., I.B. and M.B. designed experiments, analyzed and interpreted results, and wrote the manuscript, whereas all the authors contributed to its revision; K.P. performed the muscle

analyses and NOS immunohistochemistry; K.K. performed the cytophotometrical measurements; A.O. handled the subjects and muscle biopsies and established the anthropometric parameters; V.A. measured the metabolic parameters of the subjects.

Competing financial interests

The authors declare no competing financial interests.

Figure legends

Figure 1 Serial sections of vastus lateralis muscle of a T2D patient immunoreacted for NOS1, NOS2 and NOS3. A similar labelling pattern related to different fibre types was observed for all three isoforms. As an example, some fibres were identified in each section. For fibre typing see Fig. 2.

Figure 2 Fibre typing in serial sections of vastus lateralis muscle of a T2D patient. Immunostaining with fast myosin heavy chain (FAST), NOS 1 and activity of ATPase after acid (ATPase 4.3) or alkaline (ATPase 10.4) preincubation, GPDH, and SDH. The NOS1immunostaining was demonstrated on behalf of all NOS isoforms which were coexpressed in the same fibre (see Fig. 1). As an example, 13 fibres were identified and typed in each serial section: fibres 10-13 are slow fibres (type I, SO), fibres1- 9 are fast fibres (type II). Subtypes of fast fibres: 1- 3 = IIAX = FOGII , 4 and 5 = IIX= FOGII, 6-9 = IIA = FOGI

Figure 3 Representative Western blots of NOS 1-3 expression in vastus lateralis muscle from NGT and T2D groups.

Figure 4 Cytophotometrical assessment of NOS 1-3 immunoreactivity in vastus lateralis muscle from the NGT and T2D groups.

Methods

Subjects and muscle biopsies

Muscle biopsies were obtained from 25 Caucasian men (n = 12) and women (n = 13) that have been divided into 15 subjects (8 male and 7 female) with normal glucose tolerance (NGT) and 10 patients (4 male and 6 female) with type 2 diabetes (T2D). The diagnosis of NGT and T2D was based on oral glucose tolerance tests (OGTT) following the ADA-criteria [21]. Patients with malignant diseases or any acute or chronic inflammatory disease as determined by a leucocyte count > 7000 Gpt/l, C-reactive protein (CrP) > 5.0 mg/dl, or clinical signs of infection were excluded from the study. The study was approved by the ethics committee of the University of Leipzig. All subjects gave written informed consent before taking part in the study. Body mass index (BMI) was calculated as weight (in kilograms) divided by the square of height (in meters). Waist and hip circumferences were measured and waist-to-hip ratio (WHR) was calculated. Percentage body fat was measured by dual-energy X-ray absorptiometry (DEXA). All subjects completed a graded bicycle-ergometer test to volitional exhaustion and had maximal oxygen uptake measured with an automated open circuit gas analysis system at baseline. The highest oxygen uptake/minute reached was defined as the maximal oxygen uptake (VO_2max). Participants were admitted into the study at 9 am after 14 hours of fasting. They had been asked to avoid vigorous exercise for at least 3 days before the muscle biopsy. After administration of local anesthesia, muscle biopsy specimens were obtained from the vastus lateralis muscle using a biopsy device (SOMATEX, Teltow, Germany). Muscle samples were powdered with talcum and immediately frozen in liquid nitrogen. The samples were diveded into two parts. One of the pieces was used for Western blotting, the other was transferred into a cryostat 1800 (Reichert Jung, Vienna, Austria). 10 µm thick cross sections were cut which were used for enzyme histochemical and immunohistochemical analysis. To avoid possible variations in section thickness and incubation conditions, samples of NGT and T2D were mounted together on a cryostat chuck.

Enzyme histochemistry

Activity of succinate dehydrogenase (SDH, E.C. 1.3.5.1) was measured according to Lojda and Gutmann [22]. In a modified way, the method of Lojda and Gutmann [22] was used to

demonstrate activity of mitochondrial glycerol-3-phosphate dehydrogenase (GPDH, E.C. 1.1.99.5): the medium consisted of 0.1 M phosphate buffer, pH 7. 4,5 mM DL-3-glycerophosphate (disodium salt), 0.01% menadione and 0.5 mg/ml nitroblue tertazolium chloride (NBT). The incubation took 20 min at 37°C. The myofibrillar adenosine triphosphatase (ATPase, E.C. 3.6.1.32) activity was demonstrated after acid and alkaline preincubations at pH 4.35, 4.55 and 10.4, respectively [23]. Each time, control reactions were done on serial sections by missing substrate.

Myosin heavy chain (MHC) isoforms- Immunohistochemistry

Mouse monoclonal antibodies against slow and fast MHC isoforms were used following the guidelines recommended by the manufacturers (Novocastra Laboratories Ltd, Newcastle, UK). In brief, serial sections were incubated with the primary MABs 60 min at 37° C followed by incubation with rabbit-anti-mouse secondary AB (Jackson Immuno Research Laboratories, Inc, USA) for 60 min at 37°C and mouse PAP-complex (Jackson Immuno Research Laboratories, Inc, USA) 60 min at room temperature. The visualization was performed by incubation with 3,3-diaminobenzidine. After dehydration, the sections were mounted in Entellan (Merck, Darmstadt, Germany).

Fiber typing

Fibres were typed into slow and fast according to their slow or fast MHC-isoforms detected immunohistochemically as described above. According to the different acid and alkali labilitiy of the ATPase activity of the MHC isoforms demonstrated with enzyme histochemistry, four fibre types I, IIA, IIAX, IIX were differentiated. After acid preincubation at pH 4.25 and 4.55, respectively, type I fibres showed strong ATPase activity, IIAX fibres were weakly stained, IIX fibres were moderate and IIA fibres were not stained. IIAX fibres are hybrid fibres, their staining intensity was intermediate between that of IIA and IIX. After alkaline preincubation, type I fibres showed weak, IIA, IIAX and IIX fibres strong ATPase reaction. The physiological-metabolic fibre typing into SO (slow oxidative), FOG (fast oxidative glycolytic) and FG (fast glycolytic) has been described previously (Punkt 2002), based on cytophotometrically quantified activities of SDH (marker of oxidative activity) and GPDH (marker of glycolytic activity) and the immunoreactivity against slow and fast myosin in serial cross sections of the same fibre. SO fibres showed high SDH and low GPDH activity, FG fibres showed high GPDH and very low SDH activity. FOG fibres were

subdivided into FOG I (moderate SDH and high GPDH activity) and FOG II (high SDH and moderate GPDH activity).

Cytophotometry

End-point measurements were performed with a computer-controlled microscope photometer MPM 200 with a scanning table (Carl Zeiss, Oberkochen, Germany). The absorbance of the NOS-immunostained muscle fibres of NGT and T2D muscles arranged arranged in pairs on the same glass slide were measured. Three sections per muscle biopsy were evaluated. By mounting samples of NGT and T2D muscles on one and the same cryostat chuck the sections to compare were subjected the same cutting step and identical staining conditions. Differences in absorbance data between NGT and T2D muscle fibres reflect changes in NOS immunoreactivity in T2D muscles.

NOS–Immunohistochemistry

Cryostat sections were fixed in cold acetone for 15 min and thoroughly air dried for 30 min. After rinsing in PBS, nonspecific binding sites were blocked by incubation in PBS containing 10% goat serum for 30 min. PBS was used for all rinses and dilutions. Cryostat sections were incubated overnight at 4°C with rabbit primary polyclonal Abs recognizing NOS 1, NOS 2, NOS 3 (Transduction Laboratories, Lexington, KY). Primary Ab was diluted to a final concentration of 1.0 µg/ml. To quench endogeneous peroxidase activity, sections were treated with methanol containing 1.2% H_2O_2 for 15 min. Bound primary Abs were detected by employing horse radish peroxidase-conjugated goat-anti-rabbit secondary Abs which was followed by tyramide signal amplification (TSA-kit, NEN, Cologne, Germany) and ABC technique using the Vectastain ABC-kit (Vector Laboratories, USA). The visualization was performed using NovaRed substrate kit for peroxidase (Vector). The reaction was observed under the microscope and was stopped by rinsing with water after 4-10 minutes. Control incubations were: i) omission of primary Ab; and ii) substitution of primary Ab by rabbit IgG (Dianova, Hamburg, Germany) at the same final concentration as the primary Ab.

NOS-Western blotting

Total protein of skeletal muscle biopsies harvested in PBS and sedimented by centrifugation was homogenized in 10 mM Hepes (pH 7.5) containing 0.2 mM phenylmethylsulfonylfluoride (PMSF) and 0.1 mM dithiothreitol (DTT) using an Ultra Turrax (IK, Staufen, Germany) at 30.000 rpm for 3x5 sec. The final pellet was suspended in 0.75 ml

10mM Hepes (pH 7.5), 250 mM sucrose, 0.2 mM PMSF and 0.1 mM DTT and was stored at –80°C until use. 40 µg protein samples solubilized in SDS sample buffer were separated by 7.5% Laemmli-polyacrylamide gel electrophoresis. The electro transfer of the separated proteins was performed onto polyvinylidenedifluoride membranes (Boehringer, Mannheim, Germany). Primary anti-NOS antibodies were diluted according to the manufacturer's instructions (Transduction Laboratories, Lexington, KY). As positive controls for NOS 1-3 cerebellum, macrophage and endothelial lysates (Transduction Laboratories, Inc.) were used. The secondary antibody was peroxidase-labeled anti-rabbit IgG (Sigma, St. Louis MO, USA; dilution 1:15.000). The visualisation of the immunoreaction was performed by using an ECL-Kit (Amersham, Buckinghamshire, UK).

Statistical analyses

Data are shown as means ± SD. The NGT and T2D groups were compared using the paired Student's *t* test. Statistical analysis was performed using SPSS version 12.0 (Chicago, IL). *P* values < 0.05 were considered to be statistically significant.

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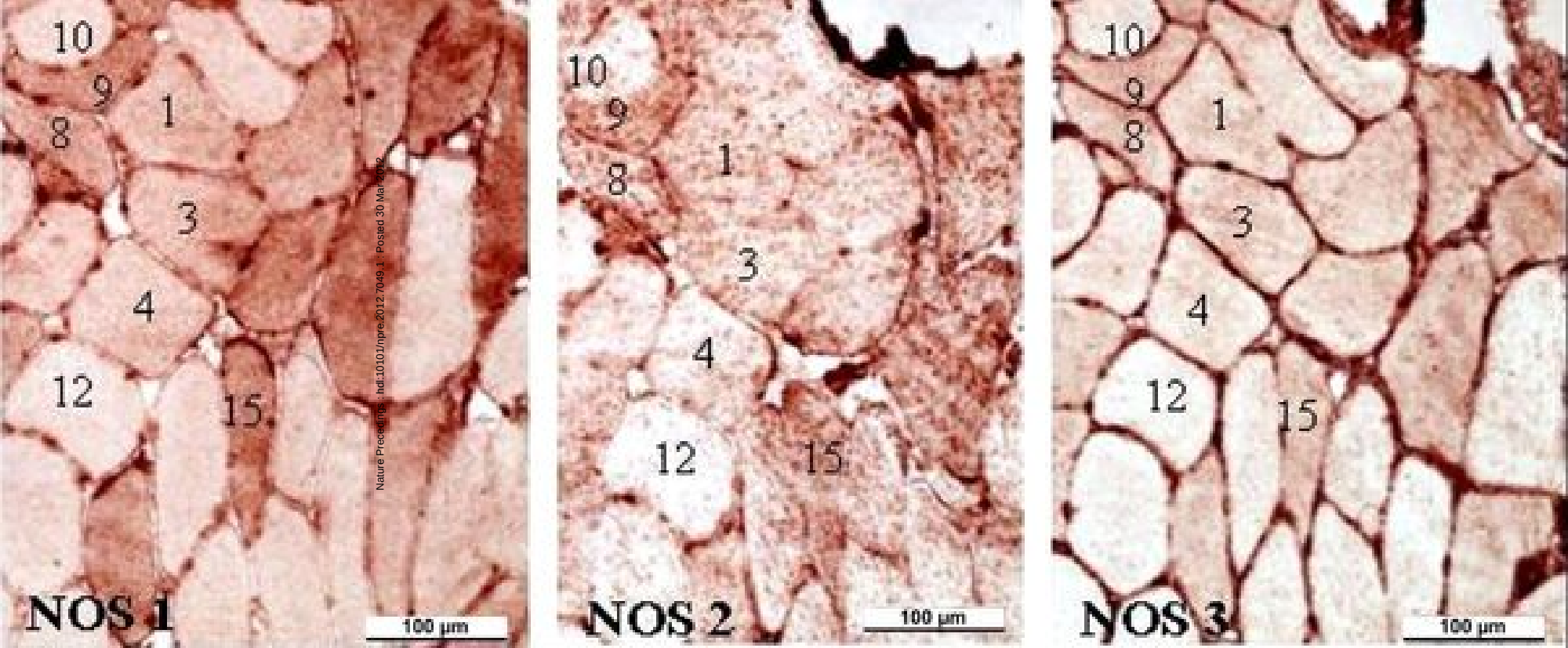

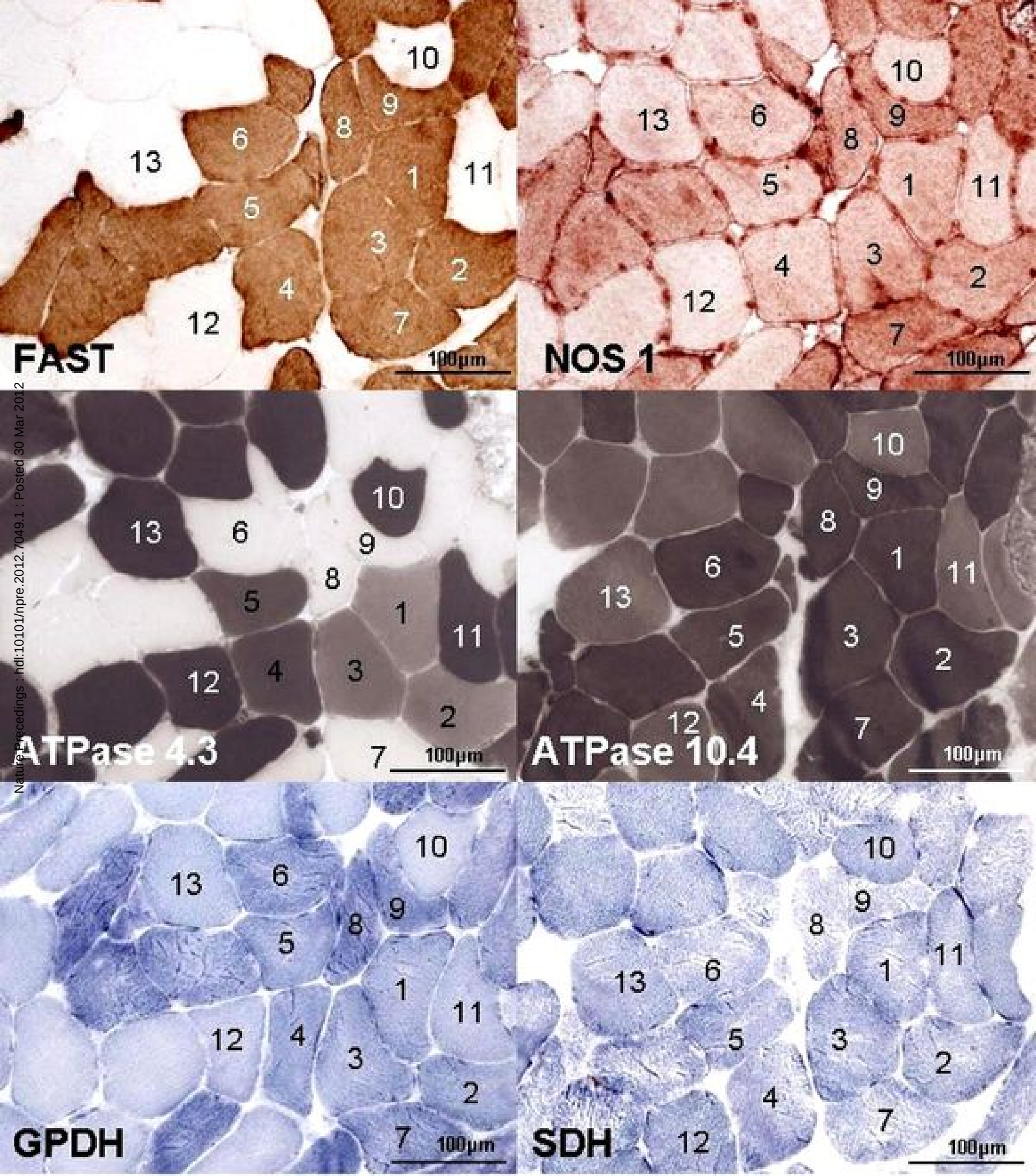

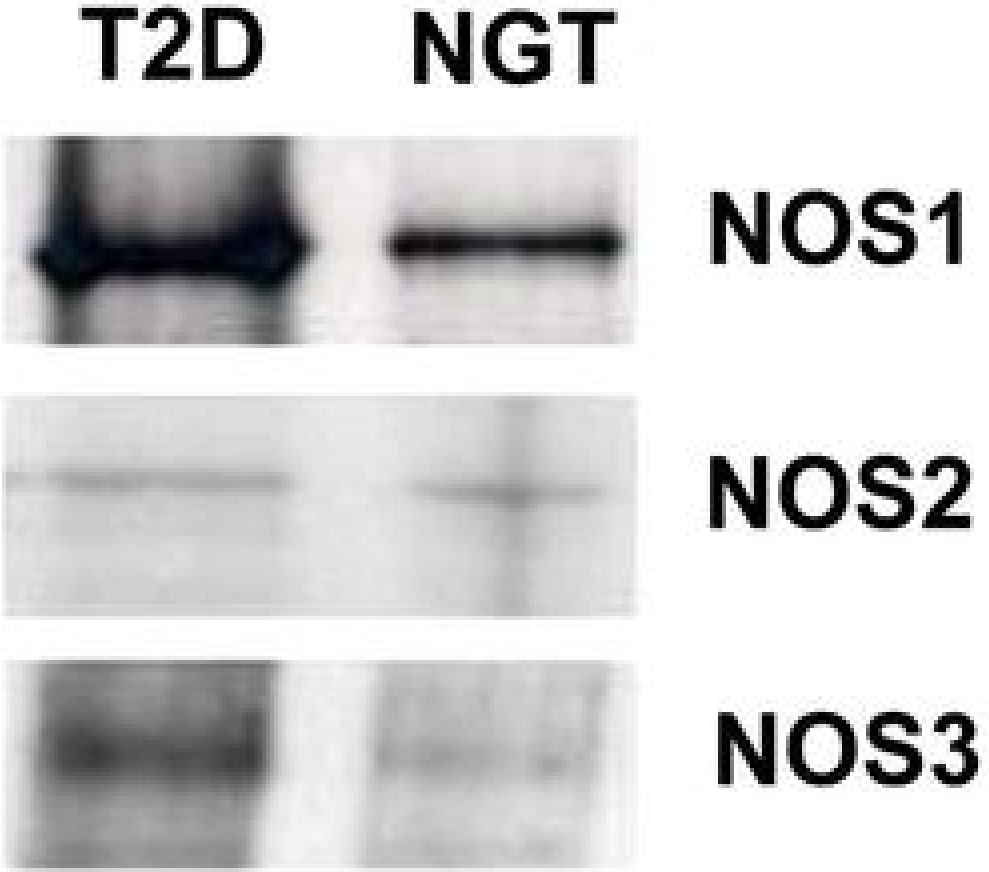

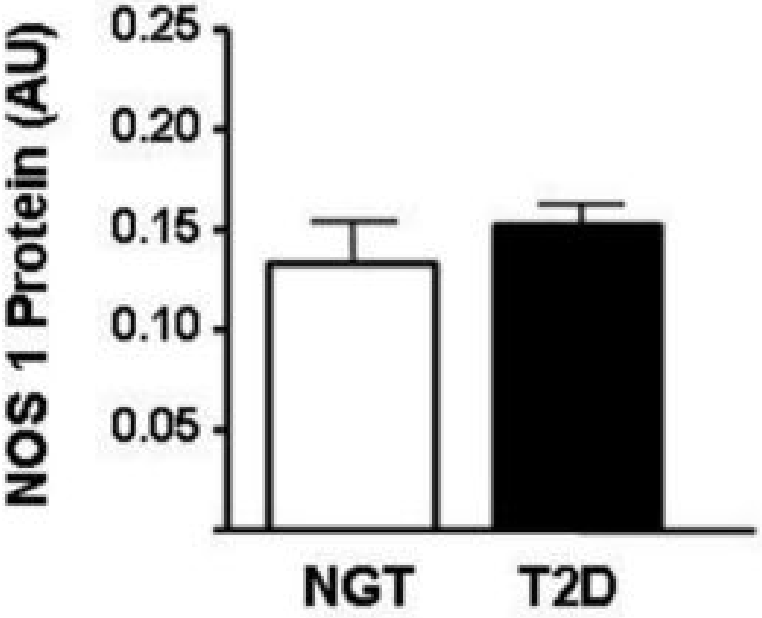

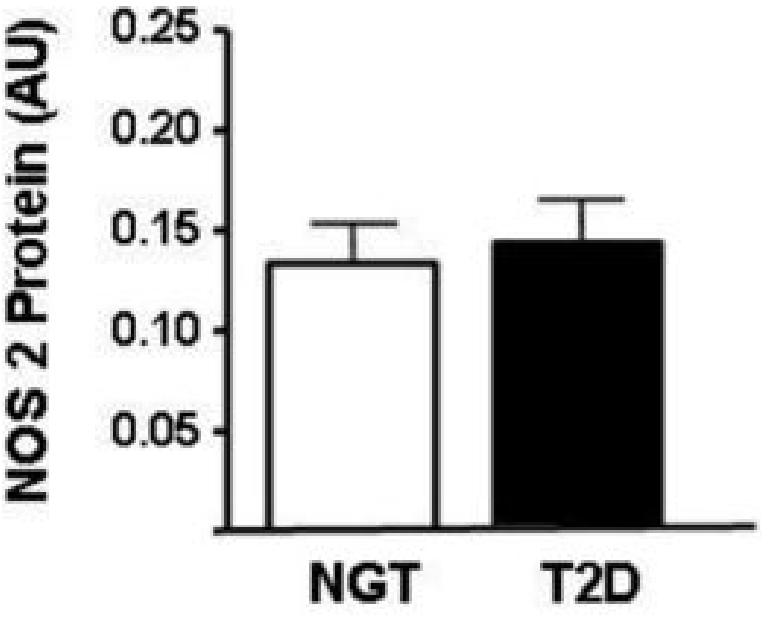

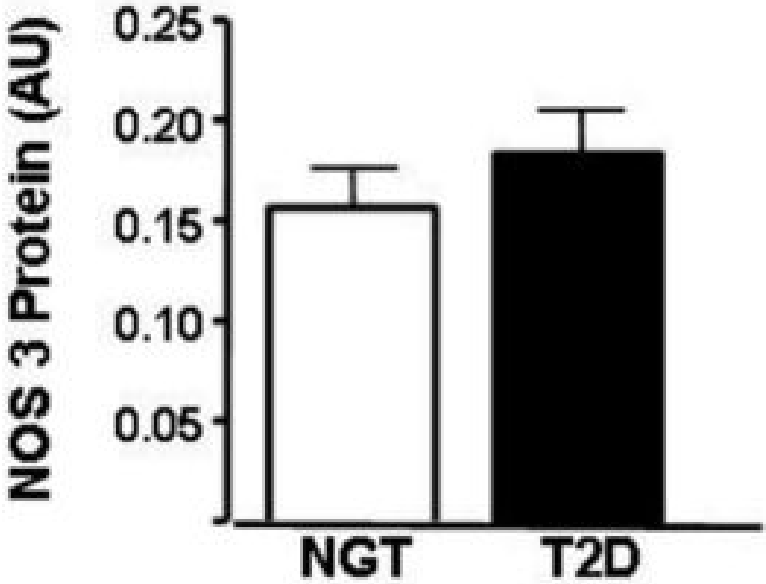

Table 1 Anthropometric and metabolic parameters of the subjects

Parameter	NGT	T2D
n	15	10
Sex (male/female)	8/7	4/6
Age (years)	56.8±7.9	58.7±6,4
BMI (kg/m^2)	31.4±3.2	32.1±3.4
Body fat content (%)	33.6±6.6	35.1±8.1
Waist-to-hip ratio	1.24±0.12	1.27±0.17
VO_{2max} ($ml\ kg^{-1}min^{-1}$)	23.5±2.7	17.4±3.8*
Fasting plasma glucose (mmol/l)	5.34±0.4	6.55±0.9 *
HbA1c (%)	5.15±0.31	6.6±0.4*
Fasting plasma insulin (pmol/l)	91.9±42	157± 51*
Glucose infusion rate, clamp (ml/min)	87.5±13	29±12 *

Data are means ± SD. * $p < 0.05$ for NGT vs. T2D subjects