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Title: The effects of aerobic exercise training at two different intensities in obesity and type 2 diabetes: Implications for oxidative stress, low grade inflammation and nitric oxide production

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ABSTRACT

Aims: To investigate the effect of 16 weeks of aerobic training performed at two different intensities on nitric oxide (tNOx) availability and iNOS/nNOS expression, oxidative stress (OS) and inflammation in obese humans with or without Type 2 Diabetes Mellitus (T2DM). **Methods:** Twenty-five sedentary, obese (BMI >30kg/m^2) males (52.8±7.2 yrs); 12 controls vs. 13 T2DM were randomly allocated to four groups that exercised for 30 minutes, three times per week either at low (Fat-Max; 30-40% VO_{2max}) or moderate (Tvent; 55-65% VO_{2max}) intensity. Before and after training, blood and muscle samples (v. lateralis) were collected. **Results:** Baseline erythrocyte glutathione was lower (21.8±2.8 vs. 32.7±4.4 nmol/mL) and plasma protein oxidative damage and IL-6 were higher in T2DM (141.7±52.1 vs. 75.5±41.6 nmol/mL). Plasma catalase increased in T2DM after Tvent training (from 0.98±0.22 to 1.96±0.3 nmol/min/ml). T2DM groups demonstrated evidence of oxidative damage in response to training (elevated protein-carbonyls). Baseline serum tNOx were higher in controls than T2DM (18.68±2.78 vs. 12.34±3.56 μmol/L). Training at Tvent increased muscle nNOS and tNOx in the control group only. Pre-training muscle nNOS was higher in controls than in T2DMs, while the opposite was found for iNOS. No differences were found after training for plasma inflammatory markers. **Conclusion:** Exercise training did not change body composition or aerobic fitness, but improved OS markers, especially when performed at Tvent. Non-diabetics responded to Tvent training by increasing muscle nNOS expression and tNOx levels in skeletal muscle while these parameters did not change in T2DM, perhaps due to higher insulin resistance (unchanged after intervention).

Introduction

The World Health Organization (WHO) states that being overweight or obese is the fifth leading risk factor for global mortality (James 2008). Low-grade inflammation is a feature of both obesity and type 2 diabetes mellitus (T2DM) (Degens 2010; Krause et al. 2012a), which are characterized by a chronic pro-inflammatory state associated with an increased release of key pro-inflammatory cytokines, e.g. tumor necrosis factor alpha (TNF-α) from different sources (Degens 2010; Krause et al. 2012a). TNF-α signalling also induces activation of several pathways which initiate the production of free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Newsholme and Krause 2012), which promote impairment of insulin signalling (Newsholme and Krause 2012).

Disrupted redox signalling or elevated oxidative stress (from prolonged periods of hyperglycaemia and/or elevated pro-inflammatory cytokines) is thought to underlie the vascular dysfunction observed in individuals with glucose intolerance and diabetes (Cersosimo and DeFronzo 2006). Likewise, it has been shown that individuals with T2DM have more pronounced systemic inflammation and oxidative stress than those with normal glucose tolerance, leading to decreased bioavailability of nitric oxide (a key mediator of vessel tone, glucose uptake and β-cell function) (Krause et al. 2012b; Krause et al. 2011; Newsholme et al. 2009; Newsholme et al. 2012).

Regular moderate intensity aerobic exercise is widely recommended for sedentary individuals due to its beneficial effect on insulin sensitivity and glycaemic control (Boule et al. 2001). It is also known that such exercise can improve glycaemic and lipid control in diabetes and consequently, reduce cardiovascular disease (CVD) risk factors through improvements in fasting blood lipids, postprandial glycaemia and lipaemia, blood pressure and body mass (Duncan 2006; Harding 2006). However, it is unclear which type, frequency

and intensity of exercise will be of most benefit for obese and/or T2DM individuals (Thomas et al. 2006). Previous work has reported a reduced capacity for fat oxidation, a shift from fat to carbohydrate utilisation at lower intensities during exercise and a lower exercise intensity associated with the maximal rate of fat oxidation in people with T2DM relative to healthy controls (Boon et al. 2007; Brun et al. 2007; Ghanassia et al. 2006). These findings have contributed to the promotion of low intensity aerobic exercise training (at the intensity corresponding to maximal rate of fat oxidation - "Fat-Max training") so as to increase fat oxidation capacity (Brun et al. 2007), rather than moderate intensity training prescriptions (corresponding to either 60-70% of VO_{2max} (Boon et al. 2007) or based on ventilatory thresholds (Tvent; (Belli et al. 2007; Fujita et al. 1990). While fat oxidation may be elevated under this intensity of exercise, information on the impact on other parameters of metabolic status or oxidative stress in T2DM patients is lacking.

Therefore, the aim of this study was to investigate changes in nitric oxide availability (and iNOS/nNOS expression), oxidative stress and inflammatory markers in response to exercise training at two different intensities (Fat-Max *vs*. Tvent intensities) in obese males with and without T2DM. Specifically, we analyzed cytokines and adipokines that are known to modulate the availability of $NO^{\cdot}$ and also be involved in the development of insulin resistance.

Research Design and Methods

Participants Characteristics

Twenty-five sedentary (not engaged in any regular physical activity for the last six months) non-smoking male participants (52.8±7.2 years old), with body mass index (BMI)

105 >27 kg/m^2 volunteered for this study (12 obese controls *vs.* 13 obese T2DM, previously
106 diagnosed by their personal physicians). BMI ranged from 27.05 to 38.08 kg/m^2 for control
107 subjects and 27.3 to 37.5 kg/m^2 for T2DM. Informed consent was obtained from all
108 volunteers prior to the study. Research assessments and protocols were approved by the UCD
109 Dublin Human Research Ethics Committee. Participants were free from secondary
110 complications of diabetes at the time of recruitment.

111

112 **Experimental Design**

113 Participants were recruited via advertising in local newspapers and workplaces.
114 Participants attended the university laboratory for testing on five occasions. In session one,
115 body composition was measured and a submaximal incremental treadmill test was performed
116 to estimate aerobic capacity (VO_{2max}) and to measure ventilatory threshold. Total body
117 composition was assessed using dual energy X-ray absorptiometry (DEXA - Lunar iDXA,
118 GE Healthcare, Buckinghamshire, United Kingdom). In session two, performed one week
119 later, resting blood and skeletal muscle biopsy samples were taken following an overnight
120 fast. The participants then completed a six-minute constant load exercise bout at an intensity
121 corresponding to 35% estimated VO_{2max}, followed by a second six-minute bout at an intensity
122 corresponding to 25% estimated VO_{2max}. The format of session three which was performed
123 one week later was identical to session two, except for the intensity of the 2 six-minute
124 constant load exercise bouts which corresponded to 60% and 45% of estimated VO_{2max}
125 respectively. Participants were then randomly assigned to train (not supervised) at either low
126 (Fat-Max training) or moderate intensity (Tvent training), giving four groups: Obese Control
127 training at Fat-Max (Control Fat-Max); Obese Diabetic training at Fat-Max (T2DM Fat-

Max); Obese Control training at Tvent (Control Tvent) and Obese Diabetic training at Tvent (Control Tvent).

The unsupervised training programme (16 weeks) consisted of a continuous outdoor 30 min walking, 3 times per week. Participants were provided with a heart rate (HR) monitor (Polar RS400) with their individual zone of training pre-programmed. Midway (week 8) through the training programme, participants returned to the laboratory (session four) and completed both the incremental and four submaximal constant load exercise bouts, for adjustment of their target HR training zone. Finally, after completing the 16 week programme, participants attended the laboratory for a fifth testing session, during which resting fasted blood and muscle samples were obtained and the exercise and body composition tests were repeated. The experimental design is described in Figure 1.

Estimated aerobic power (VO_{2max})

Maximal oxygen consumption (VO_{2max}) was estimated from a sub-maximal incremental walking test. Briefly, participants walked on a motor driven treadmill (Venus 200, HP Cosmos, Nussdorf-Traunstein, Germany) with a protocol of simultaneous changes in speed and slope, designed to achieve a linear increase in workload (Porszasz et al. 2003). The specific increments were calculated for each participant based on their age, body weight and predicted aerobic capacity (Fujita et al. 1990). Expired gases were collected and analysed using a breath by breath analyser (Quark B2, Cosmed, Rome, Italy). The test was terminated when the subjects reached the 85% of their age-predicted maximal heart rate (220- age in years). All participants were able to reach this point of exertion. VO_{2max} was then estimated by linear regression of the VO_2 – heart rate relationship to the predicted maximal heart rate.

During the test, particular attention was paid to the VCO_2 and VE trends to verify that Tvent was reached by every participant.

Sub-Maximum Constant Load Tests and Fat-Max determination

Each participant completed four 6-minute submaximal constant load treadmill exercise bouts, at intensities corresponding to 25%, 35%, 45% and 60% of their estimated VO_{2max}, as determined from the incremental test (Achten et al. 2002; Ghanassia et al. 2006).

Determination of training intensities

'Fat-max' training intensity: For each participant, the intensity corresponding to the maximal rate of fat oxidation was calculated using the data obtained from the four submaximal constant load bouts, as described previously (Ghanassia et al. 2006; Venables et al. 2005). Briefly, data from the 5^{th} and 6^{th} minute of each bout were averaged and rate of lipid oxidation calculated from VO_2 and VCO_2 (Peronnet and Massicotte 1991). This was plotted against exercise intensity, and a third-order polynomial curve was fitted to the data (Stisen et al. 2006). The intensity at the maximal point of the resultant curve was identified, and the heart rate corresponding to this intensity was then used for prescription of the participant's training zone. 'Tvent' training intensity: ventilatory threshold was identified from the incremental exercise test, using the modified V-slope method (Sue et al. 1988). Two experimenters calculated this point independently, and the average of the two VO_2 values was used. Where the two VO_2 values differed by more than 100ml, a second method was used to verify the VO_2 corresponding to Tvent (method of ventilatory equivalents; (Noonan and Dean 2000).

Biochemical analysis

Venous blood samples were obtained from an antecubital vein in heparin coated (for plasma acquisition) or clotting activator (for serum) Vacutainer™ tubes using standard aseptic techniques. Samples were immediately centrifuged (at 4°C and 1000g for 15min), after which plasma or serum were removed and stored at −80°C for further analysis. Concentrations of glucose, total cholesterol and high-density lipoprotein cholesterol (HDL-C) were measured using a portable Cardiochek Analyser (Polymer Technology Systems, Indianapolis, Indiana, USA). Triglyceride (TG) concentration was quantified using a commercially available colorimetric assay (Tryglicerides Liquicolor, Wiebsbaden, Germany). Estimates of low-density lipoprotein cholesterol (LDL-C) concentration were calculated using the Friedewald formula (Friedewald et al. 1972). Glycosylated haemoglobin (% HbA1c) was assayed using DCA Vantage Serum Analyser (Siemens, Dublin, Ireland). Plasma glycerol was assessed using a colorimetric assay (Cayman Chemical Company, USA, Catalogue 10010755) according to manufacturer's instructions. Highly sensitive, enzyme-linked immunosorbent assay (EIA) methods were used to determine the concentration of leptin (Mercodia, Catalogue 10-1199-01, Uppsala, Sweeden) and adiponectin (Mercodia, Catalogue 10-1193-01) in plasma according to manufacturer's instructions. Quantification was made using a microplate reader (Molecular Devices SpectraMax Plus 384, Sunnyvale, California, United States). Serum high-sensitivity C-reactive protein (hsCRP) was assayed using a CRP high sensitivity assay kit (Cayman, Ireland). Blood was centrifuged in heparin-coated tubes and the red blood cell pellet homogenized in cold metaphosphoric acid. Redox state and glutathione metabolism was measured in erythrocytes by a modification of the 5,5'-dithiobis(2-nitrobenzoic acid) [DTNB]/GSSG reductase recycling method, using the N-ethylmaleimide conjugating technique for GSSG sample preparation (Krause et al. 2007). Total antioxidant activity was measured using a colorimetric assay (Catalogue No: 709001,

Cayman, Ireland). Catalase activity was assessed using a Cayman Chemical Catalase Assay Kit (Catalogue No: 707002) and oxidative damage in proteins by using the Cayman Chemical's Protein Carbonyl Colorimetric Assay Kit (catalogue 10005020) (Fayh et al. 2012). Serum concentrations of IL-6 and TNF were quantified using a Human IL-6 and TNF-alpha Quantikine ELISA Kit (Catalogue No: D6050 and DTA00C, R&D Systems, USA). The choice between plasma or serum samples for the analysis was based on the assay performed, technique sensitivity and kit manufacturer instructions. Serum was used for IL-6, TNF-α and hsCRP; plasma for adiponectin, leptin, catalase, protein carbonyls, total antioxidant activity, triglycerides and glycerol and whole blood for glucose, LDL, HDL, HbA1c and total cholesterol measurements.

Skeletal muscle microbiopsy and NOS protein expression

Muscle biopsy samples were obtained using a spring-loaded and reusable instrument (Magnum reusable core biopsy instrument MG1522; Bard, Dublin, Ireland). This technique has been assessed in terms of patient tolerance and causes minimal or no discomfort (Hayot et al. 2005). The muscle samples (~15 mg) were taken from the medial part of the vastus lateralis muscle under local anaesthesia (1% lidocaine). The samples obtained from each biopsy were immediately frozen in liquid nitrogen and stored at -80°C until required. After removal from -80^{o}C storage, tissue samples were thawed and homogenized in lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 10 mM Na-pyrophosphate, 100 mM NaF, 2 mM Na_3VO_4, 10μg/ml Aprotinin, 10 μg/ml leupeptin, 3 mM benzamidine and 1 mM PMSF) using an automated homogenizer (TissueLyser LT, Qiagen, Dublin Ireland) . The homogenate was rotated at 4°C for 10 min, followed by centrifugation at 14,000g at 4°C for 10 min, after which the supernatant was collected. Cellular protein concentration was determined using a

BCA protein Assay (Pierce, Rockford, IL, USA Catalogue No: 23225). Protein samples (15μg) were denatured in sample buffer and separated by 10% SDS PAGE. The proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences, Ireland), blocked in 5% (BSA) and probed with the appropriate polyclonal antibodies; Anti-nNOS (1:10000 dilution, Cell Signalling Technologies, USA), Anti-iNOS (1:10000 dilution, Cell Signalling technologies, USA) and Anti-GAPDH (1:10000 dilution, Cell Signalling technologies, USA) over night. Following overnight incubation, membranes were washed and incubated for 60 min at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:30000 dilution, Cell Signalling technologies, USA). The blots were washed and visualized with a horseradish peroxidase-based Supersignal West Pico chemiluminescent substrate (Pierce). Results of digitalized images were expressed as mean ± S.D. using anti-GAPDH as an expression control. The densities of the bands were quantified using ImageJ version 1.44p (National Institutes of Health, USA).

Nitric oxide product determinations (tNOx) in serum and skeletal muscle samples

Frozen muscle samples were weighed, and homogenized in sterile PBS (phosphate buffer saline, GIBCO, Ireland) using an automated homogenizer (TissueLyser LT, Qiagen, Dublin Ireland). The homogenised tissue (and serum samples) were placed in an eppendorf tube and centrifuged at 10,000g for 20minutes. The supernatant was ultra-centrifuged using molecular weight cut-off filters (30kDa, Amicon, Millipore, Ireland) for 30 minutes at 100,000g. The supernatant was used for the remainder of the assays. Nitric oxide levels were determined indirectly by quantification of their oxidised products of degradation, nitrates and nitrites, using nitrate reductase and the Greiss reagent technique by a colorimetric kit

(Cayman Chemical Company, USA, Catalogue No: 780001). The inter-assay and intra-assay coefficients of variation for the Greiss reaction were 5.6% and 3.6% respectively.

Statistical Analysis

Data are presented as mean and SD. Ryan-Joiner normality test was applied prior to all analyses. For the following variables: glycaemia, HbA1c, insulin, HOMA-IR, HOMA-beta, HDL, triglycerides, CRP, VO_{2max}, TNF-α, IL-6, total antioxidants and glycerol, which were not-normally distributed, a logarithmic transformation was applied (Log_{10}). An ANOVA General Linear Model (GLM) was performed to compare the four sub-groups with three factors of interaction: 1) the presence or not of T2D (Diabetes vs. Control); 2) Time (before and after exercise training); 3) exercise intensity (Fat-Max or Tvent). Post-hoc Tukey test was further applied when appropriate. Finally, age, body fat percentage and estimated VO_{2max} were added as co-variants in the ANOVA GLM. The alpha level was set at $P<0.05$. Data were analysed using Minitab 16 software.

Results

Adherence to Training

After the completion of the training, the data from the HR monitors were downloaded and individually analysed. Adherence to training was high (92%) with participants completing 44±7 of the prescribed 48 walking sessions. In addition, all participants were successful in training at the prescribed heart rate; the average difference between the prescribed and recorded training heart rate was small (-0.5 bpm).

Comparison of the baseline blood biochemical variables between the obese control and the obese T2DM subjects

As expected, glycaemia (5.2±0.4 vs. 8.36±1.2 mmol/L) and HbA1C (5.6±0.1 vs. 7.3±0.9, % OR 38±1 mmol/mol vs. 56±2 mmol/mol) were higher in the T2DM group. Indicators of both insulin resistance (HOMA-IR) and beta-cell function (HOMA-β) were altered in T2DM, indicating that diabetic participants were insulin resistant and also may present an abnormal beta-cell function. Total cholesterol and LDL cholesterol (139.7±33.8 vs. 77.6±35.6 mg/dL) were lower in T2DM subjects. This was expected since the diabetic subjects were taking cholesterol-lowering statin drugs. VO_{2max} was lower in T2DM than obese controls (31.2±6.45 vs. 38.9±5.27 $ml.kg^{-1}.min^{-1}$, $P<0.05$). No differences were found between Control and T2DM for leptin, adiponectin, TNF-α and total antioxidants. IL-6 was significantly higher in the T2DM group, although body fat percentage as a co-variant affected this result. Significant differences were found in protein carbonyls between control and T2DM individuals (75.5±41.6 vs. 141.7±52.1 nmol/mL respectively). In addition, reduced glutathione (GSH) was lower in diabetic patients (32.7±4.4 vs. 21.8±2.8, nmol/mL).

Analysis of blood biochemistry parameters and body composition in response to exercise training

General metabolic variables such as glycaemia, lipid metabolism and HbA1c are shown in Table 1. No differences were found between pre- and post-training in any of the training groups. Similarly, no changes were observed in adiponectin, leptin or TNF-α (Table 2). With the exception of IL-6, the co-variants (% body fat, age and VO_{2max}) did not affect

any other variable. Body composition, VO_{2max} and blood pressure were also unchanged after the interventions.

Antioxidants and oxidative protein damage in response to exercise training

As shown in Table 2, the major differences regarding oxidative stress markers and antioxidants were related to the levels of protein carbonyls, being higher in T2DM subjects. Catalase activity increased in diabetic subjects only after Tvent training intervention, but not at Fat-Max (0.98±0.22 at basal levels vs. 1.96±0.3nmol/min/ml after Tvent training, Table 2). The diabetic subjects responded to exercise training with increased protein oxidative damage at both training intensities (Fat-Max and Tvent), as evidenced by decreased protein carbonyls, particularly in the Tvent group (147.2±52.6 at baseline and 66.57±19.5nmol/ml after Tvent training intervention). There were no changes in markers of oxidative stress or protein damage in either control training group. No differences in glutathione metabolism were found between pre- and post-training samples (Table 2). However, baseline values for GSH were higher in the control group than in T2DM group and the difference remained after exercise training at both intensities.

Serum levels of nitric oxide metabolites in response to exercise training

Regarding serum tNOx, in all groups and intensities, no significant differences were found after the exercise training intervention (Table 2). On the other hand, baseline values for tNOx were higher in the control group than in T2DM group (18.68±2.78 vs. 12.34±3.56μmol/L,) and this difference remained following exercise training at both intensities.

Skeletal Muscle iNOS/nNOS Expression and nitric oxide metabolites levels in response to exercise training

We analysed the responses of iNOS/nNOS expression and the tNOx production in skeletal muscle samples to chronic (training) exercise (Figures 2A-D). Following training, the only significant change observed was an increase in the levels of nNOS and tNOx in the control group trained at Tvent intensity (Figure 2A). At basal levels, as expected, skeletal muscle nNOS was higher in controls than in diabetics and the opposite was found for iNOS expression levels, but this did not impact on the baseline tNOx levels between the groups (Figures 2A-D).

Discussion

The main finding of this study was that endurance exercise training, particularly at moderate intensity (Tvent), increased catalase activity and decreased protein oxidative damage in individuals who are obese with T2DM. However, this improvement was not accompanied by changes in glycaemia or body composition. These findings are in accordance with the observation that prescription of non-supervised physical activity alone does not improve glycaemic control in T2DM (Wisse et al. 2010). A further possible reason for not observing significant changes in variables such as HbA1c with exercise training is the regular use of anti-diabetic drugs which would improve many metabolic parameters and thus reduce the scope for further change. The exercise training program used in our intervention did not result in any detectable changes in nitric oxide production in obese T2DM subjects, although the levels of skeletal muscle nNOS expression and tNOx concentration were increased in the control obese group in response to moderate aerobic exercise training.

Structured exercise is considered an important cornerstone for achieving good glycaemic control and reducing cardiovascular risk in type 2 diabetes (O'Hagan et al. 2013; Praet and van Loon 2009) Several different protocols, intensities and types of exercise have previously been promoted for the diabetic population (for review, read (O'Hagan et al. 2013; Praet and van Loon 2009)). In the present study, we analysed the chronic effects of aerobic training under two different intensities, which was performed three times per week, with sessions lasting 30 minutes, for a period of 16 weeks.

It was decided to compare Fat-Max vs. Tvent intensities because it has previously been suggested that low intensity protocols (which favour the use of fatty acids rather than carbohydrates as a source of energy) might improve metabolic, inflammatory and oxidative stress markers by inducing higher levels of fatty acid oxidation (Brun et al. 2007). This would be beneficial for T2DM subjects, since the disorder is associated with an earlier shift from fat to carbohydrate utilisation during exercise (Boon et al. 2007) and a reduced fat oxidative capacity (16; 17). It has been reported previously that a single bout of low intensity exercise, as opposed to high intensity exercise, substantially reduces the prevalence of hyperglycaemia throughout the subsequent 24h post-exercise period in longstanding T2DM patients [35]. In our hands, Fat-Max training did not change glycaemic or lipid profiles. Nevertheless, our findings do not exclude possible beneficial effects of this type of training for T2DM subjects, since we only evaluated fasting glycaemic values and not continuous daily variations, as described by Manders et al [35]. Furthermore, while significant training effects were not observed for most variables (glycaemia, lipid profile, HbA1c), calculation of Cohen's effect sizes (results not shown) showed medium-to-large effects of training. Therefore it is possible that including larger group sizes might have resulted in a significant training effect.

While NO· is an essential molecule for many physiological functions such as skeletal muscle glucose uptake and vasomotricity (Newsholme et al. 2009), its availability is

decreased in diabetes. We aimed to analyse the nitric oxide production response of obese T2DM to exercise training. Our results indicated that exercise at Fat-Max or Tvent intensities, did not produce detectable change in NO· production within the T2DM groups. However, the control group that trained at Tvent intensity responded by increasing the production of tNOx and expression of nNOS. We have previously demonstrated that skeletal muscle nNOS and iNOS expression levels are different between healthy obese, T2DM obese and lean T2DM (Krause et al. 2012b). This may indicate that obesity, rather than diabetes alone is the cause of the differential levels of tNOx intracellularly (muscle) and extracellularly (serum). Increased circulating IL-6 may promote increased levels of iNOS within the skeletal muscle of the diabetic subjects (Krause et al. 2012a). The fact that T2DM subjects did not respond to the moderate intensity training (in contrast to controls) would suggest that their lack of response may be caused by the insulin resistant state itself, since cell-based insulin signalling is required for normal nNOS expression and nitric oxide production (Krause et al. 2012b; Newsholme et al. 2009). Despite the lack of change in nitric oxide production in our T2DM subjects, the possible beneficial effects of our exercise protocol on the cardiovascular system of these patients cannot be excluded. For instance, numerous interventions based on exercise and/or dietary changes have shown improved vascular responses, such as an increase in blood flow without any significant changes to nitric oxide production (Fayh et al. 2012; Monti et al. 2012). In addition, recent findings suggest that vasodilatory prostanoids are important in determining endothelial response to Ach in diabetic and non-diabetic subjects (Meeking et al. 2000). Thus increased prostaglandin-mediated vasodilation may compensate for attenuated responses to NO·, as has been previously reported in diabetic subjects (Meeking et al. 2000).

We have also confirmed that serum tNOx level (nitric oxide availability) is reduced in patients with diabetes. We speculate that this response is due to the reduced availability of

386 circulating L-arginine (Newsholme et al. 2009; Newsholme et al. 2012). Decreased blood
387 levels of insulin, increased angiotensin II, hyper-homocysteinaemia, increased ADMA
388 (asymmetric ω-NG,NG-dimethylarginine), low plasma L-arginine and tetrahydrobiopterin
389 (BH4) are all conditions likely to decrease NO· production and which are also associated with
390 diabetes and cardiovascular disease (Newsholme et al. 2009). Recently, adipose tissue has
391 been implicated in the regulation of vascular function in humans via the release of vasoactive
392 cytokines called adipokines, including adiponectin and leptin (Antonopoulos et al. 2011). In
393 our hands, however, no differences were found in theses adipokines after the exercise
394 intervention and, for this reason, we cannot attribute the differences in nitric oxide to
395 alterations in adiponectin levels.

396 In insulin-resistant populations, several adipokines as well as muscle contraction-
397 induced factors, so-called myokines (i.e., IL-6), have been shown to modulate insulin
398 resistance and inflammatory status (Pedersen and Febbraio 2008). Although there is a
399 consensus that weight loss is associated with an increase in adiponectin and decreased levels
400 of leptin, TNF-α and high sensitivity C-reactive protein (hsCRP) (Madsen et al. 2008;
401 Monzillo et al. 2003), studies on medium-term effects of exercise without concomitant
402 weight loss are limited and produce somewhat inconsistent results (De Feyter et al. 2007;
403 Lambert et al. 2008; Stewart et al. 2007). Indeed, we did not find any changes in adipokines
404 (adiponectin and leptin), or cytokines (TNF-α) following training irrespective of exercise
405 intensity in any group, which may reflect the importance of weight loss and adiposity levels
406 on the low-grade inflammation found in obesity and diabetes. On the other hand, IL-6 was
407 higher in T2DM subjects. However, increments in IL-6 were correlated with greater body fat
408 content in this group compared with the non-diabetic group. Thus higher concentrations of
409 IL-6 in T2DM may be a consequence of greater body fat content, rather than diabetes itself.

Free radicals (i.e. ROS and $NO^{\cdot}$), are known to be involved in the pathogenesis of diabetes (Brownlee 2001). Besides inhibiting intracellular insulin signalling, the aforementioned metabolic disturbances in glucose and fat metabolism increase the formation of Amadori-glycated proteins and advanced glycation end-products (AGE), which impair receptor function for AGE (RAGE) and enhance ROS levels in almost all organ systems (Brownlee 2001; Goldin et al. 2006). Chronic exposure to Amadori products, AGE and ROS can cause vasculopathy, glomerulopathy (Brownlee 2001; Vincent et al. 2007) and potentially also induce nerve cell damage (Vincent et al. 2007). In accordance, nutritional (Vincent et al. 2007) and/or exercise interventions (Ji 2002) that modulate AGE, RAGE and/or ROS formation have been reported to improve insulin sensitivity in experimental rodent models. Despite the fact that our exercise intervention did not induce changes in inflammatory profiles or body composition, T2DM patients who trained at Tvent improved their antioxidant defences (catalase), resulting in lower oxidative damage, as represented by the levels of protein carbonyls. On the other hand, glutathione metabolism did not change, although at baseline levels, we demonstrated that diabetic subjects had lower levels of reduced glutathione. This may suggest that production of this antioxidant, perhaps as a consequence of decreased key amino acid availability for glutathione synthesis, was compromised, as previously suggested (Newsholme et al. 2012).

Conclusions and Perspectives

In summary, moderate-intensity exercise may promote alternative health benefits compared to exercise at Fat-Max intensities and, in the long term, may result in visible changes in glycemic control and cardiovascular improvement resulting in fewer diabetic complications. Obese, non-T2DM participants can respond to moderate intensity training by

increasing nNOS levels and tNOx levels in skeletal muscle cells, which may represent an adaptative mechanism to exercise. However, NO related responses in T2DM patients did not respond in the same positive direction as observed for the control group, which may be attributable to their level of insulin resistance (unchanged after the exercise intervention). We suggest that it is important to evaluate other types of exercise, including different intensities and volumes of training in order to find the most appropriate exercise for obese and T2DM population to improve cellular and metabolic function. Despite the limitations of this study (absence of diet control and the small sample size), it is important to highlight that our intervention, in spite of the low training volume intervention, induced positive modifications among obese individuals with T2DM.

Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Authors Contribution Statement

MK, JRK, COH, GDV, DS, and PM completed all sample collection. MK completed all the biochemical and molecular analysis. JRK, COH and PM performed all body composition and cardiorespiratory analysis. MK, GDV and PN co-wrote the manuscript. MK, GDV, PN, CB, CM, DS, GD and COH provided experimental advice and helped with manuscript revision. CM, GDV and PN were responsible for grant support with respect to TSR: Strand III – Core Research Strengths Enhancement (Ireland).

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Tables and Figures Legends

Table 1. Effects of exercise training on Subjects` Anthropometric, biochemical and physiological characteristics.

Table 2. Effects of exercise training on Subjects` hormone, inflammatory and Oxidant/Anti-oxidant profile.

Figure 1. Study design.

Figure 2. Representative Western blot of human skeletal muscle biopsies for nNOS/iNOS and intracellular tNOx. **A**: in Obese Control Subjects training at 60% VO_{2max} intensity. **B**: in Obese Control Subjects training at 35% VO_{2max} intensity. **C**: in T2DM Subjects training at 60% VO_{2max} intensity. **D:** in T2DM Subjects training at 35% VO_{2max} intensity. Levels of nNOS and iNOS expression in the skeletal muscle of all subjects were measured. Total levels nitrites and nitrates (tNOx) were measured as an indication of nitric oxide production within the cells. * $P<0.05$ when compared with baseline levels.