Eur J Appl Physiol (2017) 117:1175–1180
DOI 10.1007/s00421-017-3604-2

ORIGINAL ARTICLE

The effect of nitric oxide synthase inhibition with and without inhibition of prostaglandins on blood flow in different human skeletal muscles

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Received: 22 November 2016 / Accepted: 22 March 2017 / Published online: 21 April 2017

Abstract
Purpose Animal studies suggest that the inhibition of nitric oxide synthase (NOS) affects blood flow differently in different skeletal muscles according to their muscle fibre type composition (oxidative vs glycolytic). Quadriceps femoris (QF) muscle consists of four different muscle parts: vastus intermedius (VI), rectus femoris (RF), vastus medialis (VM), and vastus lateralis (VL) of which VI is located deep within the muscle group and is generally regarded to consist mostly of oxidative muscle fibres.
Methods We studied the effect of NOS inhibition on blood flow in these four different muscles by positron emission tomography in eight young healthy men at rest and during one-leg dynamic exercise, with and without combined blockade with prostaglandins.
Results At rest blood flow in the VI (2.6 ± 1.1 ml/100 g/min) was significantly higher than in VL (1.9 ± 0.6 ml/100 g/min, $p = 0.015$) and RF (1.7 ± 0.6 ml/100 g/min, $p = 0.0015$), but comparable to VM (2.4 ± 1.1 ml/100 g/min). NOS inhibition alone or with prostaglandins reduced blood flow by almost 50% ($p < 0.001$), but decrements were similar in all four muscles (drug × muscle interaction, $p = 0.43$). During exercise blood flow was also the highest in VI (45.4 ± 5.5 ml/100 g/min) and higher compared to VL (35.0 ± 5.5 ml/100 g/min), RF (38.4 ± 7.4 ml/100 g/min), and VM (36.2 ± 6.8 ml/100 g/min). NOS inhibition alone did not reduce exercise hyperemia ($p = 0.51$), but combined NOS and prostaglandin inhibition reduced blood flow during exercise ($p = 0.002$), similarly in all muscles (drug × muscle interaction, $p = 0.99$).
Conclusion NOS inhibition, with or without prostaglandins inhibition, affects blood flow similarly in different human QF muscles both at rest and during low-to-moderate intensity exercise.

Keywords Muscle fibres · Blood flow · Nitric oxide · Prostanoids · Exercise · Humans

Abbreviations

NOS	Nitric oxide synthase
VI	Vastus intermedius
RF	Rectus femoris
VM	Vastus medialis
VL	Vastus lateralis

Introduction

The continuous supply of oxygen via blood is of paramount importance for proper function of the muscle, especially during exercise (Heinonen et al. 2014, 2015;

Communicated by: Keith Phillip George

Bengt Saltin: Deceased.

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Koga et al. 2014; Laughlin et al. 2012). One of the key regulators of blood flow in skeletal muscles is postulated to be nitric oxide (NO). Animal studies show that with prior inhibition of prostaglandins, the inhibition of nitric oxide synthesis (NOS) by L-NAME during exercise or by selectively inhibiting neuronal nitric oxide synthase (without prior COX inhibition) decreases blood flow more in glycolytic than in oxidative muscles (Copp et al. 2010a, b). On the other hand, Hirai et al. (1994) found that NOS inhibition with L-NAME (with prior COX inhibition) administered prior to exercise reduced hind limb skeletal muscle blood flow in the rat during treadmill exercise to the greatest extent in primarily oxidative skeletal muscles (Hirai et al. 1994). The quadriceps femoris (QF) muscle group, which is one of the most studied muscle groups in humans, consists of four different muscles: vastus intermedius (VI), rectus femoris (RF), vastus medialis (VM), and vastus lateralis (VL). VI is located deep within a muscle group and usually consists of more oxidative muscle fibres than the other three muscles (Edgerton et al. 1975; Johnson et al. 1973). Therefore, we hypothesized based on the animal studies (Hirai et al. 1994) that the inhibition of NOS, especially when combined with the inhibition of prostaglandins formation prior exercise, will decrease muscle blood flow to a larger degree in VI than in the other three QF muscles at rest and during exercise. Positron emission tomography was used to measure the effect of NOS inhibition with and without combined blockade with prostaglandins on blood flow in these four different muscles at rest and during one leg dynamic exercise.

Methods

Subjects

Eight healthy untrained young men (26 ± 2 years, 184 ± 4 cm, 82 ± 8 kg, 24.2 ± 1.9 kg/m^2) volunteered to participate in the study. The purpose, nature, and potential risks of the study were explained to the subjects before they gave their written informed consent to participate. None of the subjects had chronic diseases, were taking regular medication or were smokers. The study was performed approximately at 4 h after the subjects had eaten their normal breakfast. The subjects abstained from caffeine-containing beverages for at least 24 h before the experiments. The subjects were also requested to avoid strenuous exercise within 48 h prior to the study. The study was performed according to the Declaration of Helsinki and was approved by the Ethical Committee of the Hospital District of South-Western Finland and National Agency for Medicine.

Study design

Before the PET experiments, the antecubital vein was cannulated for tracer administration. For blood sampling, a radial artery cannula was placed under local anaesthesia in the contralateral arm. Additionally, a cannula was placed under local anaesthesia in the femoral artery for local drug infusions. Subjects were then moved to the PET scanner with the femoral region in the gantry and the right leg was positioned in an in-house designed leg exercise dynamometer (Heinonen et al. 2007, 2010a, b, 2012c, 2013b; Ruotsalainen et al. 1997). Subjects were laying supine both during resting and exercising measurements. PET measurements were first performed at resting baseline and thereafter during exercise without any drug infusion, but only during control saline. 30 min later, resting and exercising measurements were performed during NOS blockade with N^G-monomethyl-L-arginine (L-NMMA) (Clinalfa, Laufelfingen, Switzerland). L-NMMA was infused intra-arterially with a concentration of 1.0 mg min^{-1} kg leg mass^{-1} (Heinonen et al. 2011; Mortensen et al. 2007). The infusion of the drug started 10 min before the scanning (blood flow measurement) and continued until the end of the experiments. To inhibit cyclooxygenases, indomethacin (Confortid, Alphapharma, Denmark) was infused with a concentration of 50 µg min^{-1} kg leg mass^{-1} (Heinonen et al. 2011; Mortensen et al. 2007). Systemic mean arterial pressure (MAP) was measured (Omron, M5-1, Omron Healthcare, Europe B.V. Hoofddorf, The Netherlands) on every occasion studied.

Perfusion measurements and analysis

Radiowater positron-emitting tracer [^{15}O]-H_2O was produced as previously described (Sipilä et al. 2001) and the ECAT EXACT HR+ scanner (Siemens/CTI, Knoxville, TN, USA) was used in 3D mode for image acquisition to measure muscle blood flow. The oxygen-15 isotope was produced with Cyclone 3 cyclotron (IBA Molecular, Belgium). Photon attenuation was corrected by 5-min transmission scans performed at the beginning of the PET measurements performed at rest and during exercise. All data were corrected for dead time, decay and measured photon attenuation, and the images were reconstructed into a 256 × 256 matrix, producing 2.57 × 2.57 mm in-plane dimensions of voxels with 2.43 mm plane thickness. For the measurement of perfusion at rest, scanning began simultaneously with the infusion, and consisted of the following frames; 6 × 5, 12 × 10 and 7 × 30 s at rest and 6 × 5 and 12 × 10 s during exercise. During exercise, scanning was started 5 min after exercise onset to obtain a metabolic steady-state situation and continued until the end of the exercise bout, e.g. 2.5 min (7.5 min totally). Arterial blood

radioactivity was also sampled continuously with a detector during imaging for perfusion quantification. Exercise consisted of dynamic m. quadriceps femoris (~2.5 kg muscle mass) one-legged exercise at 40 rpm with an average work load of 4.5 kg and with a knee angle range of motion of ~75–80° (Heinonen et al. 2010). Local muscle blood flow was measured separately in the four different muscle parts of the m. quadriceps femoris. All of the experiments and the data analysis were performed using the standard models and methods (Heinonen et al. 2007, 2010a, b, 2012b, 2013b; Ruotsalainen et al. 1997).

Magnetic resonance imaging

Structural magnetic resonance imaging (MRI) was performed about one week before the PET study as described earlier (Heinonen et al. 2010a), when subjects were also accustomed to the one-leg knee extension exercise model in a PET scanner. MRI scanning was performed to obtain total leg volume of the working leg since NOS inhibiting drug infusions were based on effective concentrations per litre leg volume (Heinonen et al. 2011; Mortensen et al. 2007).

Statistical analysis

Statistical analyses were performed with SAS 9.2 program (SAS Institute, Cary, NC). Statistical analyses were performed using two-way ANOVA for repeated measures, with drug (control, NOS inhibition, combined inhibition) and muscle (VI, VM, VL, and RF) as factors. If a significant main effects or interactions were found, pairwise differences were identified with the Tukey–Kramer post hoc correction. Results are expressed as mean ± SD. A p value <0.05 was considered statistically significant.

Results

General hemodynamic (heart rate and blood pressure) but not any other data reported here have been published previously (Heinonen et al. 2011). Briefly, resting baseline heart rate was 56 ± 6 bpm and was not affected by NOS blockade (53 ± 8 bpm) but was significantly lower during NOS and COX (44 ± 3 bpm, $p < 0.001$) inhibition likely due to baroreceptor activation as mean arterial blood pressure during double blockade (105 ± 9 mmHg) was higher ($p < 0.05$) compared to resting baseline (98 ± 11 mmHg) while mean arterial pressure during single NOS inhibition (103 ± 9 mmHg) did not differ significantly from the other two conditions. At rest blood flow in the VI (2.6 ± 1.1 ml/100 g/min) was significantly higher than in VL (1.9 ± 0.6 ml/100 g/min, p = 0.015) and RF (1.7 ± 0.6 ml/100 g/min, $p = 0.0015$), but comparable to VM (2.4 ± 1.1 ml/100 g/min). NOS inhibition alone or with prostaglandins reduced blood flow by almost 50% ($p < 0.001$), but decrements were found to be similar in all four muscles (drug × muscle interaction in two-way ANOVA, $p = 0.43$) (Fig. 1).

During exercise blood flow was also the highest in VI (45.4 ± 5.5 ml/100 g/min) as compared to VL (35.0 ± 5.5 ml/100 g/min), RF (38.4 ± 7.4 ml/100 g/min), and VM (36.2 ± 6.8 ml/100 g/min) (Fig. 2). NOS inhibition alone did not reduce exercise hyperemia ($p = 0.51$), but during combined NOS and prostaglandin inhibition it was reduced ($p = 0.002$), to a similar extent in all muscles (drug × muscle interaction, $p = 0.99$). Similarly as at rest, heart rate during exercise was lower during double blockade (63 ± 7 bpm, $p < 0.001$) compared to control condition (80 ± 11 bpm) but was not significantly affected by single NOS inhibition (73 ± 10 bpm). No changes in mean arterial pressure were noticed (113 ± 15, 117 ± 13, and 116 ± 13 mmHg in control, NOS inhibition, and double blockade, respectively).

Discussion

The present study demonstrates that inhibition of endogenous NO formation alone or in combination with prostaglandin blockade does not differentially affect blood flow to the different regions of quadriceps femoris, either at rest or during low-to-moderate exercise intensity used in this study. These results are in contrast to studies on rodents, which have documented muscle fibre type specific effects of NOS inhibition on blood flow. Differences between these results and results of animal model reports in the literature (Copp et al. 2010a, b; Hirai et al. 1994) are likely explained by the fact that human muscles have less spatial stratification of muscle fibre types in their muscles than do rodents, and on the other hand to the fact that only one exercise intensity was examined in this study.

Although single inhibition of NOS does not affect blood flow in exercising skeletal muscles in humans (Bradley et al. 1999; Frandsenn et al. 2001; Heinonen et al. 2011; Radegran and Saltin 1999), NO has been shown regulate glucose (Bradley et al. 1999; Kingwell et al. 2002) and free fatty acid uptake (Heinonen et al. 2013a), and even oxygen consumption in the resting state (Heinonen et al. 2011). Furthermore, the effects of NO are not limited to skeletal muscle, as it also regulates blood flow in subcutaneous adipose tissue (Heinonen et al. 2011) in addition adenosine formation, which also regulates adipose tissue blood flow (Heinonen et al. 2012a). Studies in rodents have, however, also elucidated that NO may contribute to muscle blood flow, even

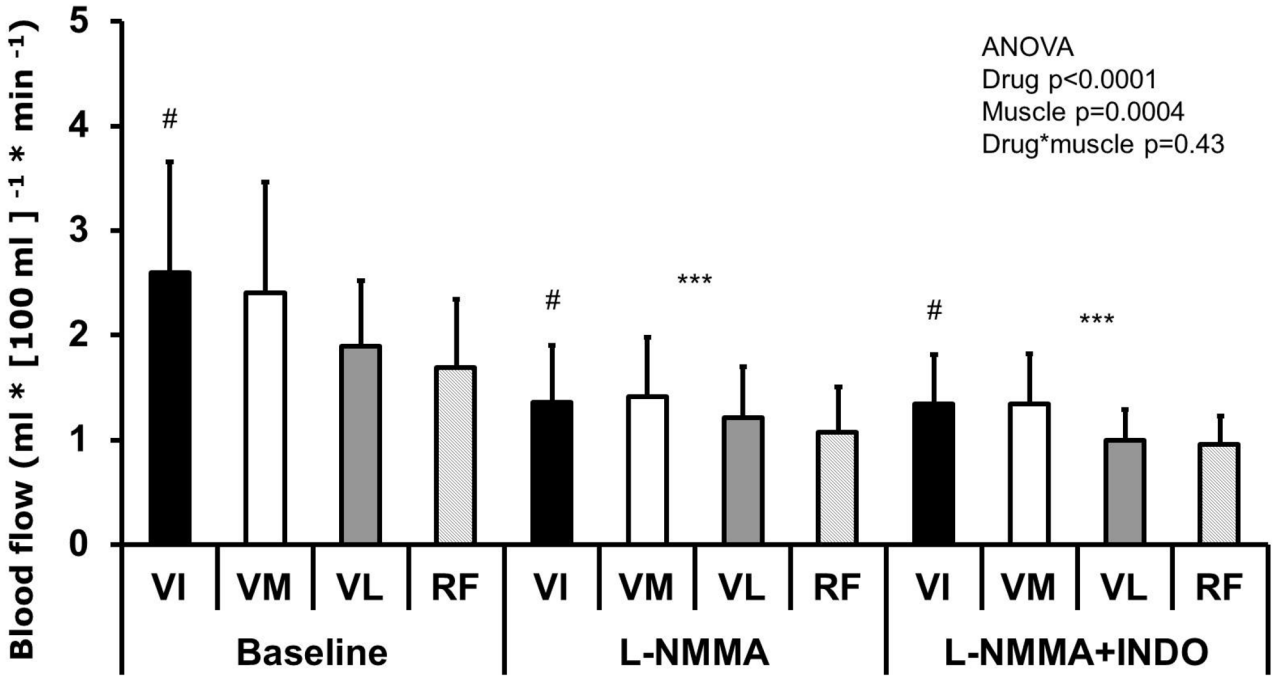

Fig. 1 Resting blood flow and the effect of nitric oxide synthase inhibition alone (L-NMMA) or in combination with inhibition of cyclooxygenase (L-NMMA + INDO) on different muscle parts of m. quadriceps femoris at rest. *VI* m. vastus intermedius, *RF* m. rectus femoris, *VM* m. vastus medialis, and *VL* m. vastus lateralis. $^{\#}p < 0.05$ as compared to VL and RF, $^{***}p < 0.001$ drug effect compared to control

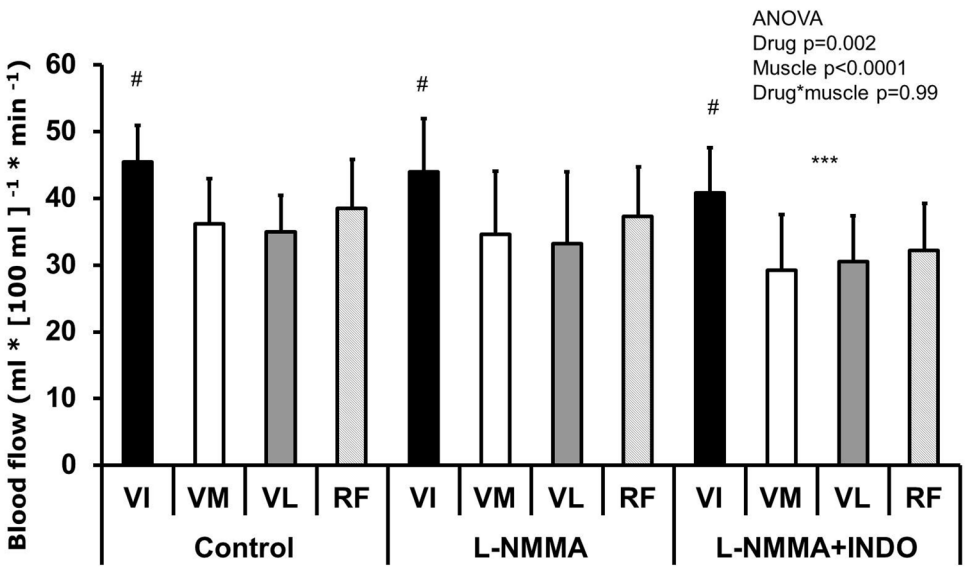

Fig. 2 Exercise blood flow and the effect of nitric oxide synthase inhibition alone (L-NMMA) or in combination with inhibition of cyclooxygenase (L-NMMA + INDO) on different muscle parts of m. quadriceps femoris during exercise. *VI* m. vastus intermedius, *RF* m. rectus femoris, *VM* m. vastus medialis, and *VL* m. vastus lateralis. $^{\#}p < 0.05$ compared to all other muscle parts, $^{***}p < 0.001$ drug effect compared to control

during exercise, in a muscle fibre type specific manner (Copp et al. 2010a, b), and these effects have recently been extended to involve also dietary nitrate supplementations, which increase NO levels in the muscle (Ferguson et al. 2015; Jones et al. 2016). In contrast to these animal studies, muscle fibre type specific effects of NOS inhibition were not documented in the present study. There are several plausible explanations for the differences between these animal and human studies.

One very obvious reason as to why muscle fibre type specific effects of NOS inhibition can be seen in rodents but not in humans is that in contrast to rodent muscles, human skeletal muscles are generally not either solely slow type or fast twitch muscles, but rather a mixture of

different types. This lessens the possibility to detect muscle specific effects in humans. This is analogous to the effects of functional sympatholysis (Duncker and Heinonen 2012; Heinonen 2014) and could be perhaps resolved in the future by investigating human calf musculature which has more distinct fibre types in its muscle parts in contrast to QF musculature, which was assessed in the present study as we did not have a PET-scanner suitable exercise-device available to strain calf musculature within the scanner. Additionally, one important difference between our study in humans and previous rodent studies is that in our analyses topic could mostly be focused on the possible differences between m. vastus intermedius, consisting mostly of type I muscle fibres (Edgerton et al. 1975; Johnson et al. 1973), and three other QF muscle parts that consist more of a mixture of type I and II fibres. On the other hand, in rodent studies the inhibition of NOS has been shown to be the most prominent in the muscles consisting mostly of type II muscle fibres (Copp et al. 2010a, b). This difference also points to the conclusion that in the future human studies calf musculature should be investigated, as it is plausible that if existing, possible muscle fibre type specific effects of NOS inhibition could be detected with some other imaging technique in m. gastrocnemius muscle, which consists mostly of type II muscle fibres in contrast to soleus, which is well-known to consist mostly of type I muscle fibres.

While the aforementioned reasons are likely to be the most obvious to explain why muscle-specific effects can be documented in rodents but not in humans, other possible explanations may contribute. One of these is exercise intensity, as in the present study only low intensity exercise recruiting mostly only slow type muscle fibres was applied due to limitations to perform exercise within the PET scanner, while recruitment of type II fibres is well-known to increase with increases in exercise intensity and muscle force requirements. As muscle blood flow and flow heterogeneity, as well as glucose uptake, are dependent on exercise intensity (Heinonen et al. 2007, 2012b, d), muscle specific analyses of NO contribution should be extended to include higher exercise intensities. Furthermore, in animal studies the effects of NOS inhibitions have been documented using specific inhibitors of neuronal NOS (Copp et al. 2010b) or other effective NOS inhibitions (Copp et al. 2010a), while in the present study more general NOS inhibition was applied, which likely blocks mostly endothelial NOS. The influence of neuronal NOS on muscle blood flow may be coupled with the recruitment of type II muscle fibres and the effect of more specific neuronal NOS inhibition should definitely also be tested in the future in humans as well. However, it is also evident in the case of significant and marked general reductions in blood flow, particularly at rest in the current study, applied NOS inhibition was also effective in affecting blood flow in all muscle parts and the effectiveness of the NOS inhibition is likely not the major contributor to the findings. Future studies may also focus on assessing muscle type specific flow in ageing or certain disease states such as in diabetes, where there is a shift in muscle fibre type composition for more type II fibres due to the loss of type I fibres.

In conclusion, NOS inhibition, with or without prostaglandins inhibition, affects blood flow similarly in different human quadriceps femoris muscles, likely due to the fact that muscles in humans in this muscle group are not as defined with respect to solely oxidative or glycolytic fibres as clearly as seen in animals. Nevertheless, it still remains to be investigated whether differences can be documented during higher intensity exercise that recruits also type II muscles fibres to a larger extent, or in muscles which are known to consist mostly of these fast twitch muscles fibres.

Acknowledgements The study was conducted within the Finnish Centre of Excellence in Molecular Imaging in Cardiovascular and Metabolic Research—supported by the Academy of Finland, University of Turku, Turku University Hospital and Abo Academy. Authors want to thank the contribution of the personnel of the Turku PET Centre for their excellent assistance during the study. The study was financially supported by the Ministry of Education of State of Finland, Academy of Finland, the Finnish Cultural Foundation and its South-Western Fund, the Finnish Sport Research Foundation, the Finnish Foundation for Cardiovascular Research, the Finnish Diabetes Research Foundation, Turku University Hospital (EVO funding), Novo Nordisk Foundation and the Danish Medical Research Council.

Compliance with ethical standards

Conflict of interest None of the authors had personal or financial conflict of interest.

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