PURPOSE: We have previously shown that local infusion of a nitric oxide synthase (NOS) inhibitor attenuates increases in leg glucose uptake during exercise in humans. We have also shown that infusion of the NOS substrate, L-arginine (L-Arg), increases glucose clearance, although the mechanisms involved were not determined. A potential mechanism for NO-mediated glucose disposal is via interactions with NOS and the energy sensor AMP-activated protein kinase (AMPK). The aim of this study was to determine the mechanism(s) by which L-Arg infusion increases glucose disposal during exercise in humans by examining total NOS activity and AMPK signaling. METHODS: Seven males cycled for 120 min at 64 ± 1% VO peak, during which [6,6-2H]glucose tracer was infused. During the final 60 min of exercise either saline alone (Control, CON), or saline containing L-Arg HCl (L-Arg, 30g at 0.5g min) was co-infused in a double-blind, randomized, counter-balanced order. RESULTS: L-Arg increased the glucose rate of disappearance and glucose clearance rate during exercise, however this was accompanied by a 150% increase in plasma insulin concentration from 65-75 min (P<0.05) that remained significantly elevated until 90 min of exercise. Skeletal muscle AMPK signaling, nNOS1/4 phosphorylation by AMPK and total NOS activity increased to a similar extent in the two trials. CONCLUSION: The increase in glucose disposal following L-Arg infusion during exercise is likely due to the significantly higher plasma insulin concentration.
Title: EFFECT OF L-ARGININE INFUSION ON SKELETAL MUSCLE METABOLISM DURING EXERCISE IN HUMANS

Short Title: L-arginine infusion and exercise in humans

Article Type: Original Investigation

Keywords: nitric oxide; contraction; AKT; insulin

Abstract: Purpose: We have shown that local infusion of a nitric oxide synthase (NOS) inhibitor attenuates increases in leg glucose uptake during exercise in humans. We have also shown that infusion of the NOS substrate, L-arginine (L-Arg), increases glucose clearance, although the mechanisms involved were not determined. Therefore, we examined the effect of L-Arg infusion during exercise in humans on skeletal muscle NOS activity and AMPK signaling as well as on plasma glucose kinetics and insulin concentration. Methods: Seven males cycled for 120 min at 64 ± 1% VO2 peak, during which [6,6-2H]glucose tracer was infused. During the final 60 min of exercise either saline alone (Control, CON), or saline containing L-Arg HCl (L-Arg, 30g at 0.5g min⁻¹) was co-infused in a double-blind, randomized, counter-balanced order. Results: L-Arg increased glucose rate of disappearance and glucose clearance rate during exercise, however this was accompanied by a rapid increase in plasma insulin by ~150% at 65, 70 and 75 min (P<0.05) which remained significantly elevated until 90 min of exercise. Skeletal muscle AMPK signaling, nNOSµ phosphorylation by AMPK and NOS activity increased to a similar extent in the two trials. Conclusion: As L-Arg infusion during exercise had little effect on skeletal muscle NOS activity, nNOSµ phosphorylation or AMPK signaling, the increase in glucose disposal was likely due to the significantly higher plasma insulin concentration in response to L-Arg infusion.
EFFECT OF L-ARGININE INFUSION ON SKELETAL MUSCLE METABOLISM DURING EXERCISE IN HUMANS

Kelly. C. Linden¹,², Glenn D. Wadley¹,³, Andrew P. Garnham³, and Glenn K. McConell¹,⁴

¹ Exercise Physiology and Metabolism Laboratory, Department of Physiology, The University of Melbourne, Victoria, 3010, Australia
² School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, New South Wales, 2650, Australia
³ School of Exercise and Nutrition Sciences, Centre for Physical Activity and Nutrition Research, Deakin University, Burwood Victoria, 3125, Australia
⁴ Institute of Sport, Exercise and Active Living and School of Biomedical and Health Sciences, Victoria University, Victoria, 8001, Australia

Running title: L-arginine infusion and exercise in humans

Correspondence: Dr. Glenn McConell

Institute of Sport, Exercise and Active Living and Biomedical and Health Sciences, Victoria University, Victoria, 8001, Australia

PH: 61-3-9919-9472

FAX: 61-3-9919-4891

Email: glenn.mcconell@vu.edu.au
ABSTRACT

Purpose: We have shown that local infusion of a nitric oxide synthase (NOS) inhibitor attenuates increases in leg glucose uptake during exercise in humans. We have also shown that infusion of the NOS substrate, L-arginine (L-Arg), increases glucose clearance, although the mechanisms involved were not determined. Therefore, we examined the effect of L-Arg infusion during exercise in humans on skeletal muscle NOS activity and AMPK signaling as well as on plasma glucose kinetics and insulin concentration. Methods: Seven males cycled for 120 min at 64 ± 1% VO\(_2\) peak, during which [6,6\(^{-2}\)H]glucose tracer was infused. During the final 60 min of exercise either saline alone (Control, CON), or saline containing L-Arg HCl (L-Arg, 30g at 0.5g min\(^{-1}\)) was co-infused in a double-blind, randomized, counter-balanced order. Results: L-Arg increased glucose rate of disappearance and glucose clearance rate during exercise, however this was accompanied by a rapid increase in plasma insulin by~150% at 65, 70 and 75 min (P<0.05) which remained significantly elevated until 90 min of exercise. Skeletal muscle AMPK signaling, nNOS\(_\mu\) phosphorylation by AMPK and NOS activity increased to a similar extent in the two trials. Conclusion: As L-Arg infusion during exercise had little effect on skeletal muscle NOS activity, nNOS\(_\mu\) phosphorylation or AMPK signaling, the increase in glucose disposal was likely due to the significantly higher plasma insulin concentration in response to L-Arg infusion.

Keywords: nitric oxide, contraction, AKT, insulin
INTRODUCTION

Paragraph number 1 Insulin and contraction both increase skeletal muscle GLUT4 translocation and glucose uptake however the mechanisms involved differ (25). We have evidence from rodents (33) and humans (5, 22) that nitric oxide (NO) production during contraction may be essential for the regulation of skeletal muscle glucose uptake during exercise. We have previously reported that local femoral artery infusion of the competitive NOS inhibitor, \( \text{N}^\text{Gl} \)-monomethyl-L-arginine (L-NMMA) into healthy men during exercise attenuates the increase in leg glucose uptake, without influencing leg blood flow or plasma insulin concentration (5). Interestingly, the attenuation in leg glucose uptake appeared to be reversed by co-infusion of the NOS substrate L-arginine (L-Arg) (5). In a follow up study in people with type 2 diabetes and age matched controls, L-NMMA infusion attenuated leg glucose uptake to a greater extent in the diabetic than the control participants (22).

Paragraph number 2 Our laboratory has also shown that infusion of L-Arg increases glucose disposal during exercise in well-trained men (26). We proposed that L-Arg infusion, by increasing the substrate availability for NOS (3), may have increased NO production and therefore mediated the increase in glucose uptake via a NO-dependent mechanism (26). Indeed, systemic infusion of L-Arg at rest in humans increases the content of the NO second messenger cGMP in both plasma and urine and elevates urine levels of the NO breakdown product, nitrate (2). In addition, L-Arg incubation increases NO production of isolated rodent skeletal muscle (1).

Paragraph number 3 Given that local infusion of the NOS inhibitor \( \text{N}^\text{Gl} \)-nitro-L-arginine methyl ester (L-NAME) attenuates the increase in NOS activity (and skeletal muscle
glucose uptake) during \textit{in situ} muscle contractions in rats (33) we have assumed that contraction increased NOS activity and L-NMMA attenuated increases in NOS activity during exercise in our human studies (5, 22). We have also assumed that skeletal muscle NOS activity is increased to a greater extent during exercise when L-arg is infused during exercise in humans (5, 26). Two studies have examined human skeletal muscle NOS activity at rest (9, 20), however no study to date has examined skeletal muscle NOS activity during exercise in human skeletal muscle. Therefore, in the current study we examined whether skeletal muscle NOS activity was increased during exercise and also whether L-arg infusion further increased NOS activity during exercise in humans.

\textit{Paragraph number 4} It is also possible, however, that the increased glucose disposal observed in our previous study (26), was due, at least in part, to increases in plasma insulin concentration. Although not statistically significant there was a tendency for L-Arg infusion to increase plasma insulin concentration during exercise in our previous human study (26). Indeed, L-Arg is a known insulin secretagogue and infusion of L-Arg at rest significantly increases plasma insulin concentration and insulin-stimulated glucose disposal in humans (13). The effect of L-arg infusion on plasma insulin concentration during exercise in humans has not previously been closely examined.

\textit{Paragraph number 5} Therefore, the aim of the current study was to examine the mechanisms by which L-Arg infusion increases glucose disposal during exercise in humans by assessing skeletal muscle NOS activity and also closely examining the time course of plasma insulin concentration changes during exercise. A potential mechanism for NO-mediated glucose disposal is the ubiquitously expressed energy sensor, AMP-
activated protein kinase (AMPK). AMPK activity increases during contraction and is reported to increase skeletal muscle glucose uptake (7, 15). There is also evidence for interactions between AMPK and NOS (7, 33). Therefore, we also examined AMPK activity and AMPK phosphorylation and the phosphorylation status of the major skeletal muscle isoform of NOS, neuronal NOS (nNOS) by AMPK during exercise. It has shown that high levels (5 mM) of the NO donor, sodium nitroprusside, increases basal AMPK activity and glucose uptake (18) and as such, it may be that L-Arg infusion during exercise increases skeletal muscle NO availability which activates AMPK and increases glucose uptake. Since the serine-threonine kinase AKT (protein kinase B, PKB) is an important signaling protein for insulin-stimulated glucose uptake (21) we also examined skeletal muscle AKT signaling during exercise with and without L-Arginine infusion. We hypothesized that L-Arg infusion would increase glucose disposal by causing greater increases in skeletal muscle NOS activity during exercise, independently of AMPK and insulin signaling in humans.

MATERIALS AND METHODS

Paragraph number 6 Participants. Seven recreationally active, non-smoker males provided informed written consent to participate in this study, which was approved by the Human Research Ethics Committee of The University of Melbourne, and conducted in accordance with the Declaration of Helsinki. Participants characteristics were, 23 ± 1 years; 72 ± 4 kg; and peak pulmonary oxygen consumption during cycling (VO2 peak) was 3.3 ± 0.3 l.min⁻¹ (mean ± SEM).

Paragraph number 7 Preliminary testing and diet control. Participants were required to attend the laboratory on four separate occasions. The first visit involved a cycling
VO₂ peak test which was determined during a graded exercise test to voluntary exhaustion on an ergometer (Lode, Groningen, The Netherlands). Participants returned to the laboratory on a separate day for a 30 min familiarization ride at a workload calculated to be ~65% VO₂ peak. Approximately one week later, participants returned to the laboratory for the first of two exercise trials, which involved cycling for 120 min at ~ 65% VO₂ peak (128 ± 8 W).

Paragraph number 8 All participants were instructed to refrain from any formal exercise and to avoid ingesting alcohol and caffeine for 24 h prior to each exercise trial. To ensure the energy intake before each trial was consistent, subjects were asked to complete a food diary over the day prior to their first experimental trial, which was photocopied and returned. They were asked to match the food diary for the second trial and to consume water ad libitum. Participants were instructed to finish eating by 10pm the evening prior to the experimental trial and attended the laboratory in a fasted state. Between trials, participants were instructed to maintain their regular exercise patterns.

Paragraph number 9 Experimental Trials. On the morning of each exercise trial a catheter was inserted into an antecubital forearm vein for blood sampling and another into the contralateral arm for infusion of the stable isotope of glucose ([6,6-²H]glucose; Cambridge Isotope Laboratories, MA, U.S.A.) and co-infusion of L-arginine or saline control. Following a basal blood sample, a bolus of 45.2 ± 0.4 µmol.kg⁻¹ of the glucose tracer was administered intravenously and was immediately followed by a constant (0.72 ± 0.02 µmol.kg⁻¹), 240 min infusion, which was continued until the end of exercise. Following 120 min of rest, the exercise protocol consisted of cycling for 120 min. Blood was sampled at -120, -30, -10 min and immediately prior to the
commencement of exercise and every 15 min during exercise for the measurement of plasma glucose and [6,6-2H]glucose. Plasma glycerol, free fatty acids and insulin were measured at rest and every 30 min of exercise. In addition, from time 60 to 90 min blood was sampled every 5 min to closely examine insulin concentration, which has been reported to peak 10 min after the commencement of L-Arg infusion at rest (13). Expired air was collected into Douglas bags for 3 min every 15 min during exercise and heart rate (Polar Favor, Oulu, Finland) was recorded every 30 min. Muscle was obtained from the vastus lateralis under local anesthesia, using the percutaneous needle biopsy technique, with suction. Prior to the commencement of exercise two separate incisions were made ~1 cm apart and muscle was sampled in a distal to proximal order at rest and at the conclusion of exercise (120 min). One leg (randomly selected) was used for each of the experimental trials. Participants received 8 ml.kg\(^{-1}\) body weight of water at the start of exercise, followed by a further 2 ml.kg\(^{-1}\) body weight every 15 min of exercise and were fan cooled throughout the trial. In a double blind, randomized cross-over design, participants received a co-infusion over the second 60 min of exercise of either 30 g of L-arginine Hydrochloride (Ophthalmic Laboratories for Pharmalab, Brookvale, NSW, Australia) mixed with saline (L-Arg; 0.5g/min \textit{i.v.}), or a placebo control treatment of 0.9% saline (0.5 g/min \textit{i.v.; CON}). Infusion of 30 g of L-arginine over 30-60 min in humans raises plasma L-arginine concentration from ~0.1 to ~6.2-7.2 mM (3).

\textbf{Paragraph number 10 Blood Analysis.} Plasma glucose, lactate and glycerol were determined using enzymatic fluorometric procedures, plasma NEFA by an enzymatic colorimetric method (NEFA-C test, Wako, Osaka, Japan) and plasma insulin using a human insulin-specific radioimmunoassay kit (Linco Research, St. Charles, MO).
Glucose kinetics were estimated at rest and during exercise using a modified one-pool non-steady-state model as previously described (24, 26). Briefly, the rapidly mixing portion of the glucose pool was assumed to be 0.65 and the apparent glucose space was estimated to be 25% of body mass. The technique utilized in this investigation estimates the rates of plasma glucose appearance (glucose \( \text{R}_a \)) and disappearance (glucose \( \text{R}_d \)) from the changes in the % enrichment of \([6,6-^2\text{H}]\)glucose and the plasma glucose concentration. Glucose clearance rate (glucose \( \text{CR} \)) was calculated by dividing the glucose \( \text{R}_d \) by the plasma glucose concentration.

**Muscle Analysis.**

**Paragraph number 11 Preparation of skeletal muscle lysate.** Frozen muscle was homogenized in ice cold lysis buffer on ice (10 µl/mg tissue; 50 mM Tris-HCl, pH 7.5 containing 1 mM EDTA, 1 mM EGTA, 10% vol/vol glycerol, 1% vol/vol Triton X-100, 50mM NaF, 5mM Na₄P₂O₇, 1mM DTT, 1mM PMSF, 1 µl.ml⁻¹ trypsin inhibitor and 5µl.ml⁻¹ Protease Inhibitor Cocktail (P8340, Sigma)), incubated on ice for 20 min and centrifuged at 16,000 g for 20 min at 4°C. The protein concentration of samples was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) with BSA as the standard.

**Paragraph number 12 Antibodies.** Polyclonal rabbit antibodies specific for phospho–AKT Thr₃₀₈, total AKT and total AMPK α protein were purchased from Cell Signaling Technology, (Beverly, MA). The phospho-specific antibodies AMPKα Thr¹⁷² and ACCβ Ser²²¹ were purchased from Upstate (Lake Placid, NY), and AMPK α1 and α2 antibodies were purchased from BD Transduction Laboratories (Franklin Lakes, NJ). The predominant skeletal muscle nNOS isoform (23), nNOSµ, was detected with a
monoclonal mouse antibody (BD Transduction Laboratories) while the phospho-
specific nNOS\textsubscript{µ} Ser\textsuperscript{1451} phosphorylation site was detected with a polyclonal rabbit
antibody (7). ACC\textbeta \textsuperscript{β} was detected using IRDye\textsuperscript{TM} 700-labelled streptavidin (Rockland).

**Paragraph number 13 Immunoblotting.** Skeletal muscle lysates (80µg) were boiled in
Laemmli sample buffer and subjected to SDS-PAGE. Binding of purified proteins was
detected by immunoblotting following an overnight incubation with primary antibody.
Membranes were incubated in Odyssey anti-rabbit IRDye\textsuperscript{TM} 800- or anti-mouse
IRDye\textsuperscript{TM} 700- labeled secondary antibody (Rockland, Gilbertsville, PA.), and were
scanned for infra-red fluorescence using an Odyssey Infrared Imaging System (LI-COR
Biosciences, Lincoln, NE, U.S.A.). When both total protein and protein
phosphorylation were measured, membranes were probed first for total protein, stripped
of antibodies (2% SDS in 25 mM glycine, pH 2.0) and successful stripping was verified
by incubation with secondary antibody followed by infrared detection. Stripped
membranes were then re-probed with the anti-phospho antibody. Phosphorylation was
expressed relative to total protein.

**Paragraph number 14 NOS activity.** NOS activity was determined in tissue extracts by
measuring the production of radiolabelled L-citrulline from radiolabelled L-arginine.
Frozen muscle was homogenized in ice cold homogenizing buffer (10 µl/mg tissue; 250
mM Tris-HCl, pH7.4, 10 mM EDTA, 10mM EGTA). NOS activity of the supernatant
was then determined using a NOS Activity Assay Kit (Cayman Chemicals, Cat 781001)
as per the manufacturers instructions. Ten µl of the supernatant was combined with 40
µl reaction mix (in final concentrations, 25 mM Tris HCL (pH7.4), 3µM
tetrahydrobiopterin, 1µM flavin adenine dinucleotide, 1 µM flavin adenine
mononucleotide, 1.25 mM NADPH, 0.75 mM CaCl$_2$ and 3 µM L-$^{14}$C Arginine monochloride (Amersham Biosciences, Piscataway, NJ,) for 30 min at 37°C in the presence or absence of 1 mM of the NOS inhibitor NG-nitro-L-arginine (L-NNA). The concentration of L-NNA was sufficient to fully block NOS activity (data not shown). NOS activity was calculated as the difference between samples incubated in the presence or absence of L-NNA and values are expressed as pmol of L-$^{14}$C citrulline formation per minute per mg of protein.

**Paragraph number 15 AMPK activity.** Skeletal muscle lysates (50 µg) were combined with 15µl of Protein A sepharose beads (Pierce), bound to either AMPK α1 or AMPK α2 polyclonal antibodies and incubated for 2 hours at 4°C. Immunocomplexes were washed in lysis buffer containing 0.5M NaCl and resuspended in 25 µl of 0.05M Tris buffer (pH 7.5). To commence the assay, 25 µl of reaction mixture containing (in final concentrations) 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol, 10 mM magnesium acetate, 0.1 mM [$^{32}$P]ATP (~200 cpm/pmol, Perkin Elmer Life and Analytical Sciences, Boston, M.A.), 30 µM AMARA peptide (Upstate Biochemicals) and 200µM AMP was added to each sample at 30°C for 20 min with agitation. 40µl of each sample was then transferred onto P81 chromatography paper and washed 3 x 10 min in 75mM H$_3$PO$_4$, once in 100% ethanol, and air dried. P81 paper was then placed in organic scintillation fluid (Opti-Fluor O, Perkin Elmer) and radioactivity was counted on a β counter (Perkin Elmer). AMARA peptide has the same AMPK phosphorylation site as ACCβ, therefore AMPK activities were calculated as units of γ-[$^{32}$P]-ATP incorporated into the AMARA peptide (ACCα (73-87)A$^{77}$) min$^{-1}$ per mg$^{-1}$ total protein subjected to immunoprecipitation (27).
Paragraph number 16 **Statistical analysis.** Data are expressed as mean ± SEM. The two experimental trials were compared using two factor (trial x time) repeated measures ANOVA using the SPSS statistical package and if there was a significant interaction, a post hoc comparison using Filcher's least significant difference (LSD) test was conducted. Statistical significance was set at P < 0.05.

**RESULTS**

Paragraph number 17 **Pulmonary Gas Measurements, Substrate Oxidation and Heart Rate.** During the first 60 min of exercise (before the commencement of the CON or L-Arg infusion) there was no difference between oxygen consumption, RER or HR between the two trials (data not shown). During the second 60 min of exercise, there was no difference in oxygen consumption (2.23±0.14 vs 2.23±0.13), ventilation (53.3±4.4 vs 55.9±5.8), respiratory exchange ratio (0.91±0.2 vs 0.91±0.01), or heart rate (154±5 vs 152±5) between the CON and L-Arg infusion trials (P>0.05).

Paragraph number 18 **Plasma glucose, lactate, insulin, glycerol and NEFA concentrations.** In both trials plasma glucose decreased while plasma lactate and glycerol increased during the 120 min of exercise. However, during the period of CON and L-Arg infusion, there was no significant difference in plasma glucose or lactate (P>0.05, Figure 1A&B) or plasma glycerol concentration (Figure 2B) trials. Plasma NEFA increased in both trials during exercise however it was significantly lower in the L-Arg trial at 90 min (P<0.05, Figure 2B). In the CON trial, plasma insulin levels decreased during the 120 min of exercise (P<0.05, Fig 1C). Infusion of L-Arg resulted in a rapid increase in plasma insulin concentration by~150% at 65, 70 and 75 min, which remained significantly elevated by~50% of CON values until 90 min (P<0.05,
At 105 min there was a trend for increased plasma insulin concentration in the L-Arg trial compared with the CON trial (Figure 1C, P=0.06).

**Paragraph number 19 Glucose kinetics.** Glucose $R_a$, glucose $R_d$ and glucose CR increased during exercise in both trials (P<0.05, Figure 3). Infusion of L-Arg significantly increased glucose $R_a$ over the final 45 min, glucose $R_d$ over the final 30 min and glucose CR at 90 and 105 min of exercise above that of the CON trial (P<0.05).

**Paragraph number 20 AMPK signaling and AKT Thr$^{308}$ phosphorylation.** Exercise significantly increased AMPK α1 activity in both the CON (62%, P<0.05, Figure 4A) and L-Arg (85%, P<0.05, Figure 4A) trials with no significant difference between trials. AMPK α2 activity was significantly elevated by 140% after exercise in the CON trial (P<0.05, Figure 4B), and, although 73% higher after exercise than at rest in the L-Arg trial, this was not significant (P=0.06). Exercise significantly increased AMPK αThr$^{172}$ phosphorylation by 180% in the CON trial (P<0.05), and by 48% in the L-Arg trial (P<0.05, Figure 5A), with no significant difference between trials. ACCβ-Ser$^{221}$ phosphorylation increased to a similar extent in both trials (P<0.05, Figure 5B). Exercise significantly increased AKT Thr$^{308}$ phosphorylation in both trials with no significant difference between the trials (P<0.05, Figure 5C)

**Paragraph number 21 Skeletal muscle NOS activity and nNOSµ Ser$^{1451}$ phosphorylation.** NOS activity during exercise was significantly increased from rest by~90% in both trials with no significant difference between the trials (P<0.05, Figure 6A). nNOSµ Ser$^{1451}$ phosphorylation also increased significantly in both trials with no significant difference between trials (P<0.05, Figure 6B).
DISCUSSION

**Paragraph number 22** This is the first study to demonstrate that skeletal muscle NOS activity is increased with exercise in humans. It appears unlikely, however, that the increased rate of glucose disposal observed with L-Arg infusion is a direct result of increased NO availability since NOS activity increased similarly during exercise in both the CON and L-Arg infusion trials. In addition, L-Arg infusion did not affect AMPK signaling during exercise so greater AMPK activation could not have accounted for the greater glucose disposal with L-Arg infusion. It is more likely that the large insulin response observed during the L-Arg infusion mediated the greater glucose disposal during exercise in that trial. However, despite the higher insulin levels during exercise, skeletal muscle AKT phosphorylation at the conclusion of exercise was not higher in the L-Arg trial.

**Paragraph number 23** Exercise is characterized by a fall in plasma insulin concentration, due to catecholamine inhibition of insulin secretion (12), and indeed in the current study plasma insulin concentration decreased during the first 60 min of exercise in both trials (Figure 1C). L-Arg is a potent insulin secretagogue at rest, acting directly on pancreatic β cells to stimulate insulin secretion (16). Somewhat surprisingly, despite the inhibitory effects of exercise on insulin secretion, L-Arg infusion rapidly caused a very large increase in plasma insulin levels that peaked at approximately 150% above CON levels (Figure 1C). In our previous study involving endurance-trained individuals we saw only a trend for an increase in plasma insulin with L-Arg infusion (26). It is possible that there was a greater insulin response in the current study because active, not specifically endurance trained individuals were
involved while in our first study well trained endurance subjects were involved. Indeed, endurance training increases hepatic insulin clearance in rodents (39) and a single bout of exercise has been observed to increase whole body insulin clearance for up to 15 hours following the cessation of exercise (28). However, it is also possible that in our previous study a higher level of plasma insulin may have been missed within the first 5-10 min of L-Arg infusion because blood samples were only collected every 15 min after the commencement of the L-Arg infusion in that study. Indeed, in the current study plasma insulin concentration increased within 5-10 min after the commencement of the L-Arg infusion (Figure 1C).

Paragraph number 24 Interestingly, the timing and pattern of the insulin response in the current exercise study was similar to what has been observed with L-Arg infusion in humans at rest (13). For example, a 30 minute L-Arg infusion (1g/min) at rest into young, healthy men and women has been shown to elicit a monophasic insulin response, peaking at 10 min and followed by a plateau that remained elevated for the duration of the 30 min infusion (13). Therefore, it appears that the inhibitory effect of elevated plasma catecholamines on insulin secretion during exercise can be overcome by L-Arg infusion.

Paragraph number 25 It is known that the addition of insulin has an additive effect on the increase in skeletal muscle glucose disposal during exercise in humans (38). It is therefore likely that the substantial increase in the concentration of plasma insulin during the L-Arg trial in the present study would have had an additive effect on glucose uptake during exercise, resulting in the significant increases in glucose $R_d$ and glucose CR (Figure 3B &C) compared with the CON trial. To verify this potential additive
signaling effect, we measured skeletal muscle AKT Thr$^{308}$ phosphorylation, since it closely parallels increases in insulin-stimulated glucose uptake following exercise (11) and is an appropriate marker of skeletal muscle insulin-signaling (6). As has been shown in some (19), but not all (34), studies, we found that exercise increased AKT Thr$^{308}$ phosphorylation in human skeletal muscle (Figure 5C). Surprisingly, however, AKT Thr$^{308}$ phosphorylation at 120 min of exercise was similar in the two trials, despite the higher plasma insulin levels in the L-arg trial (Figure 5C). It is possible, however, that the lack of difference in AKT Thr$^{308}$ phosphorylation was because plasma insulin levels in L-Arg had returned to normal levels by the end of exercise when the muscle biopsy was conducted (Figure 5C). Our decision to collect the exercise muscle biopsy at 120 min was based on our previous finding that glucose CR was augmented by L-Arg infusion during exercise at this time (26). However, the results of the present study show that most of the “surge” in plasma insulin occurs prior to this and within the first 15 min of L-Arg infusion (Figure 1C). Based on these findings, future exercise studies should examine if there is an additive effect on skeletal muscle AKT phosphorylation within the first 15 minutes of L-Arg infusion during exercise.

Paragraph number 26 An important novel finding of the present study is that skeletal muscle NOS activity increases during exercise in humans (Figure 6). Although NO has been implicated as a potential signaling intermediate in contraction mediated glucose uptake in humans, previous studies have used indirect measures of NO accumulation/NOS activity (5, 22). Previously, human studies had only examined skeletal muscle NOS activity at rest (9, 20) and it has been assumed that skeletal muscle NOS activity increases during exercise in humans. Our findings in humans are in line
with similar results during exercise (32) and in situ contractions (33) in rats and increased NO release during contraction of isolated rat skeletal muscle (1).

Paragraph number 27 We hypothesized that infusion of the NOS substrate, L-Arg, during exercise would augment the increased NOS activity during exercise above that observed in the control exercise trial. Indeed, others have shown that L-Arg incubation increases NO production in non-contracting rat muscle (1). However, we found no greater increase in NOS activity during exercise when L-Arg was infused than exercise alone. This may have been because the NOS enzymes in skeletal muscle could have been already saturated with L-Arg substrate before the infusion of L-Arg (31). The $K_m$ for nNOS (1.4-2.2µM) and eNOS (2.9µM) are much less than the plasma concentration of L-Arg in healthy humans (~100µM), and as such NOS should have been saturated in the control trial (8). Elevating plasma L-Arg, commonly with 30g i.v., such as in the present study, increases the plasma concentration in humans to ~7000µM (31).

Paragraph number 28 Despite this, surprisingly, numerous studies (4, 5, 17) have reported increases in indirect indices of systemic NO production following L-Arg infusion such as increased leg blood flow, cGMP accumulation and NOx production. This phenomenon, where increased L-Arg availability causes effects despite the NOS enzymes already likely to have been saturated with substrate, is known as the L-arginine paradox. Interestingly, it has also been suggested that many of the vascular effects of L-Arg infusion, such as vasodilation, inhibition of platelet aggregation and reduced blood viscosity are mediated by L-Arg-stimulated insulin release and not by L-Arg itself (13). It is not known if L-Arg infusion during exercise exerts its effects solely through increased insulin release. However results from the present study indicate that within
human skeletal muscle during exercise, systemic L-Arg infusion does not increase NOS activity above that of control exercise.

Paragraph number 29 Although L-Arg is a precursor for the vasodilator NO, it has previously been shown that L-Arg infusion during exercise has no effect on either leg blood flow (5) or skeletal muscle blood flow (17) in humans. Therefore, it is unlikely that the L-Arg infusion increased glucose uptake during exercise by increasing muscle blood flow, and, therefore glucose delivery. It is, however, possible that the increase in plasma insulin concentration observed during the L-Arg infusion had a vasodilatory effect of its own (13). Although high levels of plasma insulin are required for an increase in total blood flow, lower, more physiological increases in plasma insulin increase blood flow through muscle capillaries (37). It is possible that the higher L-Arg availability and higher plasma insulin levels in the current study may have combined to increase capillary flow. It is known that increases in plasma insulin phosphorylates AKT which then phosphorylates and activates eNOS in endothelial cells (29). Furthermore NOS inhibition can prevent the increases in muscle capillary flow in response to increases in insulin (36).

Paragraph number 30 It is well known that AMPK is phosphorylated and activated during exercise, and there is evidence to suggest that AMPK mediates glucose uptake during exercise (7). However a number of studies have demonstrated dissociations between skeletal muscle AMPK activity and glucose uptake during exercise/muscle contraction (10). Interestingly, in the present study, AMPK α2 activity (Figure 5B), which increased during exercise in the control trial, only tended to increase in the L-Arg trial (P=0.06). It is not clear why AMPK α2 activation during exercise was blunted in
the L-Arg trial. It is possible that the increases in plasma insulin with L-Arg infusion inhibited AMPK α2 activity, however there is limited evidence to suggest that AMPK activity is attenuated in conditions of high insulin (30). In addition, insulin has no effect on rat skeletal muscle AMPK activity in vitro (15), or in humans with carbohydrate ingestion-mediated hyperinsulinemia during exercise (24).

Paragraph number 31 There was a small, transient, yet significant attenuation of the increase in plasma NEFA levels at 90 min in the L-Arg trial (Figure 2B). We found more pronounced reductions in plasma NEFA in our previous study during exercise in endurance-trained individuals infused with L-Arg (26). We think it is unlikely that the lower plasma NEFA concentrations in L-Arg accounted for the higher glucose disposal rate in that trial. Some (40), but not all (14), studies have shown that raising plasma NEFA levels has no effect on glucose disposal during exercise in humans. In addition, very large reductions in plasma NEFA concentration during exercise have no (35) or only modest (40) effects on glucose disposal during exercise in humans.

Paragraph number 32 In summary, we have shown for the first time that exercise increases skeletal muscle NOS activity in humans. L-Arg infusion during exercise increased glucose uptake, but it appears that this was not via an increase in skeletal muscle NO production since there was no further increase in skeletal muscle NOS activity with L-Arg infusion during exercise. L-Arg infusion also had little effect on skeletal muscle AMPK activation. However, L-Arg infusion resulted in a very large increase in plasma insulin concentration during exercise, and since exercise and insulin are additive on skeletal muscle glucose uptake, it is likely that L-Arg infusion increases glucose uptake during exercise via increases in plasma insulin concentration.
GRANTS

This work was supported by a grant from the National Health and Medical Research Council (NHMRC) of Australia (GKM: 237002). The results of the present study do not constitute endorsement by the American College of Sports Medicine.

ACKNOWLEDGEMENTS

We thank the participants for taking part in this study and acknowledge the technical assistance of Professor Benedict Canny, Dr Sean McGee and Vince Murone.

CONFLICTS OF INTEREST

None to declare.
REFERENCES


FIGURE LEGENDS

Figure 1
Plasma glucose (A), plasma lactate (B) and plasma insulin concentration (C) at rest and during 120 min of exercise at 64±1% VO₂ peak with infusion of either L-arginine (L-Arg) or saline (CON). Data are mean ± SEM, N=7. * P<0.05 vs CON.

Figure 2
Plasma glycerol (A) and plasma non-esterified fatty acid (NEFA; B) concentration at rest and during 120 min of exercise at 64±1% VO₂ peak with infusion of either L-arginine (L-Arg) or saline (CON). Data are mean ± SEM, N=7. * P<0.05 vs CON.

Figure 3
Rate of glucose appearance (glucose Ra, A), rate of glucose disappearance (glucose Rd, B) and glucose clearance rate (glucose CR, C) at rest and during 120 min of exercise at 64±1% VO₂ peak with infusion of either L-arginine (L-Arg) or saline (CON). Data are mean ± SEM, N=7. * P<0.05 vs CON.

Figure 4
Skeletal muscle AMPK α1 (A) and AMPK α2 activity (B). Muscle samples obtained before exercise (rest) and immediately following 120 min of exercise at 64±1% VO₂ peak with infusion of either L-arginine (L-Arg) or saline (CON). Data are mean ± SEM, N=7 (A) and 6 (B). # P<0.05 vs rest. †, P=0.06 vs rest.
Figure 5

(A) Representative immunoblot of AMPKα Thr$^{172}$ phosphorylation measured by immunoblotting using a phospho-specific antibody to AMPKα Thr$^{172}$, normalized to total AMPKα (pan) protein (B) ACCβ-Ser$^{221}$ phosphorylation measured using a phospho-specific antibody to ACC-β Ser$^{221}$, normalized to total ACC-β and (C) Representative immunoblot of AKT Thr$^{308}$ phosphorylation measured by immunoblotting using a phospho-specific antibody specific to AKT Thr$^{308}$, normalized to total AKT protein. Muscle samples were obtained before exercise (rest) and immediately following 120 min of exercise at 64±1% VO$_2$ peak with infusion of either L-arginine (L-Arg) or saline (CON). Data are mean ± SEM, N=7 (A), N=6 (B) and N=6 (C). # P<0.05 vs rest.

Figure 6

Skeletal muscle nitric oxide synthase (NOS) activity (A) and representative immunoblot of neuronal nitric oxide synthase (nNOS)µ Ser$^{1451}$ phosphorylation (B) measured using a phospho-specific antibody to nNOSµ Ser$^{1451}$, normalized to total nNOSµ protein. Muscle samples were taken before exercise (rest) or following 120 min of exercise at 64±1% VO$_2$ peak with infusion of either L-arginine (L-Arg) or saline (CON). Data are mean ± SEM, N=6. * P<0.05 vs CON. # P<0.05 vs rest.
Figure 1

A

Plasma glucose (mmol.l⁻¹)

B

Plasma lactate (mmol.l⁻¹)

C

Plasma insulin (pmol. l⁻¹)

Steady state exercise

Time (min)

Saline or L-Arg

CON

L-Arg

Copyright Transfer Agreement (can attach or send offline to Editorial Office via postal service or fax)
Figure 2

A

Plasma Glycerol (μ mol l⁻¹)

0
100
200
300
400
500
600

CON
L-Arg

Time (min)

0
15
30
45
60
75
90
105
120

B

Plasma NEFA (mmol l⁻¹)

0.0
0.5
1.0
1.5

CON
L-Arg

Time (min)

0
15
30
45
60
75
90
105
120

Steady state exercise

Saline or L-Arg
Figure 3

A

Glucose Ra
(μmol·kg⁻¹·min⁻¹)

B

Glucose Rd
(μmol·kg⁻¹·min⁻¹)

C

Glucose CR
(ml·kg⁻¹·min⁻¹)

Steady state exercise

Time (min)

Saline or L-Arg

Copyright Transfer Agreement (can attach or send offline to Editorial Office via postal service or fax)
Figure 5

A. AMPK α pThr<sup>172</sup>

AMPK α pan

B. ACC-β pSer<sup>221</sup>

ACC-β Protein

C. AKT pThr<sup>308</sup>

AKT protein
Figure 6

A

NOS Activity
(ppm min⁻¹ mg protein)

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>L-Arg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Exercise</td>
<td>600</td>
<td>600</td>
</tr>
</tbody>
</table>

B

nNOS pSer⁺¹⁴⁵¹
nNOS protein

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>L-Arg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Exercise</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Copyright Transfer Agreement (can attach or send offline to Editorial Office via postal service or fax)