Endogenous nitric oxide as a modulator of rabbit skeletal muscle microcirculation *in vivo*

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- 1 Intravital microscopy of rabbit tenuissimus muscle microvasculature was used for *in vivo* studies of the role of endogenous nitric oxide (NO) in local vascular control. Derivatives of arginine were applied topically in order to modulate the formation of NO from L-arginine.
- 2 L-N^G-monomethylarginine (L-NMMA) (10–100 μ M), but not D-N^G-monomethylarginine (D-NMMA), dose-dependently reduced microvascular diameters. The vasoconstriction induced by L-NMMA (100 μ M) was prevented by pretreatment with L-arginine (1 mM) but not with D-arginine (1 mM). Intravenous infusions of L-arginine (300 mg kg⁻¹) reversed the effect of L-NMMA (100 μ M). L-Arginine or D-arginine applied topically at 1 mM *per se* had no effect on microvascular diameters.
- 3 Vasodilatation by acetylcholine (0.03–3 μ M) was significantly inhibited by L-NMMA (100 μ M), whereas vasodilatation by adenosine (0.1–100 μ M) or sodium nitroprusside (100 nM) was not affected.
- 4 The hyperaemic response after tenuissimus muscle contractions induced by motor nerve stimulation was unaffected by the presence of L-NMMA (100 μ M).
- 5 Aggregates of platelets and white blood cells were seen in venules during superfusion with L-NMMA (100 μ M), but not with D-NMMA (100 μ M).
- 6 Our results suggest that endogenous NO formed from L-arginine is a modulator of microvascular tone and platelet and white cell-vessel wall interaction *in vivo*. Nitric oxide does not, however, appear to play a role in the mediation of functional hyperaemia in this tissue.

Introduction

Since Furchgott & Zawadzki (1980) reported that acetylcholine-induced vasodilatation is mediated by an endothelium-derived relaxing factor (EDRF) it is becoming increasingly clear that the endothelium plays a crucial role in the regulation of vascular tone. Vasodilatation by a number of endogenous vasodilators (e.g. histamine, bradykinin, thrombin, adenosine 5'-triphosphate (ATP), acetylcholine and substance P) (Furchgott, 1983) and flow-dependent dilatation (Holtz *et al.*, 1984) are now known to be mediated via EDRF, which induces vascular smooth muscle relaxation by increasing intracellular levels of guanosine 3':5'-cyclic monophosphate (Rapoport & Murad, 1983).

Recently, EDRF has been identified as nitric oxide (NO) (Ignarro *et al.*, 1987; Kahn & Furchgott, 1987; Palmer *et al.*, 1987), which is produced by vascular endothelium (Amezcuza *et al.*, 1988; Sakuma *et al.*, 1988). Macrophages also produce nitrite and nitrate (Iyengar *et al.*, 1987), which are now known to be formed via the generation of NO (Marletta *et al.*, 1988). More recently the central nervous system (Garthwaite *et al.*, 1988; Knowles *et al.*, 1989) and neutrophils (Rimele *et al.*, 1988; McCall *et al.*, 1989) have also been shown to produce NO.

The NO formed from these different sources is synthesized from the amino acid L-arginine, and in all studied tissues L-N^G-monomethylarginine (L-NMMA) is a stereospecific inhibitor of the enzyme(s) responsible for this synthesis (Furchgott & Vanhoutte, 1989; Moncada *et al.*, 1989). Using L-NMMA, Rees *et al.* (1989) demonstrated that endothelium-derived NO plays a role in the regulation of blood pressure and in the hypotensive action of acetylcholine in the anaesthetized rabbit.

We have used L-NMMA to study the involvement of endogenous NO in the regulation of skeletal muscle microcir-

ulation under resting conditions and in the mediation of functional hyperaemia *in vivo*.

Methods

Male New Zealand White rabbits (0.8–1 kg, $n = 24$) were anaesthetized with 20% Urethane (1.5 g kg⁻¹ b.w., i.v.). Breathing was facilitated by the insertion of a tracheal cannula. Catheters were inserted in the carotid artery and the jugular vein for blood pressure recordings and administration of additional anaesthetic, respectively.

The tenuissimus muscle of the left hindleg was prepared for intravital microscopy according to Lindbom *et al.* (1982). Briefly, an incision was made along the saphenous vein, the fascia was cut and the overlying tissue lifted to expose the tenuissimus muscle. An incision caudally to the saphenous vein was made for the insertion of a transillumination system. Intravital microscopy was performed by means of a Leitz Biomed microscope with a movable stage and a salt water immersion lens (SW X25, NA 0.60).

The muscle was continuously superfused with a modified Krebs-Henseleit solution (composition, mM: KH₂PO₄ 1.2, NaHCO₃ 25.0, NaCl 118.4, KCl 4.8, CaCl₂ 2.5, MgSO₄ 1.19) at 37°C, equilibrated with 5% CO₂ in nitrogen. The hind part of the animal was kept in a sealed plastic box continuously flushed with nitrogen so as to keep tissue oxygen tensions at normal, relatively low levels. Drugs were dissolved in water and administered diluted in the superfusate proximal to the muscle by a microinjection pump (CMA 100, Carnegie Medical, Stockholm). For intravenous administration, drugs were dissolved in 1 ml of superfusion medium and administered via the jugular cannula.

To obtain access to the tenuissimus motor nerve, an incision was made slightly caudal to the left hip joint. The sciatic nerve bundle was exposed under the anterior limit of the biceps femoris muscle. The nerve to the tenuissimus muscle

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was isolated, and crushed proximally between a pair of forceps. Platinum electrodes were inserted under the nerve and insulated by a plastic cover. The electrodes were connected to a Grass Instruments S4 stimulator via a stimulus isolation unit.

The vascular structures were filmed on videotape by a Panasonic VW-1550 videocamera and a Panasonic AG-6010 videorecorder, yielding a final magnification on the TV screen of $1250\times$. Vessel inner diameter was measured off-line from the videotape recordings.

Measurements of red cell velocity in the arterioles was performed on-line by means of cross correlation technique (Wayland & Johnson, 1967). Two small fibreoptic filaments were interposed in the light path of the phototube immediately before the camera, thus guiding the two samples of light to two photomultiplier diodes. The time delay between identical photometric signals was computed by a cross-correlator, IPM Velocity Tracker Mod-102B. The volume flow was calculated from the velocity and diameter values.

The preparation was allowed to rest for 30 min to obtain stable microcirculatory conditions. After a 1 min control period, drugs were added to the superfusion solution and allowed to exert their effects until a steady state had been obtained (6–20 min). The mean of 6 diameter measurements during 1 min was used for further calculations. Dose-response curves were cumulative.

For studies of functional hyperaemia the animal was positioned in the experimental setup and allowed to rest for 30 min to obtain stable microcirculatory conditions. Control values were obtained by measuring vascular parameters for 2 min immediately before motor nerve stimulation. Stimulation (5 Hz, 0.05 ms, 0.5–5 V) was then applied for 60 s. Voltage was set at 1.0 V above the threshold for muscular contractions as observed macroscopically. During the 1 min stimulation no measurements could be made due to the movements of the muscle. Measurements were obtained every 10 s during the first 2 min following stimulation, thereafter at every 30 s up to 5 min and at 10 and 15 min after the end of stimulation. The paired *t* test was used for statistical calculations of data. Data are expressed as mean $\pm$ s.e.mean.

Drugs

Urethane, acetylcholine, adenosine, L-arginine hydrochloride and D-arginine hydrochloride were purchased from Sigma, St. Louis, Missouri, U.S.A.; heparin from Kabi Vitrum, Stockholm, Sweden; L-N^G-monomethylarginine (L-NMMA) and D-N^G-monomethylarginine (D-NMMA) were synthesized as described (Patthy *et al.*, 1977), and used as acetate salts. Sodium nitroprusside was from F. Hoffman-La Roche & Co., Basel, Switzerland.

Results

L-NMMA (10–100 μ M), applied topically, caused dose-dependent reductions of vascular diameters in the skeletal muscle microcirculation. At 100 μ M the diameter of transverse arterioles (control diameter 8–25 μ m) and their first order side branches, the terminal arterioles (control diameter 2–7 μ m), decreased to $64 \pm 4\%$ ($P < 0.01$, $n = 9$) and $80 \pm 6\%$ of control ($P < 0.05$, $n = 6$), respectively (Figure 1). Blood flow in transverse arterioles was reduced to $48 \pm 8\%$ of control ($P < 0.005$, $n = 8$) by the presence of L-NMMA (100 μ M). Venules (control diameter 10–40 μ m) within the microvascular network of the tenuissimus muscle constricted (to a diameter of $82 \pm 6\%$ of control, $P < 0.05$, $n = 10$) in response to L-NMMA (100 μ M). The maximal effect of each concentration was obtained within 15 min after starting the superfusion. D-NMMA (100 μ M) did not affect the tone of arterioles (Figure 2) or venules (data not shown).

Figure 1 Log dose-response curves for the vasoconstriction induced by topically applied L-N^G-monomethylarginine (L-NMMA) (10–100 μ M) in transverse (a) and terminal arterioles (b) of the rabbit tenuissimus muscle. Data (means with s.e.mean shown by vertical bars) are values obtained at the end of a 15 min superfusion and expressed as % of control. The asterisks denote a significant difference (* $P < 0.05$ and *** $P < 0.001$) from control values. Numbers in parentheses indicate number of observations.

The natural amino acid L-arginine (1 mM), administered topically 15 min prior to and during superfusion with L-NMMA (100 μ M), prevented the constrictor effect of L-NMMA. In contrast, topical D-arginine (1 mM), the unnatural isostere, did not alter the vasoconstrictor effect of L-NMMA (Figure 2). Topically applied L-arginine or D-arginine (1 mM) *per se* were without effect on transverse arteriolar diameters ($98 \pm 2\%$, $n = 4$ and $97 \pm 1\%$ of control, $n = 3$, respectively).

In four experiments, L-arginine (300 mg kg⁻¹) was administered systemically via a cannula in the jugular vein after the vasoconstrictor effect of topical L-NMMA (100 μ M) had been established. The vasoconstriction by L-NMMA ($77.5 \pm 3\%$ of control diameter in transverse arterioles, $P < 0.01$) was promptly reversed upon i.v. infusion of L-arginine (to $94 \pm 4\%$ of control diameter). The infusion of L-arginine had no discernible effect on systemic blood pressure.

Topical application of acetylcholine (0.03–3 μ M) evoked dose-dependent arteriolar dilatation which was progressively

Figure 2 Effects of arginine derivatives on diameters of transverse arterioles in rabbit tenuissimus muscle. Data expressed as % decrease from control diameters induced by 15 min of topical superfusion of either L-N^G-monomethylarginine (L-NMMA) alone (100 μ M; open column), or combined with L-arginine (1 mM; hatched column) or D-arginine (1 mM; crosshatched column). Also shown is the lack of effect of D-NMMA (100 μ M; solid column). Mean values with s.e.mean shown by vertical bars, $n = 4$. The asterisks denote significant differences from control values (** $P < 0.01$).

Figure 3 Log dose-response curves for the vasodilatation induced by acetylcholine (a) and adenosine (b), expressed as diameter relative to control, in transverse arterioles in the absence (○) and presence (●) of L-N^G-monomethylarginine (100 μM). Mean values with s.e.mean indicated by vertical bars, $n = 5-6$. The asterisks denote significant differences between the two curves (** $P < 0.01$; * $P < 0.05$).

antagonized by L-NMMA (100 μM) (Figure 3a). In contrast, the dose-response curve for vasodilatation induced by topical adenosine (0.1–100 μM) was not significantly altered by the presence of L-NMMA (100 μM) (Figure 3b). Likewise, L-NMMA (100 μM) failed to affect sodium nitroprusside-induced dilatation of transverse and terminal arterioles.

The effect of L-NMMA treatment on functional hyperaemia was investigated by use of motor nerve stimulation (1 min at 5 Hz, 0.05 ms and 0.5–5 V) which induces selective skeletal muscle contractions in the tenuissimus and a submaximal hyperaemic response (Persson *et al.*, 1988). During resting conditions the blood flow was $1.3 \pm 0.7 \text{ nl s}^{-1}$ (nanoliters per second) in transverse arterioles. After stimulation the blood flow increased to a peak flow of $7.6 \pm 4.6 \text{ nl s}^{-1}$ ($507 \pm 63\%$ of control, $P < 0.001$, $n = 7$) in transverse arterioles and returned to resting values within 15 min. After motor nerve stimulation in the presence of topical L-NMMA (100 μM), peak blood flow increased to $6.1 \pm 3.7 \text{ nl s}^{-1}$, a value not significantly different from control stimulation. Furthermore, L-NMMA did not alter the subsequent post-contraction hyperaemia (Figure 4).

In resting conditions some leukocytes moving off the axial stream can be seen to roll along the venular endothelium or even stick to it for a short while. Topical application of L-NMMA (100 μM) did not change the number of cells rolling or sticking. However, during superfusion with L-NMMA, aggregates of white blood cells and platelets were occasionally formed, adhering to the vessel wall and sometimes occluding the vessel before they were spontaneously washed away by the blood stream. Aggregates, which were observed only in venules, were formed at a rate of 3–6 per 10 min.

Figure 4 Blood flow in transverse arterioles of tenuissimus muscle, including a brief period (60 s as indicated by horizontal bar) of motor nerve stimulation (0.05 ms, 5 Hz, 0.5–5 V) in the absence (○) or presence (●) of L-N^G-monomethylarginine (100 μM). Mean values with s.e.mean indicated by vertical bars, $n = 7$.

Discussion

Recent studies have shown that L-arginine is the precursor of endothelium-derived NO and that its derivative L-NMMA is a stereospecific inhibitor of NO formation (Palmer *et al.*, 1988; Sakuma *et al.*, 1988). Thus, the findings that L-NMMA, but not D-NMMA, dose-dependently reduced microvascular diameters and that L-arginine, but not D-arginine, either by topical pretreatment or intravenous administration, abolished the effect of L-NMMA in skeletal muscle microcirculation strongly indicate the presence of endogenous NO derived from L-arginine in the rabbit skeletal muscle microcirculation. The stereospecific interaction by L-arginine and its methylated derivative L-NMMA on the putative NO formation is in good agreement with earlier studies (Palmer *et al.*, 1988; Sakuma *et al.*, 1988). The finding that L-arginine itself had no effect on vascular parameters implies either that the synthesis of NO from L-arginine is a substrate-inhibited process or that there are sufficient amounts of L-arginine to saturate the NO-forming enzyme in the anaesthetized rabbit (Rees *et al.*, 1989). Another possibility is that endothelium-dependent vasodilators might act via an allosteric activation of the enzyme. The arteriolar constriction *in vivo* by topical L-NMMA, observed in our experiments emphasizes that the blood pressure-increasing effect of systemic L-NMMA (Rees *et al.*, 1989) must, to a large extent, be dependent on an actual change in peripheral vascular tone rather than on alterations in central cardiovascular regulation.

The inhibition of acetylcholine-induced vasodilatation by L-NMMA in the present study agrees well with *in vitro* studies in which NO was detected in the effluent from the acetylcholine-treated rabbit heart (Amezcuca *et al.*, 1988) and from *ex vivo* measurements from vascular tissue obtained from animals treated with L-NMMA (Rees *et al.*, 1989). In the present study, the major part of the acetylcholine-induced response was inhibited, although some of the response remained (Figure 3a). This might be attributable either to an insufficient local concentration of L-NMMA or to the presence of an endothelium-derived hyperpolarizing factor, different from NO (Feletou & Vanhoutte, 1988; Chen *et al.*, 1988).

Vasodilatation by nitroprusside or adenosine was unaffected by the presence of L-NMMA, suggesting that vasoconstriction by L-NMMA was not due to a general, unspecific smooth muscle contractile action, and confirming an intact responsiveness of the vascular smooth muscle to nitrous vasodilators such as NO in the presence of L-NMMA (Rees *et al.*, 1989).

Endogenous adenosine influences basal vascular tone and partly mediates functional hyperaemia in the tenuissimus muscle (Gustafsson *et al.*, 1987; Persson *et al.*, 1988). Since L-NMMA failed to modify the vasodilatation by exogenous adenosine, the L-NMMA effect on vascular tone must be independent of basal adenosine action, which is in agreement with the suggestion that adenosine is an endothelium-independent vasodilator (Furchgott, 1983). Although adenosine partly mediates functional hyperaemia in the tenuissimus, the greater part of the hyperaemic response is intact in the presence of specific and effective adenosine antagonists (Persson *et al.*, 1988). Since L-NMMA lacked effect on functional hyperaemia, NO appears a less likely candidate as a mediator in the non-adenosine part of the hyperaemic response, either directly or as a consequence of adenosine release. However, additional studies are required to exclude such a role for NO.

In vitro data have shown that endothelium-dependent relaxation is more prominent in arteries than in veins. In agreement with this, our data indicate that the resting tone of venules in the microcirculation *in vivo* is dependent on endothelium-derived NO, but to a lesser extent than that of arterioles. The aggregates induced by L-NMMA in venules were similar to those observed during application of substance P. Such aggregates have been shown to consist of leukocytes and platelets (Öhlén *et al.*, 1989). This suggests either that NO

has an antiaggregatory role or that a charged compound like L-NMMA can induce release of tachykinins. Since D-NMMA lacked the aggregatory effect, an unspecific release of tachykinins seems unlikely. Furlong *et al.* (1987) and Hogan *et al.* (1988) have shown that EDRF has an antiaggregatory effect on platelets *ex vivo*.

In summary, our observations indicate that endogenous NO might be a significant modulator of the microvascular tone in skeletal muscle. Furthermore, our data extend the

observations which indicate that L-arginine is the precursor for endothelium-derived NO, and that vasodilatation induced by acetylcholine, but not adenosine or nitroprusside, is dependent on the generation of NO from L-arginine.

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