Importance of nitric oxide in canine femoral circulation: comparison of two NO inhibitors

Knut Arvid Kirkebøen, Pål Aksel Naess, Geir Christensen, Fredrik Kiil

Objective: The aim was to assess the importance of endothelium derived nitric oxide (NO) in the regulation of vascular tone in the limbs. Changes in the canine femoral circulation were investigated after inhibition of NO synthesis. Methods: The effects of two NO inhibitors, Nω-monomethyl-L-arginine (LNMMA) and Nω-nitro-L-arginine (NOARG), were compared on basal femoral blood flow and on endothelium dependent (acetylcholine) and endothelium independent (glyceryl trinitrate) vasodilatation in 15 pentobarbitone anaesthetised mongrel dogs. An electromagnetic flow probe was placed on the femoral artery to measure femoral blood flow. One catheter was advanced into the femoral artery proximal to the flow probe for blood pressure recording and another catheter distal to the flow probe for drug infusions. Results: LNMMA (0.28 μmol·ml⁻¹) reduced basal femoral blood flow by 44(±3)%, NOARG (0.07 μmol·ml⁻¹) by 21(±4)%, and NOARG (0.56 μmol·ml⁻¹) by 29(±3)%. The flow responses to acetylcholine were reduced after LNMMA by 27(±8)%, unaltered after NOARG (0.07 μmol·ml⁻¹), and reduced after NOARG (0.56 μmol·ml⁻¹) by 60(±7)%. The flow response to glyceryl trinitrate was unaltered. L-arginine re-established femoral blood flow after infusion of LNMMA and NOARG (0.07 μmol·ml⁻¹), but L-arginine did not re-establish femoral blood flow after NOARG (0.56 μmol·ml⁻¹), even when infused in a 60-fold molar excess. Conclusions: There is a continuous basal release of NO in the canine femoral circulation. The results obtained by infusing LNMMA suggest that more than 40% of basal femoral blood flow is mediated by endothelium derived NO. Whereas LNMMA was more potent than NOARG in reducing basal NO release, NOARG (0.56 μmol·ml⁻¹) reduced acetylcholine induced vasodilatation by as much as 60%.

The vascular endothelium seems to play an important role in modulating vasomotor tone through synthesis and metabolism of various vasodilating and vasoconstricting agents. Furchgott and Zawadzki first described the effect of an endothelium derived relaxing factor (EDRF) in aortic ring preparations. Although the identity of EDRF remains uncertain, nitric oxide (NO) appears to account, at least in part, for the biological activity of EDRF.

The terminal guanidino nitrogen of the amino acid L-arginine is the precursor of nitric oxide. Derivatives of L-arginine with modifications at the guanidino terminus, as Nω-monomethyl-L-arginine (LNMMA) and Nω-nitro-L-arginine (NOARG), inhibit the synthesis of nitric oxide. These L-arginine analogues cause endothelium dependent contractions and inhibit endothelium dependent relaxations in isolated vessels or vessel ring preparations. Given intravenously LNMMA and NOARG increase arterial blood pressure and inhibit endothelium dependent relaxations. These observations suggest that endothelium derived nitric oxide plays a role in regulating vascular smooth muscle tone in both conductance and resistance vessels.

Only one in vivo study has previously examined the role of endothelium derived nitric oxide in limbs. Vallance and coworkers showed that infusion of LNMMA into the forearm of human volunteers decreased plethysmographically measured resting blood flow and inhibited endothelium dependent vasodilatation. In vitro and in vivo studies have shown that NOARG is 4-100 times more potent than LNMMA as a specific inhibitor of EDRF formation. To our knowledge the effects of NOARG in limbs in vivo have not previously been described.

The purpose of this study was to evaluate the role of nitric oxide in the femoral arterial circulation by directly measuring femoral blood flow in anaesthetised dogs. The effects of the two L-arginine analogues, LNMMA and NOARG, were examined on basal femoral blood flow and on endothelium dependent (acetylcholine) and endothelium independent (glyceryl trinitrate) vasodilatation.

Methods

Animal preparation
Fifteen mongrel dogs of either sex (16-27 kg body weight) were anaesthetised with intravenous pentobarbitone sodium (25 mg·kg⁻¹) with maintenance doses (1-3 mg·kg⁻¹ intravenously) when required. The dogs were intubated with an endotracheal tube and ventilated by a volume regulated ventilator (model 101, Princeton Medical Instruments, Natick, MA, USA) to keep pH within the normal range. We kept body temperature constant by a heating pad, and drained the urinary bladder through a urethral catheter.

An electromagnetic flow probe (Nycotron, Norway) was placed on the femoral artery for measuring femoral blood flow. We advanced one polyethylene catheter through a side branch into the femoral artery proximal to the flow probe to the flow probe for drug infusions. We advanced one polyethylene catheter through a side branch into the femoral artery proximal to the flow probe to the flow probe for drug infusions.
Experimental procedure
In the first group of dogs acetylcholine and glyceryl trinitrate were infused into the femoral artery to select the dose of each agent which increased femoral blood flow to a similar extent. Each drug was infused until a stable femoral flow was reached (1-2 min). Both basal femoral flow and sensitivity to acetylcholine and glyceryl trinitrate varied considerably between dogs. The selected doses of acetylcholine (range: 0.1-1 μg·min⁻¹) and glyceryl trinitrate (range: 5-50 μg·min⁻¹) increased femoral flow by 66(SEM 10)% and by 56(10)%, respectively. The intra-arterial infusion rate was kept constant at 1 ml·min⁻¹. Because basal femoral blood flow varied from 80 ml·min⁻¹ to 190 ml·min⁻¹ between dogs, the dose of the arginine analogues was adjusted to give a fixed femoral arterial blood concentration. Before administration of LN MMA, D-arginine and L-arginine were infused into the femoral artery for 5 min to yield a concentration of 1.0 μmol per ml arterial blood (1.0 μmol·ml⁻¹), to exclude a direct vasoactive effect of these arginine enantiomers.

In all 12 dogs LN MMA (0.28 μmol·ml⁻¹) was infused into the femoral artery for 5 min. Pilot experiments showed that after stopping the LN MMA infusion, femoral blood flow remained low and stable for at least 20 min. After discontinuing the LN MMA infusion the selected doses of acetylcholine and glyceryl trinitrate were infused in random order within 5 min. Eleven dogs received intra-arterial infusion of acetylcholine and 10 dogs received an infusion of glyceryl trinitrate. Thereafter, L-arginine (1.0 μmol·ml⁻¹) was infused in all dogs until femoral flow was normalised. In five dogs we infused D-arginine (1.0 μmol·ml⁻¹) before the infusion of L-arginine, to determine whether the normalisation of femoral blood flow was stereospecific. After administration of L-arginine the selected doses of acetylcholine or glyceryl trinitrate were infused in random order for a third time.

The effects of NO ARG were examined in six dogs, in which the experiments were continued after examination of the effects of LN MMA. An identical experimental procedure as with LN MMA was followed, first with a low dose of NO ARG (0.07 μmol·ml⁻¹) for 5 min, and then with a high dose of NO ARG (0.56 μmol·ml⁻¹) for 5 min. For both doses of NO ARG, the selected doses of acetylcholine and glyceryl trinitrate were infused in random order before and after administration of NO ARG and after administration of D- and L-arginine. Infusion of NO ARG (0.07 μmol·ml⁻¹) started 66(12) min after onset of LN MMA infusion. Infusion of NO ARG (0.56 μmol·ml⁻¹) started 63(11) min after onset infusion of NO ARG (0.07 μmol·ml⁻¹).

In the second group, consisting of three dogs, the ability of L-arginine to reverse fully the effect of NO ARG (0.56 μmol·ml⁻¹) on femoral blood flow was examined. L-arginine was infused for 5 min in concentrations of 5.6 μmol·ml⁻¹ and 33.6 μmol·ml⁻¹. NO ARG (0.56 μmol·ml⁻¹) was then infused for 5 min, and when femoral blood flow had stabilised, the two doses of L-arginine were infused for a second time.

Animals were maintained and housed throughout under the conditions set by the Norwegian Council for Animal Research.

Drugs
N⁵-nitro-L-arginine, D-arginine hydrochloride, and L-arginine hydrochloride were purchased from Sigma, St Louis, MO, USA; acetylcholine from Dispersa, Switzerland; and glyceryl trinitrate from Hydro Pharma, Norway. N⁵-monomethyl-L-arginine was kindly provided by Dr H Hodson, Wellcome Research Laboratories, Beckenham, UK. All drugs were dissolved in saline and infused into the femoral artery at a rate of 1 ml·min⁻¹. To dissolve NO ARG and the high concentrations of L-arginine, sodium hydroxide was added to the solutions and pH adjusted to 8.0 after NO ARG had dissolved and to 7.4 after L-arginine had dissolved.

Statistical analysis
Data are presented as mean(SEM). Each dog served as its own control and the Friedman test was used to calculate the probability values for multiple comparisons. The Wilcoxon two tailed signed rank test was used for paired statistical analysis. A probability value p<0.05 was considered statistically significant.

Results
Effects of LN MMA and NO ARG on basal femoral blood flow
L-arginine (1.0 μmol·ml⁻¹) had no effect on femoral blood flow when infused before LN MMA. Infusion of LN MMA (0.28 μmol·ml⁻¹) for 5 min reduced basal femoral flow by 44(SEM 3)% (p<0.001). Pilot experiments showed that neither longer infusion time, nor higher concentration of LN MMA increased the effect on basal femoral flow. As shown in fig 1, L-arginine (1.0 μmol·ml⁻¹) infused for 5 min completely re-established femoral flow in all experiments. Infusion of the low dose of NO ARG (0.07 μmol·ml⁻¹) for 5 min reduced basal femoral flow by 21(4)% (p<0.05). Subsequent infusion of L-arginine (1.0 μmol·ml⁻¹) for 5 min completely re-established the flow.

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/26/4/357/493550/1)

**Figure 1** Effect of LN MMA (0.28 μmol·ml⁻¹) on basal femoral blood flow in 12 dogs. Note that LN MMA substantially decreased basal femoral blood flow and that L-arginine (1.0 μmol·ml⁻¹) re-established femoral blood flow.
Nitric oxide and canine femoral circulation

Figure 2 Reduction in basal femoral blood flow in the six dogs in which both LNMMa (dotted column) and NOARG (hatched column) were infused. Values are means, bars=SEM. *p<0.05 v both doses of NOARG; †p<0.05 v low dose NOARG

Infusion of the high dose of NOARG (0.56 μmol·ml⁻¹) for 5 min reduced basal femoral blood flow by 29(3)% (p<0.05). As shown in fig 2, this reduction was significantly greater (p<0.05) than the reduction after infusion of the low dose of NOARG, but significantly smaller (p<0.05) than after infusion of LNMMa. L-arginine did not completely reverse the reduction in femoral flow after administration of the high dose of NOARG. In three dogs flow was partially reversed (approximately 50%), and in three dogs flow was unaltered even though the femoral arterial blood concentration of L-arginine was increased to 4.5 μmol·ml⁻¹.

To examine the ability of larger doses of L-arginine to reverse fully the effect of NOARG (0.56 μmol·ml⁻¹) on femoral blood flow, additional experiments were performed in three dogs. Infusion of L-arginine 5.6 μmol·ml⁻¹ (10-fold molar excess) and 33.6 μmol·ml⁻¹ (60-fold molar excess) increased femoral flow by 30% and 100%, respectively. Similar increments were observed when L-arginine was infused after administration of NOARG (0.56 μmol·ml⁻¹). However, when the infusions of these high doses of L-arginine were discontinued, femoral flow rapidly declined to preinfusion levels (after NOARG) or almost to preinfusion levels (after NOARG). Thus, whereas L-arginine (1.0 μmol·ml⁻¹) completely reversed the effects of the low dose of NOARG, a 60-fold molar excess of L-arginine only reversed the vasoconstrictor effect of the high dose of NOARG by approximately 40%.

D-arginine did not alter femoral blood flow either before or after infusion of the two nitric oxide inhibitors. Systemic blood pressure was unaltered during and after the infusions of LNMMa and NOARG.

Effect of LNMMa and NOARG on femoral blood flow responses to acetylcholine and glyceryl trinitrate

Figure 3 shows that the selected dose of acetylcholine (range: 0.1-1 μg·min⁻¹) caused an increase in femoral blood flow which was reduced by 27(8)% (p<0.001) after infusion of LNMMa (0.28 μmol·ml⁻¹). Administration of L-arginine (1.0 μmol·ml⁻¹) completely re-established the flow response to acetylcholine. The selected dose of glyceryl trinitrate (range: 5-50 μg·min⁻¹) caused an increase in femoral flow which was unaltered by infusion of LNMMa and L-arginine.

As shown in fig 4, the flow responses to acetylcholine and glyceryl trinitrate were not significantly altered by infusion of the low dose of NOARG (0.07 μmol·ml⁻¹). However, after infusion of the high dose of NOARG (0.56 μmol·ml⁻¹) the flow response to acetylcholine was reduced by 60(7)% (p<0.05). Even though the high dose of NOARG did not reduce basal femoral blood flow to the same extent as LNMMa, the flow response to acetylcholine was significantly more reduced with the high dose of NOARG than with LNMMa. L-arginine did not re-establish the flow response to acetylcholine, even when the femoral arterial blood concentration was increased to 4.5 μmol·ml⁻¹. The flow response to glyceryl trinitrate was unaltered by infusion of NOARG (0.56 μmol·ml⁻¹). Systemic blood pressure was not affected during infusions of acetylcholine and glyceryl trinitrate.

Discussion

This study is to our knowledge the first to compare the effect of the two nitric oxide inhibitors, N⁶-monomethyl-L-arginine
and N\textsuperscript{\textgreek{n}}-nitro-L-arginine, in limbs in vivo. Inhibiting femoral nitric oxide synthesis in anaesthetised dogs reduced basal femoral blood flow to a substantial degree. The two nitric oxide inhibitors LNMMA and NOARG exerted different effects on basal femoral blood flow and on the flow response to acetylcholine.

**Effect of LNMMA and NOARG on basal femoral blood flow**

LNMMA induced the greatest reduction in basal femoral blood flow. The increase in peripheral vascular tone after infusion of LNMMA reduced femoral blood flow by 45%. Vallance and coworkers\textsuperscript{19} infused LNMMA into the forearm of human volunteers. Measured with strain gauge plethysmography, basal forearm blood flow was reduced by approximately 40% after administration of LNMMA. As blood flow in their preparation was measured indirectly, the absolute concentrations of the drugs used could not be determined precisely.

NOARG reduced basal femoral blood flow by 5%. Surprisingly, this reduction was considerably less than after infusion of LNMMA, even though the concentration of the high dose of NOARG was twice the molar dose of LNMMA. Studies on preconstricted endothelial cells and isolated vessels have shown that NOARG is much more potent (10-100 times) than LNMMA as an inhibitor of nitric oxide formation.\textsuperscript{18,20-22,26-28} The results of a study performed on isolated perfused rat kidneys\textsuperscript{29} indicated that NOARG was more potent than LNMMA in increasing renal vascular resistance. Why LNMMA is a more potent inhibitor of basal nitric oxide synthesis than NOARG in the femoral circulation of dogs is not clear. Differences in sensitivity for the two nitric oxide inhibitors in different species and tissues must be considered. Other possibilities are differences between conductance and resistance vessels and differences between in vitro and in vivo conditions.

The great decrease in basal femoral blood flow after administration of the nitric oxide inhibitors is in striking contrast to the minor changes after the inhibition of other vasoactive substances such as angiotensin, prostaglandins, histamine, and serotonin.\textsuperscript{35,36} Thus nitric oxide seems to be unique in determining resting blood flow in limbs and may be an important regulator of arterial blood pressure during various physiological conditions.

Several investigators have found an increase in arterial blood pressure when LNMMA or NOARG was infused intravenously.\textsuperscript{13,14,22,28} Cardiac output was measured in two of these studies and was found to be unaltered\textsuperscript{29} or moderately decreased.\textsuperscript{30} It remains to be thoroughly examined how inhibition of nitric oxide synthesis affects the vascular resistance in different organs. The present study suggests that an increase in vascular tone in limbs contributes to the hypertensive effect observed after systemic administration of LNMMA and NOARG.

That the vasoconstrictor effects of LNMMA and the low dose of NOARG in the femoral arterial bed of dogs can be counteracted by L-arginine but not by D-arginine is consistent with the previously described stereospecificity of the nitric oxide forming enzyme(s).\textsuperscript{1-9} L-arginine re-established basal femoral blood flow and the flow response to acetylcholine after administration of LNMMA. This supports the hypothesis that the inhibitory effect of LNMMA on nitric oxide synthesis can be explained by competitive inhibition.\textsuperscript{14}

L-arginine did not re-establish femoral blood flow or the flow response to acetylcholine after administration of the high dose of NOARG. Even though L-arginine was infused at a 60 molar higher concentration than NOARG, femoral blood flow was only reversed by approximately 40%. Previous reports concerning the reversibility of NOARG are conflicting. In rabbit aortic ring preparations Moore and coworkers\textsuperscript{31} reported complete reversal of the effects of NOARG by giving L-arginine. On the other hand, Kobayashi and Hattori\textsuperscript{32} reported that relaxations induced by acetylcholine were almost irreversibly inhibited after treatment with NOARG in rabbit aortic ring preparations. A 30- to 100-fold molar excess of L-arginine was required to fully reverse the blood pressure increasing effect of NOARG in rats,\textsuperscript{32} and Midgley and coworkers\textsuperscript{33} showed that a 30-fold higher concentration of L-arginine only partially reversed the effect of NOARG in rabbit femoral arteries. Similarly, Elnes and coworkers\textsuperscript{31} in conscious dogs, reported that L-arginine failed to normalise systemic blood pressure after intravenous infusion of NOARG.

Poor reversibility of NOARG mediated effects may be related to high affinity of NOARG for the enzyme(s) synthesising nitric oxide. Furthermore, the nitroguanidino moiety of NOARG is more hydrophobic than the cationic guanidino moiety of L-arginine. NOARG may therefore be taken up and "trapped" more rapidly than L-arginine in endothelial cells. In addition to competitive inhibition of the enzyme(s) synthesising nitric oxide, NOARG may induce irreversible or allosteric inhibition or alter the uptake and/or utilisation of L-arginine in the endothelium.

**Effect of LNMMA and NOARG on femoral blood flow responses to acetylcholine and glyceryl trinitrate**

The high dose of NOARG reduced the flow response to acetylcholine by 60%. Although the high dose of NOARG did not reduce basal femoral blood flow to the same extent as LNMMA, the flow response to acetylcholine was significantly more reduced with the high dose of NOARG than with LNMMA. This dissociation between the two nitric oxide inhibitors has not previously been described. Different mechanisms for nitric oxide synthesis may exist during basal release and during receptor stimulation with acetylcholine, and the two nitric oxide inhibitors may affect these two ways of synthesising nitric oxide differently.

LNMMA inhibited the flow response to acetylcholine in the present study by 27%. In the human forearm Vallance and coworkers\textsuperscript{19} also reported that LNMMA reduced the flow response to acetylcholine. The modest inhibition of the flow response to acetylcholine compared to the substantial effect of LNMMA on basal femoral blood flow found in our study, agrees with the finding of Rees et al.\textsuperscript{9} These investigators reported that the concentration of LNMMA which produced 50% contraction of vessel rings was six times lower than the concentration required to inhibit acetylcholine induced relaxation by 50%.

In agreement with previous studies\textsuperscript{8,9,19} the nitric oxide inhibitors used in the present study failed to completely antagonise the flow response to acetylcholine. It is not clear whether the proportion of acetylcholine induced vasodilatation that is resistant to the nitric oxide inhibitors is mediated by nitric oxide arising from a pool of L-arginine inaccessible to the inhibitors, or by nitric oxide arising from a source other than L-arginine. The presence of endothelium derived relaxing factor(s) different from nitric oxide, such as endothelium dependent hyperpolarising factors\textsuperscript{34} or vasodilating prostaglandins,\textsuperscript{35} has also been suggested.

Vasodilatation by glyceryl trinitrate was unaltered after infusion of LNMMA and NOARG, suggesting that the decrease in basal femoral blood flow was not due to non-
specific smooth muscle contraction. The responsiveness of the vascular smooth muscle to nitrous vasodilators such as nitric oxide is therefore intact after administration of LNMMA and NOARG.

In summary, our findings indicate that in the femoral circulation of dogs there is a continuous basal release of nitric oxide from L-arginine that promotes vasodilatation. The results obtained by infusing LNMMA suggest that more than 40% of basal femoral blood flow is dependent on nitric oxide. LNMMA was more potent than NOARG in reducing basal femoral blood flow, whereas the high dose of NOARG reduced the flow response to acetylcholine by as much as 60%.

Received 10 June 1991; accepted 12 November 1991

This study was supported by Anders Jahre’s Fund for Promotion of scientific research, the Norwegian Council for Cardiovascular Diseases and Professor Carl Semb’s Medical Research Fund. We thank Bjørn Amundsen, Bjørn Aasbø, Heidi Bråten, Kjersti Eriksen, Ivar Bjarne Hansen, Unni Lille Henriksen, Mette Reh Hølthe, Severin Leraand, Ove Moen, Annie Cristin Nilsen, Gry Steinsæter Jensen, Gerd Tørgersen, and Turid Verde for their skilled assistance.

Key terms: acetylcholine; arginine; dog; endothelium derived relaxing factor (EDRF); femoral circulation; glyceryl trinitrate; N\(^-\)monomethyl-L-arginine; N\(^-\)nitro-L-arginine; nitric oxide.

---