Experimental Physiology

Response of isolated ruminant mammary arteries to the long R_3 analogue of insulin-like growth factor I

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Isolated mammary arteries from ruminants were used in a conventional organ bath system. Acetylcholine relaxed bovine but not ovine mammary arteries; both types responded to sodium nitroprusside. Noradrenaline (NA) caused a dose-dependent increase in generated tension. An analogue of insulin-like growth factor I (long R_3-IGF-I) caused a rightward shift in the NA response curve in bovine vessels with intact endothelium ($P < 0.02$), and also in sheep arteries ($P < 0.01$). In bovine vessels, this effect was abolished when the endothelium was removed. The effect of long R_3-IGF-I in bovine vessels was abolished by N^{ω}-nitro-L-arginine methyl ester (L-NAME) an inhibitor of nitric oxide synthase, suggesting the effect of IGF-I on mammary arteries *in vitro* requires NO generation. *Experimental Physiology* (2000) **85.3**, 275–279.

The sensitivity of mammary gland blood flow to exogenous biogenic amines such as noradrenaline has been known for half a century (Linzell, 1950; Hebb *et al*. 1951), yet the exact nature of the interaction is still not well-documented. This is surprising, given the correlation between milk yield and mammary blood flow (Linzell, 1960), which emphasises the importance of being able to regulate vascular tone with respect to the ability to produce milk. It is not clear, however, whether increased mammary gland blood flow causes increased milk production, or comes about as a result of increased metabolic demand by the gland. Evidence to suggest that the rate of mammary blood flow *per se* can regulate milk production comes from experiments where infusion of known vasorelaxants has increased both blood flow and milk yield. Nitric oxide is a potent vasodilator, and infusion of the nitric oxide donor NONOate directly into the pudic artery of lactating goats increased blood flow and ipsilateral milk production (Lacasse *et al*. 1996). Conversely, hormones can have indirect effects on mammary blood flow: administration of growth hormone (GH) to ruminants increases milk production by mechanisms which do not involve the direct action of GH on the udder (Collier *et al*. 1984). Plasma levels of insulin-like growth factor I (IGF-I) are elevated following GH injections (Davis *et al*. 1987), therefore it is possible that IGF-I is involved in the associated increase in mammary gland blood flow in ruminants. Evidence to support this comes from experiments where infusion of IGF-I into the pudic artery in goats resulted in an increase in blood flow and milk yield in the infused side (Prosser *et al*. 1990, 1994). Peripheral injection of the peptide into the jugular vein produced no significant changes, possibly because IGF-I had been bound by endogenous circulating binding proteins (Davis *et al*. 1989). In order to determine whether or not IGF-I acts directly on mammary blood vessels, we have studied the *in vitro* response of arteries isolated from the udders of lactating/non-lactating ruminants to the IGF-I analogue, long R_3-IGF-I. This analogue was chosen since it retains the biological activity of the native peptide, yet is not readily bound by endogenous binding proteins (Francis *et al*. 1992).

METHODS

The normal Tyrode solution (perfusion solution) contained (mM): NaCl, 134; KCl, 6; Hepes, 10; $CaCl_2$, 1; $MgCl_2$, 1 and glucose, 10, pH was adjusted to 7.4 at 37 °C with 4 M NaOH. Solutions were made up using double-distilled water; all buffer reagents were Analar grade or better. Noradrenaline bitartrate, acetylcholine chloride, sodium nitroprusside, and N^{ω}-nitro-L-arginine methyl ester (L-NAME) were all purchased from Sigma. Human recombinant long R_3-IGF-I was purchased from Peninsula Laboratories, Europe Ltd (Merseyside, UK). Concentrated stocks of the drugs were made up in Tyrode solution with no added magnesium, calcium or glucose, and stored as aliquots at −20 °C. Drugs were thawed only once on the day of use.

Bovine mammary artery collection

Udders from freshly slaughtered cows (mixed breeds in mid to late lactation) were collected at the local slaughterhouse, weighed and transported to the Institute. Sections (2–3 cm × 2–3 mm) of a branch of the cranial mammary artery (mammaria cranialis; usually the first branch after the external pudic artery bifurcates into caudal and cranial mammary arteries), were quickly dissected out, and transferred to the laboratory in perfusion medium (i.e. normal Tyrode solution as above) kept at room temperature. The perfusion medium was not aerated during transfer from the dissection site to the laboratory (a time of 5–10 min).

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Sheep mammary artery collection

Non-lactating ewes (Finn × Dorset) were anaesthetised by intravenous injection of sodium pentobarbitone (20–30 ml of 60 mg ml^{-1}), and once the required plane of anaesthesia had been reached (no response to corneal stimulation), were slaughtered by exsanguination. The udder was removed, sections of main mammary artery (2–3 cm × 2–3 mm) were excised and removed to the laboratory as above.

Artery preparation and mounting

The arteries were gently cleaned of fat and connective tissue, and cut into rings of 2–3 mm in length. The rings were mounted on silver stirrups in a standard, heated, 10 ml organ bath (Linton Instrumentation, Norfolk, UK), and connected to a Grass FT103 force transducer. The force transducer was connected to a four channel bridge amplifier (Department of Physiology, Monash University, Australia), and the output fed to a chart recorder and computer fitted with an A/D board for data logging. Preparations were allowed to equilibrate in perfusion medium gassed with 100 % O_2 with no applied tension for 30 min. Arteries were then washed, and resting tension set to 4 g, since preliminary experiments with ruminant mammary arteries had shown the contractile response to be maximal and constant with resting tensions of between 2–8 g. Once a steady baseline had been established, doses of potassium chloride (KCl; 80 mM final concentration) followed by wash steps were applied until a reproducible contraction was obtained. The presence or absence of endothelium was tested by applying noradrenaline (NA; final concentration 1 μM), followed by acetylcholine (ACh; final concentration 10 μM), and finally sodium nitroprusside (SNP; final concentration 10 μM). A preparation was deemed to have intact endothelium if ACh induced >50 % relaxation, maximal contraction being 0 %, and baseline 100 %.

The preparations were then washed several times, and once baseline had been re-established, NA was added and cumulative response curves were plotted. During recovery from contractions, a second wash was given 5 min after the first, followed by further washes every 15 min. For experiments with long R_3-IGF-I alone, or L-NAME in combination with long R_3-IGF-I, the drugs were added after the second wash (i.e. after 5 min), and re-added after each of two subsequent washes so that the pre-incubation time with each drug was 30 min prior to the start of data collection for cumulative response curves (the drugs remained present throughout the dose–response experiments). To minimise the effect of different preparation masses between experiments, contractions were expressed as a percentage of the maximum force generated within a response curve. The estimated concentration of agonist required to give a response which was 50 % of maximal contraction (EC_{50}) was calculated from equations derived by fitting a curve to the points of the dose–response curve using 'Curve Expert' software (version 1.34; author, Daniel Hyams, Starkville, MS, USA). Differences between curves were assessed by two-way ANOVA, and differences between $-\log EC_{50}$ values (pEC_{50}s) were examined using Student's paired or unpaired *t* test where appropriate. Values are expressed as means ± S.E.M., *n* indicates the number of animals from which preparations were used. A *P* value of <0.05 was taken to be statistically significant.

RESULTS

Bovine vessels showed an ability to relax in the presence of ACh and SNP (Fig. 1*A*) whereas arteries from sheep udders always responded poorly to ACh, yet relaxed with SNP (Fig. 1*B*). The more stable analogue of ACh, carbachol had little effect either (Fig. 1*B*); this may reflect a lack of response to these agents in this preparation, rather than a denuded endothelium (see Discussion). Thus only in bovine preparations could we determine with any certainty the existence of a functional endothelium.

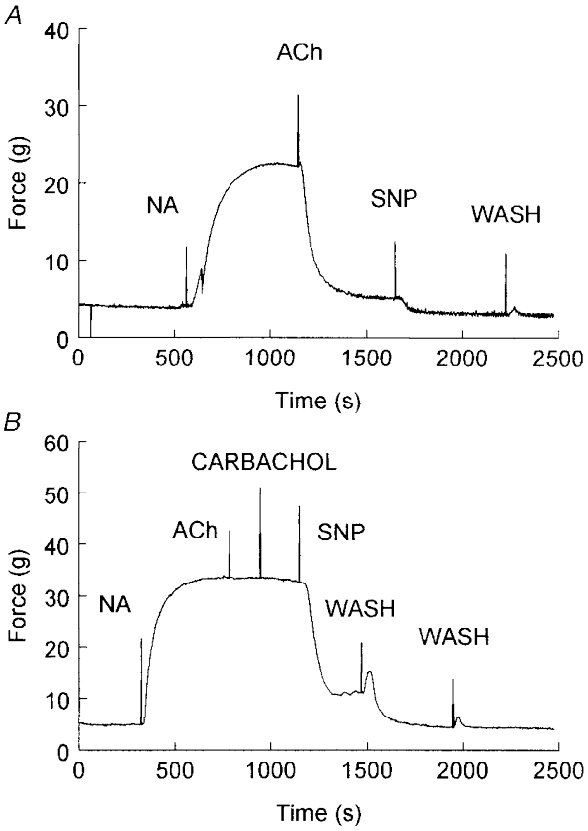

Figure 1

Response of bovine (*A*) and ovine (*B*) mammary arteries to vasodilators. ACh, acetylcholine (10^{-5} M); NA, noradrenaline (10^{-6} M); SNP, sodium nitroprusside (10^{-5} M).

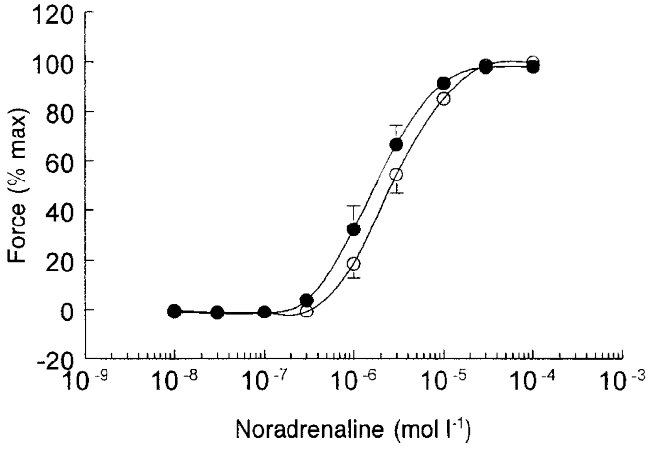

Figure 2

Mean dose–response curves to NA of bovine mammary arteries with (●, *n* = 13 animals) or without (○, *n* = 15 animals) endothelium.

Increasing doses of NA caused a dose-dependent response in bovine preparations with or without endothelium (Fig. 2; $n = 13$ and $n = 15$, respectively). Pre-incubation with long R_3-IGF-I shifted the NA response curve to the right in bovine arteries with intact endothelium (Fig. 3*A*; $P < 0.02$, two-way ANOVA; $n = 13$), but not in denuded vessels (Fig. 3*B*; $n = 15$). In sheep mammary arteries, R_3-IGF-I had the same effect on the NA dose–response curve as in bovine vessels with intact endothelium (Fig. 4; $P < 0.01$, two-way ANOVA; $n = 3$). Similarly, the pEC_{50} with NA in bovine arteries with intact endothelium was decreased following pre-incubation with long R_3-IGF-I (Fig. 5*A*; 5.71 ± 0.08 *vs.* 5.49 ± 0.05; $P < 0.002$, $n = 13$), but not in denuded vessels (Fig. 5*A*; 5.67 ± 0.01 *vs.* 5.65 ± 0.13; $n = 15$). Pre-incubation of bovine arteries with long R_3-IGF-I and the nitric oxide synthase inhibitor L-NAME abolished the decrease in pEC_{50} obtained with long R_3-IGF-I alone in arteries with intact endothelium (Fig. 5*B*; $P < 0.05$, two-way ANOVA; $n = 8$), but had no effect in denuded vessels (Fig. 5*B*; $n = 5$). In both cases, the responses of arteries treated with long R_3-IGF-I together with L-NAME were not significantly different from the control values (intact endothelium: control 5.78 ± 0.1 *vs.* L-NAME 5.72 ± 0.1, $n = 8$; denuded endothelium: control 6.03 ± 0.1 *vs.* L-NAME 6.09 ± 0.1, $n = 6$).

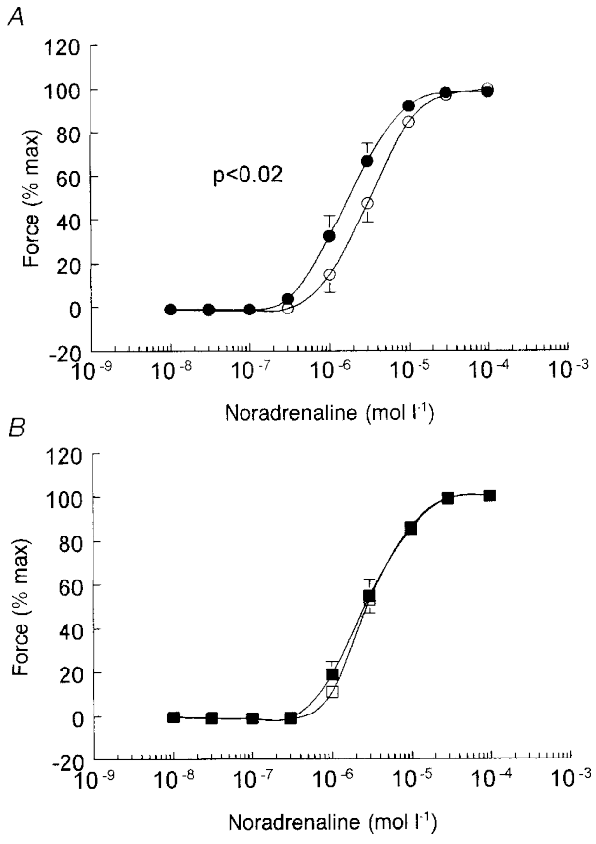

Figure 3

Effect of long R_3-IGF-I (100 nM) on NA-induced contraction in bovine mammary arteries with (*A*; ●, control; ○, long R_3-IGF-I; $n = 13$ animals), and without (*B*; ■, control; □, long R_3-IGF-I; $n = 15$ animals) endothelium.

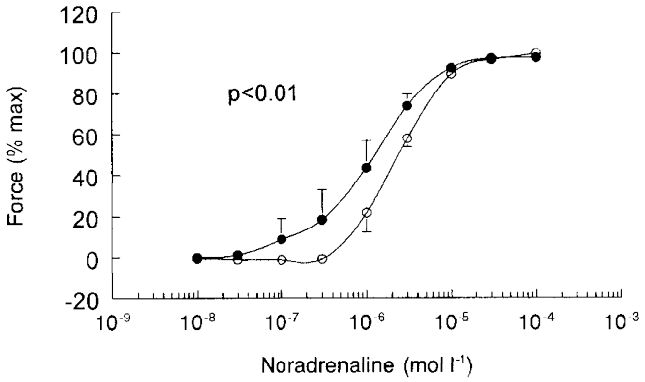

Figure 4

Effect of long R_3-IGF-I (100 nM) on NA-induced contraction in ovine mammary arteries (●, control; ○, long R_3-IGF-I; $n = 3$ animals)

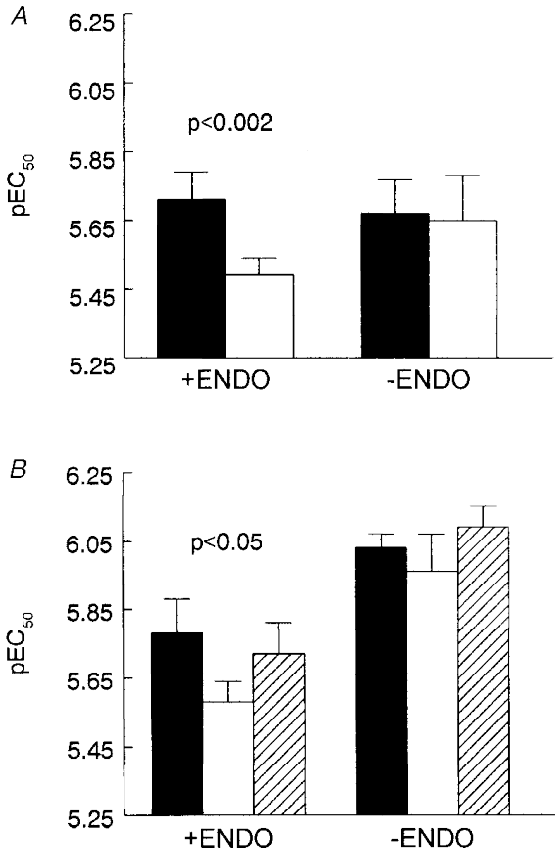

Figure 5

Bovine mammary artery pEC_{50} for NA alone (■) or with long R_3-IGF-I (□) in the presence (+ENDO; $n = 13$ animals), or absence (–ENDO; $n = 15$ animals) of endothelium (*A*), and NA alone (■) with long R_3-IGF-I with (▨) and without (□) L-NAME in the presence (+ENDO; $n = 8$ animals), or absence (–ENDO; $n = 5$ animals) of endothelium (*B*). $pEC_{50} = -\log EC_{50}$, where EC_{50} is the estimated concentration of agonist giving a response which was 50 % of maximal contraction.

DISCUSSION

We have demonstrated that isolated mammary arteries from cows or sheep respond to NA, with a pEC_{50} of 5.71 ± 0.08 and 5.95 ± 0.22, respectively. These values suggest that the preparation described here is slightly less sensitive to NA than the only other published value for bovine intramammary arteries (6.86 ± 0.17; Pereira *et al*. 1997). It is not clear why the arteries in sheep showed no response to ACh; it is unlikely that this was due to significant cholinesterase activity, since the more stable analogue, carbachol, had no effect either. Neither was it an inability of the vessels to relax, since SNP invariably produced a pronounced vasodilatation. A similar lack of response to ACh has been demonstrated in sheep pulmonary arteries, where ACh failed to relax arteries < 3 mm in diameter, although the vessels were still responsive to SNP (Kemp *et al*. 1997). The authors suggest that this reflected the regulation of resistance in that particular vascular bed, and indeed, a similar situation may occur in the mammary vessels. Given that in the present study the sheep mammary arteries were collected sooner after death and excised more rapidly than the bovine equivalents, and intact endothelium could regularly be demonstrated in the latter, this indicates that it is unlikely the endothelium was destroyed during experimental manipulation, hence perhaps the poor response to ACh is an intrinsic property of the ovine mammary artery.

Very little information exists about the behaviour of ruminant mammary arteries *in vitro*. Trakranrungsie & Will (1997) and later Pereira & Will (1997) used isolated bovine intramammary arteries in a conventional organ bath apparatus similar to the one we describe. They also found that the optimal resting tension for small (1.5–2.5 mm diameter) ruminant mammary arteries was 4 g, and that the vessels were sensitive to NA. From results obtained using the non-selective antagonists prazosin (α_1) and yohimbine (α_2), they suggest that most of the noradrenaline effect is mediated via the α_1 receptor (Pereira & Will, 1997). However, further classification of the adrenergic receptor subtypes remains to be carried out using more selective and specific agonists and antagonists. Experiments with isolated goat pudendal arteries (Jakobsen *et al*. 1994) also suggest that the *in vitro* ruminant mammary artery preparation is highly sensitive to NA, thus providing further evidence that NA is an important physiological mediator of vascular tone in ruminants.

Infusion of IGF-I into the pudic artery of goats causes an increase in blood flow presumably by vasodilatation (Prosser *et al*. 1990; Prosser & Davis, 1992), but such *in vivo* experiments could not determine whether the peptide acted directly or indirectly on blood vessel tone. This is not a unique response of the mammary artery since other effects of IGF-I on vascular tone and blood flow have been reported: IGF-I increased blood flow through iliac, renal or mesenteric vascular beds (Pete & Dunbar, 1998), and low doses (< 30 nM) attenuated NA-induced contraction in rat aorta (Wu *et al*. 1994). These vascular responses to IGF-I are not thought to be due to changes in glucose utilisation, since, at least in rats or humans, recombinant human IGF-I (rhIGF-I) produced vascular effects with no changes in glucose disposal (Pete *et al*. 1996; Pendergrass *et al*. 1998). Analogues of IGF-I such as R_3-IGF-I act in a manner similar to the native polypeptide by increasing blood flow though an organ (e.g. the skin, Harris *et al*. 1993). Our observations that long R_3-IGF-I attenuated NA-induced contraction *in vitro* suggest that the *in vivo* effect on mammary blood flow is due, at least in part, to a direct action on the mammary vasculature. We found the response to be dependent on the presence of an intact endothelium (where the presence of such could be demonstrated), and sensitive to the nitric oxide synthase inhibitor L-NAME. Together, these observations strongly suggest that long R_3-IGF-I acts on isolated ruminant mammary artery via nitric oxide (NO). Further evidence for IGF-I-mediated NO effects have been found in other studies: for example, the effect of IGF-I on rat aorta was also endothelium dependent (Wu *et al*. 1994). Furthermore, rhIGF-I attenuated the NA-induced contraction in rat tail artery by a mechanism sensitive to L-NAME (Walsh *et al*. 1996). Another NOS inhibitor L-NMMA reduced the rhIGF-I-stimulated increase in forearm blood flow in humans (Fryburg, 1996). Finally, in experiments with isolated endothelial cells from human umbilical veins or rat renal arteries (Tsukahara *et al*. 1994), rhIGF-I stimulated NO production, and this was inhibited by L-NAME. Dexamethasone, an inhibitor of the inducible form of NOS (iNOS; Radomski *et al*. 1993) had no effect suggesting rhIGF-I acts in those preparations by stimulating the constitutive form, cNOS. In conclusion, our study has shown that bovine and ovine mammary arteries respond in a dose-dependent manner to NA, and this response is attenuated by incubation with long R_3-IGF-I by a mechanism which is likely to depend on NO production.

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