Acta Oncologica Vol. 34, No. 3, pp. 373–377, 1995

THE INFLUENCE OF NITRIC OXIDE ON TUMOUR VASCULAR TONE

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Acetylcholine and sodium nitroprusside, which vasodilate via release of NO by endothelium-dependent and endothelium-independent mechanisms respectively, had little effect on tumour vascular resistance when administered to tissue-isolated tumours perfused in their normal state. However, under phenylephrine-induced vasoconstriction, sodium nitroprusside induced vasodilation whilst acetylcholine induced a small vasoconstriction. Phenylephrine itself induced an oscillatory change in tumour perfusion pressure. The nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine (L-NNA) caused a dose-dependent increase in vascular resistance in ex vivo perfused tumours which was greater than that in normal perfused hindlimbs. Systemic administration of L-NNA caused a 50% decrease in tumour blood flow which was a larger effect than in any of the normal tissues studied except spleen and skeletal muscle. Modification of NOS activity in tumours is a promising means for selective tumour blood flow modification. Investigation of endothelium-dependent versus endothelium-independent methods for modifying tumour blood flow may provide methods for further selectivity.

Nitric oxide (NO) is recognized as the primary endothelium-derived relaxing factor (EDRF) (1, 2). Recently, inhibitors of nitric oxide synthase (NOS) have been shown to reduce blood flow and energy status in tumours (3, 4) suggesting that tumour vascular tone can be influenced by endothelial-derived factors.

The aims of the present study were to determine 1) whether tumour vascular tone can be influenced by endothelium-dependent mechanisms, 2) whether tumours are under the influence of a NO-induced vasodilator tone, and 3) whether NOS inhibition has a selective effect on tumour blood flow.

Ex vivo perfusions of 'tissue-isolated' tumours were carried out in order to measure tumour vascular response to drugs without the complication of systemic effects. The relative effects of acetylcholine and sodium nitroprusside on tumour vascular tone were determined. These compounds vasodilate by increasing NO levels in vascular smooth muscle, acetylcholine via receptor activation (endothelium-dependent) and sodium nitroprusside via direct release of NO (endothelium-independent). The effect of inhibition of NOS by N^{ω}-nitro-L-arginine (L-NNA) on tumour vascular tone was also determined and compared to the effect on vascular tone of normal hindlimb during ex vivo perfusion. The selectivity of tumour blood flow modification by L-NNA was tested using systemic administration of L-NNA and measurement of blood flow to tumour and various normal tissues using uptake of ^{125}I-labelled iodoantipyrine (^{125}I-IAP).

Received 7 October 1994.
Accepted 21 November 1994.
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Paper presented at the Tumour Microenvironment Workshop in Granada, Spain, September 23–25, 1994.

Material and Methods

Tumours

Early generations (3rd to 10th away from the primary tumour) of the P22 transplanted rat carcinosarcoma, were used for these experiments (5). Subcutaneous tumours were grown in the left flank of 8–9-week-old male BD9 rats. Tumours were used for blood flow measurements when they reached a mean external diameter of 13–14 mm (2 to 3 weeks). Tissue-isolated tumours were grown in the

 ISSN 0284-186X

right inguinal fat pad of 10–11-week-old male BD9 rats essentially as described previously (6) except that no attempt was made to enclose the growing tumour. Tumours were only used for further study when the microcirculation was seen to derive solely from the epigastric vascular pedicle (approximately 50% of preparations). Tumours were used after 2 to 3 weeks' growth when they weighed 0.5–2.8 g.

Drugs

^{125}I-IAP for blood flow measurements was obtained from ICN Biomedicals Ltd. (Thame, U.K.). Acetylcholine and sodium nitroprusside (Sigma Chemical Company Ltd., Poole, U.K.) were administered to ex vivo perfused tumours by constant infusion into the perfusate line at concentrations of 0.5 μM to 100 μM. For each preparation, they were first sequentially administered to tumours in their normal state and subsequently during constriction induced by phenylephrine (Sigma Chemical Company Ltd., Poole, U.K.). Phenylephrine was administered by constant infusion at a concentration of 1 to 2 μM.

L-NNA and L-arginine (Sigma Chemical Company Ltd., Poole, U.K.) were administered to ex vivo perfused tumours and hindlimbs by constant infusion into the perfusate line at concentrations of 1.0 μM to 100 μM and 200 μM respectively. L-NNA was administered systemically to rats used for IAP blood flow measurements by bolus intravenous injection at a dose of 10 mg kg^{-1}.

Surgical preparation for ex vivo perfusions

The surgical procedure was essentially the same as that described elsewhere (6). Briefly, rats were anaesthetised with Hypnorm (Janssen Animal Health, Oxford, U.K.) and midazolam (Roche Products Ltd., Welwyn Garden City, U.K.) and the femoral artery and vein catheterized for connection to the perfusion apparatus. A tail artery and vein were also catheterized. Mean arterial blood pressure (MABP) of the rats was monitored using a physiological pressure transducer connected to the tail artery catheter.

Ex vivo perfusion set-up

Tumours and hindlimbs were perfused with Krebs-Henseleit (KH) buffer as prepared by Sigma with added bovine serum albumin (5%), insulin (50 iu/l), sodium pyruvate (0.2 mM) and heparin (7 000 USP units/l). Delivery, oxygenation and warming of the buffer have been described previously (7). Pressure was monitored distal to a bubble trap in the afferent perfusion line using a physiological pressure transducer.

Following catheterization, rats were heparinized (100 units per rat) before connecting the vascular supply of the tumour or hindlimb to the ex vivo circuit. Rats were killed by bolus injection of 0.3 ml Euthatal (RMB Animal Health Ltd., Dagenham, U.K.) into the tail vein catheter and the perfusion continued with the tissue in situ. Temperature at the surface of the tumour / limb was monitored using a thermocouple and maintained at 36°C using a lamp. Preparations were covered with gauze and kept moist with warmed saline.

Analysis of results from ex vivo perfusions

A constant perfusate flow rate was maintained throughout each experiment and perfusion pressure was monitored continuously. Vascular resistance was calculated from pressure ÷ flow rate and used as a measure of vascular tone. Results are generally expressed as a percentage change in vascular resistance following drug treatment.

Measurement of blood flow using ^{125}I-IAP

The method for measuring blood flow using tissue uptake of IAP has been described previously (5). Briefly, animals were anaesthetized with Hypnorm and midazolam and MABP was monitored via a tail artery catheter up to the point of blood flow measurement. Animals were heparinized. L-NNA (10 mg kg^{-1}) or the vehicle for the drug (acidified water) was administered by bolus injection into a tail vein (1.7 mls kg^{-1}). After 20 min, 0.37 MBq (10 μCi) of ^{125}I-IAP in 0.8 ml saline was infused into a second tail vein over 30 s. Simultaneously, free-flowing arterial blood from the tail artery was fractionated into pre-weighed vials. At the end of the 30 s the rat was killed by intravenous injection of Euthatal. Tumours and normal tissues (skin adjacent to the tumour, skin from the contralateral flank, skeletal muscle from the leg, small intestine, spleen, kidney, brain and heart) were excised, weighed and counted on a well-counter (Wallac, Milton Keynes, U.K.). Blood samples were weighed and counted. Blood flow to all the tissues was calculated from the tissue counts, the equilibrium partition coefficient of IAP in the different tissues (8) and the arterial input function derived from the arterial blood counts. This method is based on the Kety method (9) and is described in detail elsewhere (6).

Results

Ex vivo perfusions

Tumour vascular resistance measured in vivo was 279 ± 23 res. units (n = 7; 1 res. unit = 1 (mmHg) . ml^{-1} . g^{-1} . min^{-1}). Transfer to the ex vivo perfusion set-up caused a 50% reduction in resistance which is consistent with the difference in viscosity between blood and the perfusate buffer (10).

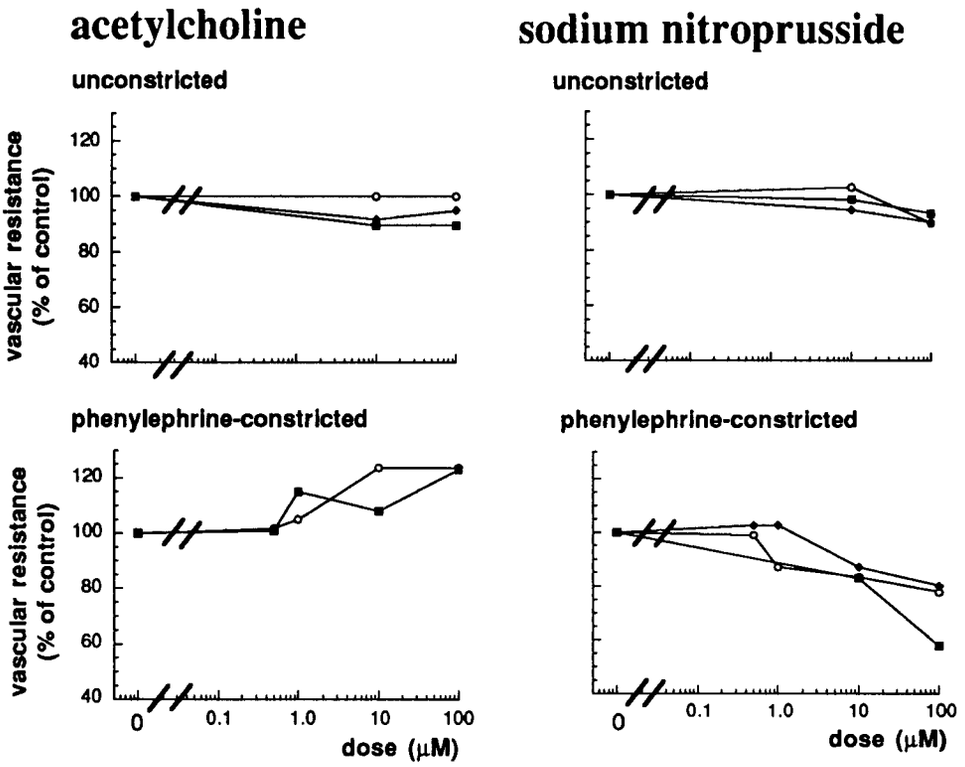

Fig. 1. Dose response for the effect of acetylcholine and sodium nitroprusside on tumour vascular resistance in ex vivo perfused tumours. Each set of symbols represents a single tumour. Phenylephrine-induced vasoconstriction was induced by constant infusion of the drug into the perfusate at a concentration of 1–2 μM.

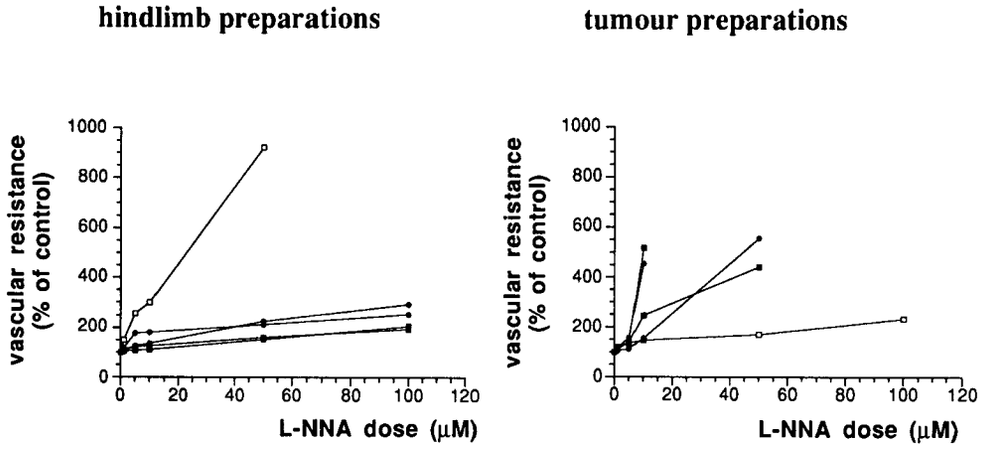

Fig. 2. Dose response for the effect of L-NNA on tumour and hindlimb vascular resistance in ex vivo preparations. Each set of symbols represents a single experiment.

The dose response for the effect of acetylcholine and sodium nitroprusside on tumour vascular resistance is shown in Fig. 1. This shows that in the unconstricted state neither drug has much effect. 1–2 μM phenylephrine induced a 45–70% increase in tumour vascular resistance. During phenylephrine-induced vasoconstriction, sodium nitroprusside induces a significant dose-dependent vasodilation whilst acetylcholine results in an anomalous further vasoconstriction. These results show that, at least in the preconstricted state, the tumours are incapable of endothelium-dependent vasodilation in response to acetylcholine. However, they are sensitive to the endothelium-independent vasodilating effects of NO released by sodium nitroprusside. It was also observed that phenylephrine induced a large oscillatory change in tumour vascular resistance (results not shown). The reason for this is not known although a similar effect has been reported for arteries supplying the P22 tumour growing in the inguinal fat pad (11).

Fig. 2 shows the effect of L-NNA administration on vascular resistance in the tumour preparations compared

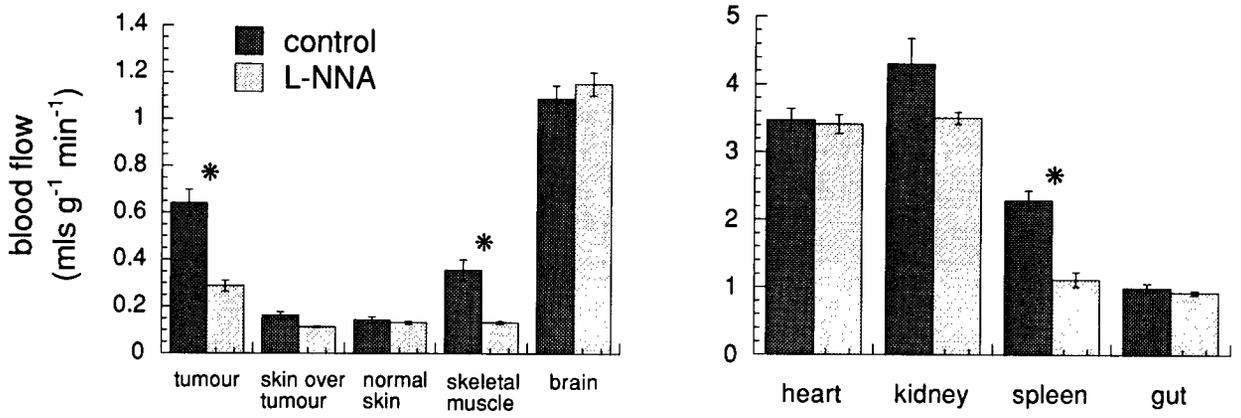

Fig. 3. Effect of intravenous administration of 10 mg kg^{-1} L-NNA on blood flow to subcutaneous P22 tumours and various normal tissues in the BD9 rat. Values are means ± 1 SEM (n = 8 in each group). * represents a significant difference ($p < 0.05$) between control group and L-NNA treated group (Students' t-test for unpaired data).

with the hindlimb preparations. Administration of L-NNA caused a very slow response in the tissues such that a plateau effect was never observed at any dose used. Therefore, in order to obtain the dose responses shown in Fig. 2, vascular resistance was measured at the end of 60-min infusion for the lowest dose (1.0 μM) and then at the end of the 30 min infusion for all subsequent doses. Results show that the tumour is more sensitive to L-NNA-induced vasoconstriction than the normal hindlimb under the conditions of this experiment. Although both tissues showed a dose response, vascular resistance increased more than 4-fold in 4 out of 5 tumour preparations whereas this level of vasoconstriction was only attained in 1 out of 5 hindlimb preparations. Infusion of L-arginine (200 μM) at the end of the experiments only partially reversed the effects of L-NNA (results not shown). This was true for both tumour and hindlimb preparations.

In vivo blood flow studies

L-NNA at 10 mg kg^{-1} induced a 40% increase in MABP and a 40% decrease in pulse rate in anaesthetised BD9 rats bearing subcutaneous P22 tumours. The effect on blood flow to tumours compared with normal tissues is shown in Fig. 3. A significant decrease in blood flow (>50%) was observed for tumour, skeletal muscle and spleen. The other tissues were unaffected. If we consider MABP to be equivalent to the perfusion pressure for each tissue, then these results are equivalent to a 40% increase in vascular resistance for most tissues and approximately a 100% increase for tumour, skeletal muscle and spleen. Thus, there is some tumour selectivity for L-NNA-induced blood flow reduction with a few important exceptions. Under the conditions of this experiment, the skeletal muscle was as sensitive to L-NNA as the tumour. This is in contrast to the ex vivo experiments described above.

Discussion

The response of the tumours to infusion of acetylcholine and sodium nitroprusside shows that 1) tumours perfused ex vivo are near-maximally dilated, 2) tumours are capable of vasodilation in response to NO, and 3) there is no endothelium-dependent vasodilation in response to acetylcholine. At this stage, we do not know whether the lack of acetylcholine-induced vasodilation was due to a lack of NO synthesis. We are currently investigating this possibility by measuring nitrate/nitrite levels in the venous outflow from tumours. The results certainly suggest that tumours may respond poorly to vasodilators which act via the endothelium. The vasoconstriction observed with the higher doses of acetylcholine can occur in normal tissues. It is known that receptors for acetylcholine exist on vascular smooth muscle cells as well as on endothelial cells and that activation of these receptors induces vasoconstriction (12). In tumours, the endothelial cell lining is often incomplete such that vasoconstriction may well dominate over endothelium-dependent vasodilation. This is analogous to the situation which occurs in normal vessels after damaging the endothelium (12). Our results suggest that vasoactive agents which act via the endothelium could provide a means of selectively modifying tumour blood flow although it is unclear as to why vasoconstriction with acetylcholine only occurred during phenylephrine infusion.

Tumours vasoconstricted more in response to L-NNA administration than the normal hindlimbs when perfused ex vivo. Results for the hindlimbs were very similar to those reported for administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME, considered to be the pro-drug of L-NNA) to the normal rat kidney perfused ex vivo (13).

We do not know whether higher doses of L-NNA would have achieved more vasoconstriction in the hindlimb preparations. Andrade et al. (3), using the NOS inhibitors L-NAME and N^{ω}-monomethyl-L-arginine (L-NMMA), also found a greater reduction of blood flow in mouse tumours growing in sponge implants than in similar preparations consisting of non-neoplastic granulation tissue. The heterogeneity of response observed in our experiments may be due to the variation in tumour size used (0.5–2.8 g). In contrast to Andrade et al. we found that L-arginine did not completely reverse the effects of L-NNA. This is unlikely to be due to excessive perfusion pressures since the same effect occurred in the hindlimb preparations where only a moderate increase in perfusion pressure was observed.

Systemic administration of a high dose of L-NNA to tumour-bearing rats produced slightly larger changes in MABP and pulse rate than those reported for other NOS inhibitors in a different strain of non-tumour-bearing rats (14). L-NNA produced a selective reduction in blood flow to the tumour compared to all the normal tissues studied except for the spleen and skeletal muscle which showed a similar reduction to the tumour. Skeletal muscle is the main component of the hindlimb perfusions and so these results are quite surprising. Ten mg kg^{-1} L-NNA is a very high dose. A lower dose may reveal the difference in response between the tumour and skeletal muscle observed in the ex vivo preparations since the effect in the hind limbs had not saturated at the doses used. Sensitivity of tumours to modification of NOS activity could be related to high levels of the inducible isoform of the enzyme as suggested by Kennovin et al. (15)

In conclusion, modification of NOS activity in tumours is a promising means for selective tumour blood flow modification. Furthermore, investigations into endothelium-dependent versus endothelium-independent methods for modifying tumour blood flow could provide a means for further selectivity.

ACKNOWLEDGEMENTS

We would like to thank CRC Gray Laboratory staff for care of the animals and Sheila Wordsworth for technical assistance. This work was supported by the Cancer Research Campaign.

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