Endothelium-dependent vasodilatation produced by the L-arginine/nitric oxide pathway in normal and ischemic bone

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We used an experimental model of the perfused isolated rabbit tibia to investigate the vasodilatation produced by nitric oxide in the circulation of bone. Tibiae were perfused at a constant flow rate while the perfusion pressure was monitored continuously. Perfusion pressure was raised by the addition of noradrenaline to the perfusate, and dose responses were measured for bolus doses of acetylcholine and sodium nitroprusside. N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthesis, was then added to the perfusate at a concentration of 10^{-4} M, and the dose responses to acetylcholine and sodium nitroprusside were repeated. Measurements were performed on groups of bones after 0, 6, 12, and 24 hours of normothermic ischemia (n 5, 4, 6, and 9, respectively). Both acetylcholine and sodium nitroprusside produced significant vasodilatation after 0 and 6 hours' ischemia, but no significant response was observed after 12 or 24 hours of ischemia. The vasodilatation produced by acetylcholine was significantly attenuated when L-NAME was added to the perfusate, but the vasodilatation produced by sodium nitroprusside remained unchanged. These findings confirm endothelial production of NO by stimulation of muscarinic receptors on the endothelial cells in bone and indicate that vasodilatation via the L-arginine/NO pathway remains viable for 6 hours after normothermic ischemia.

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There is evidence that bone vasculature can modify bone formation through changes in venous pressure (Kelly and Bronk 1990), and that endothelial cells release factors that modulate osteoclast and osteoblast function (Collin-Osdoby 1994). The endothelium can no longer be regarded as an inert semipermeable barrier between blood and vascular smooth muscle. Endothelial cells are targets for local regulatory factors and circulating hormones; in addition, endothelial cells secrete immunoregulatory and neuromodulatory factors. In bone, vascularization precedes osteogenesis, and this observation prompted the suggestion that vessels play an important role in osteogenesis (Trueta 1963).

One of the most significant advances in vascular biology in recent years has been the realization that the endothelium is an important source of local factors that control vascular tone. It has been demonstrated that acetylcholine (ACh) would produce vasodilatation only in vessels with an intact endothelium (Furchgott and Zawadzki 1980). It was hypothesized that a local factor, termed endothelium-derived relaxating factor (EDRF), caused the vasodilatation, and in 1987 EDRF was identified as nitric oxide (NO). Vasodilatation is mediated by the release of NO from endothelial cells (Palmer et al. 1987). NO is a powerful vasodilator, which is synthesized from L-arginine by nitric oxide synthase (NOS). The endothelium also produces prostacyclin and endothelium-derived hyperpolarizing factor (EDHF), which contribute to local vasodilatation, and the endothelins which contribute to local vasoconstriction. NO, endothelin, and prostacyclin have all been shown to exert an important effect on bone cell biology (Collin-Osdoby 1994).

The existence of NO-dependent vasodilatation has been shown in bone (Brinker et al. 1990, Davis et al. 1992). Brinker et al. demonstrated vasodilatation in response to ACh and nitroglycerin (an exogenous source of NO), without showing that ACh acted by the NO pathway. It has been demonstrated that ACh would diminish the vasoconstrictive effects of noradrenaline and that this effect was abolished in the presence of an inhibitor of NO production (Davis et al. 1992). However, neither of these papers studied in detail the response of the vascular bed in bone to endogenous and exogenous sources of NO.

It is now becoming accepted that endothelial cells also have an important role in the response of tissue to ischemia/reperfusion injury (Burnstock and Ralevic

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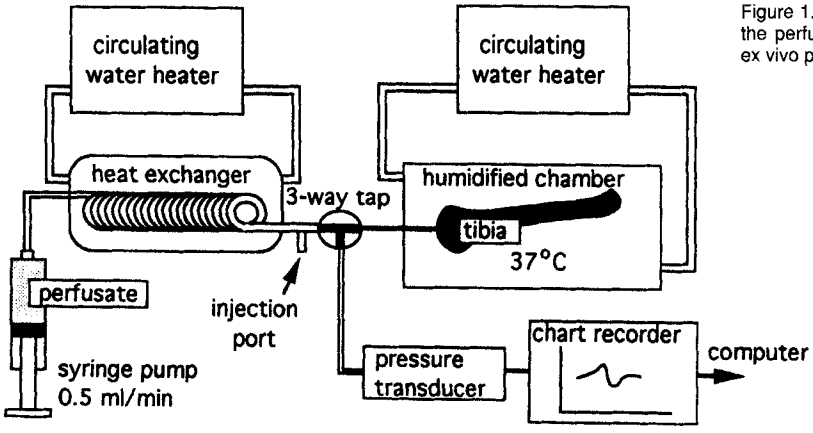

Figure 1. Schematic representation of the perfusion apparatus used for the ex vivo perfusion of the tibia.

1994, Grace 1994). Recently, it has been shown that not only normal endothelial cells can have an effect on bone, but that 4.5 hours of ischemia to a hind limb of a rat can produce a periosteal proliferative response 72 hours after the ischemic insult in the tibia of that limb (Svindland et al. 1995). Studies by Wood and coworkers have investigated the response of bone vasculature to periods of ischemia and found differing responses for different receptor types (Davis et al. 1992, Moran and Wood 1992, Roorda and Wood 1993).

We examined the ability of endothelial cells in bone to release NO to produce vasodilatation in response to ACh injection, and compared this to the vasodilatation produced by sodium nitroprusside (SNP). SNP is an exogenous source of NO that will produce relaxation of vascular smooth muscle, without stimulating release of NO from the endothelium. These experiments were performed after various periods of ischemia, to determine the duration of retained endothelial function after a prolonged ischemic insult. An understanding of the control of bone vasculature is important for many aspects of orthopedics, including fracture treatment, avascular necrosis, bone ingrowth to biomaterials, and bone grafting.

Methods

Experimental preparation

For these experiments, an isolated perfused rabbit tibia preparation was used. Male New Zealand white rabbits (weight approximately 3.5 kg) were injected with heparin (300 units/kg) and then sacrificed with an overdose of anesthetic (Euthetal). A longitudinal skin incision was made from knee to ankle and the tibia was disarticulated at the knee and ankle. The tibia and associated muscles were removed. An incision was made to expose the fibula, which was removed to expose the anterior tibial artery. Under operating microscope vision, the artery was cannulated with a 2FG (green) luer lock cannula (outside diameter 0.75 mm) (Portex Ltd, Hythe, Kent). The cannula tip was positioned just proximal to the bifurcation with the tibial nutrient artery. As soon as the cannula was secured in position, the tissue was flushed slowly with 1 ml Krebs buffer. The buffer was oxygenated with $95\%O_2/5\%CO_2$. All vessels distal to the nutrient artery were then carefully ligated. Remaining muscles were then removed, leaving the periosteum intact, before placing the tibia in the perfusion apparatus.

A schematic diagram of the perfusion apparatus is shown in Figure 1. It consists of an organ bath maintained at 37 °C, a syringe pump (Braun Instruments, Germany) to infuse the tibia at a constant rate of 0.5 mL/min, a water bath to maintain the temperature of the perfusate, and a pressure transducer (Harvard Apparatus, Edenbridge, Kent) to monitor continuously the perfusion pressure of the preparation. There is also an injection port for bolus injections into the perfusate. Preliminary experiments demonstrated that the oxygenated buffer maintained pO_2 in the syringe.

Experimental groups studied

4 groups of animals were studied in the main part of these experiments. In group 1 ("No ischemia"), the tibia was placed in the perfusion apparatus as rapidly as possible after sacrifice of the animal. Typically, 35 minutes elapsed between death and initially flushing the bone with oxygenated buffer, and a further 20 minutes before the tibia was placed in the perfusion apparatus.

In groups 2–4, the tibia was removed about 1 hour after death. The cannulation was performed as before,

the bone being flushed with buffer once the cannulation was complete. These bones were then wrapped in moist swabs and left at room temperature until either 6 hr (group 2), 12 hr (group 3) or 24 hr (group 4) after death. Then, the tibia was placed in the perfusion apparatus, as before. One other group (group 5) was studied as a time control with no ischemia (vide infra).

Experimental protocol

Perfusion pressures were allowed to stabilize before the experimental protocol was started. About 15–20 minutes was usually required. In the first part of the experiment, a dose-response to noradrenaline (NA) was measured. 25 microliter boluses of buffer containing NA in a range of concentrations from 2×10^{-7}M to 2×10^{-2}M were injected and the change in perfusion pressure was measured. These concentrations are equivalent to the injection of 5×10^{-12} to 5×10^{-7} mol NA. NA was then added to the perfusate in a concentration that raised the perfusion pressure to 120–140 mmHg. The concentration of NA required to raise the tone varied from experiment to experiment. Typically, a concentration of 10^{-5}M was required. Vasodilatation due to ACh was then studied by injecting 25 microliter boluses of ACh in concentrations of 10^{-7}M to 10^{-2}M. This was followed by an injection in the same range of concentrations of SNP. In groups 1–4, the NO synthesis inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) was added to the perfusate at a concentration of 10^{-4}M. After 30 minutes of perfusion with L-NAME, the dose responses to both ACh and SNP were repeated. In group 5 (the time control group), L-NAME was not added to the perfusate, but the dose responses to ACh and SNP were repeated after a similar interval. All chemicals were supplied by Sigma (Poole, Dorset, U.K.).

The degree of vasodilatation produced by ACh or SNP was then calculated as the percentage change in perfusion pressure, defined by the following equation:

$$\% \text{ change in pressure (pressures in mmHg)} = \frac{\text{change in pressure} \times 100}{(\text{pressure at raised tone} - \text{initial basal pressure})}$$

Results were calculated in this way as the experiment was designed to achieve similar values of perfusion pressure for all assessments of vasodilatation. Since there were differences in the initial perfusion pressure between the groups, the amount by which the pressure was raised varied from group to group.

At the end of the experiment, the tibia was perfused with Zamboni's fixative. Samples of the nutrient artery were then embedded in paraffin wax and sectioned for histological study.

The effect of ischemia time on initial basal perfusion pressure. Mean (sem)

Group	Ischemia time (h)	Basal perfusion pressure (mmHg)	
1	0	38.8	(1.3)
2	6	66.7	(6.2)
3	12	81.0	(20.6)
4	24	138.8	(20.8)
5	0	34.3	(3.5)

Statistics

Data are presented as mean (SEM). The effects of ischemia on vasoconstriction and vasodilatation were analyzed using analysis of variance (ANOVA). The effects of L-NAME on ACh and SNP vasodilatation were analyzed by the Student's paired t-test. All statistical analyses were performed with the Stata statistical software package.

Results

Basal tone

The initial basal tone was elevated with increasing periods of ischemia (Table). Comparing groups 1–4, ANOVA demonstrates significant differences between the groups ($p < 0.001$). All 3 ischemia groups showed significantly higher pressure than group 1.

NA response

The mean values for the responses to bolus injection of NA after different periods of ischemia are shown in Figure 2. There is evidence of a dose-dependent response at all times, and it appears that the response is smaller after ischemic periods. The differences in responsiveness to NA after different periods of ischemia are not, however, statistically significant. All preparations in the 0, 6-, and 12-hour groups showed responsiveness to noradrenaline. In the bones studied at 24 hours, 5 out of 9 showed very high and unstable initial basal perfusion pressure, with no subsequent response to NA injections. Of the other 4 bones in this group, the mean initial perfusion pressure was 116 (23) mmHg, and the mean responses to noradrenaline were 6.0 (7.7) mmHg, 54 (27) mmHg, and 99 (100) mmHg, at doses of 5×10^{-10}, 5×10^{-9}, 5×10^{-8} mol, respectively.

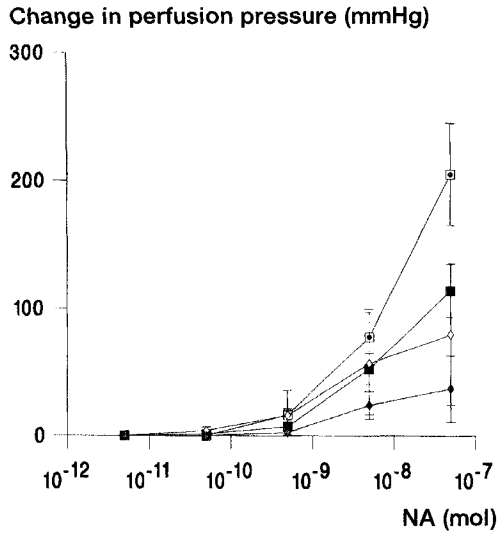

Figure 2. The increase in perfusion pressure over initial basal pressure due to increasing doses of noradrenaline injected in 25 microliter boluses. ◙ 0 h, ■ 6 h, ◇ 12 h, and ◆ 24 h.

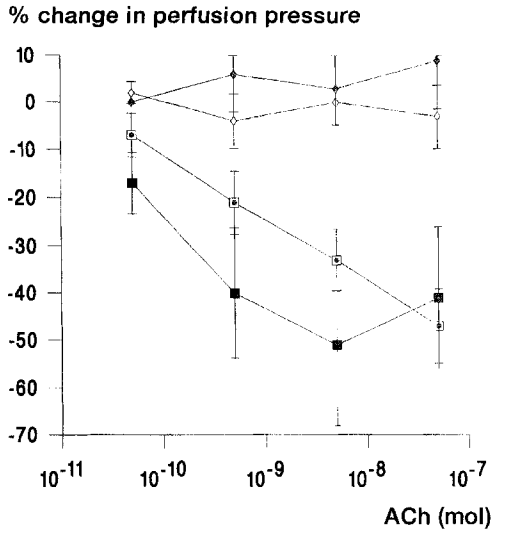

Figure 3. The response of the perfused tibiae to acetylcholine injected as 25 microliter boluses after 0, 6, 12, and 24 hours of ischemia. The vascular tone had been raised by addition of noradrenaline to the perfusate so that the vasodilatory response could be observed. ◙ 0 h, ■ 6 h, ◇ 12 h, and ◆ 24 h.

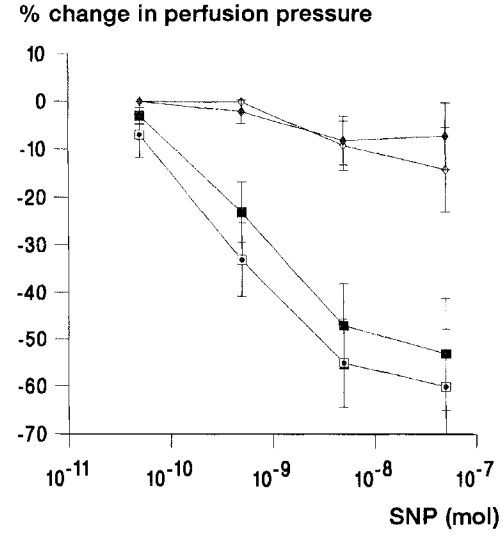

Figure 4. The response of the perfused tibiae to sodium nitroprusside injected as 25 microliter boluses after ◙ 0 h, ■ 6 h, ◇ 12 h, and ◆ 24 h of ischemia.

ACh and SNP responses

The responses to ACh and SNP after different periods of ischemia are shown in Figures 3 and 4, respectively. Both ACh and SNP produced dose-dependent vasodilatation for up to 6 hours of ischemia. Vasodilatation after 12 or more hours of ischemia was virtually absent. The differences in response analyzed by ANOVA are statistically significant at doses from 5×10^{-10} mol to 5×10^{-8} mol for both ACh and SNP.

Effect of L-NAME on ACh vasodilatation

Figure 5 demonstrates the effect of the addition of L-NAME to the perfusate on the vasodilatation produced by ACh after no ischemic insult. When L-NAME was added to the perfusate, the response to ACh was considerably reduced. The differences are statistically significant for 10^{-4}M ($p = 0.02$) and 10^{-3} M ($p = 0.01$). After 6 hours of ischemia, the vasodilatation was again reduced by L-NAME at statistically significant levels, as shown in Figure 5. No vasodilatation was observed at 12 and 24 hours, and the addition of L-NAME had no effect at these times.

Effect of L-NAME on SNP vasodilatation

The responses to SNP with or without L-NAME in the perfusate are shown in Figure 6. There were no significant differences in the responses after either 0 or 6 hours' ischemia, which showed that L-NAME had no effect on the vasodilatation produced by SNP. These measurements, performed after the ACh measurements, confirm the viability of the preparation at this time and support the observations that the attenuation of ACh vasodilatation by L-NAME is a real inhibition of NO production in the endothelium.

Time control experiments

In the experiments to assess any changes in the responsiveness of the preparation over time, no differences were observed in either the ACh or SNP responses repeated after time intervals similar to the

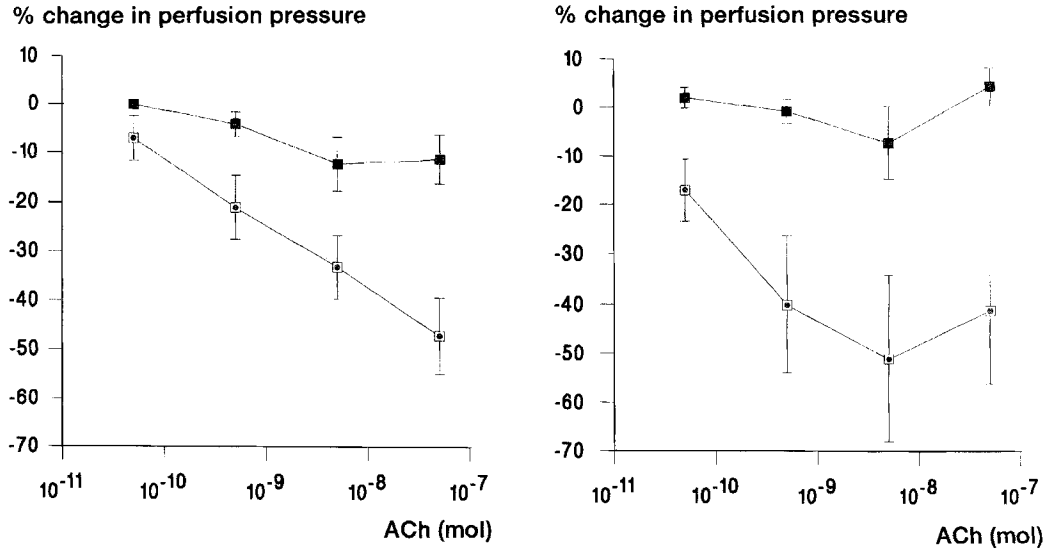

Figure 5. The effect of L-NAME (■; and initial ◙) on the vasodilatory action of ACh. 0-hour response (left) and 6-hour response (right). There were no observed effects at 12 and 24 hours.

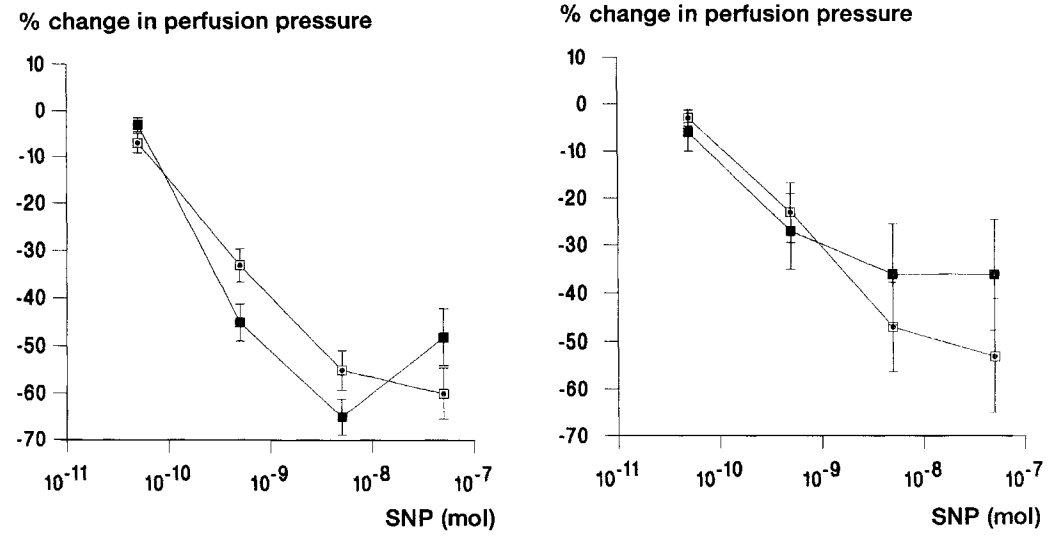

Figure 6. The effect of L-NAME (■; and initial ◙) on the vasodilatory action of SNP. 0-hour response (left) and 6-hour response (right). There were no observed effects at 12 and 24 hours.

above experiments as compared to the initial responses of the preparation (Figure 7).

Histological appearance

Histological sections of the nutrient artery, removed at the end of experiments starting after 6 or 12 hours' ischemia, are shown in Figure 8. After 6 hours of ischemia, the endothelium appears still intact, but after 12 hours of ischemia, it appears that the endothelial cells are starting to detach from the basement membrane.

Discussion

Endothelial function in normal bone

This paper demonstrates in detail the mechanism for local control of vascular resistance in bone by the production of NO in the endothelial cells of bone. The vasodilatation resulting from ACh injection is significantly attenuated when 10^{-4} M L-NAME is added to the perfusate, confirming that ACh was stimulating the production of NO in the endothelium. L-NAME inhibits the production of NO from L-arginine (Rees

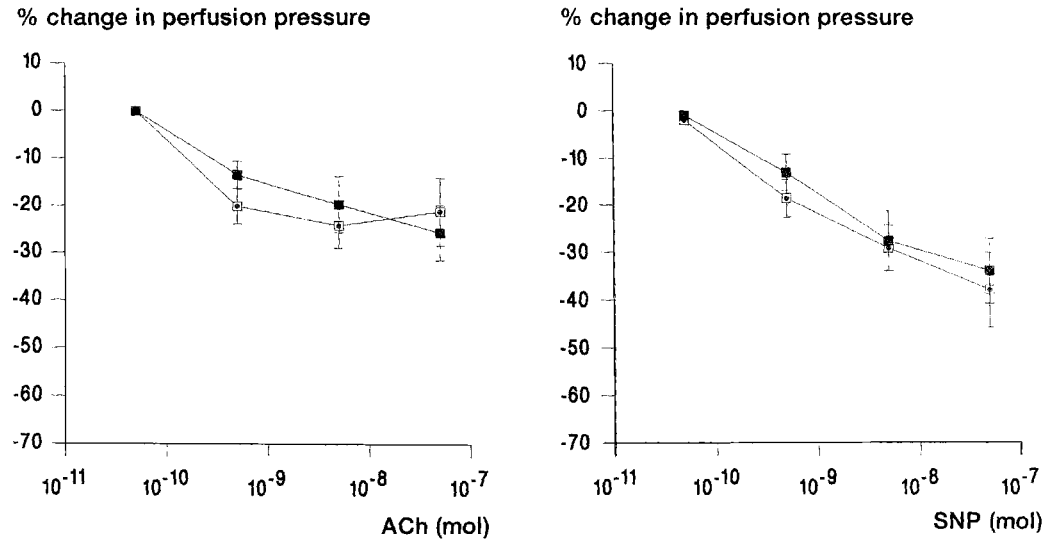

Figure 7. Time control experiments (◙ 0 h and ■ 6 h) for ACh (left) and SNP (right). Time did not have a direct effect on vasodilatation.

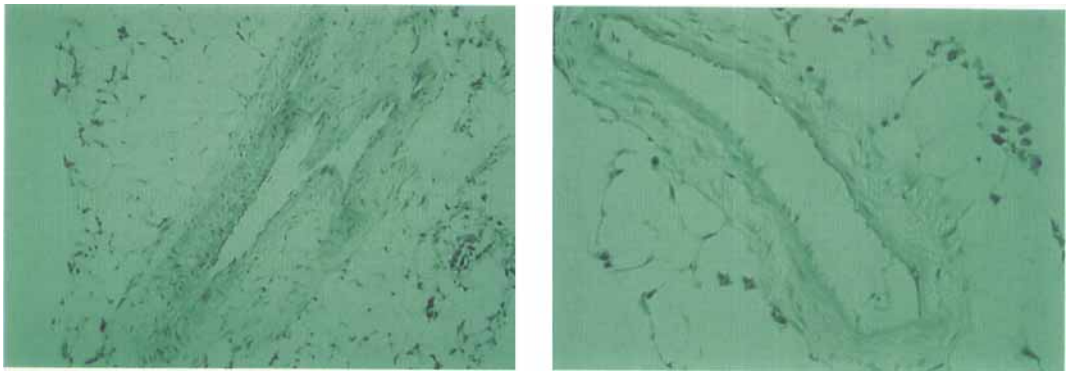

Figure 8. Histological sections of the tibial nutrient artery after 6 hours' (left), and 12 hours' ischemia (right). At 12 hours, it can be seen that endothelial cells are starting to detach from the basement membrane.

et al. 1990). The demonstration of vasodilatation to SNP, in the presence of L-NAME, confirmed that the preparations were still able to respond to NO. Brinker et al. (1990) have previously shown that ACh vasodilatation in bone is attenuated by atropine, confirming the existence of muscarinic receptors on the endothelial cells of bone. Therefore, as occurs in other tissues, ACh produces vasodilatation in bone by stimulation of muscarinic receptors on the endothelium, and thus the production of NO in the endothelial cells.

Studies of the innervation of bone have shown that most nerves are perivascular, particularly in cortical bone. Substance P, calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), and vasoactive intestinal peptide (VIP) have all been identified in perivascular nerves in bone. These observations suggest that bone, although relatively avascular, is capable of complex local control of vascular resistance, with local endothelial factors and substances released from perivascular nerves providing a dual control mechanism for the vascular smooth muscle.

It is interesting that several recent studies have suggested that endothelial cells in bone can release several substances with osteogenic actions. Both NO and endothelin-1 have been shown to modulate osteoclast activity (MacIntyre et al. 1991, Alam et al. 1992), and receptors to oestrogen and insulin-like growth factor have been identified on bone endothelial cells (Brandi et al. 1993, Fiorelli et al. 1994). It is possible, therefore, that factors involved in the local control of blood flow also modulate bone cellular activity.

MacIntyre et al. (1991) demonstrated that NO inhibited osteoclast activity, and postulated that endothelial cells may have a role in modulating osteoclast

activity, arguing that endothelial cells are abundant in bone marrow and in close proximity to osteoclasts. It has also been shown that the potent vasoconstrictor, endothelin-1, inhibits osteoclastic bone resorption by a direct effect on cell motility (Alam et al. 1992). Receptors to estrogen and insulin-like growth factor have been identified on bone endothelial cells, which also suggests additional mechanisms for endothelial modulation of bone metabolism (Brandi et al. 1993, Fiorelli et al. 1994). The possible interactions of endothelial cells with bone have recently been reviewed (Collin-Osdoby 1994).

Effect of ischemia on endothelial function

In the present study, there was no detectable difference between the ACh responses after 0 or 6 hours' ischemia, and similar results were obtained for SNP. Neither ACh nor SNP showed any vasodilatation after 12 or 24 hours of ischemia. As the NA data suggest that the smooth muscle is still functional after 12 hours of ischemia, the lack of ACh response could be due to endothelial cell damage, but as there was no response to SNP it could indicate dysfunction of the NO/guanylate cyclase pathway in smooth muscle.

It has been shown by Roorda and Wood (1993) that ACh does not attenuate the vasoconstrictive response to NA after 24 hours of normothermic ischemia. However, there was significant potentiation of NA response in the presence of N^G-monomethyl-L-arginine (L-NMMA), suggesting at least partial damage to the endothelium. It has been demonstrated that continuous perfusion of an isolated bone could maintain ACh responsiveness for up to 4 hours, but that there was evidence of loss of function after this time (Moran and Wood 1992). Alpha-1 and alpha-2 adrenergic receptors in bone have been shown to remain viable after at least 24 hours' ischemia (Davis et al. 1992).

It has been proposed that, during ischemia, ATP is released from endothelial cells to act on endothelial P_2-purinoceptors, leading to release of NO and subsequent hypoxic vasodilatation (Burnstock and Ralevic 1994). In vessels in which the endothelium is damaged, vasoactive substances released from endothelial cells can act directly on receptors on the smooth muscle, causing vasoconstriction. The histological sections in our study show damaged endothelium after 12 hours of ischemia, indicating that the vascular smooth muscle could have been stimulated directly. Certainly, the NA responses showed that the smooth muscle was still viable.

Reperfusion following ischemia can result in greater local tissue damage than ischemia alone. There are many factors involved in reperfusion injury, including oxygen free radicals, neutrophils, and endothelial factors. Xanthine oxidase, prostacyclin, thromboxane, endothelin, adhesion molecules, and NO may all contribute to the endothelial response to reperfusion injury. NO production not only causes vasodilatation, but also inhibits platelet aggregation and platelet adhesion to endothelium. However, NO may also react with superoxide ions to yield secondary cytotoxic species via the peroxynitrite anion.

It can be difficult to separate the effects of ischemia and reperfusion, and perfusion of bones with Krebs buffer may not represent the true in vivo effect of reperfusion. In our study, the initial perfusion pressure increased with increasing periods of ischemia, until after 24 hours of ischemia. The perfusion pressure was then very high, and often unstable in many of the preparations studied. This may be an indication of the no-reflow phenomenon in reperfusion injury.

Changes in bone blood flow have been cited as a major contributing factor in the failure of free vascular bone transplants. Despite patent anastomoses, graft flow is lower than normal bone, and a 15% failure rate has been observed in free vascularized fibula grafts (De Boer and Wood 1989). Although NO has a dual role in ischemia/reperfusion injury, the findings of the present study suggest that therapies to maintain endothelial cell viability are useful in controlling vascular resistance in this situation. It is possible that modification of the endothelial response observed in these experiments are produced by ischemic preconditioning. A number of investigators have shown that a series of short ischemic episodes, followed by reperfusion, make the heart more resistant to a further prolonged period of ischemia (Walker and Yellon 1992). It has also been shown that ischemic preconditioning protects against coronary endothelial dysfunction (Richard et al. 1994). The preconditioning probably involves the release of endogenous factors (adenosine, bradykinin, NO, and prostacyclin) from ischemic tissue.

In summary, the present studies suggest that vasodilatation via the L-arginine/NO pathway remains viable for at least 6 hours, but that it is abolished by 12 hours of normothermic ischemia.

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