Nifedipine increases microvascular permeability via a direct local effect on postcapillary venules

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NIFEDIPINE is a dihydropyridine calcium antagonist that is prescribed widely for angina and hypertension. It acts primarily as a vasodilator but is also a negative inotrope.

A common side effect of nifedipine is peripheral edema, often in patients with normal cardiac function. In those with preexisting heart failure, edema may be made worse. The edema cannot be explained by vasodilation alone because it is not a class effect of all vasodilators (2, 44). Angiotensin-converting enzyme inhibitors are primarily vasodilators but do not have edema as a side effect. Co-administration of the angiotensin-converting enzyme inhibitor captopril may even mitigate the edema effect of nifedipine (9). The negative inotrope effect of nifedipine may contribute to, but does not explain, the edema because other negative inotropes such as β-adrenoceptor antagonists do not cause edema to the same extent (18).

Previous work has shown that nicardipine, a dihydropyridine derivative calcium-channel antagonist, has a direct effect on the microcirculation of anephretic rats to increase transvascular fluid leak. This leads to a shift of fluid from the intravascular to the extravascular compartment with a consequent increase in hematocrit (39–41).

It has been proposed that the effects of calcium-channel antagonists in the microcirculation can be explained by their preferential vasodilator activity on the arterial side of the microcirculation and inhibition of local microvascular autoregulatory mechanisms to increase capillary hydrostatic pressure and thus transcapillary flux (10, 30, 45). These are hypothetical explanations because the microvascular site of the leakage of edema caused by nifedipine has not been investigated experimentally.

In the current work we set out to show that nifedipine can act locally to induce microvascular leakage. Evans blue injected systemically in rats leaked out at skin sites where agents injected locally caused plasma extravasation. This visual demonstration of increased vascular permeability was confirmed by injecting animals systemically with radiolabeled albumin, which like the dye, accumulates in edematous skin sites and can be quantified.

To identify the anatomical site of the microvascular leak caused by nifedipine, Monastral blue dye was injected into the systemic circulation. This dye has been used extensively to investigate increased vascular permeability (13, 25, 27). Early experiments using Monastral blue or colloidal carbon identified the post-capillary venule as the segment of the microcirculation that controls inflammatory edema (13, 19). The present study was designed to determine whether this site is also involved in the microvascular permeability response to nifedipine.

METHODS

Animals. Male Wistar rats weighing 200–250 g were anesthetized with 50 mg/kg ip of pentobarbitone sodium. The relevant skin area was shaved with electric clippers. During the experiment, anesthesia was maintained with pentobarbitone sodium (10 mg·kg−1·h−1) in an air-conditioned room at 20–23°C. All injections into skin sites with test agents were given in 100 µl of buffer via a 27-wire gauge needle.

Drugs. The nifedipine used was the generous gift of Bayer (Berkshire, UK). The solution of 2% nifedipine in ethanol was diluted 100-fold with saline. Calcium chloride was added to give a final concentration of 1 mM.

Human serum 125I-labeled albumin was from Amersham International (Bucks, UK). Monastral blue and other drugs and chemicals were obtained from Sigma (Poole, UK).
anesthetized rats via the tail vein. Nifedipine 10\(^{-7}\) mol in 100 \(\mu\)l was injected subcutaneously in the shaved scrotal skin, and 0.1 ml ethanol (1%) was injected subcutaneously in the opposite testis as a control. After 1 h the rats were euthanized with an overdose of pentobarbitone sodium, while clamps were applied to the root of the scrotum to minimize blood loss from the vessels so as to preserve the pattern of the vasculature. The cremaster muscle was excised and left for 24 h in 10% Formalin. The thin fascia on the cutaneous side of the muscle was removed under a dissecting microscope, and the muscle was left for an additional 24 h in glycercin. Finally, the preparation was trimmed, dipped in warm glycerin jelly for a few minutes, and mounted for microscopy.

To identify the microvascular segment of leak in rat mesenteric vessels, the fur on the abdominal skin of anesthetized rats was shaved, and an incision was made along the midanterior abdominal wall. Two loops of intestine were pulled out and laid on a flat board. The loops of intestine were moistened throughout the experiment by the superfusion of saline. Monastral blue was injected intravenously, and there-after 100 \(\mu\)l of nifedipine (10\(^{-7}\) mol) was applied on the mesentery of one loop and 100 \(\mu\)l of ethanol (1%) on the opposite loop. After 1 h, 5 ml of Formalin were applied on the mesentery, and animals were euthanized with an overdose of pentobarbitone sodium. The mesentery was excised and left in Formalin for 24 h. The tissue was trimmed, rinsed in distilled water, floated in warm glycerin jelly, and mounted for microscopy.

**Quantification of permeability change.** Local plasma extravasation was measured as the intradermal accumulation of human microvascular 125I-labeled albumin (1.5 \(\mu\)Ci/kg), which had been injected intravenously 5 min before the test agents were given in rat skin. This method was used because multiple skin sites can be injected, allowing a dose response and control response to be measured in each animal. Test agents were dissolved in 100 \(\mu\)l of saline and injected intradermally in a balanced site pattern. The set of injections was performed in duplicate in each rat. After 30 min, the animal was euthanized with an overdose of pentobarbitone sodium, and a 5-ml blood sample was taken by cardiac puncture into heparin (10 U/ml final concentration). The skin was removed, and the injection sites were excised with a 17-mm diameter punch. Skin and plasma samples were placed in tubes and counted in an automatic gamma counter. Plasma extravasation was expressed by dividing each skin 125I count by the radioactivity in 1 \(\mu\)l of plasma at the time of death. This method is widely used to measure edema (42, 43, 45), and it correlates with other methods such as rat paw swelling (16, 23, 26).

**Copper assay.** The accumulation of the copper-containing dye Monastral blue in rat cremaster muscle was visible at nifedipine-injected sites as a blue stain and was quantified by measuring the muscle copper content. Iron and zinc were measured as controls.

Rats were anesthetized, and Monastral blue (0.1 ml/100 g, 3% suspension) was injected intravenously via the tail vein followed immediately by the local subcutaneous injection of nifedipine (0.1 ml, 10\(^{-7}\) mol/100 \(\mu\)l) on one side of the scrotal skin and 0.1 ml ethanol (1%) on the opposite side. One hour later, to allow for the clearance of dye from the circulation, the animals were euthanized with an overdose of pentobarbitone sodium. An incision was made over the midscrotal skin, and the cremenster of both testes was dissected out and placed on a filter paper moistened with sterile water. The tissue samples were weighed and dried out to constant weight, digested in 0.4 ml of concentrated nitric acid, made up to 5 ml with water, and then the copper concentration was measured by a flame atomic absorption technique (D. Baldwin, Kings’ College, London, UK). The assay detection limit was 2–3 \(\mu\)g dry tissue (21).

**RESULTS**

Local leakage of radiolabeled plasma albumin response to nifedipine. Figure 1 shows the dose response of plasma leakage to locally injected nifedipine over the dose range of 10\(^{-10}\)–10\(^{-7}\) mol/site. Nifedipine 10\(^{-10}\), 10\(^{-9}\), 10\(^{-8}\), and 10\(^{-7}\) mol/site caused 9.9 ± 2.5, 17.0 ± 2.4, 24.3 ± 5.9, and 23.3 ± 5.4 \(\mu\)l plasma leakage, respectively, compared with the control of 5.5 ± 1.1 \(\mu\)l. The plasma leakage response to each dose of nifedipine was significantly greater than control (P < 0.05, n = 7).

Visualizing plasma protein extravasation with Evans blue. The local injection of 0.1 ml of nifedipine 10\(^{-7}\) mol/100 \(\mu\)l caused obvious blueing of the scrotal skin in rats injected intravenously with Evans blue. The blue-
ing appeared ~10 min after the injection and became maximal by 20–30 min. In comparison the control side injected with ethanol (1%) was devoid of blueing. No other skin area stained blue, confirming the localization of the response to the nifedipine-injected site. With doses of nifedipine as low as \(10^{-10}\)–\(10^{-7.2}\) mol/site, there was also obvious blueing compared with control, although the response was not quite as intense as with \(10^{-7.2}\) mol/site.

Quantification of macromolecular permeability with copper assay. The copper content of Monastral blue (copper phthalocyanine) was used as a surrogate marker to quantify dye accumulation in tissue as an indication of increased endothelial cell permeability. Because of the short half-life (3 min) of Monastral blue in the circulation, virtually no dye remains in the lumen of vessels after 1 h, and the amount measured in the tissue corresponds mainly to intramural dye.

Figure 2 shows the concentration of copper (µg/g dry wt tissue) in the cremaster muscle of six rats. Zinc and iron concentrations were measured as controls. In each of the six rats the concentration of copper in the cremaster injected with nifedipine \((10^{-7.2} \text{ mol/0.1 ml})\)-exposed muscle was higher than the control (ethanol 1%). This method underestimates the difference between control and nifedipine-exposed preparations, because the copper content was measured in the whole sample, not just the affected site, and includes all vessels, not just postcapillary venules.

The mean ± SE of copper concentration (µg/g dry tissue) was 15.0 ± 2.4 in nifedipine-injected cremaster compared with 5.3 ± 0.7 in control \((P < 0.05)\). The mean concentrations of zinc (131 vs. 131 µg/g dry tissue) and iron (80 vs. 82 µg/g dry tissue) remained unchanged.

**Fig. 1.** Plasma leakage response to intradermal injection of nifedipine was demonstrated in dorsal skin of rats. Local leakage of \(125^\text{I}\)-labeled albumin was measured at site of injection and expressed as volume of plasma (µl). Nifedipine at doses of \(10^{-10}\), \(10^{-9}\), \(10^{-8}\), and \(10^{-7.2}\) mol/site significantly increased (*\(P < 0.05\) for each dose) plasma leakage compared with control. Data are means ± SE of 7 rats, two replicates per animal.

**Fig. 2.** Concentration of copper (µg/g dry tissue) in cremaster muscles of 6 male rats was measured by flame atomic absorption to quantify the accumulation of dye. Monastral blue (copper phthalocyanine) was injected intravenously and nifedipine \((10^{-7.2} \text{ mol and control (1% ethanol)}\) were injected subcutaneously in scrotal skin. Concentrations of tissue zinc and iron were also quantified as additional control measures. Concentration of copper, used as an indicator of Monastral blue extravasation, was significantly (*\(P < 0.05\)) higher in nifedipine-exposed cremaster muscle compared with control (3-fold rise). Concentrations of iron and zinc were not different in nifedipine and control tissues.
The application of the vascular-labeling method to rat mesenteric vessels showed similar results with nifedipine. Exposure of rat mesentery to nifedipine $10^{-7.2}$ mol caused heavy staining of the postcapillary venule (Fig. 3D), whereas the adjacent arteriole of similar size (28 µm) remained clear from the dye. Again there was no trace of dye in the larger venules or capillaries (Fig. 3E). The extravascular connective tis-
sue was also free of dye under the light microscope, because the dye particles are too large to pass through the basement membrane and are trapped in the vessel wall.

Confirmation of intramural entrapment of Monastral blue dye. Figure 4A shows an electron micrograph from a cross section of a vessel stained with Monastral blue. The deposits of dye are scattered unevenly around the vessel wall and localized between endothelial cells and the basal lamina. In some segments it forced the separation of the endothelial cell from the basement membrane by >1 µm. There were no foreign particles in the endothelial cells and no alteration in the cellular structure in this study. There were no definite intercellular gaps between endothelial cells. Because the rats were euthanized 1 h after the local administration of nifedipine, any structural changes induced by it may have reversed during this time. The mural structure of the vessels with deposit comprised endothelial cells and the underlying basement membrane. The vessel walls were relatively devoid of smooth muscle consistent with these being venules. The diameter of the vessels was consistent with them being postcapillary venules.

The X-ray spectrum produced by microanalysis of ultrathin sections of a postcapillary venule confirms the high copper content of particles lodged in the basement membrane region compared with sites within or outside the vessel wall (Fig. 4B). There is an osmium peak on each trace as the tissue was fixed with osmium tetroxide.

**DISCUSSION**

This study shows that the direct application of the calcium-channel antagonist nifedipine to the microcirculation increases vascular permeability. This was visualized as a blue stain of the skin at the site of injection in animals dosed with Evans blue dye systemically. The accumulation of the copper-based dye Monastral blue in skeletal muscle was confirmed by measuring the tissue copper content. The effect of nifedipine was quantified further by showing an increase in radioactivity in nifedipine-injected skin sites in animals previously given radiolabeled albumin systemically.

This is the first demonstration that nifedipine increases microvascular permeability, although earlier work suggested that the dihydropyridine nicardipine can cause a shift of plasma fluid from the intravascular space to the interstitium (41). In anephretic rats nicardipine increased the extravasation of plasma protein-bound Evans blue dye into skeletal and cardiac muscle, the loss of intravascular fluid causing a rise in hematocrit. A study with nifedipine in hypertensive patients also suggested a shift of fluid from the intravascular to the extravascular space (37).

In the present experiments both skin and skeletal muscle permeability increased with doses as low as $10^{-10}$ moles per site. The injection volume was 0.1 ml. After diffusion of the drug into the adjacent tissue was allowed (perhaps a 10-fold dilution), an approximate tissue concentration of the drug may be 0.1 nmol/ml. This is close to the approximate therapeutic range of

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**Fig. 4. Intramural accumulation of Monastral blue dye was examined by electron microscopy and X-ray microanalysis.**

**A:** electron micrograph ($\times 14,000$) of a venule cross section from a cremaster muscle exposed to nifedipine ($10^{-7.2}$ mol). There are dark deposits scattered around vessel wall that are localized between endothelium (E) and basal lamina (B). There are no foreign particles within vessel lumen (L). Absence of smooth muscle around vessel confirms its identity as postcapillary venule. Scale bar = 1 µm. **B:** X-ray microanalysis from different sites of tissue cross section of postcapillary venule used for localization of dye particles. Vertical lines mark spectrum position of Cu (from Monastral blue dye) and Os (from osmium tetroxide used for tissue fixation). Whereas copper is evident in spectrum from subendothelial sample, it is absent from intraluminal and surrounding skeletal muscle control sites.
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nifedipine concentrations in humans in plasma of 0.1–0.2 nmol/ml (14). In both plasma and extracellular fluid, nifedipine is ~95% protein bound. It should be stressed that these calculations are only an estimate of tissue concentrations that may occur with systemic dosing.

The mechanism of the side effect of edema with calcium-channel antagonists is not known, although the vasodilator and negative inotrope activity of this class of compound are usually quoted as the cause. The edema is not usually caused by heart failure, is not accompanied by weight gain, and is frequently diuretic resistant (15, 17, 24). These features are compatible with an increase in microvascular permeability. A case report where intravascular cardiovascular parameters were measured in a patient with primary pulmonary hypertension who developed pulmonary edema with nifedipine suggested the likely cause was a change in microvascular permeability (28). Increased microvascular permeability is also likely to explain the case reports of periorbital edema caused by nifedipine and diltiazem (5, 32, 38). It may explain why not all negative inotropes or vasodilators cause edema and the intravascular to extravascular shift of fluid reported with nicardipine.

In the absence of topographical data, the site of fluid leakage in response to nifedipine has been presumed to be the capillary. This was thought to be secondary to a rise in capillary hydrostatic pressure from preferential dilation of the precapillary vessels. This hypothesis was based on studies showing that dihydropyridines, as well as verapamil and diltiazem, preferentially dilate cat skeletal precapillary vessels and the afferent glomerular arteriole (4, 10, 11).

We show for the first time that the extravasation of Monastral blue dye caused by nifedipine occurs through the postcapillary venule. This segment of the microcirculation controls the inflammatory edema response (6, 19). Specialized endothelial cells contract in response to inflammatory mediators. Agents such as bradykinin, histamine, or endothoxin contract the actin and myosin of the endothelial cytoskeleton to open intercellular hydraulic clefts allowing extravasation of fluid between the interendothelial cell junctions (8, 12, 20, 22, 31). This postcapillary venule endothelium also controls leukocyte adhesion and migration (7) but has not been implicated previously in the edema caused by cardiovascular drugs.

In other work we investigated whether an increase in macromolecular permeability occurs with calcium antagonists. Using similar experimental techniques to the present work, we found that over the dose range studied, diltiazem injected locally increased permeability, whereas verapamil did not. In contrast, verapamil increased microvascular blood flow, but diltiazem did not, suggesting that for these two calcium antagonists at least an increase in permeability is independent of microvascular vasodilatation (34). We went on to compare the microvascular effects of prostaglandin E2 with nifedipine and again showed dissociation between vasodilator and permeability effects with prostaglandin E2 being the more potent vasodilator for similar effects on permeability (35).

The microanatomical demonstration of an increase in postcapillary venule permeability in this study is consistent with our previous pharmacological findings that the increase in plasma extravasation caused by nifedipine could be suppressed by positive inotropes such as isoprenaline (36). In many models of inflammatory edema, β-adrenergic agonists or other agents that increase endothelial cell cAMP suppress the edema response (33, 42).

The mechanism whereby nifedipine increases permeability is not known but may be a direct effect on the endothelium. It has been shown that calcium-dependent mechanisms in endothelial cells modulate the permeability of venular microvessels to water and macromolecules (3). However, voltage-operated calcium channels have not been observed on endothelium (1). Interestingly, it has been suggested that the calcium-channel antagonist nitrendipine is in fact a calcium-channel opener on isolated endothelial cells (29), and this increases intracellular calcium possibly via activation of shear stress cation-selective channels. Other agents such as bradykinin, which increases endothelial cell permeability, are also known to increase intraendothelial cell calcium concentrations (3). It is possible that an intracellular increase in endothelial cell calcium may be associated with the increase in permeability caused by nifedipine but this needs further investigation. The action of calcium antagonists to increase permeability might be indirect through the release of mediators such as platelet-activating factor, ATP, or 5-hydroxytryptamine from cells such as platelets. Alternatively, these drugs may initiate an interaction between leukocytes and endothelial cells by inducing adhesion molecule expression.

Although we show nifedipine increases vascular permeability in rat skeletal muscle, skin, and mesentery, we have not shown this phenomenon in humans. Nifedipine-induced edema in patients usually presents as ankle swelling, suggesting that the hydrostatic pressure of upright posture and lymphatic drainage of the legs may be contributing factors.

In conclusion, this study shows for the first time that the calcium-channel antagonist nifedipine can act directly on the microcirculation to increase postcapillary venule permeability to macromolecules. This increase in permeability may contribute to edema formation.

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