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

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Taherzadeh, Morteza, Asit K. Das, and John B. Warren. Nifedipine increases microvascular permeability via a direct local effect on postcapillary venules. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1388–H1394, 1998.—Calcium-channel antagonist drugs are prescribed widely for angina and hypertension. A limiting side effect is edema, which can make heart failure worse. We show that nifedipine, a dihydropyridine-type calcium-channel antagonist, can increase vascular permeability in rat skeletal muscle and skin when injected locally. In nifedipine-injected cremaster muscle, the copper content, used to quantify Monastral blue dye accumulation, was 15.0 ± 2.4 µg/g compared with 5.3 ± 0.7 µg/g in control preparations (P < 0.05). The injection of nifedipine in rat skin in vivo increased local plasma leakage in injected sites from 5.5 ± 1.1 µl in control sites to 9.9 ± 2.3, 17.0 ± 2.4, 24.3 ± 5.9, and 23.3 ± 5.4 µl in sites injected with 10⁻², 10⁻³, 10⁻⁴, or 10⁻⁵ mol/site, respectively (P < 0.05 in each case compared with control). Vascular labeling techniques using light microscopy, electron microscopy, and microanalysis show that the microvascular site of leakage is not from capillaries but from postcapillary venules of 12–36 µm in diameter, the same site that controls the edema response in inflammation. Nifedipine can act within the microcirculation to increase the permeability of the postcapillary venule.

METHODS

Animals. Male Wistar rats weighing 200–250 g were anesthetized with 50 mg·kg⁻¹ i.p. of pentobarbitone sodium. The relevant skin area was shaved with electric clippers. In the experiment, anesthesia was maintained with 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-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. A maximal dose of 10⁻⁷ mol/site was used. This dose contained a 1% concentration of ethanol. Nifedipine was stored in the absence of light, and all dilutions and experiments were carried out under the illumination of a sodium lamp (wave length 580 nm). This illumination avoided radiation-mediated degradation of nifedipine, which is daylight and ultraviolet light sensitive.

For all test agents, dilutions were made 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).
Visualisation of plasma extravasation. To visualize plasma extravasation, Evans blue dye at 2.5% wt/vol (1 ml/kg) was injected intravenously via the tail vein. After 5 min, nifedipine was injected subcutaneously in the shaved scrotal skin of one testis, and ethanol (1%) was injected as a control in the opposite testis. Photographs of the injection sites were taken after 30 min when the response appeared to be maximal.

Microvascular localization of permeability change. To identify the site of edema leak within the vascular wall of the microcirculation, the copper-containing dye Monastral blue was first injected intravenously (0.1 ml/100 g, 3% wt/vol) in anesthetized rats via the tail vein. Nifedipine 10^{-7.2} mol in 100 µl 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 glycerin. 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 µl of nifedipine (10^{-7.2} mol) was applied on the mesentery of one loop and 100 µl 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 albumin labeled with 125I (1.5 µCi/100 µl) 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 µl 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 µl 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^{-7.2} mol/100 µl) 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 midsartal skin, and the cremaster 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 µg/dry tissue (21).

Electron microscopy and microanalysis. The anatomical location of Monastral blue within the wall of stained vessels and the ultrastructural changes in the microcirculation were examined by transmission electron microscopy. Rats were anesthetized, and Monastral blue (0.1 ml/100 g) was injected intravenously via the tail vein followed by the subcutaneous injection of agents in the shaved scrotal skin. One hour was allowed for the clearance of Monastral blue from the circulation. The animals were euthanized with an overdose of pentobarbitone sodium, and the root of the scrotum was clamped with a hemostat. The cremaster muscles were dissected out and immersed in chilled 2% glutaraldehyde and left for 4 h. The tissue samples were rinsed in cacodylate buffer (1 M, pH 7.2), fixed with 1% glutaraldehyde, and then dehydrated in graded alcohol. The tissue samples were transferred to epoxy resin and left for 24 h in a 60°C oven for polymerization. Ultrathin sections (90–100 nm) were cut and placed on grids and examined under an electron microscope (Philip, EM201).

Microanalysis was carried out to confirm that the deposits seen within the vessel wall were Monastral blue dye by measuring the copper content. Ultrathin sections were cut and placed on grids. These unstained sections were analyzed on a Hitachi scanning transmission microscope.

Measuring vessel diameter. Photographs of the microcirculation and a calibration grid were projected onto a large screen from 35-mm photographic slides. Measurements were made on the projected image of the external diameters of the vessels stained with dye and converted to actual size by the projected image of the calibration grid.

Statistical analyses. Numerical results are expressed as means ± SE. For the measurement of microvascular permeability, all data points are the mean of six animals, each experiment performed in duplicate per rat. Statistical comparisons were made with a two-tailed test using analysis of variance and taken as significantly different if P < 0.05.

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^{-7.2} mol/site. Nifedipine 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7.2} mol/site caused 9.9 ± 2.5, 17.0 ± 2.4, 24.3 ± 5.9, and 23.3 ± 5.4 µl plasma leakage, respectively, compared with the control of 5.5 ± 1.1 µl. 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^{-7.2} mol/100 µl caused obvious blueing of the scrotal skin in rats injected intravenously with Evans blue. The blue-
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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.

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 ($10^{-7.2}$ 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.

Microscopic vascular labeling of permeability change. Figure 3A shows the microscopic appearance of Monastral blue-stained vessels that were exposed to nifedipine ($10^{-7.2}$ mol/site). The main arteriole (A) and venule (V) run in parallel. Whereas there was not trace of dye in the large or small arterioles, the small venules (postcapillary venules, Vp) adjoining the main venules were heavily stained with the dye. Staining starts to appear in vessels of mean ± SE outer diameter of 12 ± 1 µm and stops when the external diameter reaches 36 ± 1 µm ($n = 50$). The microvascular position and diameter of these vessels confirms they are postcapillary venules, the same segment of the microcirculation that responds to inflammatory stimuli. The same selective microscopic staining of postcapillary venules was seen when the technique was applied to cremaster muscle exposed to 0.1 ml histamine ($10^{-8}$ mol) in terms of diameter and of the microvascular location of the labeled vessels (Fig. 3B). The labeling of vessels in both nifedipine and histamine preparations abruptly ceased at the point of convergence with the main venules 70–200 µm in diameter. At the capillary end, the staining became less intense in vessels of less than ~15 µm in diameter and ceased entirely before reaching the capillary (~5–8 µm). The topography of stained vessels from both nifedipine- and histamine-exposed tissues were indistinguishable, suggesting a common underlying mechanism. Control tissue, injected with 0.1 ml of 1% ethanol, showed no vascular labeling (Fig. 3C).

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 $10^{-7.2}$ 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 anephritic 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 10−7.5 mol. 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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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 other 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 vasodilation (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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