Effects of hydroxyethylrutosides on the permeability of microvessels in the frog mesentery

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1 We have investigated the effects of a standardised mixture of hydroxyethylrutosides (HR, Venoruton), a mixture of five of its main components (M) and each of the five components separately (7-mono-HR, 7,4'-di-HR, 7,3',4'-tri-HR, 5,7,3',4'-tetra-HR and 7,3',4'-tri HQ) upon the permeability of single perfused capillaries and venules in the mesenteries of pithed frogs.

2 In each experiment, the hydraulic permeability (L_p) of a single perfused microvessel and the effective osmotic pressure ($\sigma\Delta\pi$) exerted by macromolecules across its walls were estimated by a microocclusion technique, first during control perfusion and then in the presence of a known concentration of test substance.

3 HR, M and 7,4'-di-HR reduced L_p in a similar concentration-dependent manner over the range of $1 \mu\text{g ml}^{-1}$ to 1 mg ml^{-1} (maximum reduction was to 40% of control L_p at 1 mg ml^{-1}). At perfusate concentrations greater than 1 mg ml^{-1} , these substances reduced L_p to a lesser extent. While the four other test substances reduced L_p significantly when their perfusate concentrations equalled or exceeded $100 \mu\text{g ml}^{-1}$, they were all less potent than 7,4'-di-HR.

4 The reduction in L_p induced by the mixture of flavonoids was only slightly reversed by subsequent perfusion with flavonoid-free solutions.

5 When permeability was increased by perfusing with protein-free solutions, both HR and 7,4'-di-HR reduced and then reversed the increase in L_p in a concentration-dependent manner over the range of $1 \mu\text{g ml}^{-1}$ to $100 \mu\text{g ml}^{-1}$. None of the other component flavonoids was effective in restoring L_p under these conditions.

Keywords: Capillaries; flavonoids; hydroxyethylrutosides; microcirculation; microvascular permeability; vascular permeability

Introduction

O-(β -hydroxyethyl)-rutosides (oxerutins, Venoruton), abbreviated to HR, is a standardized mixture of hydroxyethyl derivatives of rutin which is widely used in patients with chronic venous insufficiency to reduce leg oedema and its related symptoms (Pulvertaft, 1983; Balmer & Limoni, 1989; Nocker *et al.*, 1989; de Jongste *et al.*, 1989; Wadworth & Faulds, 1992). Although HR has been shown to lower microvascular filtration rates in human limbs (Roztocil *et al.*, 1971; 1977; Cesarone *et al.*, 1992), the mechanism of its action is obscure.

Studies on experimental animals suggest that HR reduces microvascular permeability. It has been shown to reduce the leakage of plasma macromolecules in burns in anaesthetized dogs (Arturson, 1972; Hilton, 1982) and to diminish the accumulation of albumin in rats after local injection of histamine and bradykinin (Gerdin & Svensjo, 1983). Previous studies in our laboratory (Blumberg *et al.*, 1989; Michel *et al.*, 1990) showed that HR reduced the hydraulic permeability (L_p) of single perfused frog capillaries in both normal and inflamed mesenteric tissues. In inflamed tissues where permeability to plasma proteins was increased, HR was found to raise the effective osmotic pressure ($\sigma\Delta\pi$) exerted across the microvascular walls by perfusate macromolecules (Blumberg *et al.*, 1989). In preliminary experiments we also observed that the increase in vascular permeability which follows perfusion of these vessels with solutions free of plasma proteins (Mason *et al.*, 1977; Michel & Phillips 1985), could be partially (and sometimes completely) reversed by perfusion with solutions containing HR (Michel *et al.*, 1990).

The present paper describes experiments which address three issues arising from our previous studies. The first of these is to establish the relation between the reduction in L_p

induced by HR and the concentration of HR in the perfusate. While Blumberg *et al.* (1989) showed that the effect of HR was concentration-dependent, they failed to establish a clear dose-response relation, possibly because they pooled data from both normal and inflamed vessels. To minimize the range of 'control' values of permeability in the present study, we have investigated only vessels that appeared normal at the outset of an experiment.

The second issue arises from HR's being a mixture of O-(β -hydroxyethyl)-rutosides. The potency of its separate components has not been investigated previously and in this paper we describe the permeability-reducing effects of five of the principal constituents of HR.

The third set of experiments described in this paper was designed to investigate the range of perfusate concentration of HR over which it would reverse the increase in permeability induced by perfusion with protein-free solutions. We extended these experiments to examine the effectiveness of some of the principal components of HR in reducing permeability under these conditions. The effects of 7,4'-di-O-(β -hydroxyethyl)-rutoside, the constituent that reduced permeability most effectively in our preparation, were examined also over a range of perfusate concentrations. The effects of the other principal components were examined only at a single perfusate concentration.

Like Blumberg *et al.* (1989) we carried out our experiments on single perfused frog mesenteric capillaries and venules. By using this preparation we were able to apply techniques (Michel *et al.*, 1974; Michel, 1980) which yield values for both the L_p and the molecular sieving properties of vessel walls to macromolecules ($\sigma\Delta\pi$) in the same microvessel before, during and after perfusion with HR or one of its components. Working on single perfused microvessels reduces the ambiguities involved in interpreting changes in

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blood tissue transport in terms of changes in permeability. In the capillary beds of intact organs and tissues, however, changes in blood-tissue transport may result from changes in the distribution of flow (and pressure) between different microvascular units as well as from changes in permeability.

Methods

Preparation

The experiments were performed on single mesenteric capillaries and venules of frogs (*R. temporaria* and *R. pipiens*) in which brains and upper spinal cords had been destroyed by pithing. The frog was laid in a perspex tray and a loop of intestine was delivered through a lateral incision through the abdominal wall and the mesentery laid over the polished upper surface of a short perspex pillar. The mesentery was then transilluminated through the pillar and viewed through a Wild M8 stereomicroscope while the tissue was kept cool, moist and well oxygenated by continuous superfusion of its upper surface with frog Ringer solution bubbled with 97% O₂, 3% CO₂. A thermistor bead on the upper surface of the pillar in contact with the base of the mesentery, allowed tissue temperature to be monitored continuously.

Solutions

The Ringer solution used as a superfusate had the following constituents (mM): NaCl 111.1, KCl 2.4, MgSO₄·7H₂O 1.1, glucose 5.5, CaCl₂·1.1, NaHCO₃ approximately 10 but adjusted so that pH was between 7.2 and 7.4 when the solution was bubbled with 97% O₂ and 3% CO₂. Single microvessels were perfused with control and test Ringer solutions to which had been added bovine serum albumin (10 mg ml⁻¹) and the neutral macromolecule, Ficoll 70 (40 mg ml⁻¹). The concentrations of small molecules in the control perfusates were identical to those in the superfusates except the perfusates contained no NaHCO₃ and pH was adjusted by titration with 0.23 M NaOH immediately before being used. The test perfusates were made by adding a known mass of test substance to a measured volume of control perfusate. For the experiments described below in Sections 2 and 3 of Results, seven test substances were supplied to the experimenters (S.K. and C.C.M.) under ten different code numbers.

In some cases the same substance was supplied under a different code number. The code was revealed to the experimenters only after these experiments had been completed. The test substances were: (a) a standard preparation of HR (O-(β-hydroxyethyl)-rutosides, (lot number 2634, Zyma SA); (b) 7-mono-O-(β-hydroxyethyl)-rutoside (7-mono-HR); (c) 7,4'-di-O-(β-hydroxyethyl)-rutoside (7,4'-di-HR); (d) 7,3',4'-tri-O-(β-hydroxyethyl)-rutoside (7,3',4'-tri-HR); (e) 5,7,3',4'-tetra-O-(β-hydroxyethyl)-rutoside (5,7,3',4'-tetra-HR); (f) 7,3',4'-trio-O-(β-hydroxyethyl)-quercetin (7,3',4'-tri-HQ); (g) an artificial mixture (M) consisting of the above five pure compounds, in the same proportions in which they occur in HR (3: 32: 57: 7.5: 0.5). The structures of these flavonoid derivatives are shown in Table 1.

The oncotic pressure of both control and test perfusates, measured in a Hansen type membrane osmometer fitted with an Amicon PM-10 membrane, lay in the range of 20–23 cmH₂O. A few washed human red cells were added to all the perfusate solutions to act as markers of fluid flow. For the experiments described in Section 5 of Results, the second perfusate was a Ringer solution containing Ficoll 70 (40 mg ml⁻¹) but no bovine serum albumin (BSA). The third perfusate in these experiments was a similar Ringer solution (containing Ficoll 70 but not BSA) to which the test substance was added at a known concentration. Test substances were not coded in these later experiments.

Measurement of microvascular permeability

Microvascular permeability was assessed from the hydraulic permeability coefficient (L_p) and the effective oncotic pressure exerted across the microvascular walls by perfusate macromolecules ($\sigma\Delta\pi$). Fluid movements through unit area of vessel wall (J_v/A) are related to the transmural hydrostatic pressure difference (ΔP) across them through the relation:

$$J_v/A = L_p (\Delta P - \sigma\Delta\pi) \quad \dots\dots 1.$$

Since L_p is independent of capillary hydrostatic pressure in these vessels over a wide range, the relation between J_v/A and ΔP , at constant $\sigma\Delta\pi$, is linear with a slope of L_p and an intercept of $\sigma\Delta\pi$. Thus measurements of J_v/A at two values of ΔP allow both L_p and $\sigma\Delta\pi$ to be estimated.

J_v/A and ΔP were measured by the method of Michel *et al.* (1974). In this technique a long, straight and unbranched microvessel (diameter 15–25 μm) is cannulated with a sharpened micropipette and perfused with a Ringer solution

Table 1 Structure of the flavonoid derivatives tested

No.*	R ¹	R ²	R ³	R ⁴	R ⁵
1	HOCH ₂ CH ₂	H	Rutinoside	H	H
2	HOCH ₂ CH ₂	H	Rutinoside	HOCH ₂ CH ₂	H
3	HOCH ₂ CH ₂	H	Rutinoside	HOCH ₂ CH ₂	HOCH ₂ CH ₂
4	HOCH ₂ CH ₂	HOCH ₂ CH ₂	Rutinoside	HOCH ₂ CH ₂	HOCH ₂ CH ₂
5	HOCH ₂ CH ₂	H	H	HOCH ₂ CH ₂	HOCH ₂ CH ₂
6		Mixture of compounds 1, 2, 3 and 4			

*1 7-Mono-hydroxyethyl rutoside. 2 7,4'-Di-hydroxyethyl rutoside. 3 7,3',4'-Tri-hydroxyethyl rutoside. 4 5,7,3',4'-Tetra-hydroxyethyl rutoside. 5 7,3',4'-Tri-hydroxyethyl quercetin.

containing a few suspended red cells at constant pressure. After 5–10 min of perfusion, the outflow from the microvessel is interrupted by occlusion with a fine microneedle, some distance downstream from the cannulation site. The movements of red cells in the occluded segment then reflect fluid movements through the capillary walls between the marker red cells and the site of occlusion. From the velocity of a red cell (which is determined from a video recording) and the dimensions of the microvessel, J_v/A can be calculated. Such measurements of red cell velocity are made over the period 2 s to 10 s after microocclusion when the pressure applied to the microvessel through the pipette can be switched to a second value (Michel, 1980). While changes in the volume of the occluded segment interfere with the red cell movements immediately after occlusion or immediately after step changes in pipette pressure during occlusion, these compliance changes are complete within a second providing the microvascular pressure remains higher than 10 cmH₂O (Michel *et al.*, 1974; Levick & Michel, 1977; Swayne & Smaje, 1989). No attempt to estimate microvascular compliance was made in the present experiments.

Because the pressure applied to the micropipette approximates within 0.1 cmH₂O to the pressure inside the microvessels during occlusion, values of J_v/A can be estimated at different values of microvascular pressure which in turn approximates closely to ΔP . Thus both L_p and $\sigma\Delta\pi$ may be determined.

Protocol

In each experiment a single chosen capillary or venule was perfused with the control perfusate and L_p and $\sigma\Delta\pi$ were determined. The micropipette was then removed and the vessel was recannulated with a second micropipette filled with the test perfusate. L_p and $\sigma\Delta\pi$ were redetermined after at least 5 min perfusion with the second (test) solution. In the determination of dose-response curves, estimates of L_p and $\sigma\Delta\pi$ were made on 12 different vessels in the absence and presence of a given concentration of the test substance.

To investigate the effects of test substances in the absence of plasma proteins, L_p and $\sigma\Delta\pi$ were determined during each of three successive perfusions on the same microvessel. The first perfusate was the standard control perfusate containing Ficoll 70 and BSA; the second perfusate contained Ficoll 70 but no BSA; the third perfusate contained Ficoll 70 plus a known concentration of a test substance but no BSA. L_p and $\sigma\Delta\pi$ were determined between 5 and 10 min after the start of the first and third perfusions but it was found necessary to perfuse for 20 min with the second solution (protein-free) in order to obtain a reproducible increase in L_p over its control value.

Statistics

L_p was estimated from the slope of the regression line relating J_v/A to capillary pressure during perfusion with either control or test solution. The intercept of this relation with the pressure axis was taken as $\sigma\Delta\pi$.

Paired *t* tests were used to assess the significance of differences between L_p and $\sigma\Delta\pi$ in the absence and presence of test substances.

Results

(1) Reproducibility of estimates of L_p and $\sigma\Delta\pi$ in the same vessel

At the outset of the investigation, the reproducibility of the technique was assessed by determining the values for L_p and $\sigma\Delta\pi$ in a capillary or venule perfused with a control solution and then removing the micropipette, recannulating the vessel

with a second micropipette filled with the same control perfusate and remeasuring L_p and $\sigma\Delta\pi$. Seventeen experiments of this kind were carried out. The mean value ($\pm$ s.e.mean) of L_p during the first perfusion was $3.97 (\pm 0.45) \times 10^{-7}$ cm s⁻¹ cmH₂O⁻¹ and during the second perfusion was $3.71 (\pm 0.44) \times 10^{-7}$ cm s⁻¹ cmH₂O⁻¹. A paired *t* test revealed no significant difference between the first and second estimates of L_p .

The mean value ($\pm$ s.e.mean) of $\sigma\Delta\pi$ was 12.85 (± 0.47) cmH₂O during the first perfusion and 11.35 (± 0.43) cmH₂O during the second perfusion. The difference was not significant.

(2) Reduction of permeability by varying concentrations of mixtures of flavonoids

Figure 1a–d show the effects of various concentrations of Venoruton (HR) upon the relation between J_v/A and P_c in four single perfused capillaries. Each part of Figure 1 represents the results of a single experiment at one concentration of HR. It is seen that as the test perfusate contained increasing concentrations of HR, the slope of the relation between J_v/A and capillary pressure was reduced to a greater extent relative to its control value. There was, however, no change in the intercept of the relation, $\sigma\Delta\pi$. These four experiments were representative of the general response of microvessels to perfusion with HR. A clear concentration-dependent lowering of L_p could be observed as the HR concentration in the perfusate was raised from 10 μ g ml⁻¹ to 1 mg ml⁻¹. Perfusion with a test solution containing 10 mg HR ml⁻¹, however, reduced L_p to a smaller extent than perfusion at 1.0 mg ml⁻¹. In Figure 2a, each closed circle is the mean value of 12 determinations of L_p in vessels perfused with solutions containing a given concentration of HR. The open circles represent mean control values of L_p for the same microvessels later investigated at that concentration of HR. Thus the effect of a particular concentration of HR can be assessed from the difference in L_p between the open and closed-circles at that concentration. A paired *t* test revealed that the differences were significant ($P < 0.001$) at all perfusate concentrations of HR equal to or greater than 10 μ g ml⁻¹. Figure 2b is a similar representation of the data obtained from experiments where the second perfusate contained M, the reconstituted mixture of five components of HR. Here each point is based on 24 determinations of L_p as M was investigated on two separate occasions under two different code numbers.

The general pattern is very similar to that seen for HR namely that increasing concentrations of the mixture reduce L_p to an increasing extent up to a concentration of 1 mg ml⁻¹ when L_p is lowered to less than half its control value. At a concentration of 10 mg ml⁻¹, both HR and the reconstituted mixture reduce L_p to smaller extent than at 1 mg ml⁻¹. Nevertheless a paired *t* test revealed the reduction in L_p was highly significant ($P < 0.001$) at this concentration as it was at all perfusate concentrations of M equal to or greater than 10 μ g ml⁻¹. Like HR, the reconstituted mixture did not effect the value of $\sigma\Delta\pi$.

(3) Reduction of permeability by varying concentrations of five purified components of HR

When each of the five purified components was tested on their own, they were all found to reduce L_p ($P < 0.02$, paired *t* test) once their concentration in the perfusate had exceeded 100 μ g ml⁻¹. None of the components significantly affected the values of $\sigma\Delta\pi$. There were, however, marked quantitative differences in their effectiveness. Thus at concentrations of 1 mg ml⁻¹, 7-mono-HR, 7,3',4'-tri-HR, and 5,7,3',4'-tetra-HR reduced L_p by only 10%–13% ($P < 0.001$) and at concentrations to 10 mg ml⁻¹ lowered L_p further to 14–20% below its control value ($P < 0.001$). Thus they were considerably less effective than either HR or the reconstituted

Figure 1 (a–d) The relation between fluid filtration per unit area of capillary wall (J_v/A) and capillary pressure in four experiments on single mesenteric capillaries of frog. Each diagram shows the results from a vessel which was perfused first with the control perfusate (○) and then with a perfusate containing HR (●). The concentrations of HR in the second perfusate were (a) 0; (b) $10 \mu\text{g ml}^{-1}$; (c) $100 \mu\text{g ml}^{-1}$; (d) 1.0 mg ml^{-1} .

Figure 2 The effects of varying concentrations of HR and the reconstituted mixture of five of its components upon the L_p of single microvessels of frog. (a) Mean values $\pm$ s.e. mean of L_p in the absence (○) and presence (●) of HR ($n = 12$). (b) Mean values $\pm$ s.e. mean of L_p in the absence (○) and presence (●) of M, the reconstituted mixture of five components of HR ($n = 24$).

mixture. 7,4'-di-HR, however, reduced L_p when it was present at all concentrations greater than $10 \mu\text{g ml}^{-1}$ ($P < 0.001$). When its perfusate concentration was 1.0 mg ml^{-1} , L_p was reduced to 40% of its control value (a reduction of 60%) and as with HR itself, raising the perfusate concentration of 7,4'-di-HR to 10 mg ml^{-1} reduced the magnitude by which it lowered L_p . 7,3',4'-tri-HQ was less effective than 7,4'-di-HR but more effective than the other three β -hydroxyethyl rutosides significantly reducing L_p ($P < 0.001$) when present in the perfusate at concentrations greater than $10 \mu\text{g ml}^{-1}$. At a concentration of 1 mg ml^{-1} , 7,3',4'-tri-HQ lowered L_p to 74% of its control value (i.e. a 26% reduction).

The dose-response curves for the five components are shown in Figure 3. Here the effect on L_p is shown as the mean value of L_p in the presence of a given concentration of the test substance divided by the mean control value of L_p for the same microvessels. Each point is based on paired test and control measurements on 12 microvessels.

(4) Reversibility of effects of reconstituted mixture on L_p

To investigate the reversibility of the effects of the reconstituted mixture upon permeability, 12 experiments were carried out in which a microvessel was perfused with (i) control perfusate, (ii) perfusate containing the mixture at a concentration of 1.0 mg ml^{-1} , (iii) control perfusate. Estimates of L_p and $\sigma\Delta\pi$ were made during each perfusion and continued for at least 20 min (and usually 30–40 min) during the final control perfusion. It was found that L_p which was reduced

from an initial value ($\pm$ s.e.mean) of $4.56 (\pm 0.47) \times 10^{-7} \text{ cm s}^{-1} \text{ cmH}_2\text{O}^{-1}$ to one of $0.94 (\pm 0.11) \times 10^{-7} \text{ cm s}^{-1} \text{ cmH}_2\text{O}^{-1}$ during perfusion with the mixture, was increased only slightly after 20 min–40 min of the third perfusion when it had a mean value of $1.11 (\pm 0.14) \times 10^{-7} \text{ cm s}^{-1} \text{ cmH}_2\text{O}^{-1}$. The small rise in L_p during the final perfusion was reproducible and a paired t test revealed it was significant ($P < 0.01$).

Figure 3 The effects of varying concentrations of five purified components of HR upon the L_p of frog single microvessels: (■) 7-mono-HR; (●) 7,4'-di-HR; (▲) 7,3',4'-tri-HR; (△) 7,3',4'-triHQ; (□) 5,7,3',4'-tetra-HR. Each point represents the mean ($\pm$ s.e.mean) value of L_p in the presence of a given concentration of the test substance divided by the mean value of L_p for the paired controls ($n = 12$).

(5) Effects of HR and its principal components on L_p of vessels in which permeability has been increased by protein-free perfusions

Perfusion with albumin-free solutions consistently increased L_p to approximately twice its value in the presence of 1% BSA and reduced the effective osmotic pressure exerted by Ficoll 70 in the perfusate. HR reversed the increase in L_p when added to the perfusate at concentrations equal to or greater than $6 \mu\text{g ml}^{-1}$. Figure 4a–d shows the changes in fluid filtration rate per unit area of microvascular wall (J_v/A) with changes in microvascular pressure in four experiments each with a different concentration of HR in the third perfusate. Each part of Figure 4 relates J_v/A to microvascular pressure during the three perfusions of a single experiment. The slope of each line yields the value for L_p during that perfusion while the intercept with the pressure axis gives the value for $\sigma\Delta\pi$. It is seen that removal of BSA from the perfusate (solution b) increases the slope of the relation (L_p) and reduces the value of $\sigma\Delta\pi$ in all experiments. Whereas HR has no effect when present at a concentration of $1 \mu\text{g ml}^{-1}$, it restores L_p to its control value at 0.006 mg ml^{-1} and takes it below control when its concentration is 0.1 mg ml^{-1} . HR, however, does not appear to restore $\sigma\Delta\pi$ to its initial value.

Between 8 and 10 experiments were carried out for each concentration of HR in the third perfusate and the results are summarized in Table 2. It is seen that removal of BSA from the perfusate consistently increased L_p and the increase was reversed by HR in a dose-dependent fashion. The changes in $\sigma\Delta\pi$, however, are less clear. While perfusion with albumin-

Figure 4 (a–d) The relations between fluid filtration (J_v/A) and capillary pressure in four experiments on single mesenteric capillaries of frog. Each diagram shows the results of a single experiment when the vessel was perfused first with a control perfusate containing Ficoll 70 and serum albumin (●), then with a similar solution containing Ficoll 70 but no albumin (▲) and finally with a solution containing Ficoll 70 and HR (□). The concentrations of HR in the third perfusate were as follows: (a) $1 \mu\text{g ml}^{-1}$; (b) $6 \mu\text{g ml}^{-1}$; (c) $10 \mu\text{g ml}^{-1}$; (d) $100 \mu\text{g ml}^{-1}$.

Table 2 Reversal of the effects of removal of serum albumin by hydroxethyl-rutosides (HR)

Concentrations of HR in solution (iii) (mg ml ⁻¹)		Ringer perfusate solutions							
		(i) Perfusate with 4% Ficoll 70 + 1% BSA		(ii) Perfusate with 4% Ficoll 70		(iii) Perfusate with 4% Ficoll 70 + HR		L _{p1} /L _{p2}	L _{p3} /L _{p2}
		L _{p1}	(σΔπ) ₁	L _{p2}	(σΔπ) ₂	L _{p3}	(σΔπ) ₃		
0.1	10	3.36 (± 0.31)	11.11 (± 0.38)	7.0 (± 0.88)	9.10 (± 0.73)	1.65 (± 0.73)	10.18 (± 0.48)	0.48	0.24
0.01	10	3.81 (± 0.51)	10.9 (± 0.43)	7.57 (± 0.79)	6.35 (± 0.70)	3.06 (± 0.49)	9.37 (± 0.64)	0.50	0.40
0.006	8	3.27 (± 0.34)	10.71 (± 0.81)	8.69 (± 0.45)	5.78 (± 0.70)	2.64 (± 0.29)	7.15 (± 0.52)	0.38	0.3
0.003	10	4.5 (± 0.77)	10.34 (± 0.76)	8.48 (± 0.51)	6.43 (± 0.46)	7.91 (± 0.54)	6.08 (± 0.53)	0.53	0.93
0.001	10	4.33 (± 0.31)	11.53 (± 0.36)	8.72 (± 0.51)	6.51 (± 0.87)	8.75 (± 0.38)	7.10 (± 0.62)	0.5	1.00

All figures for L_p and σΔπ are mean values ± s.e.mean. Values for L_p are expressed as 10⁻⁷ × cm s⁻¹ H₂O⁻¹; values for σΔπ are in cmH₂O.

Table 3 Reversal of permeability increase (induced by removal of serum albumin) by 7,4'-di-HR

Concentration of di-HR in solution (iii) (mg ml ⁻¹)		(i) Perfusate with 4% Ficoll 70 + 1% BSA		(ii) Perfusate with 4% Ficoll 70 (protein-free)		(iii) Perfusate with 4% Ficoll 70 + di-HR		L _{p1} /L _{p2}	L _{p3} /L _{p2}
		L _{p1}	(σΔπ) ₂	L _{p2}	(σΔπ) ₂	L _{p3}	(σΔπ) ₃		
0.1		4.58 (± 0.51)	11.33 (± 0.27)	9.44 (± 0.42)	5.86 (± 0.83)	3.15 (± 0.43)	6.94 (± 1.07)	0.49	0.33
0.01		4.58 (± 0.65)	11.30 (± 0.19)	9.13 (± 0.84)	7.61 (± 0.87)	4.4 (± 1.00)	7.91 (± 0.64)	0.50	0.48
0.001		4.05 (± 0.83)	11.00 (± 0.38)	8.04 (± 0.65)	5.70 (± 0.72)	7.86 (± 0.85)	6.03 (± 0.63)	0.50	0.98

All figures are mean values of experiments ± s.e.mean. Values for L_p are expressed as 10⁻⁷ × cm s⁻¹ cmH₂O⁻¹; values for σΔπ are in cmH₂O.

For abbreviations, see text.

Table 4 Effects of 7-mono-HR, 7,3',4'-tri-HR and 5,7,3',4'-tetra-HR in single vessels where permeability has been increased by perfusion with protein-free solutions

Test substance in perfusate (iii)		(i) Perfusate with 4% Ficoll 70 + 1% BSA		(ii) Perfusate with 4% Ficoll 70 alone		(iii) Perfusate with 4% Ficoll + test substance		L _{p1} /L _{p2}	L _{p3} /L _{p2}
		L _{p1}	(σΔπ) ₁	L _{p2}	(σΔπ) ₂	L _{p3}	(σΔπ) ₃		
mono-HR 10 μg ml ⁻¹	6	3.53 (± 0.50)	10.23 (± 0.17)	8.21 (± 0.80)	5.95 (± 0.70)	8.33 (± 0.69)	6.17 (± 0.86)	0.43	1.01
tri-HR 10 μg ml ⁻¹	6	4.22 (± 0.62)	11.48 (± 0.60)	8.28 (± 0.63)	5.07 (± 1.29)	8.50 (± 0.66)	5.20 (± 1.21)	0.51	1.03
tetra-HR 10 μg ml ⁻¹	6	3.00 (± 0.25)	10.25 (± 0.60)	8.03 (± 0.56)	4.40 (± 0.27)	7.95 (± 0.67)	4.47 (± 0.713)	0.37	0.99

All values of L_p and (σΔπ) represent means of 6 experiments ± s.e.mean. Values of L_p are expressed as 10⁻⁷ × cm s⁻¹ cmH₂O⁻¹ and σΔπ in cmH₂O.

For abbreviations, see text.

free solutions reduced σΔπ, perfusion with HR at concentrations ≥ 6 μg ml⁻¹ raised σΔπ from a mean (± s.e.mean) of 5.78 (± 0.70) cmH₂O to one of 7.15 (± 0.52) cmH₂O but did not restore it to its control value (10.71 cmH₂O).

When varying concentrations of 7,4'-di-HR were present in the third perfusate, the effects on L_p were similar to those found with HR. The data are summarised in Table 3 which also shows that 7,4'-di-HR had no significant effect upon σΔπ.

The effects of the other three principal constituents of HR were investigated at the single concentration of 10 μg ml⁻¹. Six experiments were carried out with each of these compounds as the test substance and the results are summarized in Table 4.

Although near maximal effects of HR and 7,4'-di-HR were seen when they were present at a concentration of 10 μg ml⁻¹, none of the other rutosides were found to affect permeability at this concentration.

Discussion

Effects of HR and its constituents on vessels of normal permeability

Our findings, which confirm many of the observations of Blumberg *et al.* (1989), show that HR reduces the L_p of frog

mesenteric microvessels in a concentration-dependent manner. Maximum effects of HR are seen in the range of 0.1 mg ml⁻¹ to 1.0 mg ml⁻¹. We have also shown that a mixture of five components of HR reproduces the permeability lowering properties of HR in frog mesenteric capillaries. This suggested that at least one of the constituents of the mixture was responsible for the effects of HR on permeability. An examination of the effects of the individual components revealed that 7,4'-di-HR was largely responsible for the reduction of L_p by HR. The other four components had smaller but significant effects in reducing L_p. It is, of course, quite possible that in the mixture and in HR itself, they have a synergistic influence on the actions of 7,4'-di-HR. Such synergism would account for the slightly greater potency of the mixtures than 7,4'-di-HR in reducing L_p.

The effects of a wide range of concentrations of HR and 7,4'-di-HR upon L_p are described by bell-shaped dose-response curves. It is of particular interest that the purified single compound, 7,4'-di-HR, is less effective in reducing L_p when its perfusate concentration rises above 1 mg ml⁻¹. One possible explanation would be that 7,4'-di-HR binds to two different receptors which initiate opposite effects on permeability. Providing the interaction of these effects is non-competitive and the affinity for the receptor that promotes a reduction of L_p is greater than that which leads to a permeability increase, the overall dose-response curve would be bell shaped (e.g. Young *et al.*, 1992).

We did not observe any effects of HR (or its constituents) on $\sigma\Delta\pi$ in vessels of normal permeability. This is consistent with the observations of Blumberg *et al.* (1989). While they reported that HR raised $\sigma\Delta\pi$, the effects were restricted to inflamed vessels where $\sigma\Delta\pi$ was initially low and $\sigma\Delta\pi$ was raised to values in the normal range but not above it.

We have also confirmed that the effects of HR on L_p reversed very slowly. This characteristic effect of HR has restricted our experimental protocols in that the effects of test substances on permeability had to be assessed by comparing the value of L_p (and $\sigma\Delta\pi$) for a given vessel in the presence of the test substance with its value for the same vessel during the preceding control perfusion only. Justification for this general protocol is derived from observations that there were no significant changes in L_p when the second perfusate was identical in composition to the first.

The slow reversibility of the effects of HR on permeability may be of considerable significance in maintaining therapeutic levels of these substances in the tissues during their long term administration. In this context it is worth noting that the concentrations of HR and 7, 4'-di-HR which clearly reduced permeability, are comparable with levels which might be achieved if the recommended oral dose of HR were diluted in a volume equivalent to that of the plasma or blood (500 mg in 3 litres or 5 litres). While the incomplete absorption of HR from the gastrointestinal tract and its hepatobiliary clearance from portal venous blood might seriously reduce the levels of HR in the systemic arterial blood, the slow reversibility of the effects of HR raise the possibility that repeated oral doses may allow potent levels of HR to accumulate in the target areas (Neumann *et al.*, 1992).

Effects of HR and its components on vessels perfused with protein-free solutions

In this study we have shown that both HR and 7,4'-di-HR reverse the increase in L_p which is induced by perfusion with protein free solutions. The fall in $\sigma\Delta\pi$ to Ficoll 70, which accompanied the increase in L_p , was incompletely reversed by HR and little affected by 7,4'-di-HR.

Our preliminary investigation of the effects of HR on vessels in which permeability had been increased by protein-free perfusion (Michel *et al.*, 1990), arose from the observation that HR reduced the permeability of inflamed frog microvessels in spite of the persistence of gaps in the endothelium (Blumberg *et al.*, 1989).

The increase in permeability which occurs in inflammation or that resulting from mediators such as histamine, is associated with the development of gaps in the endothelium. By contrast, perfusion with protein-free solutions raises permeability without the development of gaps (Mason *et al.*, 1979). The raised permeability associated with removal of

protein can be readily and completely reversed by perfusion with albumin containing solutions (Mason *et al.*, 1979; Michel & Phillips, 1985). Furthermore, there is strong evidence that serum albumin binds reversibly to the luminal surface of the endothelium (Schneeberger & Hamelin, 1984; Schnitzer *et al.*, 1988), and since its removal from this site is associated with the permeability increase, it has been proposed that the ultrafilter of capillary walls and a major component of the hydraulic resistance of the pathways through them, reside in a lattice-like structure formed by the interaction of plasma proteins with the luminal endothelial glycocalyx (Curry & Michel, 1980; see Michel, 1988, for review). Thus when Blumberg *et al.* (1989) observed a reduction of permeability without the disappearance of the endothelial gaps, they suggested that HR might reduce the permeability of an extracellular barrier such as the glycocalyx. Our observation that HR can reverse the rise in L_p associated with removal of albumin is consistent with this hypothesis. It seems unlikely, however, that the flavonoids act in the same way as albumin itself. The action of albumin is dependent upon its positively charged arginine residues (Michel *et al.*, 1985) and it is believed to reduce L_p by occupying space within the surface coat and by ordering the fibrous molecules of glycocalyx into an evenly spaced lattice (Michel, 1988). It is unlikely that HR and 7,4'-di-HR, which are small molecules, can have a direct action of this kind. Furthermore, we should note that HR and 7,4'-di-HR failed to reverse the fall in $\sigma\Delta\pi$.

An alternative mechanism would be for 7,4'-di-HR (and possibly other as yet unidentified components of HR) to act on the endothelial cell and for intracellular mechanisms to order the molecules of the glycocalyx or promote its secretion. It is of considerable interest that Curry & Lenz (1987) reported that noradrenaline reduced the L_p of frog mesenteric capillaries in the absence of BSA but not in its presence. Recent work by He & Curry (1991) has shown that depolarizing the endothelium with high extracellular K^+ concentrations reduces L_p . The depolarization reduces Ca^{2+} influx into the endothelial cells (He & Curry, 1991) and preliminary reports suggest that the raised L_p following protein-free perfusion may be reduced by high concentrations of extracellular K^+ (He *et al.*, 1991). At present it is unclear how changes within the endothelial cell can modify permeability except where this is associated with the development of gaps between adjacent endothelial cells. Against this background the possible mechanism of actions of HR and other flavonoids on vascular permeability remains highly speculative.

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