Effects of Endothelial Denudation and Cholesterol Feeding on In Vivo Transport of Albumin, Glucose, and Water across Rabbit Carotid Artery

Aram V. Chobanian, James O. Menzoian, Jane Shipman, Kevin Heath, and Christian C. Haudenschild

From the Evans Department of Clinical Research, Divisions of Medicine, Surgery, and Pathology, Cardiovascular Institute, Boston University School of Medicine, Boston, Massachusetts

SUMMARY. An in vivo system for studying arterial transport was developed which utilized the rabbit carotid artery perfused in vivo with Dulbecco's modified Eagle's medium containing $^{125}$I-labeled albumin, $[^3H]3$-methyl-$d$-glucose, or tritiated water. The appearance of labeled materials in jugular venous blood was measured serially over 4 hours. Vascular integrity was assessed by scanning electron and transmission microscopy. Maintenance of endothelial integrity appeared dependent on perfusion with nutrient tissue culture medium, use of papaverine to inhibit arterial spasm, and circulation of the medium under pressure. Acute endothelial denudation with a balloon catheter induced an approximate 10-fold increase in plasma concentration of labeled albumin and a 3-fold rise in plasma $[^3H]3$-methyl-$d$-glucose activity, compared with results in animals with intact endothelium. Increased appearance of tritiated water in venous blood was also observed in the rabbits with denuded endothelium, although the relative rise was less than that with albumin or glucose. Feeding rabbits a diet containing 1.5% cholesterol for periods of 16–28 weeks produced approximately 10-fold increases in plasma concentration of $^{125}$I-labeled albumin after arterial perfusion to levels comparable to those present in chow-fed rabbits with experimental endothelial denudation. The increases in albumin transport with cholesterol feeding occurred even though a relatively small fraction of the intimal surface was involved with lesions. The results suggest that the arterial endothelium provides a relative barrier to albumin and, to a lesser extent, glucose and water. The findings also suggest that cholesterol feeding markedly increases arterial permeability to albumin, to a degree that is disproportionately greater than the extent of atherosclerotic involvement of the intimal surface. (Circ Res 53: 805–814, 1983)

THE transport of macromolecules and other substances across the arterial wall has been the subject of considerable interest because of its possible relationship to the initiation or progression of atherosclerosis. Previous studies have demonstrated that the arterial endothelium provides a relative barrier to vascular uptake or transport of a variety of substances, including albumin (Siflinger et al., 1975; Nerem et al., 1976; Caro et al., 1980; Bratzler et al., 1977a), fibrinogen (Sanchez et al., 1976; Smith and Staples, 1980), low density lipoproteins (Bratzler et al., 1977b), horseradish peroxidase (Flory and Shepard, 1970), lactoperoxidase (Stein and Stein, 1972), and ferritin (Stein and Stein, 1973). Little information is available concerning the fate of most substances after entering the arterial wall. Much of the prior work has been performed with isolated arteries studied in vitro. Passage of albumin through the arterial wall has been demonstrated in canine aortic sacs (Duncan and Buck, 1961) and in cannulated rabbit common carotid artery perfused in vitro (Caro et al., 1980). Removal of arterial endothelium has been reported to increase substantially the fluid loss through the rabbit thoracic aorta (Vargas et al., 1979) and to enhance the entry of albumin into the canine carotid artery (Fry, 1973). Other mechanical manipulations also may increase albumin transport, including stretching (Duncan et al., 1962), increasing the wall shear stress (Reif et al., 1976), or subjecting the vessel to sinusoidal oscillatory changes in length (Chien et al., 1981).

In vivo studies following intravenous administration of labeled albumin into rabbits have suggested that albumin enters the arterial wall both from the endothelial surface and the adventitial side, and that transport through the media occurs primarily by diffusion (Bratzler et al., 1977a; Colton et al., 1980). Similar studies utilizing labeled low density lipoproteins (LDL) have indicated that LDL entry into aorta from the circulating blood occurs from both intimal and adventitial surfaces, with the former accounting for the major fraction (Bratzler et al., 1977b). The entry of albumin and fibrinogen into aortic wall of pigs studied in vivo has been reported to be greater in areas staining positive with Evans blue dye than in adjacent sites, and the highest concentrations of both proteins in the wall appear to be in the intima and inner media (Bell et al., 1974a, 1974b).
The current studies have been performed to examine the transport of molecules from the arterial luminal surface into the peripheral circulation. An in vivo perfusion system involving the rabbit common carotid artery has been utilized to study the influence of endothelial denudation and cholesterol feeding on the process. The results indicate that mechanical removal of the endothelium markedly increases the transport of albumin and, to a lesser extent, glucose and water, across the artery wall into the blood stream. The findings also suggest that, after cholesterol feeding, the trans-arterial movement of albumin increases to levels approximating those observed following endothelial denudation.

Methods

Adult male New Zealand white rabbits (2.8–3.2 kg) were anesthetized with pentobarbital. The right internal jugular vein was isolated, and a polyethylene catheter was inserted and advanced to the right atrium for obtaining blood samples. The left common carotid artery was exposed from the base of the neck to its bifurcation without stripping away the adventitia. All observable branches of the common carotid artery (usually 2–5 in number) were ligated with 6-0 prolene sutures, and the artery was ligated at its bifurcation with 4-0 silk sutures. An arteriotomy was performed and a Silastic catheter (0.76 mm, inner diameter), which was stretched over a polyethylene tip (0.86 mm), was inserted and kept in place with a ligature to serve as the efflux line. The common carotid was perfused with medium (see below) against arterial pressure until free of blood, and then ligated proximally at the base of the neck. The efflux line was closed with a stopcock to prevent collapse of the vessel. A proximal Silastic catheter then was inserted and tied in place, and the isolated common carotid artery (approximately 3.5 cm in length) was perfused with medium maintained at 39°C from a reservoir chamber for at least 15 minutes before addition of the isotopes. Preparations were excluded if blood appeared in the medium during this period. Throughout the procedure, the carotid artery was kept moist with saline, and papaverine (0.3% solution) was applied topically to prevent vasospasm (Haudenschild et al., 1981a).

The perfusion solution used in all experiments consisted of Dulbecco's modified Eagle's medium to which was added newborn calf serum (10%), l-glutamine (0.57 g/liter), and papaverine (0.02 mg/liter). The albumin in the medium was obtained entirely from the calf serum and its concentration, which ranged from 2.7 to 3.0 g/liter, was determined colorimetrically with bromcresol green (Doumas et al., 1971). Penicillin (96,000 U/liter), streptomycin (86,000 U/liter), and amphotericin B (0.25 µg/liter) were added to prevent bacterial and fungal contamination. The medium (10 ml) was kept in a conical glass reservoir at 39°C in an atmosphere of 95% O2, 5% CO2. The pH of the solution ranged between 7.3 and 7.4 during the study. The medium was recirculated continuously through the carotid artery at approximately 50 mm Hg pressure, with a peristaltic pump (Pharmacia P-3). The pressure was monitored intermittently, with a T-tube inserted into the efflux line and connected to a transducer and strip-chart recorder.

All isotopes were obtained from New England Nuclear, and were added to the reservoir solution after perfusion of the carotid artery had been established. Four to five animals were used in each of the groups. In the studies with labeled albumin, the 125I-albumin was present in the perfusion medium in a concentration of 5 µCi/ml. In representative experiments, aliquots of the labeled albumin were dialyzed against water for 24 hours at 4°C before use, with Spectraray 3 dialysis membrane tubing (Fisher Scientific Co.).

Blood samples (2 ml) were collected from the jugular venous catheter at the beginning of the study and after 10, 30, 60, 120, 180, and 240 minutes of perfusion. 125I radioactivities of the medium and plasma were counted directly in an auto-gamma scintillation spectrometer, both before and after precipitation with trichloroacetic acid (TCA). Aliquots of the medium were removed for assay at the initiation of the study and after 2 and 4 hours. Medium loss was measured from the decrease in reservoir levels throughout the procedure.

The nature of the 125I radioactivity in plasma was also assessed by Sephadex gel filtration. A 1-ml plasma sample obtained after 4 hours of perfusion was applied to a Sephadex G-50 column (1.5 X 30 cm) which was pre-equilibrated with 0.1 M sodium chloride, 0.02% sodium azide, and 0.01 M Tris, pH 7.4. Samples were eluted with the buffer at a flow rate of 25 ml/hr. The void volume and total volume were determined with dextran blue 2000 and H2O, respectively. The position of albumin was measured by absorbance at 280 nm. 125I radioactivity in the column eluates was assayed directed in the autogamma scintillation spectrometer.

In the experiments involving glucose and water transport, H2O (specific activity, 100 mCi/g) and [3H]-3-O-methyl-d-glucose (specific activity, 80 Ci/mmol) were present in the perfusion solution at concentrations of 250 µCi/ml and 50 µCi/ml, respectively. The activity was assayed in a liquid scintillation counter after addition of Liquiscint (National Diagnostics Co.).

No. 6, December 1983
ally performed concurrently, on the same day, with aliquots of the same medium.

The experiments involving the net flux of labeled albumin across the carotid were also performed in cholesterol-fed rabbits. Male New Zealand white rabbits (1.5–2.0 kg) were fed a diet containing 1.5% cholesterol and 5.2% corn oil over a period of 16–28 weeks. Plasma cholesterol levels were monitored at 3- to 4-week intervals, as described (Haudenschild et al., 1981b), and relative nonresponders were excluded from the studies. The carotid artery perfusions were performed in an identical manner, as stated above.

In studies involving labeled albumin, 125I radioactivity of the common carotid artery was also assayed. At the end of the experiment, the carotid artery was perfused for an additional 20 minutes with medium free of labeled material. After perfusion, the artery was removed and the adventitia was carefully stripped away as previously described (Haudenschild et al., 1981b). 125I radioactivity was measured in the intima-media preparation. Assays were also performed in the contralateral nonperfused common carotid artery.

Results

A diagram of the experimental system is shown in Figure 1. All manipulations of the vessel were performed without extensive dissection around the artery. Contraction of the artery was prevented by use of papaverine and by perfusion of the vessel at approximately the mean blood pressure of the animal. Once the experimental system was established, studies could be performed for at least 4 hours without producing evidence of endothelial cell injury. If any blood appeared in the medium during perfusion, it was assumed that all branches of the carotid artery had not been ligated during the study. Such experiments were terminated and their results were discarded. Approximately 15% of the studies were excluded for such technical reasons, but all other experimental data were utilized.

The plots of plasma 125I-labeled albumin activity during the 4-hour infusions are illustrated in Figure 2. When the carotid artery was deendothelialized with the balloon catheter, there was a marked increase in levels of 125I radioactivity in blood, compared with the values obtained when the endothelium was maintained intact. The average medium loss during perfusion was significantly greater in the vessels subjected to the balloon catheter (0.40 ± 0.05 ml/hr) than in the control arteries (0.15 ± 0.03 ml/hr) (P < 0.001).

The major fraction of plasma 125I radioactivity could be precipitated with TCA in all experiments. After 10 minutes of perfusion, the TCA precipitate accounted for 93.1 ± 1.3% of total plasma radioactivity; at 120 minutes, 92.6 ± 1.5% and at 240 minutes, 92.3 ± 1.4%. Characterization of the 125I radioactivity in representative blood samples obtained after 240 minutes of perfusion was performed by Sephadex gel filtration. The major portion of plasma radioactivity (greater than 90%) was recovered from the column in the albumin-containing fraction. Use of dialyzed or nondialyzed 125I-labeled albumin preparations produced similar results with respect to the rate of appearance of both total and TCA-precipitable radioactivity in plasma.

Calculations were made of the amount of albumin transported across the carotid artery, based on the specific activity of albumin measured at the beginning of the experiment, and assuming that all of the labeled albumin present in the TCA precipitate of blood resulted from such transport, that all of the labeled albumin in blood remained in the intravascular space and was not metabolized during the course of the experiments, and that the volume of distribution or plasma volume was approximately 5% of body weight. The estimated transport based upon these calculations averaged 7 μg/cm length per hr (18 μg/cm² intimal surface area per hr) for normal carotid artery and 68 μg/cm per hr (170 μg/cm² per hr) following endothelial denudation.

The relative disappearance of labeled albumin from the medium in the control experiments was
much less than that of the fluid. The calculated rate of loss of albumin in these studies averaged 14% of that expected if such loss occurred on the basis of leakage of the complete medium into the systemic circulation.

The increase in plasma levels of $^3$H$_2$O during perfusion is shown in Figure 3. Somewhat greater levels were observed in animals after removal of the carotid endothelium, although the differences were only significant at 3 and 4 hours.

With $[^3$H]-3-methyl-$d$-glucose, the plasma levels of $^3$H radioactivity were 2- to 3-fold greater after endothelial denudation than with intact endothelium (Fig. 4). The differences were significant at all time intervals from 1 to 4 hours.

The plasma cholesterol levels in the cholesterol-fed rabbits ranged between 1190 and 3580 mg/dl from the second month of the study to its completion, with mean levels at sacrifice of 2600 ± 206 mg/dl. In the cholesterol-fed group, the levels of $^{125}$I radioactivity in plasma following carotid artery perfusion were significantly greater than in controls (Fig. 5). However, no significant differences were observed when comparisons were made between cholesterol-fed animals and those from which the endothelium had been removed. The rate of appearance of labeled albumin in the blood of cholesterol-fed animals did not correlate with the level of plasma cholesterol. $^{125}$I radioactivity in the perfused carotid artery at the completion of the study was significantly greater ($P < 0.01$) in the deendothelialized carotid artery than in the vessels with intact endothelium (Table 1). Similar differences were observed when comparisons were made between cholesterol-fed and control animals ($P < 0.01$). The levels of carotid $^{125}$I radioactivity were not significantly different in cho-

<table>
<thead>
<tr>
<th>Effects of Endothelial Denudation and Cholesterol Feeding on Arterial $^{125}$I Radioactivity after 4-Hour Perfusion of Rabbit Common Carotid Artery with $^{125}$I-labeled Albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I radioactivity (dpm/cm length)</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Endothelial denudation</td>
</tr>
<tr>
<td>Cholesterol feeding (4-6 months)</td>
</tr>
</tbody>
</table>

* $P$ value < 0.01 for comparison between control animals and those subjected to endothelial denudation.
† $P$ value < 0.01 for comparison between control and cholesterol-fed rabbits.
lesterol-fed animals and in those subjected to balloon catheter treatment.

Negligible $^{125}$I radioactivity was present in the contralateral (nonperfused) carotid artery, averaging 1% or less of the levels present in perfused vessels.

Scanning electron microscopy of the entire intimal surface of representative carotid arteries was performed after 4 hours of perfusion. In the control animals, the endothelial surface appeared intact (Figs. 6 and 7). The specimens subjected to prior mechanical denudation demonstrated complete absence of endothelial cells.

After cholesterol feeding, intimal plaques that covered approximately 5–10% of the intimal surface (Fig. 8) were observed. Areas of endothelial cell loss were present with underlying exposed subendothelial tissue. In places, round cells that had the characteristics of blood-borne cells were adherent to these sites (Fig. 9). In intimal areas free of plaques, the endothelium appeared intact, but its surface was not as flat as in controls.

Transmission electron micrographs taken from focal raised lesions after 4 hours of in situ perfusion showed lipid droplets within monocytes and smooth muscle cells, and increase in intimal extracellular matrix. The endothelium was continuous over large areas, but showed occasional vacuolization and blebbing at junctional sites (Fig. 10). Adjacent to the lesions, the internal elastic lamina was folded (Fig. 11), and the endothelial continuity was not disrupted.

**Discussion**

We have established a reproducible in vivo system for studying the transport of substances across the common carotid artery of rabbits. The arterial endothelium has been preserved in an intact state, and no morphological evidence of vascular injury has been apparent. Important ingredients for the preservation of the endothelium have included minimal manipulation or dissection of the adventitia (other than for ligation of the arterial branches), perfusion of the artery under pressure, and use of enriched tissue culture medium.

Molecular transport across the endothelium and total arterial wall is a complex process and can involve several different pathways, including passage via intercellular junctions, pinocytotic vesicles, transendothelial channels, or diffusion through cell membranes. Under normal circumstances, macromolecules such as albumin are thought to be transported primarily in vesicles (Chien, 1978). Water, glucose, and other small molecules are thought to be transported across capillary endothelium, primarily through intercellular junctions and transendothelial channels (Chien, 1978). The current studies have indicated that the endothelium is the major...
FIGURE 7. High magnification of scanning electron micrograph of perfused control carotid artery shown in Figure 6. A continuous layer of flat endothelial cells is preserved. 1000X.

FIGURE 8. Raised lesion in the intima of common carotid artery of a cholesterol-fed rabbit. The vessel wall shows a wavy appearance adjacent to the lesion, whereas the remaining intima is indistinguishable from controls. The specimen was pressure-fixed after 4 hours of in situ perfusion. 54X.
FIGURE 9. Detail of damaged endothelium in a raised lesion in the common carotid artery of a cholesterol-fed rabbit. The rounded cells filling the gaps between endothelial cells show the morphology of blood-borne cells. 540X.

barrier to the transport of albumin (and, to a lesser extent, glucose and water) across the arterial wall. Our findings with albumin are consistent with the prior studies of Fry (1973), Siflinger et al. (1975), and Ramirez (1979), who utilized different techniques. The relative decrease in water flux after endothelial denudation is similar to that reported in human and rabbit aorta (Vargas et al., 1979; Blackshear et al., 1978), indicating that—independent of the mechanism—the endothelium contributes to the total resistance to water flux.

The major portion of the \( ^{125}I \) radioactivity recovered in plasma appeared to be albumin-bound. It could be precipitated with TCA and migrated with albumin on Sephadex gel filtration. The radioactivity not associated with albumin represented approximately 10% of total plasma radioactivity during the 4-hour period of study and, presumably, reflected albumin metabolism. Such metabolism has been observed previously in cultured cells (Ehrenreich and Cohn, 1967; Ryser, 1968) and in intact arterial tissue (Bratzler et al., 1977a). Its extent cannot be quantified in our studies, since the volume of distribution of labeled metabolites of albumin entering the circulation should be different from that of albumin. Such metabolites should be smaller in size and pass more rapidly into the extravascular compartments than albumin.

Considerable net flux of labeled albumin across the luminal surface into the blood stream was apparent, even in control carotid artery with intact endothelium. The estimated amount appearing in the blood after perfusion averaged approximately 7 \( \mu \text{g/cm} \) length of carotid per hr in control tissue and 68 \( \mu \text{g/cm} \) per hr after removal of endothelium. These values do not take into consideration the albumin metabolized either within the vessel wall or after it had been transported into the blood stream. Any loss of albumin from the intravascular compartment during the course of the studies also has not been accounted for. Thus, the calculated figures are probably underestimates of the actual rates. The values are roughly similar to albumin influx rates of 9 to 27 \( \mu \text{g/cm}^2 \) surface area per hr for porcine aorta reported by Bell et al. (1974), but their studies utilized different techniques in which \(^{131}I\)-labeled albumin was injected intravenously into the pig, and arterial radioactivity was determined at sacrifice 2 and 24 hours after the injection. Their approach could not assess the amount of labeled albumin passing through the aorta. The control values would represent overestimates of the actual rates, if unrecognized endothelial damage were present, but the differences between the control and experimental groups would then be even greater.

The influence of cholesterol-feeding on arterial albumin flux was striking, with approximately 10-fold greater levels of \(^{125}I\) radioactivity in venous...
blood of cholesterol-fed, compared with control, rabbits. The levels were comparable to those observed after experimental endothelial denudation. The mechanisms involved in this change in albumin flux have not been defined as yet, although other prior studies using different and less direct techniques have also suggested that cholesterol feeding enhances arterial uptake of albumin (Stefanovich and Gore, 1971). The increase in net flux of albumin that we observed appeared disproportionate to the extent of involvement of the carotid endothelium by atherosclerotic disease. Only 10% or less of the surface of the perfused arteries had visible plaques. At these sites, areas of endothelial denudation were present with exposed subendothelial tissue, to which occasional round cells, resembling blood borne cells, were adherent. The remaining endothelium appeared intact by scanning electron microscopy, although, adjacent to the plaques, the endothelial surface had a wavy appearance—suggesting compression or contraction of such regions. It is interesting to speculate that cholesterol feeding and/or atherosclerotic disease in the vessel wall could induce nondenuding functional alterations that
could lead to enhanced transport of albumin or of macromolecules. As an example, a change in surface charge of vascular cells could produce such an outcome. Endothelial and smooth muscle cells have a net negative surface charge which may act as a relative barrier to anionic substances such as albumin and LDL (Damon and Skutelsky, 1976), and treatment of cell cultures with cationic substances such as poly-L-lysine or protamine sulfate increases albumin uptake markedly (Ryser and Hancock, 1965; Nicolson et al., 1977). Exposure of cultured smooth muscle cells to the cationic platelet-derived growth factor enhances binding of LDL (Chait et al., 1980).

A marked increase in transport of macromolecules through the endothelium in the presence of only minimal involvement of the endothelial surface has been explained on theoretical grounds by a model proposed by Nir and Pfeffer (1979). As an example, the model predicts that if as little as 3% of the endothelial surface is damaged with circular holes involving 10% of the medial thickness, a 250% increase in uptake could occur. The relevance of this model to the in vivo situation is unknown, however.

The increase in albumin flux that we observed with cholesterol feeding could possibly be related to an increase in vascularity of the vessel wall which occurs with the development of atherosclerosis, rather than to any effect on the endothelium. Adventitial blood flow has been reported, by Heistad et al. (1981), to increase in cholesterol-fed monkeys, and such enhanced flow might affect the passage of albumin across the arterial wall may be enhanced with atherosclerosis (Bratzler et al., 1977a, b; Reif et al., 1981), to increase in cholesterol-fed monkeys, and transport of molecules through the arterial wall combined. The vesicular transport of albumin across the arterial wall is enhanced with atherosclerosis (Chien et al., 1978), with a much greater resistance to transport at this site than exists in all of the remaining areas of the vessel wall combined. The vesicular transport of macromolecules in vascular tissue appears to be a passive process, unaffected by a variety of metabolic inhibitors (Siflinger et al., 1975; Jennings and Florey, 1967), and transport of molecules through the arterial media has been thought to occur primarily through diffusion (Bratzler et al., 1977a, b; Weinbaum and Caro, 1976). Nevertheless, changes in transport of albumin across the arterial wall may not necessarily occur as a result of changes in endothelial permeability, and the possibility of a contribution of enhanced blood flow through the vasa vasorum cannot be excluded entirely.

Similarly, we cannot exclude the possibility that some effect of cholesterol feeding other than the production of arterial disease may have played a role. However, our findings suggest strongly that vascular permeability to albumin and its net flux across the arterial wall is enhanced with atherosclerosis.
Ryser HJP, Hancock R (1965) Histones and basic polypeptides stimulate the uptake of albumin by tumor cells in culture. Science 150: 501–503
Stein O, Stein Y (1972) An electron microscopic study of the transport of peroxidases in the endothelium of mouse aorta. Zellforsch Z 133: 211–222

INDEX TERMS: Arterial transport • Endothelium • Atherosclerosis and arterial permeability • Endothelial denudation
Effects of endothelial denudation and cholesterol feeding on in vivo transport of albumin, glucose, and water across rabbit carotid artery.
A V Chobanian, J O Menzoian, J Shipman, K Heath and C C Haudenschild

doi: 10.1161/01.RES.53.6.805

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/53/6/805