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Cerebral Endothelial Surface Charge in Hypertension*

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Summary. Anionic groups on cerebral arteriolar endothelium were localized using cationized ferritin (CF), and alterations in the distribution of these groups were documented in arterioles with increased permeability to horseradish peroxidase (HRP) in angiotensin-induced acute hypertension.

Normotensive animals showed a uniform distribution of anionic groups on the endothelial luminal plasma membrane when fixed or live vessels were reacted with CF. Anionic groups were localized at the mouth of pinocytotic vesicles in both preparations; however, only live cells demonstrated CF particles within vesicles, and the possibility that these represent pinocytosed CF particles cannot be ruled out. Cationized ferritin particles were not observed on the plasma membranes within interendothelial spaces in either of the preparations.

Sixty percent of hypertensive animals with pressures over 200 mmHg showed increased arteriolar permeability to HRP. At 2.5 min, permeable arteriolar segments with active vesicular transport of HRP showed marked reduction or loss of CF binding. Capillaries and venules in the adjacent cortex and nonpermeable arterioles demonstrated linear endothelial CF binding similar to controls. Most permeable vessels of animals killed 6–20 min after onset of acute hypertension when the blood-brain barrier is usually closed showed CF binding on endothelium indicating that there is rapid restoration of the net negative charge.

These studies demonstrate that increased arteriolar permeability in acute hypertension is associated with a transient alteration of surface charge. The mechanism by which charge is altered remains to be determined.

Key words: Endothelium – Experimental hypertension – Cationic ferritin – Cerebrovascular

Introduction

Studies using a variety of techniques (Danon et al. 1972; Furchgott and Ponder 1941; Gasic et al. 1968) have demonstrated that a net negative charge is present on the surface of cells indicating the presence of anionic groups on the plasma membrane. About 50% of the anionic groups are sialyl groups (Danon and Skutelsky 1976) with carboxyl groups of proteins, phosphates of phospholipid and amines of proteins contributing (Burry and Wood 1979). Introduction of the reagent cationized ferritin (CF) has allowed localization of anionic groups at an ultrastructural level and physiological pH (Danon et al. 1972). Using CF a negative charge has been demonstrated on the endothelium of noncerebral vessels in preparations using live (Skutelsky and Danon 1976; Simionescu et al. 1981; Pietra et al. 1983) or previously fixed cells (Pelikan et al. 1979; Simionescu et al. 1981). There is increasing interest in the role of surface charge in endothelial permeability to macromolecules (Cavallo et al. 1980; Melnick et al. 1981; Nagy et al. 1983; Sibley et al. 1983).

Our previous studies (Nag et al. 1977, 1979) on angiotensin-induced acute hypertension have demonstrated that some penetrating arterioles in the temporo-parietal cortex develop increased permeability to plasma proteins and protein tracers, such as horseradish peroxidase (HRP). This study was undertaken to determine the distribution of anionic groups on normal cerebral arteriolar endothelium at an ultrastructural level using cationized ferritin. Rats with angiotensin-induced acute hypertension were then studied to determine whether arterioles with increased permeability to HRP show an alteration of endothelial surface charge.

Materials and Methods

Female Wistar-Furth rats, weighing 200–220 g, were anesthetized using methoxyflurane. A PE 50 cannula was inserted into the femoral vein, and a PE 90 cannula was inserted into the aorta for blood pressure measurements and removal of blood for blood gases. Blood pressure was measured by a pressure transducer and recorded throughout experiments using a Grass polygraph. The experimental groups were as follows:

Hypertensive Animals

Twenty-one animals were injected i.v. with HRP, Type II (Sigma Chemical Co., St. Louis, MO, USA) in a dose of 30 mg/100 g.

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Hypertension was then induced by a 2-min infusion of angiotensin amide in a dose of 12.5 μg/ml per minute. Experiments were terminated by perfusion of fixative 2.5, 4, 6, 10, and 20 min after onset of the angiotensin infusion.

Normotensive Controls

Group I. Four animals received saline i.v. instead of angiotensin and HRP.

Group II. Four animals were injected with saline and the same dose of HRP as the hypertensive animals.

Fixation and demonstration of HRP reaction product was done as described previously (Nag et al. 1979). About 90 brain slices per hemisphere were examined by light microscopy. Blocks containing either permeable or nonpermeable arterioles from the temporo-parietal area were selected for CF binding studies.

Anionic Groups on Fixed Endothelium

The superficial molecular layer of brain slices of test and control animals was cut away to ensure that solutions would have access to the vascular lumina. Brain slices were washed with Dulbeccos' phosphate buffered saline (DPBS) and then with DPBS containing 0.1 M glycine to quench unbound aldehyde groups and prevent nonspecific adsorption of ferritin on the surface of membranes. They were then incubated in CF having a concentration of 0.5 mg/ml in DPBS at room temperature for 20 min to 2 h. Vessels of control animals were also incubated in solutions of cadmium-free native ferritin, pH 4.5 (horse spleen, Calbiochem-Behring, San Diego, CA, USA) for the same time periods and at the same concentration. Tissues were rinsed with DPBS and processed for electron microscopy.

All tissues for electron microscopy were postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 30 min followed by staining en bloc with uranyl acetate in sodium hydrogen maleate-NaOH buffer, 0.05 M (pH 7.0). They were then dehydrated and embedded in an epon-araldite mixture, and ultrathin sections were examined unstained or following staining with lead citrate using a Hitachi H500 electron microscope at 75 KV. Vascular segments of test and control animals were studied at approximately the same depth from the cortical surface by the same methods as described previously (Nag et al. 1979).

Anionic Groups on Living Endothelium

The external and common carotid arteries of four rats were dissected. A PE 10 cannula inserted into the left common carotid artery was not tied, so flow through the vessel was maintained. The external carotid artery was ligated. Animals were then perfused with Krebs-Ringer-bicarbonate buffer, pH 7.4, at 37° C for 1 min via a cannula in the ascending aorta to avoid interaction of the ligand with blood cells. Cationized ferritin, pH 8.4, 0.5 mg/ml per minute (Miles-Yeda, Rehovot, Israel) was then infused via the carotid cannula for 2 min followed by a 3-min infusion-free period. Two rats received one infusion of CF, while others received two infusions. Experiments were terminated by intra-aortic perfusion of fixative. Blocks of cortex containing arterioles were processed for electron microscopy using the same techniques as described above.

Results

Blood Pressure

The mean resting systolic blood pressure of the normotensive animals was 120 ± 8 mmHg (Fig. 1). The mean blood pressure rise in the hypertensive animals at 1 min was 230 ± 14 mmHg. The blood pressure reached resting levels at 4–5 min.

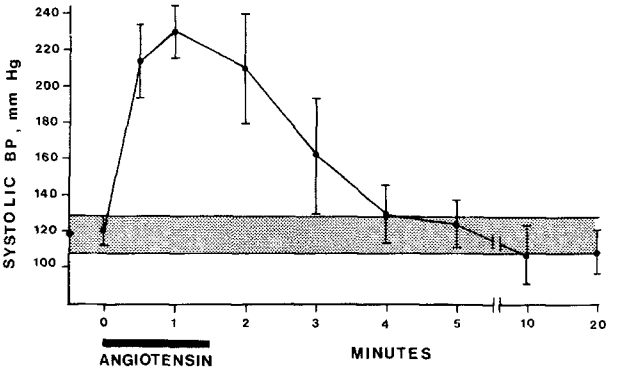

Fig. 1. Pressor response to an infusion of angiotensin amide in hypertensive animals. The *shaded area* shows the mean systolic blood pressure and the SD of the normotensive controls

Permeability Studies

Normotensive Animals. A few cortical slices of normotensive animals receiving HRP showed tracer in the walls of occasional arterioles.

Hypertensive Animals. Sixty percent of the hypertensive animals showed increased permeability to HRP in numerous arterioles of the temporo-parietal cortex.

As shown previously, animals killed at 2.5 min when blood pressure was markedly elevated, showed HRP principally in arteriolar walls (Fig. 2A). At this time interval a few capillaries and venules also showed tracer in their walls. Electron microscopy showed tracer in all layers of arteriolar walls and numerous pinocytotic vesicles containing tracer (Fig. 2B). The interendothelial junctions did not show continuous labeling from the luminal to the abluminal end.

At later time intervals after the onset of acute hypertension, when the blood pressures had returned to basal levels residual areas of tracer extravasation around arterioles were frequently seen (Fig. 3A). On electron microscopy tracer was seen in arteriolar walls and in continuity in the extracellular spaces of the surrounding neuropil (Fig. 3B). At these time intervals very little tracer remained in pinocytotic vesicles or interendothelial spaces.

Anionic Groups on Living Endothelium

Normotensive animals receiving CF via the carotid artery over a 5-min period showed patchy binding of clumps of CF particles to segments of the luminal plasma membrane leaving interpatch areas that were free of CF. After 10 min of circulation, linear continous binding of CF occurred on the luminal plasma mem-

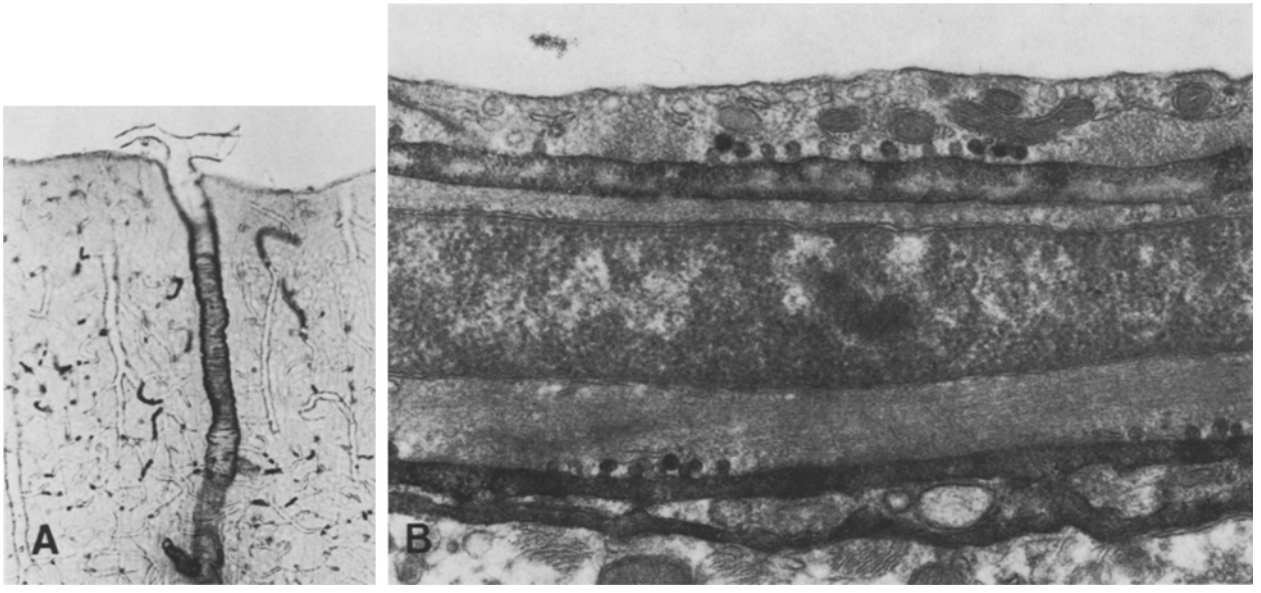

Fig. 2. A Permeable arteriole of a hypertensive rat killed at 2.5 min showing HRP in the wall. **B** Electron microscopy shows HRP in all layers of the wall with no extravasation into the surrounding neuropil. The endothelium shows numerous pinocytotic vesicles. Tracer is present in pinocytotic vesicles at the abluminal plasma membrane and in the adjacent basement which is not uniformly labeled with tracer suggesting this is an early phase of permeability alteration. No CF particles are present on the endothelium. **A** ×81, **B** ×31,500

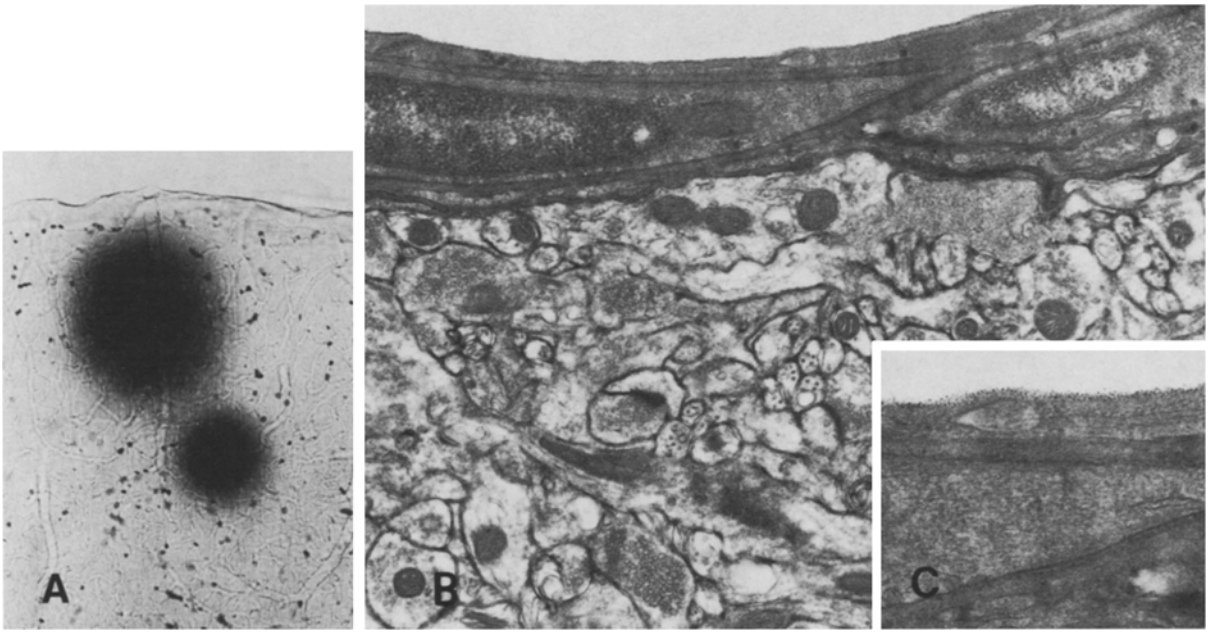

Fig. 3. A Hypertensive rat at 10 min showing focal areas of HRP extravasation in the cortex. **B** Electron microscopy shows HRP in the arteriolar wall with extravasation into the extracellular spaces of the surrounding neuropil. The uniform CF binding on the luminal plasma membrane is shown at higher magnification in the inset (**C**). **A** ×81, **B** ×16,650, **C** ×37,700

brane of arterioles, venules, and capillaries (Fig. 4). Cationized ferritin particles were observed on the plasma membrane only at the luminal end of the interendothelial spaces but not along the length of the interendothelial spaces. Anionic groups were demonstrable on stomata of pinocytotic vesicles and within vesicles that were open onto the vascular lumen (Fig. 4).

Anionic Groups on Fixed Endothelium

Normotensive Animals. The pattern of CF binding on fixed endothelium (Fig. 5) was the same as observed on live endothelium, except that no CF binding was observed within pinocytotic vesicles. Vessels incubated in CF solutions for 20 – 30 min consistently showed the

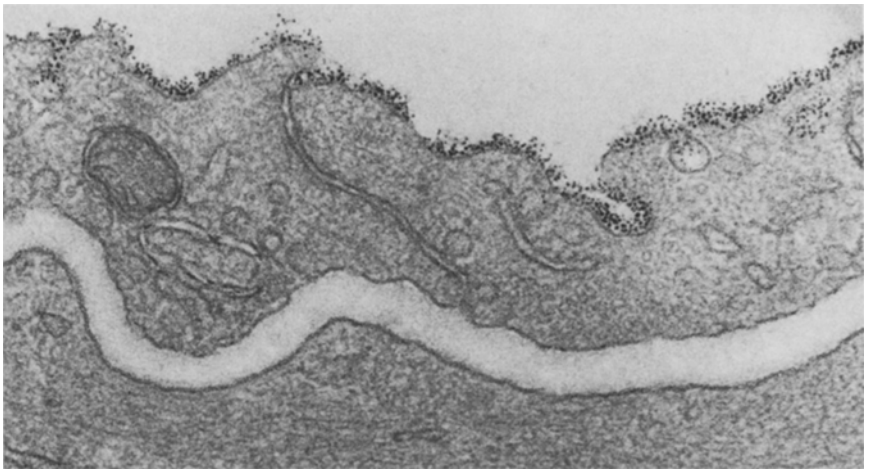

Fig. 4. Segment of arteriolar wall from a normotensive rat showing CF particles on the luminal endothelial plasma membrane and within pinocytotic vesicles prior to fixation. The plasma membranes at the luminal end of the interendothelial space shows CF, while those within the space are free of CF. × 60,000

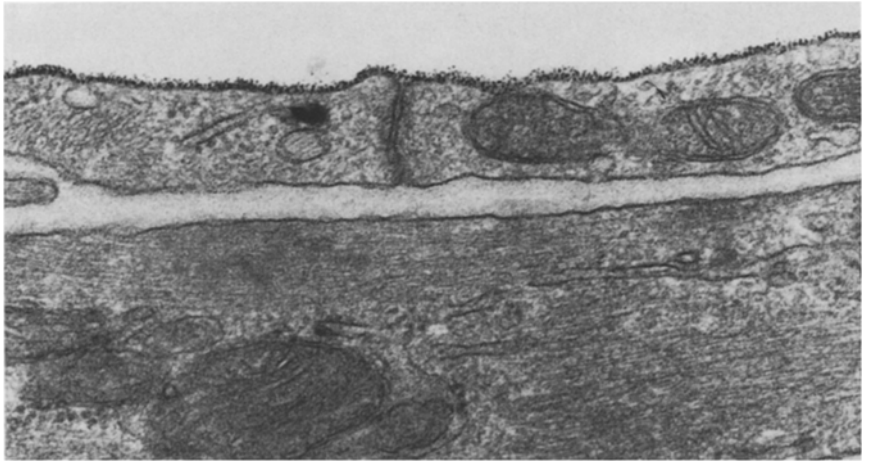

Fig. 5. Segment of arteriolar wall from a normotensive animal which received saline and HRP. Following fixation linear uniform CF binding is seen on the endothelial plasma membrane, on the stomata of pinocytotic vesicles, and the luminal end of the interendothelial space. No CF particles are seen within pinocytotic vesicles or the interendothelial space. × 54,000

same pattern of CF binding, and longer incubations did not change the pattern of binding; therefore, for all subsequent studies vessels were incubated at these time intervals. Controls receiving both saline and HRP showed the same pattern of CF binding as those receiving only saline indicating that the presence of HRP in the circulation did not alter the distribution of anionic groups.

Sections incubated in native ferritin failed to reveal binding of ferritin particles on the endothelium.

Hypertensive Animals. Endothelium of permeable arterioles at 2.5 min showed marked reduction or loss of CF binding (Fig. 2B). Nonpermeable capillaries and venules in the same section and nonpermeable arterioles showed the normal pattern of CF binding and provided a good positive control for the reaction. At 4–6 min 30 % of permeable vessels showed absence of CF binding (Table 1), while the remaining vessels and all nonpermeable vessels examined showed CF binding similar to that seen in normotensive controls. All permeable vessels examined at 10 and 20 min showed the same pattern of CF binding as the nonpermeable vessels (Fig. 3B).

Discussion

The principal finding in the present study is that increased cerebral arteriolar permeability in acute hypertension is associated with a loss or marked reduction of the anionic groups on the endothelium. At later time intervals after onset of hypertension, when the blood-brain barrier is again closed, restoration of the anionic groups on the endothelium occurs.

Although cerebral endothelium has different permeability properties to protein tracers as compared to noncerebral vessels, the distribution of anionic groups under normal conditions appears to be the same in both types of vessels. Our studies of live cerebral endothelial cells have demonstrated that after a short circulation

Table 1. Arteriolar endothelial charge

	No. of arterioles with CF binding	Total arterioles sectioned
Controls		
Saline	6/6	
Saline and HRP	6/6	
Hypertensive	Permeable to HRP	Nonpermeable to HRP
2.5 min	0/12	6/6
4 min	8/12	5/6
6 min	4/6	5/6
10 min	6/6	6/6
20 min	6/6	6/6

time large discrete patches of CF binding occur at intervals along the plasma membranes leaving interpatch areas that are free of the ligand as observed in noncerebral vessels (Skutelsky and Danon 1976; Pelikan et al. 1979; Cavallo et al. 1980; Pietra et al. 1983). However, on exposure of the endothelium to CF for longer periods there is linear uniform distribution of CF along the luminal plasma membranes as observed by others (Pelikan et al. 1979; Simionescu et al. 1981). CF binding did not occur on the plasma membrane within interendothelial spaces in any of the previous studies (Skutelsky and Danon 1976; Cavallo et al. 1980; Pietra et al. 1983) or in the present study.

The distribution of anionic groups on the luminal plasma membrane of cerebral arteriolar endothelium following fixation was the same as reported by others in live cells (Simionescu et al. 1981; Pelikan et al. 1979) and fixed cells therefore our studies of surface charge in hypertension with appropriate controls were done on previously fixed vessels. Horseradish peroxidase in the form used in this study has a neutral charge (Rennke et al. 1978) and by itself did not alter the surface charge on endothelium since there was no difference in the distribution of anionic groups of controls receiving saline only and those which received saline and HRP.

Cationized ferritin was not observed within pinocytotic vesicles following fixation. However, when CF was injected via the carotid prior to fixation CF particles were observed within vesicles at the luminal plasma membrane. Studies of systemic vessels prior to fixation have shown CF binding within pinocytotic vesicles of mesenteric (Clough 1982) and pulmonary capillaries (Pietra et al. 1983) and not within vesicles of pancreatic and intestinal capillaries (Simionescu et al. 1981). Whether the presence of CF within pinocytotic vesicles of live endothelium suggests that anionic groups are present within vesicles or that they represent pinocytosed CF particles remains uncertain. However, the finding that some pinocytotic vesicles in live cerebral endothelium were not labeled by CF fails to support the hypothesis proposed by Frøkjaer-Jensen (1980) that endothelial vesicles are part of cluster-like invaginations from the cell surface.

The pattern of HRP extravasation in acute hypertension is similar to our previous observations (Nag et al. 1979). The finding of increased numbers of pinocytotic vesicles in endothelium during the early phase of permeability and the absence of continuous labeling of interendothelial spaces at this time period supports pinocytosis as the principal route of protein transport in this model.

During acute hypertension increased arteriolar permeability is associated with loss or decreased anionic groups on endothelium. It is unlikely that we are dealing with an artifact because capillaries and venules in the adjacent areas and nonpermeable arterioles showed CF binding indicating that the vessels did have access to the CF solutions. There is increasing interest in the role of charge in cerebrovascular permeability alterations. Neutralization of the negative charge on endothelium by intracarotid injection of the polycation protamine sulfate has been reported to be associated with increased cerebrovascular permeability to Evans blue (Harbedo and Andersson 1983), and HRP (Nagy et al. 1983). In the latter study decreased anionic groups on cerebral endothelium were demonstrated in vitro by decreased colloidal iron binding to previously fixed brain tissue. Furthermore, a study of glomerular vessels has reported decreased anionic groups on endothelium of hybrid New Zealand mice with chronic immune complex disease, prior to proteinuria (Melnick et al. 1981).

An additional finding in the present study is that previously permeable arterioles of animals killed after return of pressures to normal show restoration of the surface anionic groups. At these time intervals after induction of hypertension by a single dose of a pressor agent, the blood-brain barrier is known to be restored to normal (Johansson and Linder 1978; personal observation). Therefore, the vessels showing HRP in the neuropil indicate spread of tracer which leaked during the hypertensive episode. Evidence that anionic groups can be regenerated is obtained from the in vitro studies (Skutelsky and Danon 1976) of aortic endothelial cells. Incubation of vascular segments with CF results in rapid aggregation of most anionic groups on the luminal front of the endothelium followed by detachment of the CF patches leaving most of the luminal surface devoid of anionic sites. Further incubation of such vessels without CF results in regeneration of binding capacity for the polycationic label.

Whether the decrease in the anionic groups observed in acute hypertension precedes the permeability alteration observed in cerebral vessels or occurs follow-

ing the passage of HRP across the endothelium remains unclear at the present time. It is known that on contact with proteins, such as albumin, there is decrease of the anionic groups on the endothelial surface (Cavallo et al. 1980). Possibly, acute hypertension disturbs the laminar flow of macromolecules allowing proteins to come into contact with the endothelium decreasing the surface charge and promoting permeability of endothelium to proteins. Alternatively, hypertension in some manner yet unknown neutralizes the net negative charge on endothelium resulting in increased vascular permeability to proteins.

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