Intracerebral Arteriolar Permeability to Lanthanum

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Lanthanum, an electron-dense tracer, has been used extensively in the study of the structure of cell junctions. The present study was undertaken to determine whether the interendothelial junctions of normal intracerebral arterioles allow passage of lanthanum and to document the alterations occurring in these structures in acute hypertension. Perfusion of lanthanum for 12–40 minutes in control animals resulted in passage of tracer into arteriolar walls and into the extracellular compartment of the surrounding brain. The two principal mechanisms associated with tracer extravasation into the brain were diffuse passage through endothelial cytoplasm and through interendothelial spaces bypassing tight junctions. The latter finding has not been previously reported in normal cerebral arterioles and suggests that the tight junctions of these vessels are different from those of capillaries and consist of a meshwork of closely arranged maculae occludentes rather than complete circumferential occluding bands as was previously believed. Hypertensive animals showed accelerated passage of lanthanum, it being demonstrable not only in arteriolar walls but in capillary and venular walls and the surrounding neuropil after only 5 minutes of circulation. Passage of tracer through vessel walls occurred by the same routes as in controls. In addition, increased numbers of pinocytotic vesicles were observed in the endothelium, confirming our previous studies that increased vesicular transport occurs in cerebral arteriolar endothelium in acute hypertension. (Am J Pathol 1982, 107:336–341)

NEUTRALIZED LANTHANUM solutions have proven useful in distinguishing gap junctions from tight junctions and distinguishing incomplete tight junctions (maculae occludentes) from complete circumferential tight junctions (zonulae occludentes).[1–6] Lanthanum solutions are perfused following prior tissue fixation with aldehyde mixtures. Glutaraldehyde fixation results in formation of intermolecular and intramolecular links between amino acids, yielding rigid heteropolymers of protein.[7] Presumably, the protein subunits forming the intercellular junctions are stabilized, and differences found in the intercellular junctions in control and experimental circumstances are representative of actual biologic differences.

Brightman and Reese,[8] using neutralized lanthanum, observed that the junctions between endothelial cells of intracerebral capillaries excluded passage of this tracer from the vascular lumen into the vessel wall and therefore were of the zonulae occludentes type. The latter study did not mention whether the interendothelial junctions of intracerebral arterioles were similar. In recent years there has been increasing awareness of the important role of arterioles in blood-brain permeability alterations in diverse experimental situations.[9–11] The present study was undertaken to determine whether interendothelial junctions of cerebral arterioles normally exclude lanthanum and to determine whether alterations occur in these structures in acute hypertension.

Materials and Methods

Female Wistar-Furth rats weighing 200–220 g were anesthetized by the open-drip method, using methoxyflurane. A PE90 cannula inserted into the abdominal aorta was connected to a pressure transducer for a continuous recording of the blood pressure and for obtaining blood for blood gases prior to the termination of the experiment. Only animals with pH and blood gases in the normal range were included in the study.

Acute hypertension was induced by a 2-minute infusion of angiotensin amide (Ciba) in a dose of 20 μg/minute. Controls received saline instead of angio-

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tensin. Three minutes after the angiotensin or saline infusion animals were perfused with an aldehyde mixture[12] via the ascending aorta at a pressure of 120 mm Hg for 3 minutes. This was followed by a 3% neutralized lanthanum solution diluted with an equal amount of the above aldehyde fixative and perfused for 5, 12, 25, and 40 minutes at the same pressure. The brains were sectioned at about 50-μ intervals with a tissue sectioner, and unstained slices were examined by light microscopy, following which blocks containing arterioles from the temporal and parietal cortex were processed for electron microscopy. The preparation of lanthanum suspension and the processing techniques for electron microscopy were similar to those described by Revel and Karnovsky.[2] In addition, tissues were stained *en bloc* with 2% uranyl acetate in 0.05 M sodium hydrogen maleate–NaOH buffer prior to dehydration. Semithin sections stained with 1% toluidine blue were examined, and arterioles containing lanthanum were selected for ultrathin sectioning and examined unstained or stained with 1% lead citrate with a Hitachi H-500 electron microscope with a goniometer stage.

Results

Control animals had a mean blood pressure of 134 mm Hg (±9). The blood pressure elevation following a 2-minute angiotensin infusion was similar to our previous observations.[13] After 1 minute the systolic blood pressure averaged 80 mm Hg above control levels, and at the time of sacrifice (3 minutes) the mean systolic blood pressure was 196 mm Hg (± 15), a value 48 mm Hg above control levels.

Light Microscopy

Lanthanum was identified in vessel walls of normotensive and hypertensive rats as a black deposit both in unstained fixed slices of brain and in plastic-embedded material.

Normotensive Rats

Electron Microscopy

Animals perfused with lanthanum for 5 minutes did not show tracer in walls of intracerebral vessels or the surrounding brain. The endothelium showed a few pinocytotic vesicles, and those open to the vascular lumen contained lanthanum. The extracellular spaces between endothelial cells of arterioles were divided into compartments by the presence of 3–5 tight junctions. At 5 minutes lanthanum was present at the luminal end of the interendothelial space bypassing a few tight junctions (Figure 1a).

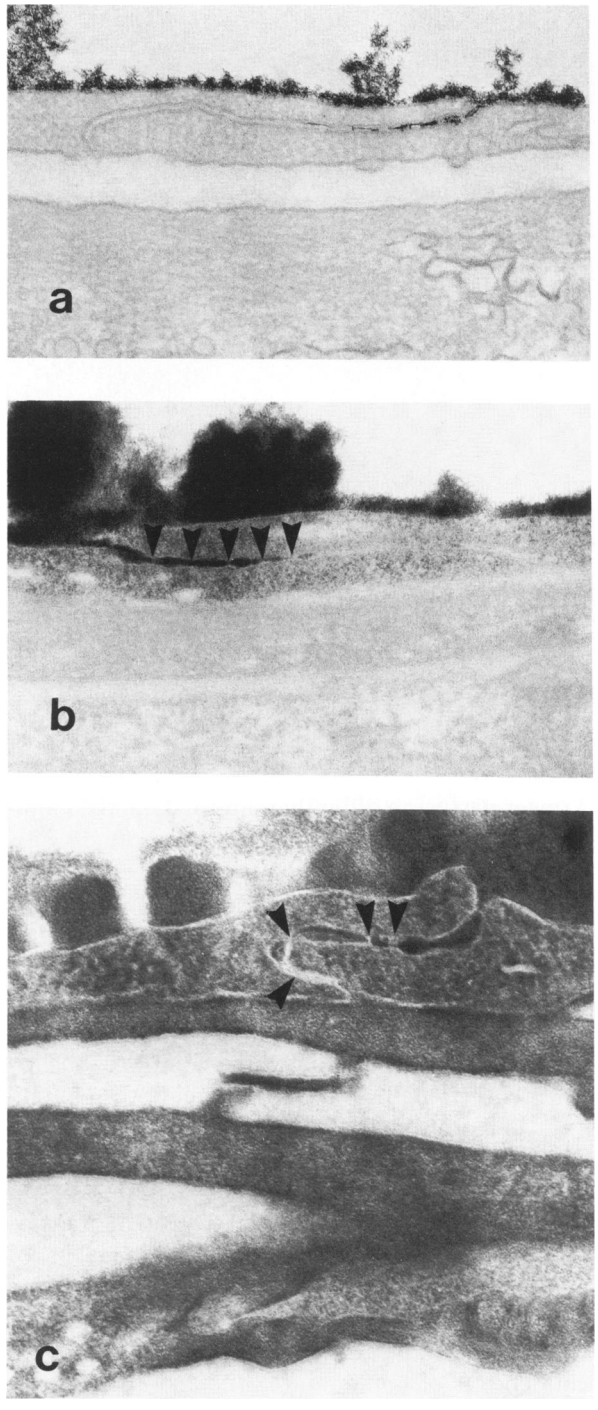

Figure 1—Normotensive rats. **a**—At 5 minutes lanthanum is present at the luminal end of the interendothelial space of an arteriole. (×60,000) **b**—A greater length of an arteriolar interendothelial space is labeled at 12 minutes, bypassing several tight junctions (*arrows*). In addition, tracer is present diffusely in endothelial cytoplasm. (×67,300) **c**—At 25 minutes lanthanum is present in all layers of an arteriolar wall and the entire length of the interendothelial space bypassing the tight junctions (*arrows*). (×88,600)

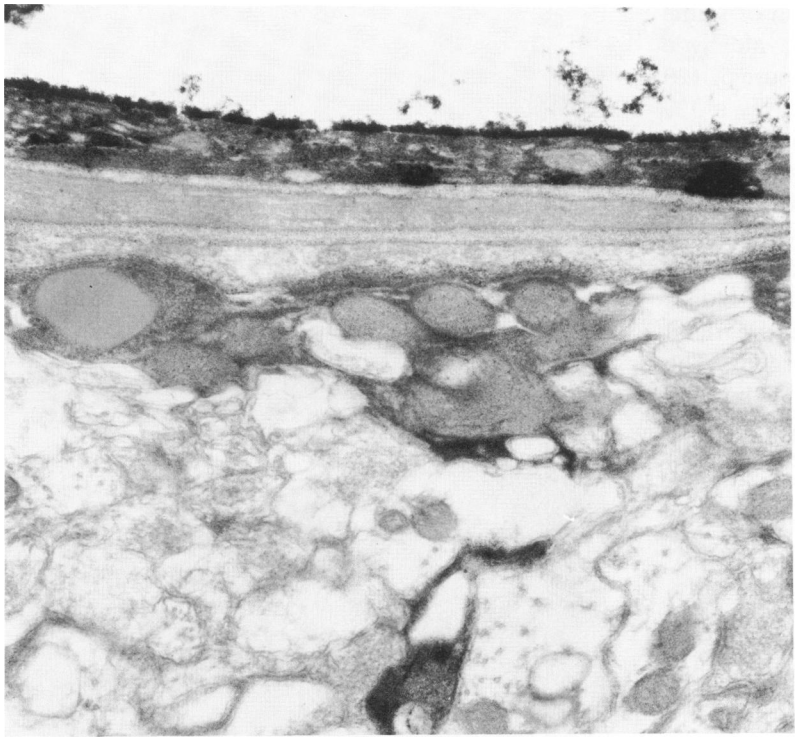

Figure 2—Normotensive rat. At 40 minutes tracer is present in all layers of the arteriolar wall and in continuity in the extracellular spaces of the surrounding neuropil. The endothelial cytoplasm is diffusely stained by tracer. (×24,000)

Longer perfusion of lanthanum for 12 and 25 minutes resulted in passage of tracer into arteriolar walls, it being demonstrable in continuity in endothelial basement membranes and in the smooth muscle, and adventitial basement membranes. At 12 minutes there was greater labeling of interendothelial spaces, bypassing many more tight junctions (Figure 1b). A few interendothelial spaces at 12 minutes and the majority at 25 minutes demonstrated complete labeling of their entire length from the luminal to the abluminal end (Figure 1c). The plasma membranes adjacent to these interendothelial spaces were intact, as were the tight junctions. At 40 minutes lanthanum was demonstrable in the extracellular spaces of the surrounding neuropil (Figure 2). Diffuse deposits of tracer in endothelial cytoplasm adjacent to intact luminal plasma membranes was also observed.

Capillaries and venules occasionally demonstrated focal deposits of lanthanum in the endothelial cytoplasm. However, no tracer was demonstrable in the endothelial basement membrane or in the brain. Interendothelial spaces of capillaries and venules showed focal collections of lanthanum at the luminal end only, and tracer was never found along the entire length of the interendothelial spaces of these vessels.

Hypertensive Rats

Electron Microscopy

Lanthanum was demonstrable in all layers of arteriolar walls and in the extracellular spaces of the surrounding neuropil as early as 5 minutes after tracer perfusion (Figure 3). The interendothelial spaces were intact, with no disruption of their membranes, but most of them demonstrated tracer in all the compartments along the entire length from the luminal end to the abluminal end (Figure 3a and b). An additional finding in the hypertensive animals was the presence of numerous pinocytotic vesicles in the endothelium (Figure 4); and, again, those open to the vascular lumen or to endothelial basement membranes containing lanthanum showed the tracer. Incomplete channels formed by fusion of pinocytotic vesicles were also observed. Diffuse deposits of tracer were observed in the endothelial cytoplasm. Longer periods of lanthanum circulation provided essentially similar findings.

After 5 minutes of tracer perfusion lanthanum was also found in capillary and venular walls and the extracellular spaces of the surrounding brain. There was no evidence of mechanical disruption of vascular

walls. These vessels also demonstrated diffuse deposits of tracer in endothelial cytoplasm. Penetration of the entire length of the interendothelial space was observed infrequently.

Discussion

The significant finding in the present study is that lanthanum penetrates the interendothelial spaces of normal intracerebral arteriolar endothelium, bypassing the tight junctions, but does not do so in capillaries and venules. Nagy et al,[14] using similar techniques, did not observe complete penetration of the interendothelial spaces of normal cerebral arteries and capillaries. These authors did not mention the total duration of lanthanum perfusion, and their failure to demonstrate tracer in interendothelial spaces may be related to a short perfusion time. Two other reports[8,15] demonstrated, as this study confirms, that the junctions of normal cerebral capillaries and venules are not penetrated by lanthanum, but arterioles were not studied.

Failure of horseradish peroxidase (HRP) to penetrate the brain via interendothelial spaces of normal cerebral vessels has led to the conclusion that the tight junctions of these vessels extend circumferentially around endothelial cells and are of the zonula occludens type.[16] Freeze fracture studies have demonstrated that these junctions form a complex interconnected network along the interendothelial space.[17,18] The entire circumference of endothelial cells cannot be visualized by either of the latter techniques. Therefore, while it is known that numerous tight junctions join adjacent endothelial cells, it remains to be proven whether or not they are circumferential. The finding in the present study of tracer in all of the interendothelial compartments between pentalaminar junctions in normal arterioles but not capillaries suggests that the arteriolar junctions are not continuous circumferential bands but may be incomplete at some locations to allow slow passage of macromolecules from the vascular lumen into the brain or in the reverse direction, as demonstrated in Figure 5. Alternatively, tracer could have entered the interendothelial compartments from the endothelial cytoplasm through the plasma membranes. However, complete labeling of interendothelial spaces was observed in areas where no tracer was present in the adjacent endothelial cytoplasm, making the latter possibility less likely. In the present study, since lanthanum is perfused following fixation, one cannot attribute labeling of interendothelial compartments to pinocytotic transport of tracer, as demonstrated in studies using HRP.[19]

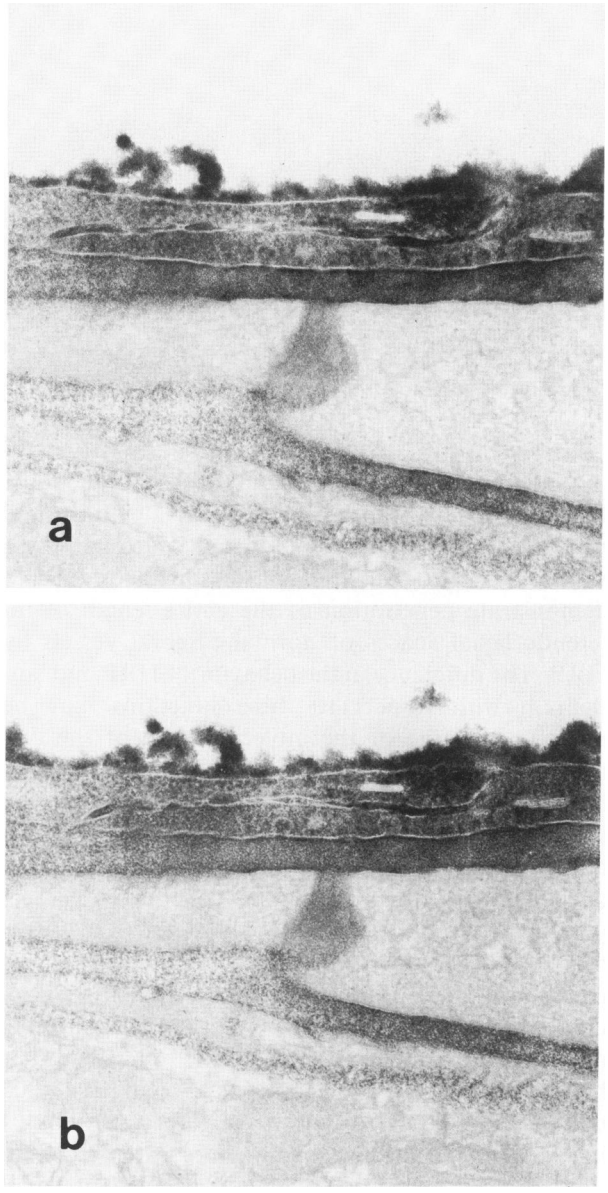

Figure 3—Hypertensive rat. At 5 minutes lathanum is demonstrable in all layers of an arteriolar wall. Notice the labeling of the entire length of the interendothelial space, in **a**, 0°, and in **b**, + 15° tilt. The central portion of the interendothelial space is better visualized in **a** and both the luminal and abluminal end are better demonstrated in **b**. (**a**, ×59,500, **b**, ×56,800)

Hüttner et al,[5] using techniques similar to those of the present study, demonstrated complete penetration of interendothelial spaces of normal aorta and other systemic arteries by lanthanum. The authors concluded that the pentalaminar fusions seen along the interendothelial spaces between consecutive pools

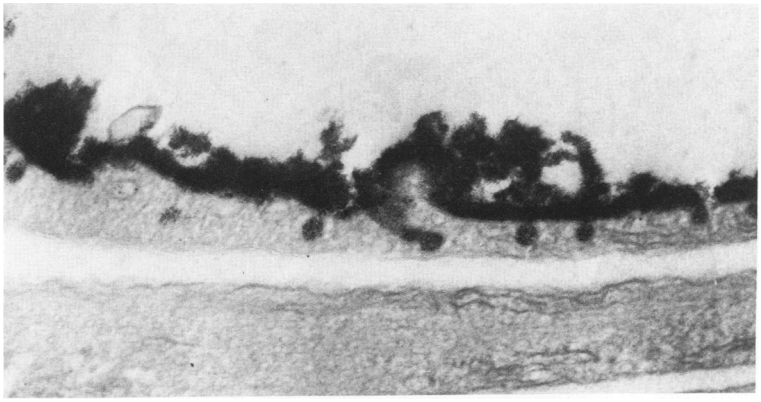

Figure 4 — Hypertensive rat. Numerous pinocytotic vesicles are observed in the endothelium, and those open to the vascular lumen contain tracer. (×56,700)

of lanthanum are parts of incomplete tight junctions. In a subsequent study these authors[6] demonstrated penetration of the interendothelial spaces of these vessels by HRP. All studies thus far have failed to demonstrate penetration of the entire length of interendothelial spaces of normal cerebral vessels by HRP. The difference in the behavior of HRP and lanthanum with respect to these junctions may be related to charge or the molecular size of the two markers. Schatzki and Newsome,[20] have reported that in neutralized lanthanum solutions prepared by the method of Revel and Karnovsky[2] 80% of the lanthanum exists as a charged particle of less than 500 daltons, while the other 20% consists of larger, possibly colloidal particles. Nonfiltered and ultrafiltered lanthanum have equally good staining and tracer properties by electron microscopy, suggesting that staining depends largely on the ultrafiltrable noncolloidal lanthanum ion. HRP, on the other hand, is known to have a molecular weight of 43,000 daltons. Possibly the endothelial tight junctions of intracerebral arterioles, being more extensive than those of systemic arteries, allow passage of small molecular weight tracers such as lanthanum but not HRP.

Diffuse intracellular deposits of lanthanum in endothelium with intact plasma membranes were observed in both control and hypertensive animals. Cytoplasmic accumulation of lanthanum was often associated with the presence of tracer in adjacent endothelial basement membranes and other components of the vessel wall, suggesting it was one of the mechanisms by which the tracer passed into the brain. There is no agreement in the literature regarding the interpretation of intracellular deposits of tracer. Hoffstein et al[21] interpreted diffuse intracellular deposits in normal myocardial cells to be an artifact, but in the test group they considered it to represent evidence of ischemic damage. Some authors have considered intracellular deposits of tracer to be a mechanism of transport,[22,23] while others feel it represents an artifact due to fixation.[12,24] In the present study the latter possibility cannot be excluded, since lanthanum was introduced after fixation.

Acute hypertension resulted in some physical or other alteration of endothelium, allowing more rapid passage of lanthanum into the brain, it being demonstrable in not only arteriolar walls but capillary and venular walls after only 5 minutes of perfusion. Tracer extravasation occurred by the same mechanism as observed in control animals, and numerous interendothelial spaces demonstrated lanthanum along their entire length. Nagy et al[14] also demonstrated successive labeling of interendothelial compartments by lanthanum after acute hypertension.

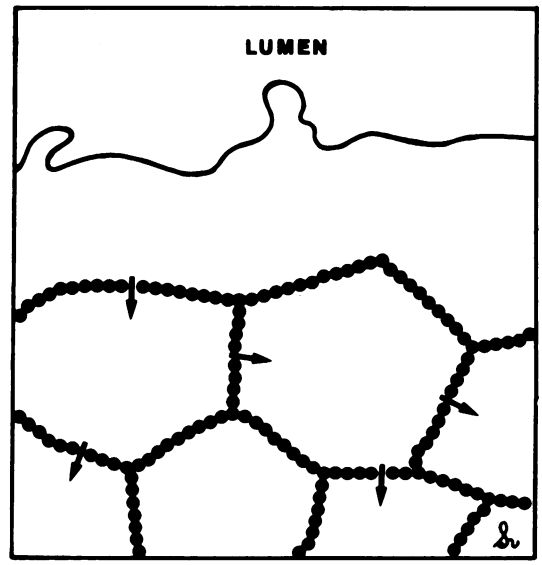

Figure 5 — En face diagram of an arteriolar interendothelial space. Junctions are shown as a network of ridges formed by fusion of the protein subunits of adjacent cell membranes. Tracers such as lanthanum possibly penetrate the interendothelial spaces at points where junctions are incomplete, as indicated by the *arrows*.

As in our previous studies,[9,13] the arteriolar endothelium of hypertensive animals showed an increased number of pinocytotic vesicles, and those vesicles open to the vascular lumen were passively filled with the marker. Occasional incomplete channels were also identified. This, in conjunction with other studies, indicates the importance of transendothelial pinocytotic transport in hypertension.

The present study demonstrates that under normal conditions the interendothelial spaces of cerebral arterioles, but not capillaries and venules, may provide a route for passage of substances having a low molecular weight, although apparently at a slow rate. In this respect cerebral arterioles differ from capillaries and venules, suggesting that endothelium throughout the cerebral vascular tree is not uniform.

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