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ORIGINAL PAPER

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Blood-brain barrier disruption in the hypothalamus of young adult spontaneously hypertensive rats

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Abstract Vascular permeability and endothelial glycocalyx were examined in young adult spontaneously hypertensive rats (SHR), stroke-prone SHR (SHRSP), and Wistar Kyoto rats (WKY) as a control, in order to determine earlier changes in the blood-brain barrier (BBB) in the hypothalamus in chronic hypertension. These rats were injected with horseradish peroxidase (HRP) as an indicator of vascular permeability. Brain slices were developed with a chromogen and further examined with cationized ferritin, a marker for evaluating glycocalyx. Staining for HRP was seen around vessels in the hypothalamus of SHR and SHRSP, but was scarce in WKY. The reaction product of HRP appeared in the abluminal pits of endothelial cells and within the basal lamina of arterioles, showing increased vascular permeability in the hypothalamus of SHR and SHRSP, whereas there were no leaky vessels in the frontal cortex of SHR and SHRSP, or in both areas of WKY. The number of cationized ferritin particles binding to the capillary endothelial cells was decreased in the hypothalamus of SHR and SHRSP, while the number decreased in the frontal cortex of SHRSP, compared with those in WKY. Cationized ferritin binding was preserved in some leaky arterioles, while it was scarce or disappeared in other leaky vessels. These findings suggest that BBB disruption occurs in the hypothalamus of 3-month-old SHR and SHRSP, and that endothelial glycocalyx is markedly damaged there without a close relationship to the early changes in the BBB.

Keywords BBB · Hypertension · Hypothalamus · SHR

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Introduction

Vascular dementia is a heterogeneous syndrome caused by the occlusion of large vessels or small artery disease (Hachinski et al. 1987; Parnetti et al. 1994; Roman 1996; Wallin and Blennow 1993). The pathogenesis of, and relationship between, the latter small artery lesions, i.e., lacunae and white matter lesions, and progressive cognitive impairment or dementia have been much debated. Wardlaw et al. (2003) recently reviewed the causative role of blood-brain barrier (BBB) impairment in vascular dementia, with leakage of serum components into and through the walls of cerebral small arterioles leading to neuronal and glial damage. Hypertension is one of the risk factors for cerebrovascular disease and also has a role in the development of vascular dementia. It is well known that hypertension causes BBB dysfunction and brain damage (Amenta et al. 2003; Fredriksson et al. 1987; Johansson 1980; Knox et al. 1980; O'Sullivan et al. 2003). Earlier changes in the BBB in chronic hypertension and the mechanism of BBB disruption remain unclear.

Spontaneously hypertensive rats (SHR), which are normotensive at birth and gradually develop hypertension in the first months of life, represent a model of chronic hypertension sharing several similarities with human essential hypertension (Fredriksson et al. 1985, 1988a, b; Hazama et al. 1975; Mueller 1982; Ogata et al. 1981; Okamoto and Aoki 1963; Okamoto et al. 1974; Sabbatini et al. 2000, 2002; Yamori et al. 1975). At present, it is known that in the early changes in the BBB of SHR, BBB damage occurs in the cerebral cortex, and in the deep gray matter in SHR at the age of 5 or 6 months or older, at which point tissue damage has already manifested (Fredriksson et al. 1987; Knox et al. 1980), while in-

creased vascular permeability appears in pial arterioles of 3-month-old SHR (Knox et al. 1980). It remains to be clarified whether the BBB in parenchymal vessels has deteriorated or not in 3-month-old SHR, and also whether the BBB has deteriorated or not in the hypothalamus of SHR. The hypothalamus is a well-known autonomic regulatory region of the brain involved in integrating several behaviors, as well as cardiorespiratory activity (Kramer et al. 2000). Several studies have revealed neuronal dysfunction in the hypothalamus of hypertensive animals (Horn et al. 1998; Kramer et al. 2000). It is hypothesized that the dysfunction in the specific neuronal system in the hypothalamus has significant neurophysiological ramifications for cardiovascular dysregulation in SHR (Kramer et al. 2000). A hypothalamic defect has been suggested to be involved in mediating elevated levels of resting arterial pressure in SHR (Fukushima 1968; Yamori and Okamoto 1969). Therefore, we examined the BBB in the hypothalamus of 3-month-old SHR, which were in a hypertensive state.

The BBB damage was evaluated in terms of endothelial glycocalyx and vascular permeability, both of which are altered in acute hypertension (Nag 1984). Glycocalyx is a surface layer lining the endothelial cells and is composed of proteoglycan. Its physiological functions are multifaceted: it is a transport barrier, a porous hydrodynamic interface in the motion of red and white blood cells in microvessels, and a mechanotransducer of fluid shearing stresses to the cytoskeleton of endothelial cells. Combined cationized ferritin (CF) binding and horseradish peroxidase (HRP) histochemical labeling techniques were used to detect the surface anionic charge based on glycocalyx (Lindner et al. 1998; Thurauf et al. 1983; Vorbrodt et al. 1986) and as a sensitive method of vascular permeability (Mesulam 1978; Ueno et al. 2000, 2001a, b, 2002), respectively.

Materials and methods

Twelve-week-old male Wistar Kyoto/izumo (WKY), SHR/izumo (SHR), and stroke-prone SHR/izumo (SHRSP) rats (purchased from Japan CL, Japan) weighing 250–300 g (n=15) were used. The experimental protocol was conducted according to the Japanese Experimental Animal Research Association Standards, as defined in the Guidelines for Animal Experiments, and was approved by the Animal Research Committee at Kagawa University. Systolic and diastolic arterial pressure was measured in awake rats by the tail-cuff method. The animals were anesthetized with sodium pentobarbital (60 mg/kg body weight), and injected with diphenhydramine (0.5 mg/100 g body weight, i.p.) 30 min before the injection of HRP to avoid an anaphylactic response possibly induced by HRP. Then, the rats were injected with HRP (50 mg per rat, type VI; Sigma, St. Louis, MO, USA) via a femoral vein 5 min before perfusion fixation.

The animals were perfused transcardially with 0.01 mol/l phosphate-buffered saline and then with a fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in a 0.1 mol/l phosphate buffer, pH 7.4 (Ueno et al. 2000). The brain was removed and immersed in 1.25% glutaraldehyde and 1% paraformaldehyde in a 0.1 mol/l phosphate buffer, pH 7.4, at 4°C for 12 h. It was placed in a sucrose buffer solution consisting of 10% sucrose in a 0.1 mol/l phosphate buffer, pH 7.4, at 4°C for 12–24 h, sectioned coronally on a microslicer into sections 50 μm in thickness, and collected in a 0.1 mol/l phosphate buffer.

For light microscopic observation, some sections were further transferred to an incubation medium composed of a 0.01 mol/l acetate buffer (pH 3.3), tetramethylbenzidine (TMB), and hydrogen peroxide, as reported previously (Mesulam 1978; Ueno et al.

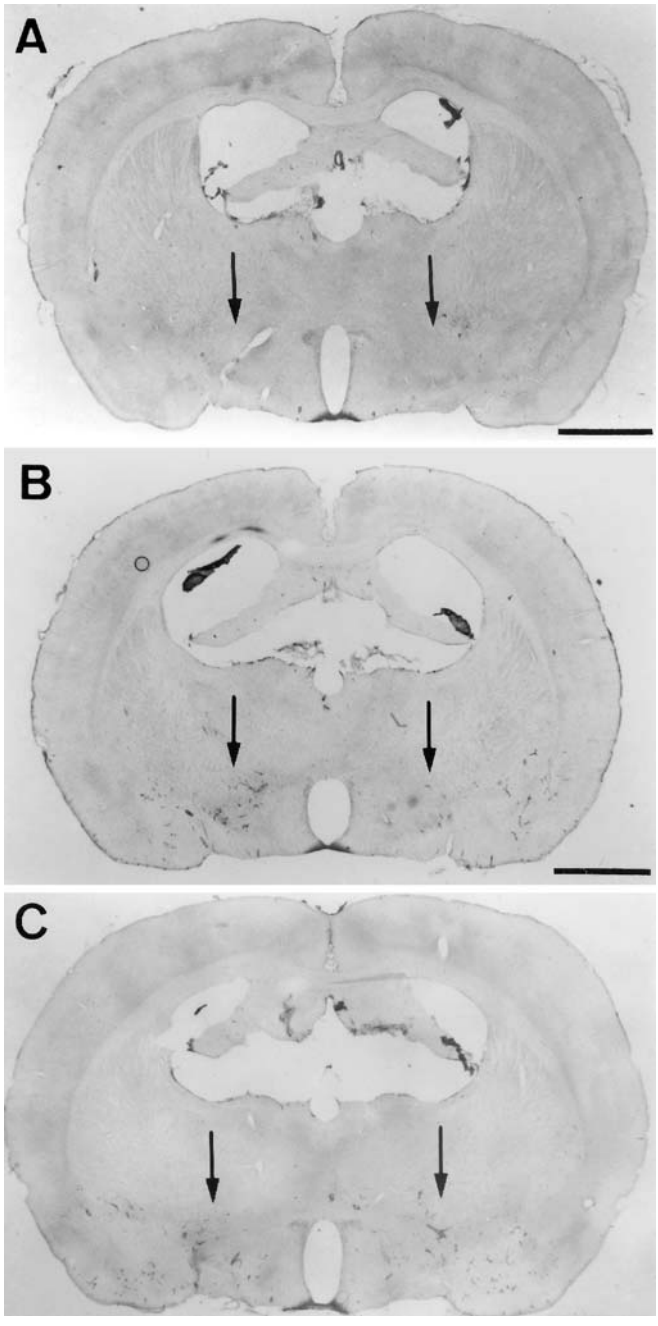

Fig. 1A–C Light microscopic photographs showing the staining for horseradish peroxidase (HRP) with tetramethylbenzidine in Wistar Kyoto rats (WKY; **A**), spontaneously hypertensive rats (SHR; **B**), and stroke-prone SHR (SHRSP; **C**). The staining reaction for HRP is seen in anterior portions of the hypothalamus and parts of the amygdala and piriform cortex in SHR (**B**) and SHRSP (**C**), but is scarce in WKY (**A**). The areas indicated by *arrows* (**A–C**) were investigated using an electron microscope. *Scale bars* indicate 2 mm

2001a). The sections incubated with TMB were mounted on gelatin-coated glass slides and a few sections were counterstained with hematoxylin-eosin.

For electron microscopic observation, the surface anionic sites in the vessel wall were first examined. The sections were immersed for 4 h in ice-cold 0.1 mol/l NH_4Cl in a 0.1 mol/l cacodylate buffer, pH 7.3, to block free aldehyde groups. This step was followed by overnight washing in the same buffer. Then, the sections were incubated for 15 min at room temperature in a solution of CF (Sigma) in 0.1 mol/l of a cacodylate buffer, pH 7.3 (1 mg/ml), according to the procedure of Thurauf et al. (1983) with some modifications (Vorbrodt et al. 1986). Some sections were incubated under similar conditions in a solution of native ferritin (Calbiochem-Novabiochem, La Jolla, CA, USA) to evaluate non-specific binding. Then, the sections were transferred to an incubation medium of a 0.1 mol/l cacodylate buffer, pH 7.3, diaminobenzidine tetrahydrochloride (DAB), and hydrogen peroxide (Reese and Karnovsky 1967; Ueno et al. 2000). The sections were washed and fixed for 1 h in 1% OsO_4 buffered with a 0.1 mol/l cacodylate buffer, pH 7.3, washed again, en bloc stained with 1% uranyl acetate for 1 h at room temperature in darkness, dehydrated in ethanol, and embedded in Epon 812. One-micrometer-thick sections were taken from each block and stained with 0.5% toluidine blue. Ultrathin sections were cut, placed on uncoated or coated grids and observed with a JEM-1200EX electron microscope. In addition, some grids were stained with uranyl acetate and Reynold's lead citrate.

Assessment of CF binding to the microvascular glycocalyx was made from photographic prints with a final magnification of ×120,000. Glycocalyx thickness was determined at 1-cm intervals along the endothelial luminal surface by measuring the distance from the cell membrane to the farthest CF molecule (Lindner et al. 1998). Statistical analysis was performed by non-parametric group tests (Mann-Whitney *U*-test) using StatView II software (Abacus Concepts) on a Macintosh computer. Blood pressure was represented by the mean of five values ±SD and statistical analysis of the blood pressure among three groups was performed by non-parametric group tests (Mann-Whitney *U*-test). The vessels, in which the staining for HRP was seen within the basement membrane, were considered to be leaky vessels with increased vascular permeability, and statistical analysis of the percentage of leaky arte-

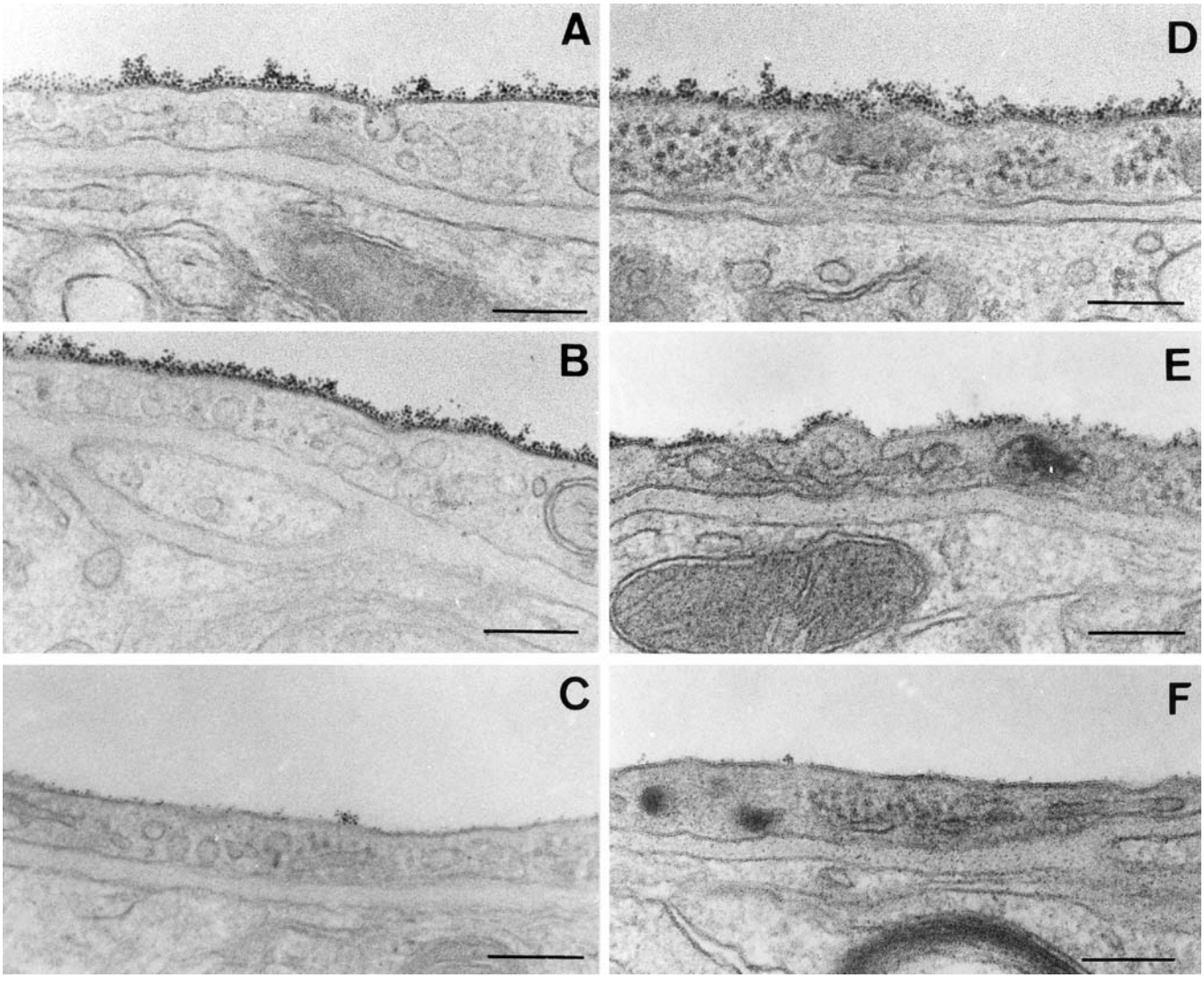

Fig. 2A–F Representative electron microscopic photographs of microvessels, showing capillary profiles in the frontal cortex (**A–C**) and the hypothalamus (**D–F**) of WKY (**A**, **D**), SHR (**B**, **E**), and SHRSP (**C**, **F**). The cationized ferritin (CF) binding to the endothelial cells in WKY is constant in both areas. The CF binding to the endothelial cells is mostly homogeneous, but partly sparse in the frontal cortex of SHR (**B**) and sparse in the hypothalamus of SHR (**E**). The CF binding to the endothelial cells is sparse in both areas of SHRSP (**C**, **F**). *Scale bars* indicate 200 nm

rioles was done for SHRSP (or SHR) and WKY using Fisher's exact probability test.

Results

Blood pressure (systolic/diastolic) was significantly higher in 3-month-old SHR (184±30 mmHg/143±23 mmHg) and SHRSP (216±27 mmHg/163±17mmHg) than in the same-aged WKY (124±9 mmHg/97±8 mmHg).

A staining reaction for HRP was seen very clearly around median eminence, a BBB-free area, in three strains of rats (Fig. 1). In addition, staining for HRP was present around vessels in anterior portions of the hypothalamus and parts of the basal forebrain, amygdala, and piriform cortex of SHR and SHRSP, while it was weak around a few WKY vessels (Fig. 1). On the contrary, no reaction product was observed in the frontal cortex of the three strains. Electron microscopic examination in capillaries of the frontal cortex and the hypothalamus revealed that there was homogeneous CF binding to the capillary endothelial cells in both areas of WKY (Fig. 2A, D), while no binding was detected with native ferritin (data not shown). The CF binding was mostly homogeneous, but partly sparse, in the frontal cortex and sparse in the hypothalamus of SHR (Fig. 2B, E). The CF binding to the endothelial cells was sparse in both areas of SHRSP (Fig. 2C, F). In quantitative analysis, the number of CF particles bound to the endothelial cells was significantly decreased in the frontal cortex of SHRSP and in the hypothalamus of SHR and SHRSP compared to those in WKY (Fig. 3). Electron microscopic examination in arterioles of both areas revealed that the reaction product of HRP was occasionally seen in the vesicular structures inside the endothelial cells in each strain. On the contrary, the extravasated reaction product was seen exclusively in arterioles of the hypothalamus of SHR and SHRSP, being localized in the vesicular structures such as abluminal pits of endothelial cells and within the basal lamina, whereas in WKY there were no vessels with increased vascular permeability. The percentage of arterioles showing increased vascular permeability was 20% (6 vessels showing increased vascular permeability/30 vessels examined, $P<0.05$) in SHRSP, 6.7% (2 vessels showing increased vascular permeability/30 vessels examined, $P>0.05$) in SHR, and 0% (0 vessel showing increased vascular permeability/30 vessels examined) in WKY. Extravascular deposition was distributed densely, but sporadically, in contact with specific endothelial cells. The CF binding to the endothelial cells was not changed and was homogenous in some arterioles showing an increased vascular permeability in SHR and SHRSP (Fig. 4B, C), while it was slightly sparse (Fig. 4D) or rare (Fig. 4E) in other arterioles showing increased vascular permeability in SHRSP. In addition, the CF binding disappeared in a few vessels, presumably venules, showing diffuse staining for HRP in the endothelial cytoplasm connected with the luminal membrane of a pit (Fig. 4F).

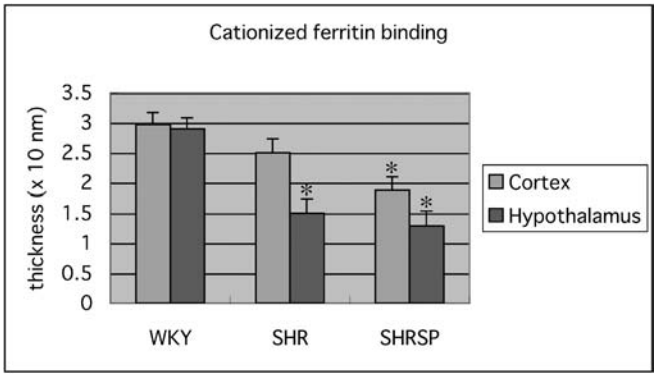

Fig. 3 The mean thickness of CF particles bound to the endothelial luminal surface. The mean thickness in the hypothalamus is significantly less in SHR and SHRSP than in WKY, while the mean thickness in the frontal cortex is significantly less in SHRSP than in WKY. Values are means±SEM. * $P<0.01$, significantly different by the Mann-Whitney *U*-test compared with the corresponding value in WKY

Discussion

Light microscopic study revealed the staining reaction for HRP around vessels in anterior portions of the hypothalamus and parts of the basal forebrain, amygdala, and piriform cortex in SHR and SHRSP. In this study, we focused on the vascular permeability in the anterior portions of the hypothalamus, because the staining for HRP was observed strongly there.

Electron microscopic findings indicated that cerebrovascular permeability to HRP increased exclusively in arterioles of the hypothalamus in 3-month-old SHR and SHRSP. The CF binding to the capillary endothelial cells deteriorated not only in the hypothalamus of SHR and SHRSP, but also in the frontal cortex of SHRSP. Accordingly, it is likely that the endothelial dysfunction in capillaries had already occurred in widespread areas of 3-month-old SHRSP brains, while the endothelial dysfunction was not related directly to increased vascular permeability in young SHRSP. It is likely that the aggravation of endothelial dysfunction and increased vascular permeability of the arterioles may lead to hyaline and fibrinoid degeneration of the vessels in adult SHR and SHRSP (Amano 1977). The CF binding to endothelial cells was preserved in some arterioles, showing increased vascular permeability, in the hypothalamus of SHR and SHRSP (Fig. 4B–D), as was also observed in the hippocampus (Ueno et al., 2004). On the other hand, the number of CF particles bound to the endothelial cells was decreased in other leaky arterioles (Fig. 4E), as was not seen in the hippocampus. It is conceivable that the decreased CF binding to the endothelial cells of arterioles may be not closely related to increased vascular permeability and that the hypertensive state may induce the endothelial damage more remarkably in the hypothalamus than in the hippocampus.

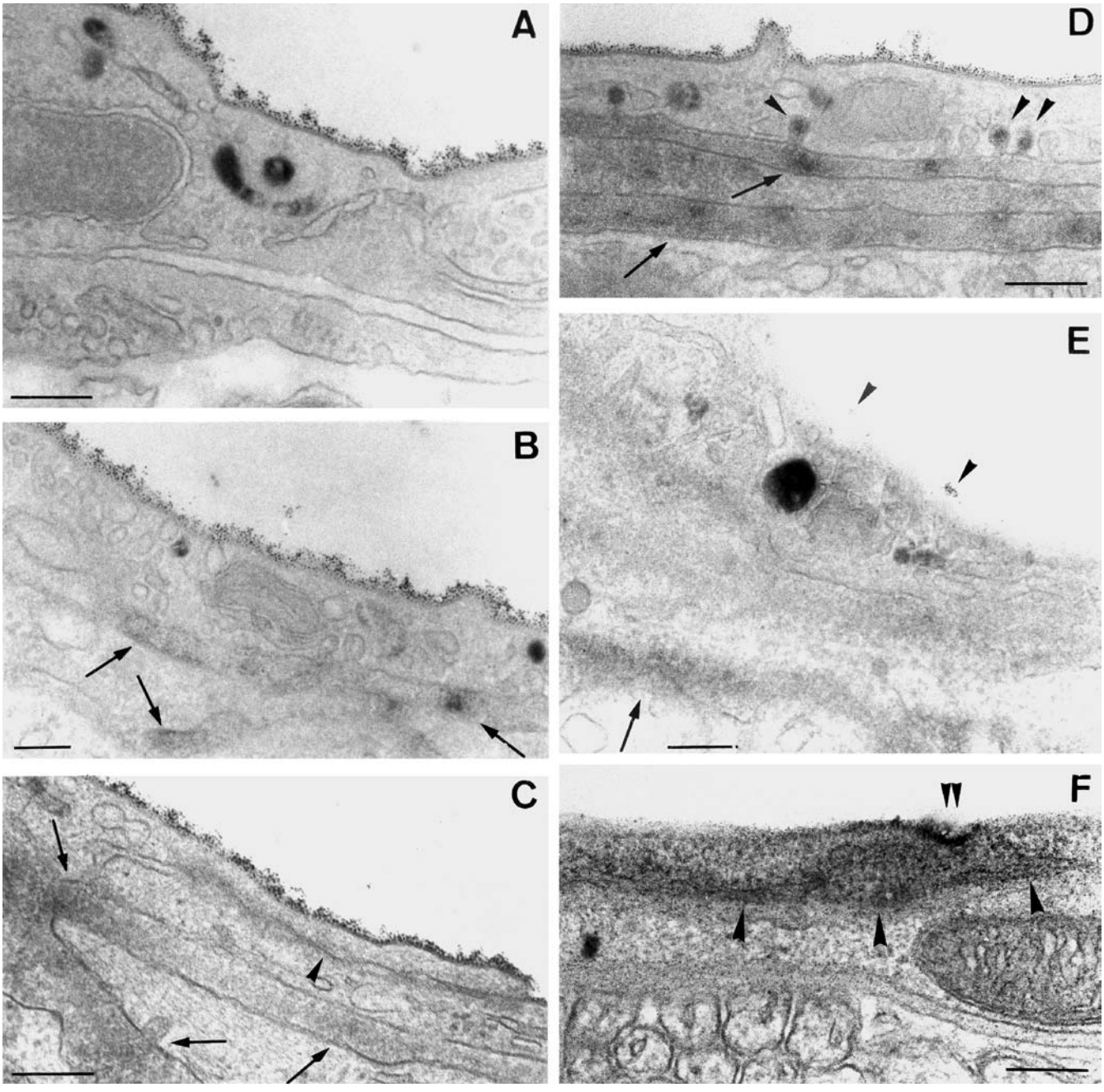

Fig. 4A–F Electron microscopic photographs of vessels in the hypothalamus of WKY (**A**), SHR (**B**), and SHRSP (**C–F**). No leakage of HRP is seen in arterioles of WKY, and CF binding to endothelial cells is uniformly observed (**A**). The reaction product for HRP is seen within the basal lamina (*arrows* in **B**) and in vesicular structures of the endothelial cytoplasm of SHR, while CF particles are uniformly observed (**B**). The reaction product of HRP is not seen in the junctional clefts of the endothelial cells (*arrowhead* in **C**) but in plasmalemmal vesicles, especially abluminal vesicles (*arrowheads* in **D**), of endothelial cells and within the basal lamina of arterioles (*arrows* in **C–E**) in SHRSP (**C–E**), indicating increased vascular permeability. The CF particles bound to the endothelial cells are uniformly observed (**C**), slightly sparse (**D**), or rare (*arrowheads* in **E**) in arterioles of SHRSP. The reaction product of HRP is diffusely seen in the cytoplasm (*arrowheads* in **F**) of the endothelial cells connected with the luminal membrane of a pit (*double arrowhead* in **F**), while the CF particles in this vessel, presumably a venule, have disappeared (**F**). *Scale bars* indicate 200 nm

The number of leaky arterioles showing staining for HRP within the basement membrane in SHRSP significantly increased compared with that in WKY. However, as the number of HRP-loaded vesicular structures in the arterioles was not calculated, the detailed mechanism of the vascular hyperpermeability in the endothelial cells has not yet been determined. It is well known, however, that enhanced vesicular transport appears to be a common response of the cerebral endothelium to a variety of stimuli (Cervos-Navarro et al. 1983). Caveolae-mediated tran-

scytosis possibly contributes to vascular hyperpermeability (Pascariu et al. 2004; Simionescu et al. 1974; Stan 2002). On the other hand, the involvement of caveolae in the transcytosis of macromolecules was recently questioned by the caveolin knockout mouse model (Drab et al. 2001). Cationized ferritin binding to HRP-loaded vesicular structures was not found in the luminal membrane of endothelial cells (Fig. 4), although the binding to vesicular structures without HRP was occasionally seen in their luminal membranes (Fig. 2A). Accordingly, these findings do not necessarily suggest that the vascular hyperpermeability observed in this study results from increased caveolae-mediated transcytosis. Transport through junctional clefts of endothelial cells may contribute to increased vascular permeability; however, this was not observed in this study (Fig. 4C). The reaction product of HRP was seen not only within the basal lamina, but also occasionally in the abluminal pits of the endothelial cytoplasm of arterioles (Fig. 4D). Accordingly, it is likely that increased vascular permeability in the arterioles appears to be accounted for by enhanced vesicular transport. In addition, we found some venules showing diffuse staining of HRP in the endothelial cytoplasm without CF binding to its luminal membrane (Fig. 4F), as was not seen in the hippocampus of SHRSP (Ueno et al. 2004). The diffuse staining of HRP in the endothelial cytoplasm of venules was also seen in the hippocampus of aged senescence-accelerated mice (SAMP8; Ueno et al. 2001a) and in the corpus callosum of rats with chronic cerebral hypoperfusion (Ueno et al. 2002). It is likely that the diffuse leakage of intravascular macromolecules through endothelial cytoplasm with endothelial glycocalyx damage may occur in venules. However, future studies with another tracer are needed to confirm the diffuse leakage of macromolecules transendothelially. It remains unclear why the hypothalamic area appears to be especially susceptible to hypertension-induced hyperpermeability. It seems that the blood in this area is supplied by proximal portions of branching vessels of the major artery and accordingly the vessels in the hypothalamus may be under higher pressure than those in the others. It is conceivable that increased vascular permeability in arterioles and venules of the anterior hypothalamic area may affect norepinephrine release in this area (Carlson et al. 2001; Peng et al. 1995, 2003).

In the present study, alterations in the surface charge of the capillary endothelial cells were not necessarily related to increased vascular permeability. Nag (1984) reported that an increase in vascular permeability in acute hypertension was accompanied by a transient alteration in surface charge. The difference may result from the fact that the BBB damage at the early stage of chronic hypertension in this study was milder than that in the acute hypertensive state.

These results suggest that the chronic hypertensive state induces BBB disruption, and that endothelial glycocalyx is markedly damaged without a close relationship to BBB disruption in the hypothalamus at an early stage.

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