Increased numbers of caveolae in retinal endothelium and pericytes in hypertensive diabetic rats

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LABORATORY STUDY

Abstract

Purpose **Long-term clinical studies have now shown that tight control of blood pressure in type 2 diabetes reduces the risk of diabetes-related death and common diabetic complications, including diabetic retinopathy. However, the mechanisms by which hypertension enhances diabetic microvascular disease, especially diabetic retinopathy, are poorly understood. We developed an experimental model of hypertension in diabetic rats and studied the early ultrastructural changes in retinal capillaries under these conditions.**

Methods **Hypertension was induced in diabetic BioBreeding (BB) rats by unilateral nephrectomy, weekly subcutaneous mineralocorticoid and 0.9% oral saline. Serial blood pressures and ultrastructural features of retinal capillaries were recorded in four groups: normotensive Wistar rats, normotensive diabetic rats, hypertensive Wistar rats and hypertensive diabetic rats.**

Results **A significant and sustained increase in systolic blood pressure occurred in both groups of nephrectomised rats. There was a significant increase in the number of caveolae (i) in both pericytes and endothelial cells in animals with hypertension and diabetes together compared with all other groups and (ii) in pericytes in animals with diabetes alone. The number of direct contacts between pericytes and endothelial cells was reduced in diabetic and hypertensive diabetic animals. Hypertension and diabetes had an interactive effect in producing retinal capillary basement membrane thickening.**

Conclusions **In the BB rat hypertension and diabetes have an interactive effect in increasing the number of caveolae in both endothelial cells and pericytes. We speculate that this may be a reflection of changes in calcium and nitric oxide metabolism in these animals.**

Key words BioBreeding (BB) rats, Caveolae, Diabetic retinopathy, eNOS, Hypertension

Patients with all forms of diabetes have an increased incidence of systemic hypertension,[1] and those with hypertension and diabetes have a higher risk of renal impairment than subjects with either condition alone.[2] There is now conclusive evidence that tight control of blood pressure reduces the risk of death related to diabetes, diabetic complications and progression of diabetic retinopathy in type 2 diabetes.[3] In the early stages of insulin-dependent diabetes microvascular pressure and flow are increased. However, the mechanisms by which hypertension enhances diabetic microvascular disease, especially diabetic retinopathy, are poorly understood.[4,5] In a study of spontaneously hypertensive rats made diabetic with streptozotocin, Hammes and colleagues[6] identified a variety of retinal vascular changes. These included arteriolar microthrombi, microaneurysm formation and vascular deposits of advanced glycation products.

In view of these findings, and because of the current clinical interest in the interaction of diabetes and hypertension, we designed an experiment to study ultrastructural changes in endothelial cells and pericytes in a model of hypertension and diabetes. We found changes in basement membranes and contacts between endothelial cells and pericytes. There were significant, and at present inexplicable, increases in the numbers of caveolae in pericytes and endothelial cells in rats that were both hypertensive and diabetic.

Materials and methods

Experimental animals

Young spontaneously diabetic BioBreeding (BB) male rats, 60–90 days old (250–300 g), were obtained from a breeding colony at the Biomedical Research Unit, Southampton University Hospitals. Age-matched male Wistar rats were used as controls. Hypertension was induced in groups of both diabetic and control rats using the DOCA salt method.[7] Under

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general anaesthesia induced with fentanyl flucanisone and diazepam a left unilateral nephrectomy was performed. Injections of deoxyhydrocorticosterone acetate (DOCA) salt (Sigma), 10 mg weekly (i.m.) were given and drinking water was replaced with 0.9% saline. We therefore studied four separate groups of animals: normotensive Wistar rats (group 1), hypertensive Wistar rats (group 2), normotensive BB rats (group 3) and hypertensive BB rats (group 4).

These procedures were approved and regulated by the United Kingdom Home Office project licence 90/909. The standard of care of all animals conforms to those of the ARVO Resolution for the Use of Animals in Research.

Periodic observations

Systolic blood pressure was measured at least once a week by tail artery sphygmomanometry (model 229, Pulse Amplifier System, Life Sciences Instruments, Palo Alto, CA).[8] Animals were weighed daily and urinary glucose and ketones estimated with BM multistix. Novo Lente porcine insulin (Novo Nordisk, Crawley, W. Sussex, UK) was given at a standard daily dose of 2.4 units for males and 2.0 units for females. This dose was adjusted daily in order to ensure that the animals received the minimum dose consistent with avoiding glycosuria and ketonuria. Human Actrapid insulin (Novo Nordisk) and saline rehydration were used as required.

Preparation of retinal tissues

After 8 weeks, animals were anaesthetised with sodium pentobarbitone (60 mg/kg i.p.) and, following a thoracotomy, perfused via the left ventricle with 3% glutaraldehyde in cacodylate buffer, pH 7.4. The eyes were removed intact and fixed in glutaraldehyde for a further 24 h at 4 °C. Radially orientated sections of the retina, approximately 5 × 1 mm, were taken from the left eye within 5 mm of the head of the optic nerve. They were left for at least 12 h in 0.1 M sodium cacodylate sucrose buffer (pH 7.4) at 4 °C and then further fixed in 2% osmium tetroxide for 2 h. The tissue sections were stained in 2% uranyl acetate for 30 min and dehydrated through graded ethanol solutions. Sections were then cleared in Histasol (30 min), infiltrated with 50:50 Histasol/Spurr's resin (1 h) and placed in 100% Spurr's resin (12 h). Appropriate areas of 1 μm sections were selected and cut at 60–90 nm.

Ultrastructural observations

Membrane-associated vesicles

The numbers of surface-associated vesicles were counted on the luminal and abluminal surfaces of both pericytes and endothelial cells. The area to be studied was defined at low magnification and photographed. Vesicle counts in this area were made directly from the screen at variable magnifications. The total cytoplasmic area was calculated by measuring the area of the pericyte or the endothelial cells and subtracting the area of nucleus included. The results were expressed as the number of vesicles per square micrometre of cytoplasm.

Pericyte–endothelial cell contacts

Pericyte–endothelial cell contacts were defined as sites where the cell membranes of pericytes and endothelial cells appeared to be in direct contact, with no intervening basement membrane. These included simple apposition contacts, where the cell membranes came together in parallel, or more complex 'peg and socket' patterns involving one or more cellular protrusion.[9] Counts were made directly from electron micrographs.

Pericyte cover

The length of the endothelial cell outer membrane in direct apposition to pericyte processes was measured from photomicrographs using a computerised image analysis system (software Colourvision 1.7.4a, Improvision, UK). The results were expressed as a percentage of the total length of the endothelial cell outer membrane.

Basement membranes

Between 1 and 12 satisfactory capillaries, with sharp basement membrane borders, were present in each grid. Electron photomicrographs were enlarged to ×10 000. Basement membrane thickness (BMT) was measured in two different ways. In method 1 a magnifying eyepiece graticule was used to measure 10 random points around the circumference of each vessel, excluding areas where pericytes were present. Method 2 was adapted from that designed by McEwan *et al.*[10] Total basement membrane area was measured from each electron micrograph using computerised image analysis. The total length of the lines delimiting the basement membrane (BM) was measured and divided by 2 to give the basement membrane length. Mean BMT was calculated as BM area divided by BM length.

Statistical methods

Results were analysed by one-way or two-way analysis of variance. A value of $p < 0.05$ was defined as significant.

Results

Periodic observations

Urinary glucose was less than 28 mmol/l in all animals throughout the 8 weeks of the study. Ketonuria, which was corrected using Actrapid insulin, was detected on a maximum of 9 days in any one diabetic animal. All DOCA salt nephrectomised animals developed increased blood pressure within 2 weeks. Peak systolic levels were sustained between 4 and 8 weeks.

Table 1. *Summary of results (mean values include standard deviations)*

Measurement	Control (group 1)	Hypertensive (group 2)	Diabetic (group 3)	Hypertensive diabetic (group 4)
Final systolic blood pressure (mmHg)				
Mean	123 ± 1.9	190 ± 2.7	124 ± 3.0	190 ± 2.2
No. of eyes (animals)	6 (5)	14 (7)	10 (5)	8 (5)
No. of capillaries studied	20	21	21	30
Endothelial cell:pericyte ratio	1:2.00	1:2.03	1:2.03	1:2.28
Total no. of pericyte vesicles per μm^2 cytoplasm				
Mean	2.7×10^{-4} $\pm 2.4 \times 10^{-4}$	4.3×10^{-4} $\pm 2.8 \times 10^{-4}$	6.2×10^{-4} $\pm 3.8 \times 10^{-4}$	10.4×10^{-4} $\pm 5.3 \times 10^{-4}$
Range	$0.0–11.0 \times 10^{-4}$	$0.7–10.6 \times 10^{-4}$	$0.0–14.90 \times 10^{-4}$	$0.0–20.1 \times 10^{-4}$
Total no. of endothelial cell vesicles per μm^2 cytoplasm				
Mean	8.6×10^{-4} $\pm 3.5 \times 10^{-4}$	9.5×10^{-4} $\pm 3.2 \times 10^{-4}$	9.6×10^{-4} $\pm 2.2 \times 10^{-4}$	14.3×10^{-4} $\pm 4.4 \times 10^{-4}$
Range	$3.3–18.7 \times 10^{-4}$	$3.6–15.2 \times 10^{-4}$	$4.3–13.0 \times 10^{-4}$	$6.7–25.4 \times 10^{-4}$
No. of pericyte abluminal vesicles per μm^2 cytoplasm				
Mean	2.3×10^{-4} $\pm 2.0 \times 10^{-4}$	3.9×10^{-4} $\pm 2.5 \times 10^{-4}$	5.8×10^{-4} $\pm 3.5 \times 10^{-4}$	9.3×10^{-4} $\pm 4.7 \times 10^{-4}$
Range	$0.0–8.6 \times 10^{-4}$	$0.7–10.1 \times 10^{-4}$	$0.0–13.3 \times 10^{-4}$	$0.0–18.8 \times 10^{-4}$
No. of endothelial cell abluminal vesicles per μm^2 cytoplasm				
Mean	6.8×10^{-4} $\pm 3.0 \times 10^{-4}$	7.4×10^{-4} $\pm 2.5 \times 10^{-4}$	8.0×10^{-4} $\pm 2.1 \times 10^{-4}$	11.7×10^{-4} $\pm 3.9 \times 10^{-4}$
Range	$2.9–14.8 \times 10^{-4}$	$2.9–11.9 \times 10^{-4}$	$3.9–12.4 \times 10^{-4}$	$4.5–21.2 \times 10^{-4}$
No. of pericyte luminal vesicles per μm^2 cytoplasm				
Mean	0.33×10^{-4} $\pm 0.54 \times 10^{-4}$	0.48×10^{-4} $\pm 0.45 \times 10^{-4}$	0.45×10^{-4} $\pm 0.45 \times 10^{-4}$	1.2×10^{-4} $\pm 1.1 \times 10^{-4}$
Range	$0.0–1.99 \times 10^{-4}$	$0.0–1.99 \times 10^{-4}$	$0.0–1.66 \times 10^{-4}$	$0.0–3.6 \times 10^{-4}$
No. of endothelial cell luminal vesicles per μm^2 cytoplasm				
Mean	1.8×10^{-4} $\pm 0.9 \times 10^{-4}$	2.0×10^{-4} $\pm 1.1 \times 10^{-4}$	1.6×10^{-4} $\pm 0.7 \times 10^{-4}$	2.6×10^{-4} $\pm 0.8 \times 10^{-4}$
Range	$0.4–3.9 \times 10^{-4}$	$0.6–4.7 \times 10^{-4}$	$0.5–3.1 \times 10^{-4}$	$1.1–4.2 \times 10^{-4}$
Pericyte–endothelial cell contacts per capillary				
Mean	3.3 ± 1.3	2.2 ± 1.5	1.9 ± 1.1	1.9 ± 1.4
Range	0–5	0–6	0–4	0–5
Mode	4	2	2	2
Percentage pericyte cover				
Mean	59.4 ± 22.3	64.2 ± 20.8	46.9 ± 22.0	54.5 ± 20.6
Range	17.6–99.3	24.7–100.0	5.4–94.1	12.0–86.4
Basement membrane thickness (nm): Method 1				
Mean	142 ± 7.8	142 ± 14.6	144 ± 31.7	167 ± 41.6
Range	109–168	123–169	117–240	126–250
Basement membrane thickness (nm): Method 2				
Mean	119 ± 21.5	130 ± 32.5	112 ± 27.5	143 ± 40.6
Range	83–178	88–220	66–172	98–269

Plasma-membrane-associated vesicles

Plasma-membrane-associated vesicles (60–70 nm diameter) were identified consistently in both pericytes and endothelial cells (Fig. 1a, b, d). These vesicles were concentrated along the part of the membrane furthest from the capillary lumen. They were not observed as free cytoplasmic vesicles. Detailed results are shown in Table 1. The following comments are made in summary:

Total number of vesicles

There was a significant increase in the total number of vesicles in both pericytes and endothelial cells in animals with hypertension and diabetes together (group 4) compared with all other groups (pericytes: group 1, $p < 0.01$; group 2, $p < 0.01$; group 3, $p = 0.03$; endothelial cells: group 1, $p = 0.01$; group 2, $p < 0.01$; group 3, $p < 0.01$). These results suggest that the effect on the increase in the total number of vesicles with

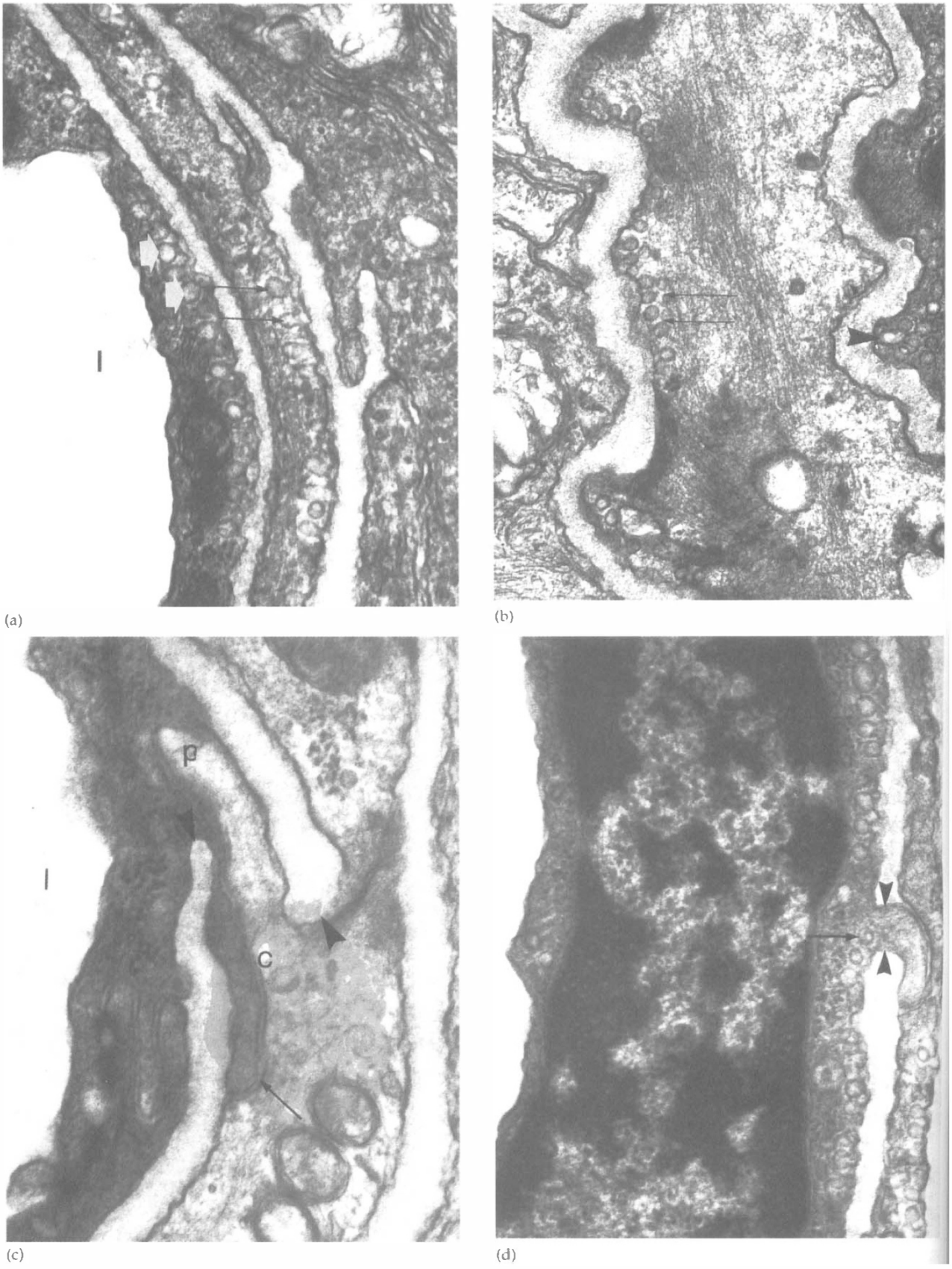

Fig. 1. *Electron micrographs of retinal capillary endothelium. (a) Plasma-membrane-associated vesicles (caveolae) in both an endothelial cell (white arrowheads) and a pericyte (black arrows). Note that caveolae are concentrated at the abluminal surface of each cell type. Normotensive Wistar rat, ×40 000. (b) Caveolae arranged at the abluminal surface of a pericyte (arrows). Note the prominent microfilaments within the cytoplasm of the pericyte. Some caveolae are visible in the cytoplasm of the adjacent endothelial cell at the left (arrowhead). Hypertensive Wistar rat, ×30 000. (c) Direct contact between an endothelial cell and a pericyte. Processes from both a pericyte (p) and an endothelial cell (arrow) protrude through a gap in the basement membrane, marked by arrowheads, and are in contact with each other (c). The capillary lumen is to the left (l). Normotensive Wistar rat, ×40 000. (d) Projection of endothelial cell cytoplasm through a gap in the basement membrane (arrowheads), forming a so-called peg and socket connection with a pericyte. Note the prominent caveolae at the abluminal aspect of the endothelial cell (arrows). Hypertensive diabetic rat, ×30 000.*

hypertension and diabetes together, in both pericytes and endothelial cells, was synergistic. In rats with diabetes alone there was a significant increase in the number of vesicles in pericytes ($p = 0.04$) but not endothelial cells. There was no significant effect of hypertension alone in either cell type (pericytes: $p = 0.22$; endothelial cells: $p = 0.47$).

Number of abluminal vesicles

There was a significant increase in the number of abluminal vesicles in both pericytes and endothelial cells in rats with hypertension and diabetes together, compared with all other groups (pericytes: group 1, $p < 0.01$; group 2, $p < 0.01$; group 3, $p = 0.04$; endothelial cells; group 1, $p = 0.01$; group 2, $p < 0.01$; group 3, $p < 0.01$). In rats with diabetes alone there was a significant increase in the number of abluminal vesicles in pericytes ($p = 0.03$) but not endothelial cells ($p < 0.08$). There was no effect of hypertension alone in either cell type (pericytes: $p = 0.21$, endothelial cells: $p = 0.50$).

Number of luminal vesicles

In endothelial cells there was a significant increase in the number of luminal vesicles in rats with hypertension and diabetes together compared with controls ($p = 0.02$) and rats with diabetes alone ($p = 0.04$) but not compared with rats with hypertension alone ($p = 0.12$). In pericytes there was a significant increase in the number of luminal vesicles in rats with hypertension and diabetes together (group 4) compared with all other groups (group 1, $p = 0.02$; group 2, $p = 0.02$; group 3, $p < 0.01$). There was no significant increase in the number of luminal vesicles in either pericytes or endothelial cells in rats with hypertension alone or diabetes alone.

Other ultrastructural changes

Direct contacts between pericytes and endothelial cells were identified in the majority of capillaries examined. These included direct appositions between processes which appeared to run parallel to each other and so-called peg and socket contacts[9] (Fig. 1c, d). These contacts were closely related to defects or gaps in the basement membrane. No junctional complexes were observed at the magnifications that we used in this study. The number of contacts was significantly greater in normotensive Wistar animals (group 1) than in either of the diabetic groups (group 3, $p < 0.01$; group 4, $p < 0.02$). There was no significant difference in the number of contacts between hypertensive Wistar animals (group 2) and either of the diabetic groups (group 3, $p = 0.83$; group 4, $p = 0.67$), nor were there significant differences between normotensive diabetic and hypertensive diabetic animals ($p = 0.77$). There were no significant inter-group differences in the percentage of the capillary circumference covered by pericytic processes.

Diabetic animals with hypertension (group 4) showed significant basement membrane thickening compared with the other three groups of animals (Table 1). Using method 1 (see Materials and methods) there was a significant increase in basement membrane thickening in rats with hypertension and diabetes compared with controls ($p = 0.04$). There was no significant increase in rats with hypertension alone ($p = 0.27$) or diabetes alone ($p = 0.31$) compared with controls. Using method 2 (see Materials and methods) there was a significant increase in basement membrane thickening in rats with hypertension and diabetes together compared with rats with diabetes alone ($p < 0.05$) and compared with controls ($p = 0.04$), but not compared with rats with hypertension alone ($p = 0.43$). With both methods it appeared that the increase in basement membrane thickening in hypertensive diabetic animals (group 4) was greater than the added individual effect in animals with hypertension or diabetes alone (groups 2 and 3).

Discussion

This experimental study in rats has demonstrated that hypertension and diabetes have an interactive effect in producing an increase in the number of membrane-associated vesicles in endothelial cells and pericytes. The size, location and ultrastructural appearances of the vesicles seen suggested that they were caveolae, non-clathrin-coated invaginations of the plasma membrane.[11,12] The number of direct contacts between pericytes and endothelial cells was reduced in diabetic and diabetic-hypertensive animals. Hypertension and diabetes had an interactive effect on basement membrane thickening.

Physiological role of caveolae

The biological functions of caveolae are the subject of much current research. They were first implicated in transcytosis of macromolecules, such as low-density lipoprotein. It is now clear that they have a major role in importing and concentrating molecules (potocytosis) and delivering them to specific locations within the cell.[12] Caveolin, a 22 kDa membrane protein, is an important structural component of caveolae. More than 60 molecules have been identified biochemically or morphologically in caveolae or caveolin-rich membrane domains. These molecules include insulin receptors, the receptor for advanced glycosylation end-products (RAGE) and endothelial nitric oxide synthase (eNOS).[11–14] In particular caveolae are rich in molecules involved in cell signalling, such as G-protein-coupled receptors, plasma membrane Ca^{2+} and protein kinase C (PKC).[12]

Although we cannot explain the increase in caveolae that we demonstrated in hypertensive diabetic animals it is possible that it is related to abnormalities in calcium metabolism. Young spontaneously hypertensive rats show an increase in the number of caveolae in capillary endothelial cells of the myocardium and some of these

caveolae are aggregated in abnormal clusters.[15] Endothelial and smooth muscle cell caveolae are thought to be the regulators of cytoplasmic free calcium, containing a pump to remove Ca^{2+} from the cell, and inositol triphosphate receptors which are thought to facilitate cellular influx of Ca^{2+}.[12,16] In healthy cells fluctuations in Ca^{2+} levels are normalised by activation of PKC. The beta and delta isoforms of PKC are preferentially activated in the vasculature of diabetic animals and this is associated with a variety of pathophysiological changes, including vasoconstriction.[17] In the context of the current experiments, what evidence there is suggests that a potent activator of PKC, phorbol-12-myristate-13-acetate (PMA), inhibits potocytosis and *reduces* the number of caveolae in mouse fibroblast and monkey kidney cell cultures.[18]

Capillary perfusion pressure is increased in both systemic hypertension[19] and diabetes[5] and the associated increase in haemodynamic shear stress is likely to produce an increase in intracellular Ca^{2+}.[20] Abnormalities in nitric oxide metabolism may also be important in our experimental model as NO synthase has been localised to caveolae[21] and is acutely stimulated by increases in endothelial intracellular Ca^{2+}.[22] In recent experiments using cultured bovine retinal microvascular endothelial cells vascular endothelial growth factor (VEGF) was shown to increase vascular permeability by an eNOS-dependent mechanism of transcytosis in caveolae.[23]

Previous studies of retinal microvasculature

There are no other comparable reports of retinal pathology in BB rats. Caldwell and Slapnick[24] studied the retinal microvasculature using freeze-fracture techniques in normal and streptozotocin-treated (insulin-dependent diabetic) rats. They found, as we did, that plasmalemmal vesicles at the abluminal aspect of pericytes were increased in diabetic animals in comparison with controls. Their studies did not include hypertensive animals. Hammes and colleagues[6] studied spontaneously hypertensive rats (SHR) made diabetic by intravenous injection of streptozotocin. In comparison with normotensive controls these animals had significantly increased numbers of thrombi and microaneurysms in retinal capillaries and reduced numbers of pericytes. Other investigators have studied direct contacts between endothelial cells and pericytes in animals in the retina, optic nerve, myocardium and granulation tissue,[25–27] and in skin biopsies from human subjects with insulin-dependent diabetes.[28] Some of these contacts have been identified as tight junctions,[29] and, although there is limited understanding of their functions, there is evidence that they may act as a pathway for the transport of epidermal growth factor.[27] Robison and his colleagues[26] produced hyperglycaemia in rats by feeding galactose and noted, as we did, a marked reduction in endothelial cell–pericyte contacts in animals with basement membrane thickening.

In conclusion, our experiments have shown that in the BB rat hypertension and diabetes have an interactive effect in increasing the number of caveolae in both endothelial cells and pericytes. This may be a response to altered intracellular calcium kinetics. The functional significance of this change could be of considerable interest.

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