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R. P. Apkarian
Emory University

N. L. L' Hernault
Emory University

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CORRELATIVE LIGHT, TRANSMISSION, AND HIGH RESOLUTION (SE-I) SCANNING ELECTRON MICROSCOPY STUDIES OF RHESUS ADRENOCORTICAL VASCULAR MORPHOLOGY

R.P. Apkarian* and N.L. L' Hernault[2]

*Scanning Electron Microscope Facility, Yerkes Regional Primate Research Center
[2]Department of Ophthalmology, School of Medicine
Emory University, Atlanta, Georgia 30322

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Abstract

A detailed correlative morphologic description using light microscopy (LM), transmission electron microscopy (TEM) and high resolution SE-I scanning electron microscopy (SEM) was conducted on the capillary endothelium of the zona-fasciculata (Z-F) in juvenile male rhesus monkeys. The glucocorticoid synthesis and release phenomena, associated with stress stimulated release of adrenocorticotropic hormone (ACTH) via the hypothalamic-pituitary axis, intimately involves capillaries of the Z-F. A comprehensive study of all the ultrastructural features implicated in the transendothelial uptake of steroidogenic precursors and release of glucocorticoids in perfused rhesus adrenals has not previously been made. This report presents correlative images of transendothelial openings that include previously described single diaphragmed fenestrae and plasmalemma vesicles, and double diaphragmed transendothelial channels. New observations of endothelial cell pockets, tight junctional complexes and membrane filled ghost sacs were recorded from perfused rhesus adrenal. Membranous ghosts associated with adrenocortical endothelium were reported in a previous TEM study of perfused rat, however the potential argument existed that ghosts were artifactual. Their role as steroid hormone releasing structures remains an open question, yet their structural characteristics appear justified based on imaging of identical profiles observed in perfused rhesus adrenocortical specimens. These structural features are considered for the potential of gating and sorting of metabolites, and release of glucocorticoids in response to ACTH stimulated stress events.

KEY WORDS: secondary electron-I imaging, Cr coating, adrenal cortex, capillary endothelium, steroidogenesis

* Address for Correspondence:
Robert P. Apkarian
Scanning Electron Microscopy and X-ray Microanalysis Facility, Yerkes Regional Primate, Research Center, Emory University, Atlanta, GA 30322. Phone No. (404) 727-7766

Introduction

The physiological response in mammals, including physical trauma and emotional stress, invariably results in increased secretion of cortisol. Glucocorticoid secretion is mediated by the hypothalamic secretion of adrenocorticotrophic hormone (ACTH) releasing factor, which stimulates ACTH release by the anterior pituitary into the blood stream. ACTH stimulates cortisol release from the cellular cords along the sinusoids of the adrenal zona fasciculata (Z-F). Characteristic morphological features imaged by transmission electron microscopy (TEM) of steroid producing cells in the Z-F are striking, showing parallel arrays of granular reticulum, agranular tubular elements, lipid droplets, and mitochondria with tubular cisternae which occupy a large part of the cell. These ultrastructural features can also be used to determine the physiological state of the cell.[5,14]

It is generally accepted that ACTH binds to a receptor on the cortical cell surface thereby promoting cAMP production, enzyme activation through protein phosphorylation, and subsequent steroid synthesis. A recent study using calcium channel blockers suggest calcium influx is essential for steroid secretion and that vanadium inhibits cortisol secretion elicited by ACTH.[7] These results suggest that there are at least two receptors mediating ACTH dependent steroid secretion.[7] The synthesis in adrenocortical cells of adrenodoxin, adrenodoxin reductase, cytochromes P-450 side chain cleavage and P-450 11β, and the availability of NADPH are increased in a coordinated manner by ACTH stimuli during steroid synthesis.[6] The cortical capillary network provides the transportation system by which ACTH and steroid precursors (low density lipoproteins, LDLs) are brought to the adrenal gland and by which glucocorticoid products are secreted into the circulation. In the Z-F of the adrenal cortex, cords of steroidogenic cells line the sinusoidal capillary plexus. The elaborate interface between steroidogenic and endothelial cells in the Z-F and zona reticularis (Z-R) contains intercellular and perivascular spaces analogous to the bile canaliculi and the Spaces of Disse respectively, in the hepatic lobules of the liver.[10] The perivascular spaces contain a discontinuous basement membrane and cortical cells project microvilli into both the intercellular and perivascular compartments.[14] The thin (60-400 nm), attenuated expanse of the capillary endothelial cell is the only vascular barrier to blood-borne metabolites such as ACTH, LDLs, and steroids that enter or leave the

peripheral circulation and percolate amongst the steroidogenic cells.[11]

Various organs, including exocrine glands of the pancreas, intestinal mucosa, kidney cortex and adrenal cortex contain fenestrated capillary endothelium in which some of the biochemical and ultrastructural features of these cells have been probed.[15] The sum of single diaphragmed vesicles and fenestrae, and double diaphragmed transendothelial channels, all known to have similar 50-80 nm diameters, constitute the total number of transendothelial openings (TO). TEM studies have produced limited descriptions of endothelial cell ultrastructure in the adrenal cortex from various animal models. Relatively little attention has been paid to the strategic regions of vascular-perivascular metabolic exchange involving one or more types of TO. Quantitative TEM studies demonstrate the distribution of TO in different tissues from Murine capillaries[9] and morphologic descriptions of rat and rhesus adrenocortical vasculature have been reported.[5,14] We have shown an *in vivo* effect of ACTH which involved an increase in the number of fenestrae and other TOs in the Z-F capillaries of the hypophysectomized rat.[3] Others have shown that ACTH does not significantly modify the surface density of fenestrae in cultures of adrenal cortical capillary endothelial cells *in vitro*.[8]

It follows that the ability of an animal to respond to stress, and the ACTH stimulated release of cortisol, may be at least partially mediated at the microvascular level in the Z-F of the adrenal cortex.

Scanning electron microscopes (SEMs), equipped with condenser/objective (c/o) lens specimen stages and high brightness electron sources operated at high accelerating voltages (20-30 kV), are capable of generating specimen specific SE-I and SE-II contrasts. Provided that an ultrathin (1-2 nm) continuous contrast film of fine grain size chromium (Cr) is used to evenly coat the specimen, SE-I enriched high magnification images will provide accurate delineation of surface features in the 1-10 nm range.[12] Three dimensional images of lumenal, perivascular and cortical cell cytoplasmic surface ultrastructure in cryofractured rat adrenals have been described using SE-I contrasts.[2,3] Filamentous fine structure of TO and membrane ectodomains were accurately imaged on the lumenal aspect of cryofractured adrenal specimens. SE-I methodologies have been described for the imaging of macromolecular membrane fine structure on the lumenal surface of mouse glomerular fenestrated capillary endothelium.[12] By this method the surface features of endothelial cell pockets have been described[13] and it was suggested that these structures are also involved in gating and sorting molecular exchanges across the endothelium.

The present study involving light, transmission, and SE-I scanning electron microscopy, combines these imaging modes to thoroughly describe vascular fine structure, perivascular architecture, and cell-to-cell interactions in the capillary bed of the zona fasciculata in juvenile rhesus adrenal glands. Prior TEM studies[5] were performed on nonperfused rhesus adrenals and therefore did not provide detailed vascular morphology. This morphological description is intended to provide insight into the vascular features which may gate metabolites thereby regulating steroidogenesis and cortisol release in response to stress factors.

Materials and Methods

Fixation Procedure

Four male juvenile rhesus monkeys (2 and 6 months old), selected for other studies, were anesthetized with ketamine and euthanized with pentobarbital overdose. The thoracic aorta was cannulated with a 12 gauge needle in the mid-thoracic region. The portal vein was ligated and the vasculature was flushed with 1L of 0.1M Na-cacodylate buffer and 4% sucrose at pH 7.4 followed by fixation with 1L of 2.5% glutaraldehyde in 0.1M Na-cacodylate, pH 7.4. Both adrenal glands were excised and quartered for overnight fixation in electron microscope grade 2.5% glutaraldehyde in cacodylate, pH 7.4.

Light and Transmission Electron Microscopy

Adrenocortical tissue was postfixed in 1.0% OsO_4 in 0.1M cacodylate buffer for 1-2 hours at room temperature. Osmication was followed by three deionized water (dH_2O) rinses in 30 minutes (adrenals from 6 month old animals were given an additional step in which they were en bloc stained with 2.0% uranyl acetate). Tissue was dehydrated in a graded ethanol series followed by two 15 minute changes of propylene oxide (P.O.). Specimens were transferred to a 1:1 mixture of P.O. and LX112 (Ladd Research) for several hours after which it was placed in pure resin for four hours. Tissue was embedded in fresh resin in BEEM capsules and placed in a 60°C oven for 48 hours to polymerize.

One micron thick sections were cut with glass knives on an LKB ultramicrotome. They were stained with a solution of 1.0% toluidine blue and 1.0% sodium borate to locate the zona fasciculata of the cortical region. Light micrographs were taken with an Olympus BH microscope. Silver-gray thin sections were cut with a diamond knife, stained with 2.0% aqueous uranyl acetate for 30 minutes followed by Reynold's lead citrate (2 min) and observed with a JEOL 100CX transmission electron microscope operated at 80 kV.

High Resolution (SE-I) Scanning Electron Microscopy

Postfixed (1% OsO_4 in 0.1M cacodylate, pH 7.4) adrenocortical tissue was rinsed in dH_2O 6 times for 30 min. followed by graded exchange into absolute ethanol and wrapped into parafilm packets. Cryofracture was performed as previously described for rat adrenals[3] and thawed back into absolute ethanol. Samples were continuously exchanged from ethanol to Freon-113 (T.F.) for 2 h and critical point dried from CO_2 using delicate handling procedures. One continuous purge of the intermediate fluid with CO_2 was conducted at an exhaust flow rate of 1.2 L/min. The CPD chamber was regulated from purging to critical temperatures at a rate of 1°C/min.

Shiny surfaces of adrenal cortex were mounted face-up onto 9mm aluminum stubs with silver paste, and degassed at 5×10^{-6} Pa in a Denton DV-602 turbo pumped deposition system with LN_2 trap. The system was backfilled with argon to 1×10^{-1} Pa, and the specimens were sputter coated with a continuous fine grain 2 nm film of chromium.[4] Specimens were imaged in-the-lens (SE-I signal mode) of an ISI DS-130 SEM equipped with a LaB_6 emitter operated at 25kV.[1]

Results

Thick plastic sections (1 μm) were used for precise localization of the zona fasciculata (Z-F) and subsequent orientation for TEM ultramicrotomy. Capsular tissue was intimately associated with the zona glomerulosa, which extended 100-200 μm into the cortical region (Fig. 1). The cortical zone, 300 μm deep to the capsularis and extending to the zona-reticularis, was located and photographed for precise location within the zona-fasciculata. All sections for TEM ultramicrotomy were selected within this region. Steroidogenic cells arranged in cords lined the capillary plexus in the Z-F of rhesus adrenals from 2 and 6 month old animals (Fig. 2) creating a system of perivascular and intercellular spaces. Notably, the steroidogenic cells of 6 mo. adrenals contained a far greater amount of lipid droplets than the 2 mo. specimens. Cryofractured SEM specimens revealed similar spacings amongst steroidogenic (cortical) and endothelial cells while empty vesicles reflected the lipid droplet content within the adrenal tissue (Fig. 3). Low magnification TEM revealed morphology associated with steroidogenic cells. This consisted of large numbers of lipid droplets and mitochondria, ordered stacks of rough endoplasmic reticulum (RER), and dense bodies (Fig. 4). Brenner (1969) previously described for nonperfused adrenals from adult male rhesus elaborate perivascular spaces (PS) which interconnected with intercortical cell spaces (IS), and that both spaces contained large numbers of cortical cell microvilli. Six month old juvenile male rhesus adrenal Z-F capillaries also displayed these same features. Low magnification SEM images displayed a similar profile as TEM in which the cryofractured specimen surface revealed large numbers of lipid droplets, intercellular and perivascular spaces crowded with cortical cell projections. Additionally, SEM images displayed the lumenal surface of the capillary (C) endothelium (Fig. 5).

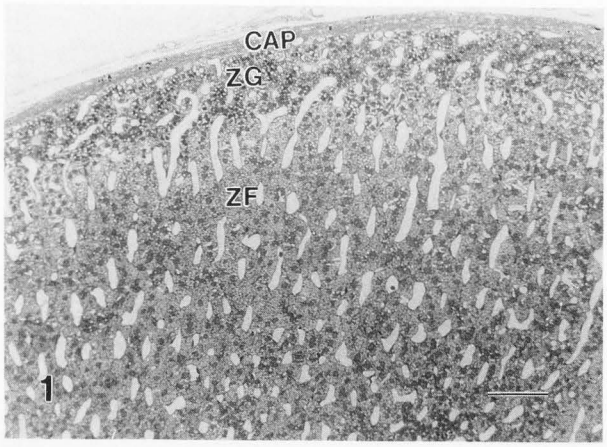
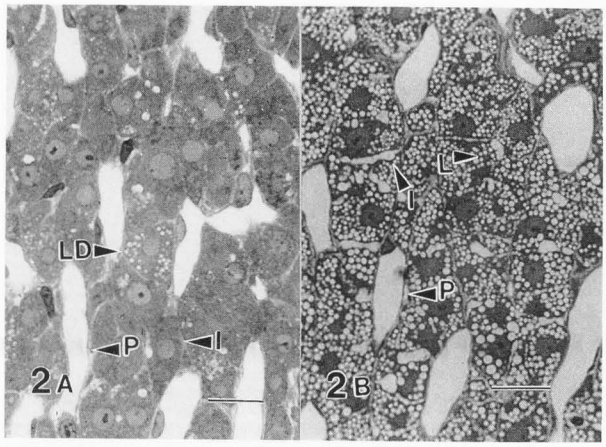


Fig. 1. Light micrograph of 6 month old male rhesus adrenal cortex. Note capsularis (CAP), and capillaries of zona-glomerulosa (ZG) and fasciculata (ZF). Bar = 100 μm.



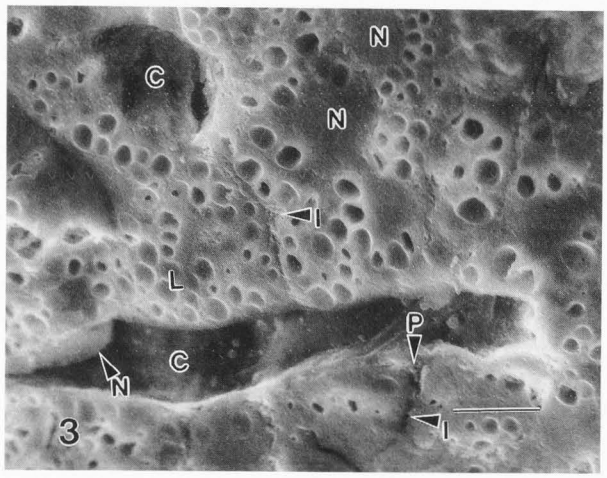


Fig. 2A. Z-F of 2 month old rhesus adrenal contain steroidogenic cells with few lipid droplets (L). Perivascular (P) and intercellular (I) spaces are seen. Bar = 25 μm.
Fig. 2B. Z-F of 6 month old rhesus adrenal contain a large amount of lipid droplets (L) and perivascular (P) and intercellular spaces (I) are prominent. Bar = 25 μm.

Fig. 3. Cryofractured SEM specimen from Z-F of 6 month old animal reveal lipid droplet content and a network of perivascular (P) and intercellular spaces (I). Note cross and longitudinal fractures through capillaries (C) lumen and endothelial and cortical cell nuclei (N), and lipid droplets (L). Bar = 5 μm.

Intermediate magnification SEM images of the endothelial cell (EC) surface contained large numbers of transendothelial cell openings (TO) in the 60-80 nm range distributed throughout the nonnucleated thin attenuated endothelial cell cytoplasm (Fig. 6). TEM was capable of differentiating the three known types of TOs. Along the thin (50-60 nm) cytoplasmic expanse of the EC, lumenal and ablumenal surfaces contained single diaphragmed vesicles and fenestrae that displayed 60-80 nm widths and contained a central density (Fig. 7). The perivascular space contained a discontinuous

basement membrane (BM). Cortical cell microvilli appeared as slender 0.1 μm diameter membrane bound profiles which contained microfilaments and were occasionally attached to one another. Diaphragmed vesicles were also located along the plasma membrane covering the EC nucleus and these vesicles occasionally abutted the nuclear membrane (Fig. 8). Double diaphragmed EC channels, whose lumenal surface diaphragms were in the 60-80 nm range, spanned the 100-150 nm cytoplasmic expanse (Fig. 9). Many PS in the Z-F contained double diaphragmed channels interdispersed amongst single diaphragmed fenestrae (Fig. 10). The discontinuous BM and cortical cell MV were located in close proximity to these diaphragmed structures. Another frequently encountered feature in the EC were cytoplasmic pockets which commonly appeared in pairs (Fig. 11). In TEM cross-section, these pockets contained diaphragmed openings which frequently showed a central density. In longitudinal section, fenestral-like diaphragms were clearly observed (Fig. 12). At high magnification some diaphragms within a pocket contained a central density. Directly associated in the PS were collagen and BM components (Fig. 13). High resolution SE-I SEM provided an image of the lumenal aspect of an EC pocket (Fig. 14). The openings were of the same range as in TEM preparation and that diaphragms displayed a central density. A fifth structure implicated in the gating of blood borne metabolites was the EC tight junctional complex (Fig. 15). Two cytoplasmic expanses which have joined and formed a tight junction, appeared as a ridge on the lumenal surface when imaged in the SE-I signal mode (Fig. 16). It was common in both TEM and SE-I SEM to observe a tangentially oriented unknown type of diaphragmed vesicle in close proximity to the junctional complex. Transendothelial openings (SEM) and fenestrae (TEM) were also seen. Previously described only in rat adrenal Z-F, sacs of membranous ghosts appeared on the ablumenal surface (Fig. 11), whereas other membranous profiles appeared as buds, pinched on the lumenal aspect of EC (Fig. 17).

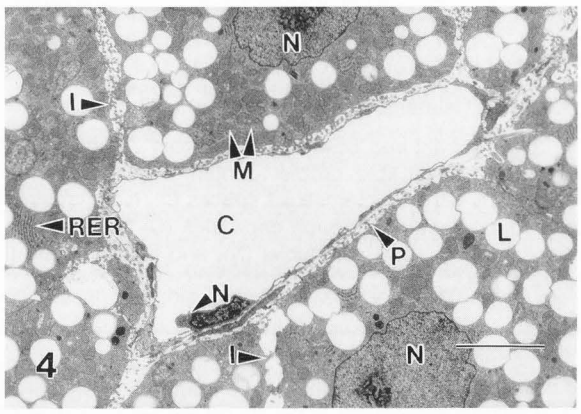
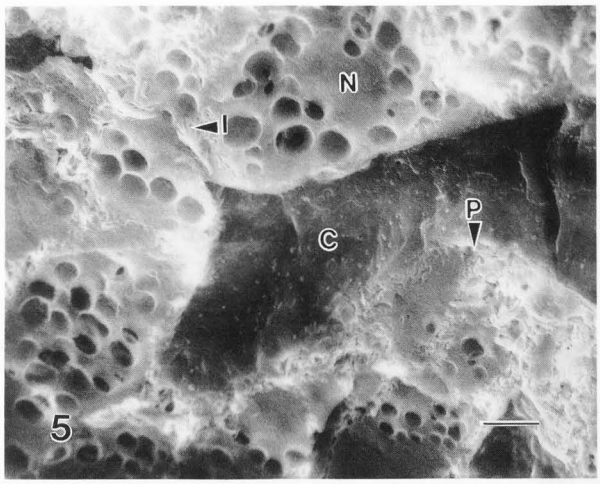
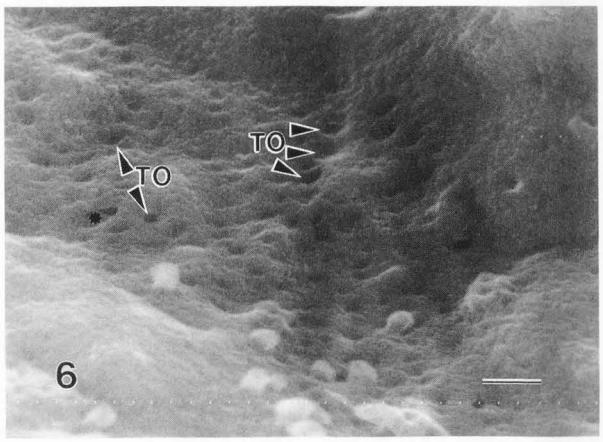


Fig. 4. TEM of capillary (C) in rhesus adrenal Z-F. The nuclei (N) of the endothelial and cortical cells were prominent. Cortical cells contained rough endoplasmic reticulum (RER) mitochondria (M) lipid droplets (L) and extended their microvilli into perivascular (P) and intercellular spaces (I). Bar = 5 μm.





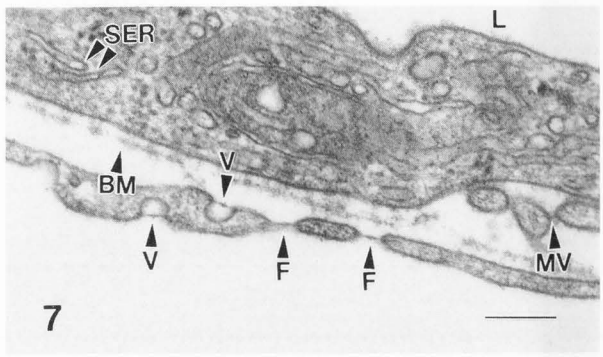
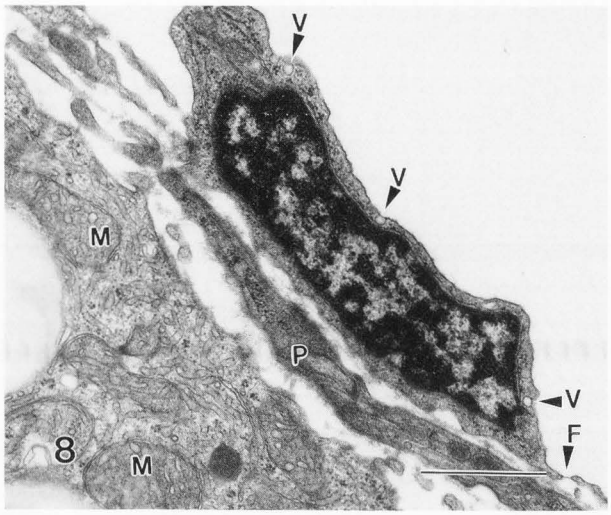
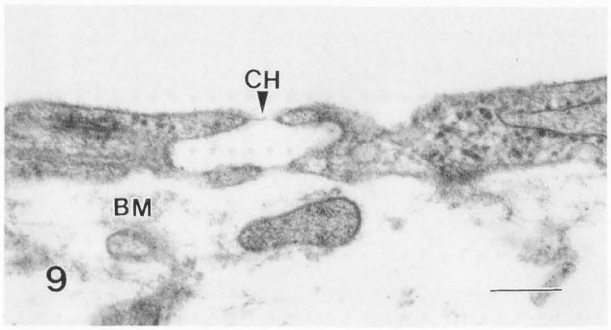


Fig. 5. Intercellular (I) and perivascular spaces (P) contained basement membrane and microvillar components. A cortical cell nucleus (N) and lipid droplets (L) were recognized adjacent to the capillary (C) lumenal surface. Bar = 2 μm.

Fig. 6. Intermediate magnification SE-I SEM of lumenal surface reveals transendothelial openings (TO) in the 60-80 nm range. Note single TO (*) with a 150 nm diameter. Bar = 200 nm.





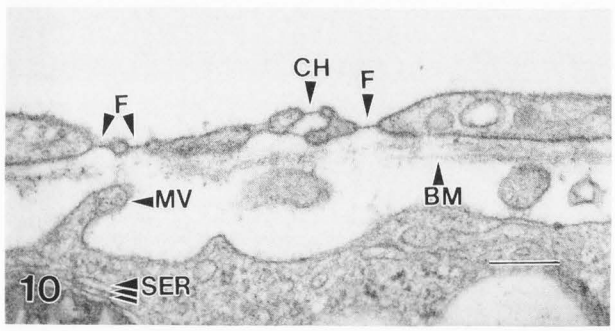
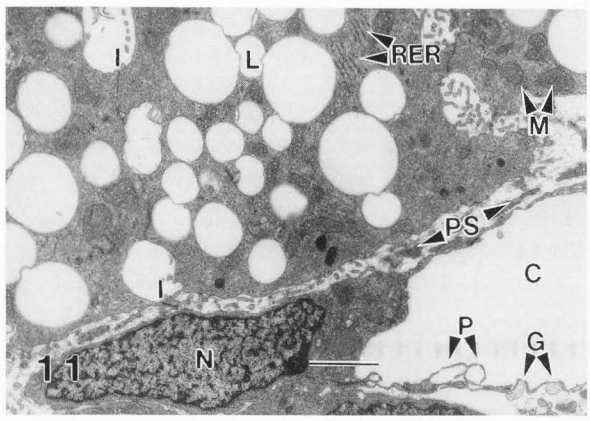


Fig. 7. Endothelial cytoplasm may attenuate as thin as 60 nm. Single diaphragmed fenestrae (F) and plasmalemma vesicles (V) on both the lumenal and ablumenal surfaces were fitted with a central density and ranged in size from 60 to 80 nm correlating with SE-I image of TO (Fig. 6). Note the discontinuous basement membrane (BM) and microvilli (MV) in the perivascular space. The cortical cell cytoplasm contained lipid (L) and smooth endoplasmic reticulum (SER). Bar = 200 nm.

Fig. 8. Diaphragmed plasmalemma vesicles (V) and fenestrae (F) are observed adjacent to the nuclear membrane of the endothelial cell. Note cortical cell mitochondria (M) and pericyte process (P). Bar = 1 μm.



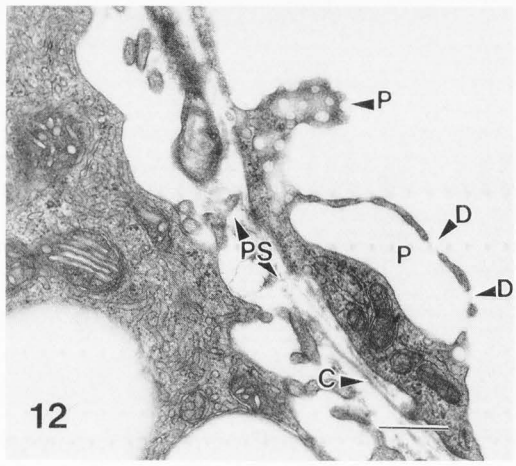
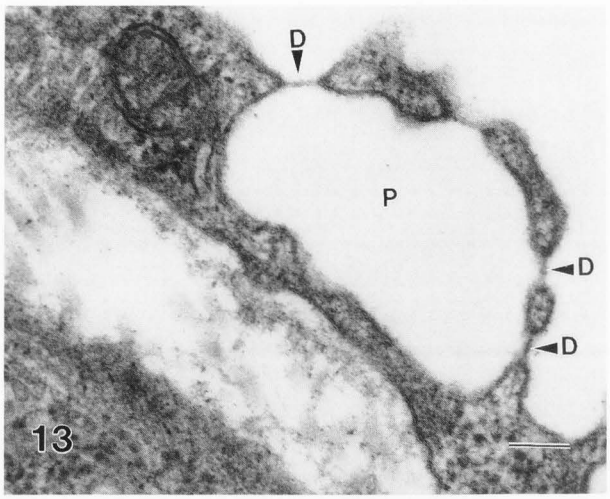


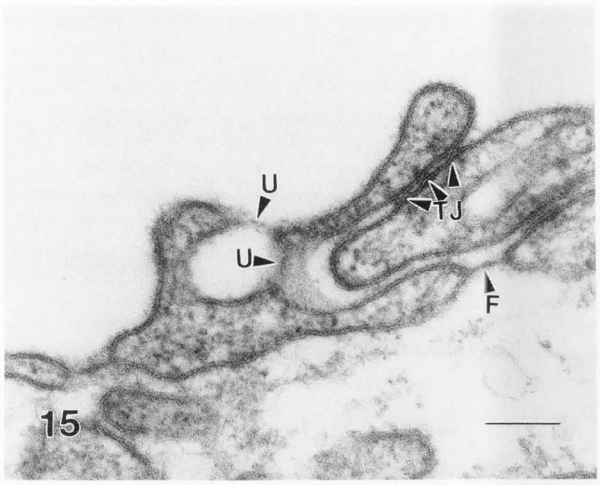
Fig. 9. Double diaphragmed channel (CH) openings (60-80 nm) directly connect lumenal and ablumenal surfaces. Note the discontinuous basement membrane (BM). Bar = 200 nm.

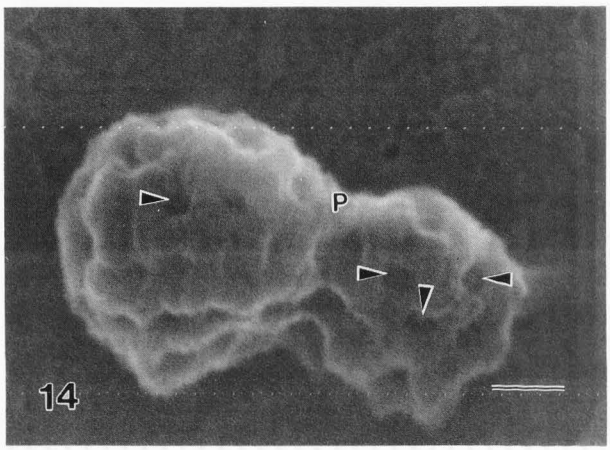
Fig. 10. Double diaphragmed channels (CH) and single diaphragmed fenestrae (F) are frequently interdispersed in the endothelium. The adjacent cortical cell cytoplasm contains smooth endoplasmic reticulum (SER), while microvilli (MV) and basement membrane (BM) components occupy the perivascular space. Bar = 200 nm.

Fig. 11. The lumenal surface of the capillary (C) display a pair of endothelial cell pockets (P) while ghosts (G) abut the ablumenal capillary surface. The endothelial cell nucleus (N) is in close proximity to perivascular (PS) and intercellular spaces (I). Note synthetic cortical cell cytoplasmic features of mitochondria (M), rough endoplasmic reticulum (RER) and lipid (L). Bar = 2 μm.

Fig. 12. Endothelial cell pockets (P) display diaphragmed (D) openings in both cross and longitudinal profiles. Perivascular spaces (PS) contained trace collagen (C) fibers. Bar = 0.5 μm.







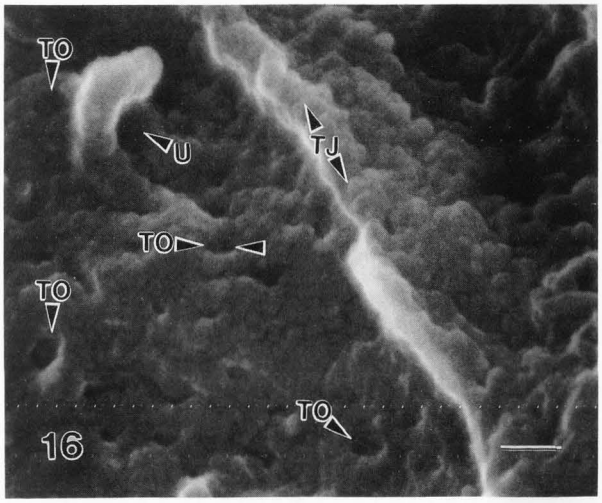


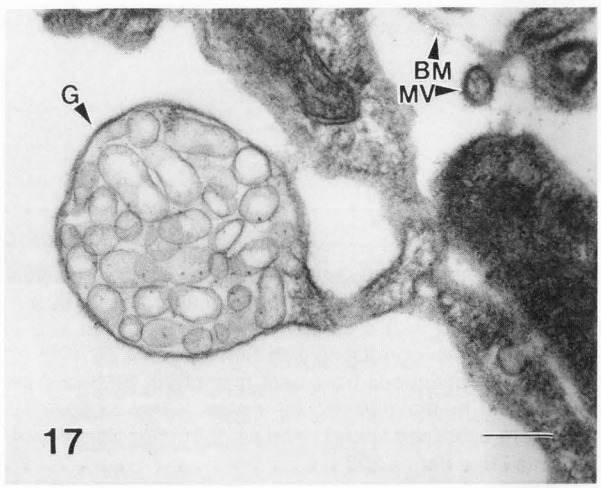
Fig. 13. High magnification TEM of a pocket (P) reveal diaphragmed openings (D) ≈55 nm in diameter which usually contain a central density (arrow). Bar = 100 nm.

Fig. 14. A high resolution SE-I image of the lumenal surface of an endothelial cell pocket (P) reveals 50 nm diaphragmed openings with central densities (arrows). Bar = 100 nm.

Fig. 15. Tight junctions (TJ) occlude the lumenal (L) surface in an overlapping of joining cytoplasmic expanses. Fenestrae (F) and unknown (U) types of openings coexist in the junctional region. Bar = 100 nm.

Fig. 16. SE-I high resolution image from the lumenal aspect of a tight junction (TJ). Transendothelial openings (TO) with central densities and diaphragms are adjacent to unknown (U) types of openings in the junctional region. Bar = 100 nm.

Fig. 17. Ghost (G) profiles appear to have traversed the endothelium into the lumen. Microvilli (MV) and basement membrane (BM) components are seen in the perivascular space. Bar = 200 nm.



Discussion

ACTH stimulated steroidogenesis and glucocorticoid secretion affect the homeostatic ability of an organism, however nonspecific stress may be detrimental to certain physiological functions and cause disease. For instance, an organism may survive due to anti-inflammatory suppression via ACTH-glucocorticoid release yet this immune suppression may cause the spread of infections. More complete knowledge of adrenocortical ultrastructure, in particular steroidogenic interaction with the vasculature, would be of great importance in determining factors which regulate glucocorticoid synthesis and release from the Z-F of the primate (rhesus) adrenal cortex.

Fenestrated capillaries in the Z-F of juvenile rhesus adrenals as well as in other visceral organs sort and gate hydrophilic macromolecules from plasma to interstitial fluid compartments. Single diaphragmed fenestrae which were the most numerous component were assumed to be the most important structural feature for capillary permeability properties although single diaphragmed vesicles and double diaphragmed channels coexisted in the same capillary beds.[9] Negatively charged fenestral diaphragms were different from the neutral diaphragms of channels and vesicles and thus created uneven distribution of anionic sites on the luminal plasmalemma.[15]

In a previous study of the Z-F capillaries in rat adrenals we showed that following hypophysectomy there was a dramatic decrease in transendothelial openings and fenestrae.[3] After only 2 hours of ACTH administration preceded by hypophysectomy 48 hours earlier there was a 2 fold increase in fenestrae. Vitamin A metabolites such as retinoic acid were shown by others to promote a threefold *in vitro* differentiation of fenestrae in adrenocortical endothelial cells in culture.[8] Adrenocortical hormones increase the serum vitamin A level and its mobilization from the liver and stimulate retinol-binding protein synthesis. It has been suggested that retinoids mediate the observed *in vivo* effect of ACTH on endothelial fenestrae, but this *in vivo* effect remains to be established.[8]

The present study utilized high resolution SE-I SEM and TEM imaging modes to more fully evaluate ultrastructural features in rhesus adrenal Z-F endothelium. Although SE-I images can not discern the type of transendothelial opening observed on the lumenal aspect of the EC, quantitative assessments of the total numbers of TO was helpful for determination of the degree of endothelial cell differentiation. TEM provided a qualitative view of single diaphragmed vesicles and double diaphragmed channels. Fenestrae were the most numerous type of transendothelial opening. Additionally, the two imaging modes served to correlate and document three other endothelial cell structures in the adrenal Z-F which were implicated in transendothelial gating and sorting. Endothelial cell pockets, first described by Peters and Milici[13] in murine peritubular capillaries, were observed herein for juvenile rhesus adrenal Z-F endothelium. TEM and SE-I imaging from cryofractured specimens revealed cytoplasmic folds which extended into the capillary lumen and displayed numerous diaphragmed openings that frequently contained a central density. This multifold transendothelial channel may provide a transport mechanism for plasma proteins or perhaps steroidogenic precursors, but at the present time it is not known what charge is present on the diaphragms. Utilizing the correlative electron imaging modes, we have demonstrated tight "occluding" junctions in the Z-F endothelium (Figs. 15, 16). SE-I images described a ridged structure that provided a dimensional correlation with the overlapped junctional complex seen in TEM images. The joining of cytoplasmic expanses into a junctional complex in adrenocortical capillary endothelium is a common observation and often elaborate interdigitation is clearly recognized in the SE-I mode.[3] Coincidentally unknown types of channels and diaphragmed TO with a central density were usually present in the vicinity of these junctional folds.

A previous TEM description of the rat adrenal cortex detailed remnant membranes from ruptured lipid droplet boundaries along with the central lamina of smooth endoplasmic reticulum that were released into the perivascular space and designated as "ghosts".[14] The ghosts reportedly appeared to have been fixed in a state of traversing the endothelial lining and appeared in the lumen of cortical capillaries. Juvenile (6 mo. old) rhesus adrenocortical perivascular spaces occasionally contained membranous sacs (ghosts) similar to those described for rat (Fig. 11). Large membrane filled sacs were observed to be continuous with the EC surface extending on into the lumen. The descriptive report of rat adrenal cortex after endocrine manipulation has elaborated on an "endoplasmocrine" or modified apocrine secretory phenomena proposed to be the glucocorticoid release mechanism.[14] The present study does not provide evidence for the aforementioned proposed phenomena that the membranous ghosts were remnants of the glucocorticoid secretory process. Careful perfusion fixation procedures utilized in the earlier adrenal study in rat and the present study in rhesus have confirmed membranous ghosts in intercellular compartments which also extend into the capillary lumen. We provide supporting evidence that membranous ghosts are not artifacts but are more likely a short lived transient structure which involves either or both the uptake of precursors and the release of product from steroidogenic to vascular compartments. Exact immunolabelling and tracing studies which utilize correlative SEM and TEM are needed to provide definitive evidence for LDL uptake and glucocorticoid release.

In this study we have reported diaphragmed plasmalemmal vesicles abutting the EC nuclear membrane. It is irresistible to speculate that blood borne chemical messages (retinoids?) may be transported via diaphragmed plasmalemmal vesicles to the endothelial cell nucleus thereby promoting genetic material to direct the state of EC cytoplasmic differentiation.

It has been reported that the lipid droplet content and intercellular and perivascular spaces in the Z-F of rat adrenals was reduced following ACTH administration.[14] In the present study we did not conduct any endocrine manipulation and therefore observed no identifiable change in cellular spacing. It should be considered that a normal state without stress stimuli would be unlikely due to the animals anesthetization and sacrifice. Therefore, variations in stress stimulus prior to the perfusates rapid fixation will inevitably occur and may influence cellular spaces as well as steroidogenic and vascular activity. Although we consistently observed relatively few lipid droplets in 2 month old rhesus

adrenals and large numbers of lipid droplets in 6 month old animals it is impossible based upon the small number of animals used in this study to state whether stress stimulus or age would account for the difference in lipid content or synthetic activity of the Z-F.

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Discussion with Reviewers

R.B. Johnson: Your SEM photographs of the capillaries of the adrenal cortex seem to indicate a very irregular endothelial surface. Is it possible that double diaphragmed channels and pockets could be artifactual due to oblique sections through this irregular endothelial surface? Could the authors speculate on the physiological significance of single and double fenestrated transendothelial openings?

Authors: The endothelial cell lumenal surface, seen with a SEM, has a generally flat membrane equipped with diaphragmed transendothelial openings located predominantly along thin cytoplasmic expanses and on pocket regions that may extend 1 μm into the lumen. The confirmed existence of double diaphragmed channels with a neutral charge has been established as an ultrastructural entity.[15] Together with vesicle diaphragms (neutral) and negatively changed fenestral diaphragms, double diaphragmed channels provide a molecular interface on the lumenal surface which gate and sort plasma-borne metabolites according to their charge. Endothelial cell pockets contain numerous diaphragms that are closely packed and do not bind catonized ferritin.[13] Figure 12 revealed two adjacent endothelial cell pockets one of which was observed in cross-section while the other was oblique to the plane of section. The result is that the obliquely sectioned pocket displayed several diaphragms with a central density and others with a ring-like structure while in the cross-sectional profile diaphragms appeared as a thin line as they do on other transendothelial openings. It is speculated that pockets represent a multifold channel involved in capillary exchange. Together with single diaphragmed vesicles and fenestrae and double diaphragmed channels, pockets provide the adrenocortical sinusoids with the selectivity to take up specific proteins and lipid metabolites involved in steroidogenesis. Since no detailed labelling study has been undertaken, no evidence exists to support or refute whether endothelial ghost and/or pockets may be involved in glucocorticoid release. Much more attention is warranted toward the study of glucocorticoid release pathway.

P.M. Motta: Have the authors observed within the primate adrenal gland perivascular space any cellular element (similar to the perivascular cells described in other organs) placed in close relation with the capillary wall? And, if these cells have been observed, are these elements contacting the endothelial fenestrations as is reported for perisinusoidal cell prolongations in the liver?

Authors: Similar to the hepatic lobule, the primate adrenal cortex displays a sinusoidal architecture composed of parenchymal and endothelial cell types networked amongst perivascular and intercellular spaces. In the liver sinusoids, the space of Disse contains numerous parenchymal cell microvilli, has no basement membrane and is associated with a thin attenuated endothelial cell lining fitted with large (100 nm) nondiaphragmed fenestrae that allow circulating plasma direct access to bathe parenchymal cells. [16,17] Within the terminal hepatic venules fibroblasts, vascular pericytes, and reticular fibers have been observed in the space of Disse. The vasculature of the adrenal cortex is arranged in cord-like channels rather than lobules yet appropriate physiological stimuli regulates perivascular spacing in both organs. The characteristics of adrenocortical perivascular spaces include a discontinuous basement membrane, scattered cortical cell microvilli and collagen, and a lining of fenestrated endothelium fitted with 60-80 nm diaphragms. Unlike the sinusoidal endothelium in liver, diaphragmed fenestrae and five other adrenocortical endothelial cells specializations described in this paper are implicated in gating and selecting metabolite access to and from circulating plasma. The perivascular spaces within the sinusoids of adrenal and liver differ in the numbers of parenchymal cell microvilli (greater in liver) and with the type of fenestrated endothelium (diaphragmed versus non-diaphragmed) they are associated. We have made only rare observations of pericytes (see Figure 8), and frequently observed scattered microvilli and discontinuous basement membrane within 100 nm proximity of a diaphragmed fenestrae (Figure 10). Although we have observed wandering macrophages in the rat adrenal cortex, we have not encountered them in juvenile male rhesus adrenals.

T.D. Allen: Your scanning micrographs and high magnification clearly demonstrate the superiority of chromium as a fine grain film. However, how does the overall signal level from an element with a relatively low atomic number compare with say gold, for overall surface morphology at lower resolution, for localisation of the sites of interest?

Authors: SEMs equipped with in-the-lens specimen stages effectively eliminate the SE-III signal component that dominates the conventional secondary electron image and realizes SE-I signal mode operation (SE-I/SE-II signal ratio).[2] The total secondary electron yield would be greater for gold decorated samples rather than those that were chromium coated due to the enhanced scattering and generation of the SE-II signal component characteristic of large grain high atomic number metal films. Only the high resolution SE-I signal component provides information from the probe impact site at an escape depth of 1-2 nm and is generated prior to a significant number of scattering events. Even though the total secondary electron yield is lower for Cr films the image is of higher resolution than a gold or platinum decorated sample because of a higher SE-I/SE-II signal ratio. Chromium film noted for their fine grain, nonmobile compact nature enrich the high resolution SE-I contrast and therefore the accuracy of information noticeable at intermediate magnification (see Figure 6).

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