Hormonal regulation of adrenal microvillar channel formation

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Abstract **This study examined the in vivo relationship between expression of the HDL receptor scavenger receptor class B (SR-BI) and corresponding structural changes in the rat adrenocortical cell microvillar compartment. Using hormonal stimulation and withdrawal protocols, we were able to manipulate adrenal SR-BI levels and carry out qualitative and quantitative measurements correlating SR-BI expression with microvillar mass and microvillar channel formation. Young male rats were used as controls or treated with adrenocorticotropin hormone (ACTH) (24 h), 17α-ethinyl estradiol (17α-E2) (5 days), or dexamethasone (DEX) (24 h). Quantitative Western blot analysis and immunocytochemistry indicated that ACTH and 17α-E2 treatment greatly increased SR-BI expression in the adrenal (especially in the microvillar compartment of adrenocortical cells), whereas DEX treatment led to a decrease of SR-BI by all measurements. At the same time, striking ultrastructural changes occurred in the adrenocortical cell microvillar compartment: e.g., microvillar area and microvillar channel formation and complexity dramatically increased (compared with control values) after ACTH or 17α-E2 treatment, whereas the same values declined after DEX treatment. These measurements illustrate the exceptional flexibility and responsiveness of the microvillar compartment to hormonal stimuli, and suggest that regulation of SR-BI expression and structural configuration of the surface of steroidogenic cells goes hand in hand.**—Azhar, S., A. Nomoto, and E. Reaven. **Hormonal regulation of adrenal microvillar channel formation.** ***J. Lipid Res.*** **2002.** 43: **861–871.**

Supplementary key words SR-BI • HDL • steroid hormone • ACTH • corticosterone • cholesterol • 17α-ethinyl estradiol • steroidogenesis

Tissues of the rat that require large amounts of exogeneous cholesterol for steroid hormone production (e.g., adrenal, ovary, and trophic hormone sensitized testes) obtain much of this cholesterol from circulating HDL. The major cholesterol uptake process used by these tissues is known as the selective cholesteryl ester (CE) uptake pathway (1), a term that indicates that HDL-CEs are released directly into cells without cell internalization of the whole lipoprotein particle (2–8). Although details of the molecular mechanisms by which cells can selectively internalize large quantities of neutral lipids are not yet clear, some aspects of the pathway have been established. Most importantly, it is known that a regulatable HDL receptor protein scavenger receptor class B type I (SR-BI) is found on the cell surface of a variety of rodent high cholesterol-requiring tissues (steroidogenic tissues, liver) (9–16), and that cells that express high levels of SR-BI can efficiently obtain lipoprotein derived CEs for use in hormone or product synthesis (7, 8, 12, 14, 15, 17–19). Indeed, work from several laboratories using different adrenal, ovarian, testicular, or liver tissue models show an exceptionally tight association between expression of SR-BI protein and the direct measure of selective CE uptake under changing physiological conditions, including hormone stimulation, hormone inhibition, lipid levels, SR-BI over-expression, or genetic ablation (11, 12, 15, 20–27).

It is of interest that rodent steroidogenic cells that express high levels of SR-BI in vivo are endowed with an intricate surface microvillar system specialized for the trapping of lipoproteins (2, 3, 5, 28–30). We refer to this general region of the steroidogenic cell as the microvillar compartment and the specialized space created between adjacent microvilli as microvillar channels (28–30). Often, inverted microvilli form double-membrane channels within the peripheral cytoplasm of the cells as they make close contact with the invaginated portion of the adjacent cytoplasm (15, 28, 29). Electron microscopic immunocytochemistry techniques show heavy labeling for SR-BI specifically in regions corresponding to such microvilli and microvillar channels (13, 15), and there is no longer any doubt that the cell surface localization of SR-BI is specifically localized to the microvillar compartment including the microvillar channels, and that a tissue exhibiting a microvillar compartment expressing high levels of SR-BI is active in selective CE uptake (5, 13, 15). In rat steroidogenic tissues, one finds that the microvillar channels are often filled with spherical particles the size of rat HDL (15, 28, 29).

Abbreviations: ACTH, adrenocorticotropin hormone; CRF, corticotropin-releasing factor; DEX, dexamethasone; SR-BI, scavenger receptor class B type I; 17α-E2, 17α-ethinyl estradiol.

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Results from a recent study using a heterologous insect cell system (31) has added new meaning to the relationship between SR-BI, microvillar channels, and "selective" lipoprotein-CE uptake. It had previously been assumed that preformed microvillar channel structures in cells secondarily acquire SR-BI protein after a stimulus (in most cases, a hormonal stimulus) activating the selective uptake of lipoprotein CEs and steroidogenesis. The over-expression of recombinant SR-BI in baculovirus infected insect ovary cells (*Sf*9) altered this story. It became clear (even in such a primitive cell system) that the expression of SR-BI by itself was the stimulus for double membrane formation. That is, non-infected or infected control *Sf*9 cells do not express SR-BI, show microvillar channels, or internalize CEs. However, in baculovirus infected *Sf*9 cells expressing high levels of SR-BI, new double membraned channels are induced, and these membranes, in turn, facilitate the binding of exogenously provided HDL and selective HDL-CE uptake. It was of interest that the newly formed double membraned structures were most often observed as a complex network of channels within the cell peripheral cytoplasm.

In the current effort, we examined the functional relationship between SR-BI expression and microvillar channel formation in a physiologically responsive steroidogenic tissue, the rat adrenal. Using hormonal stimulation and withdrawal protocols, we have been able to manipulate adrenal SR-BI expression, permitting qualitative and quantitative correlations to be made between adrenal SR-BI levels and adrenocortical cell microvillar channel formation and function.

MATERIALS AND METHODS

Materials

[1,2-^{3}H(N)]corticosterone, 40–60 Ci (1.48–2.22 TBq/mmol) was purchased from NEN Life Science Products (Boston, MA). Bovine plasma fibronectin, corticosterone, 17α-ethinyl estradiol (17α-E2), and N^6,2′-*O*-dibutylyladenosine 3′,5′-cyclic monophosphate (Bt_2cAMP) were purchased from Sigma Chemical Co. (St. Louis, MO). Collaborative Biomedical Products (Bedford, MA) supplied insulin and transferrin. Electrochemiluminescence kit Western blotting detection reagents were purchased from KPL (Gaithersburg, MD). Long acting adrenocorticotropin hormone (ACTH) gel preparation (HP Acthar gel) was the product of Armour Pharmaceuticals, (Kankakee, IL). The anti-rat SR-BI and anti-rat apolipoprotein (apo)A-I antibodies used have been described and characterized in this laboratory (12, 31). All other reagents used were of analytical grade.

Animals and hormonal treatment

Four groups of 3-month-old male Sprague-Dawley rats (Charles Rivers Laboratories, Hollister, CA) were studied: *1*) non-stressed controls (C), *2*) rats treated with long-acting ACTH gel (10 IU *sc* every 8 h for 24 h period), *3*) rats treated with 17α-E2, 10 mg/kg BW *sc* every 24 h for 5 days, and *4*) rats treated with dexamethasone (DEX) (a single injection 100 μg, *sc*) for a 24 h period.

Western blot analysis of SR-BI

The expression of SR-BI protein in whole adrenal tissues and cultured adrenocortical cells was assessed by Western blot analysis using previously described methodology (12, 13). Briefly, adrenal tissues were homogenized in 10 vol of buffer (20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.25 M sucrose, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 5 μg/ml pepstatin), centrifuged (800 × *g*) for 10 min, and supernatant centrifuged for 60 min at 100,000 × *g*. The resulting pellet was washed with buffer to remove floating lipids and membranes were used for immunoblotting of SR-BI. Similarly, cultured adrenocortical cells were washed twice in ice-cold phosphate-buffered saline, and lyzed in lysis buffer [50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% Triton X-100 (v/v), 0.5% deoxycholate (v/v), 1% SDS (w/v), 5 mM EDTA, and 1 mM dithiothreitol]. Following incubation at 37°C for 15 min, each lysate was sonicated briefly to disrupt chromatin (DNA) and then used for SR-BI immunoblotting.

Samples were mixed with equal volumes of 2× Laemmli sample buffer [120 mM Tris-HCl, pH 6.8, 2% SDS (w/v), 10% sucrose (w/v), and 1% 2-mercaptoethanol] and subjected to 7% SDS-PAGE. For each sample, a constant amount of protein (5–20 μg) was loaded on the gel. Protein standards (Myosin, 200 kDa; β-galactosidase, 116.3 kDa; Phosphorylase b, 97.4 kDa; BSA, 66.2 kDa; and ovalbumin, 45 kDa) were also loaded on the gel. After electrophoretic separation, the proteins were transferred to Immobilon PVDF membranes (Millipore Corp., Bedford, MA) using standard techniques. The protein blots were incubated with anti-rat SR-BI for 2 h at room temperature, then probed with peroxidase-labeled mouse anti-rabbit IgG and visualized using the ECL system. The resulting radiographic chemiluminescence was visualized for different time points (1–10 min), and appropriate films were subjected to densitometric scanning.

Lipoproteins and SR-BI expression in cultured adrenocortical cells

Adrenocortical cells from hypocholesterolemic (17α-E2 treated) animals were used to determine if the addition of exogenous lipoproteins could downregulate SR-BI expression. Adrenocortical cells were isolated by digestion with collagenase and DNase as previously described from this laboratory (32), maintained in serum free Dulbecco's modified Eagles medium: nutrient mixture F-12 (1:1) (32) for 24 h prior to the experimental protocol comparing untreated cells (basal) with those given apoE-free human high density lipoproteins ($hHDL_3$) (500 μg protein/ml), Bt_2cAMP (2.5 mM), or $hHDL_3$ + Bt_2cAMP for a 24 h interval.

Measurement of secreted corticosterone

Aliquots of culture media were assayed for secreted corticosterone by the radioimmunoassay (RIA) technique as previously described (5, 32).

Miscellaneous techniques

$hHDL_3$ used in the cell culture studies were isolated as described previously (30), and the procedure of Markwell et al. was used to quantify protein content of $hHDL_3$ (33). The DNA content of the cells was quantified fluorimetrically (34) while protein in membrane fractions was determined by a modification of the procedure as described by Peterson (35). Serum cholesterol concentrations were determined according to the procedure of Tercyak (36). Serum corticosterone was extracted with methylene chloride and assayed by RIA (5, 32). Adrenal free cholesterol (FC) and cholesteryl esters (CE) were extracted and quantified as described earlier (5).

Morphological techniques

Adrenals from several rats of each of the treated categories were perfusion fixed for standard light microscope immunohistochemistry (4% paraformaldehyde fixation, paraffin embedment, immunoperoxidase technology), or standard electron microscopy (2%

glutaraldehyde, eponate embedment), or immunocytochemistry at the electron microscope level (2% paraformaldehyde + 0.2% glutaraldehyde, LR Gold embedment and immunogold technology) as previously described (13, 15, 31).

For electron microscopy, care was taken to compare tissues obtained from similar regions of the adrenal cortex. In control animals, SR-BI is not found in z. glomerulosa regions of the adrenal cortex, but is present in both the z. fasciculata and z. reticularis, being most expressed in proximal regions of the z. fasciculata. Because of this zonal difference, special procedures were used to obtain random photographs for quantifying structural changes and SR-BI expression in adrenocortical cells. In brief, perfusion-fixed adrenals were sectioned to present linear sinusoids in the most proximal regions of the z. fasciculata. Appropriately situated sinusoids were targeted for photography at very low magnification. The sinusoidal surfaces of 10–12 adjacent cells were then photographed from each of two sinusoids at 14,000 K (standard microscopy sections) or 19,000 K (immunogold labeled sections), and various area and length measurements were made using a graphics tablet (Wacom, Vancouver, WA) integrated with the Scion Corporation (Frederick, MD) version of the NIH Image program. Area measurements of adrenocortical cell microvillar compartments from variously treated rats were expressed for a standard horizontal length of measured cell surface. Data for microvillar channel length or SR-BI immunogold labeling were expressed for 100 mm^2 area microvillar compartment, thereby correcting for hormone induced changes in microvillar compartment area.

RESULTS

Relationship of plasma and adrenal cholesterol levels to corticosterone production following hormonal treatment

ACTH treatment. ACTH treatment for 24 h results in a 10-fold increase in serum corticosterone levels compared with control animals (**Table 1**). While serum cholesterol is unaltered in ACTH treated rats, the amount of stored adrenal cholesteryl ester in these animals is one-half control levels (Table 1).

17α-E2 treatment. 17α-E2 given for 5 days leads to a doubling of control corticosterone levels despite a severe decline in circulating cholesterol levels. Also, stored adrenal cholesteryl esters are markedly reduced compared with control levels (Table 1).

Dexamethasone treatment. Twenty four hours of dexamethasone treatment leads to low serum corticosterone (∼10% of control levels) with no change in circulating cholesterol. Stored tissue cholesteryl esters are similar to control levels (Table 1).

TABLE 1. Serum corticosterone, cholesterol levels and adrenal cholesteryl esters (CE)

Treatment	Serum Corticosterone	Serum Cholesterol	Adrenal CE
	mg/dl	*μg/dl*	*μg/mg tissue*
Control	3.2 ± 0.4	56.0 ± 4.5	45.1 ± 5.5
ACTH	34.1 ± 4.1[b]	57.9 ± 4.3	18.0 ± 1.7[a]
17α-E2	8.9 ± 0.8[a]	9.6 ± 1.2[b]	6.6 ± 1.1[b]
DEX	0.3 ± 0.05[b]	60.8 ± 4.5	48.9 ± 6.8

Results are mean ± SE of eight separate determinations.
[a] $P < 0.005$ versus control.
[b] $P < 0.001$ versus control.

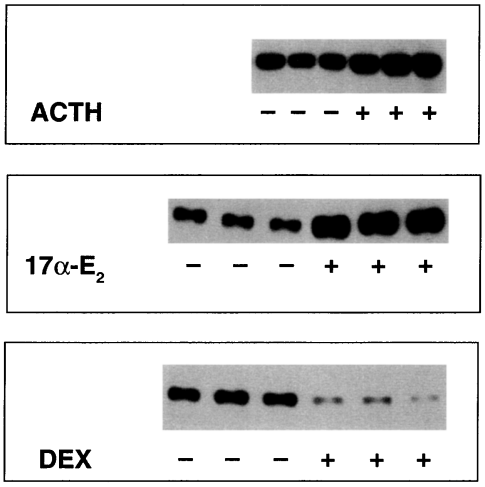

Fig. 1. Western blots comparing adrenal scavenger receptor class B, type I (SR-BI) expression from three separate control (−) and treated (+) animals following administration of adrenocorticotropin hormone (ACTH), 17α-ethinyl estradiol (17α-E2), or dexamethasone (DEX) (see animal protocols under Materials and Methods). The blots show substantial changes in SR-BI expression depending on treatment.

Adrenal SR-BI response to hormonal treatment

Western blots of the treated adrenals showing SR-BI expression (**Fig. 1**) indicate that ACTH or 17α-E2 treatment have major stimulating effects on adrenal SR-BI levels with densitometric scans of the blots, indicating that ACTH treatment leads to >2 fold increase in SR-BI expression, and 17α-E2 injections lead to ∼5-fold increase in SR-BI.

In contrast, DEX treatment is associated with a dramatic decline in SR-BI expression, with densitometric data showing levels only ∼30% SR-BI expression in control adrenals (Fig. 1).

Western blots of adrenal SR-BI (**Fig. 2**) show that SR-BI can also exist in dimeric and oligomeric/heteromeric forms as shown previously by Landschulz following the use of 17α-E2 in rats (9), and Williams, following the use of ACTH and DEX in rats (37). With the use of identical amounts of protein extract per lane, SR-BI dimer (∼160 kDa) and oligomer/heteromeric (>320 kDa) expression in adrenals of ACTH (Fig. 2A) and 17α-E2 treated rats (Fig. 2B) is 2–10-fold greater than seen in control adrenals, whereas DEX treated adrenals (Fig. 2B) show negligible SR-BI dimer expression.

In an effort to separate the influence of hormone versus cholesterol levels on the observed increase in adrenal SR-BI expression, isolated adrenocortical cells from hypocholesterolemic (17α-E2 treated) animals were subsequently maintained in culture with or without cAMP, lipoproteins, or a combination of these factors. Corticosterone secretion was measured and Western blots were used to determine SR-BI expression. **Table 2** shows that corticosterone production increased 6-fold in cells given $hHDL_3$ alone, but increased 600-fold in cells treated with $hHDL_3$ +

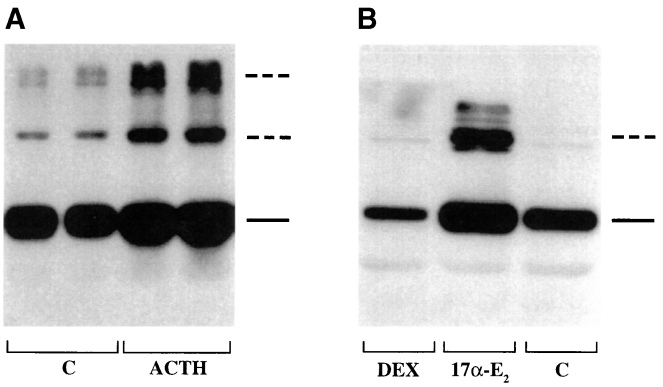

Fig. 2. Western blots from two separate experiments (A, B) showing the presence of SR-BI monomer (solid line), as well as SR-BI dimer and oligomer/heteromer expression (dashed lines) following various hormonal treatments. SR-BI dimers and oligomers/heteromers are present in control samples, but show increased formation following ACTH and 17α-E2 treatment, and decreased formation after DEX treatment.

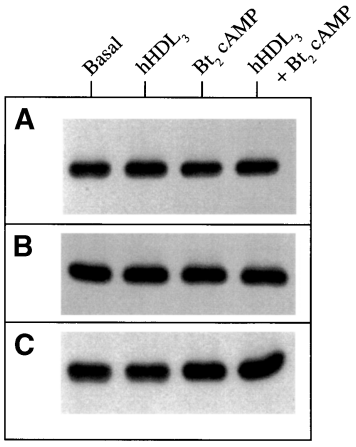

Fig. 3. Western blots of three separate experiments (A, B, C) of SR-BI expression in cultured adrenocortical cells (isolated from 17α-E2 hypocholesterolemic animals) untreated, or incubated with apolipoprotein (apo)E-free human HDL ($hHDL_3$), N^6,2′-*O*-dibutyladenosine 3′,5′-cyclic monophosphate (Bt_2cAMP), or a combination of these two agents. Such cells from the hypocholesterolemic rats show high levels of SR-BI expression, which are not further modified by treatment with exogenously supplied lipoproteins and/or Bt_2cAMP.

Bt_2cAMP. At the same time, under identical experimental conditions, SR-BI expression did not change with either treatment (**Fig. 3**).

SR-BI immunohistochemistry in response to hormonal treatment

Regardless of hormonal treatment, SR-BI staining is associated with the entire surface of cells of the z. fasciculata (and to a lesser extent with cells of the z. reticularis) so that all adrenocortical cells in these layers appear to be surrounded by SR-BI. However, in every adrenal examined, the level of SR-BI expression appears to fade gradually from the most proximal to the most distally located cells in the cortex.

In general, SR-BI staining at the light microscope level (**Fig. 4**) reflects adrenal SR-BI expression seen in Western blots; i.e., the immunostaining of adrenal tissue from ACTH treated rats (Fig. 4B) is more intense than in control rats (Fig. 4A), whereas tissue from DEX treated rats (Fig. 4C) is far less stained than adrenals from controls. These results are similar to those previously observed in adrenals of mice treated with ACTH or DEX (10, 25), and in adrenals of rats treated with 17α-E2 (9). It should also be noted that the breadth of the stained band surrounding each cell varies with hormonal treatment and is broader in cells of adrenals from ACTH treated animals than in control or DEX treated adrenals. Additionally, in ACTH treated animals, SR-BI staining is also seen in cells of the z. glomerulosa (data not shown), and in general, adrenocortical cells from ACTH treated animals are increased in size.

TABLE 2. Effect of hypocholesterolemia on corticosterone secretion by cultured adrenocortical cells in response to $hHDL_3$ and/or Bt_2cAMP

Additions	Corticosterone (ng/μg DNA ± SE)
Basal	6.8 ± 1.9
$hHDL_3$ (500 μg/ml)	39.8 ± 9.9
Bt_2cAMP (2.5 mM)	126.0 ± 24
Bt_2cAMP + $hHDL_3$	4761.0 ± 1050

Cultured adrenocortical cells were incubated with $hHDL_3$ and/or Bt_2cAMP for 24 h. Suitable aliquots of the culture media were assayed for secreted corticosterone by RIA. The results are mean ± SE of four separate experiments.

Ultrastructural changes in response to hormonal treatment

Standard electron microscopy: microvillar compartment. In analyzing the ultrastructure of adrenocortical cells from the treated animals, it was imperative to compare cells from similar cortical zones, but also from similar areas within each zone. As outlined in Materials and Methods, every effort was made to select similarly placed sinusoidal vessels (visible within 5–10 cell layers into the proximal z. fasciculata), and to randomly photograph the sinusoidal surface of underlying adrenocortical cells. When this was done it became clear that the microvillar compartment of adrenocortical cells from the variously treated animals were distinctly different from one animal group to another.

In adrenals of untreated control rats (**Fig. 5A**), the microvillar compartment of z. fasciculata cells includes a rich supply of microvilli generally protruding into the sub-endothelial space; some microvilli make close connections with adjacent microvilli, or with the cell surface forming microvillar channels. Some layering of the microvillar channels (i.e., stacks of microvilli in close contact with each other) occurs, but for the most part such stacks are superficial, occurring within the sub-endothelial space itself or embedded very superficially in cells.

In ACTH-treated or 17α-E2-treated rats, a very different picture emerges (Fig. 5B, C). After either treatment, a

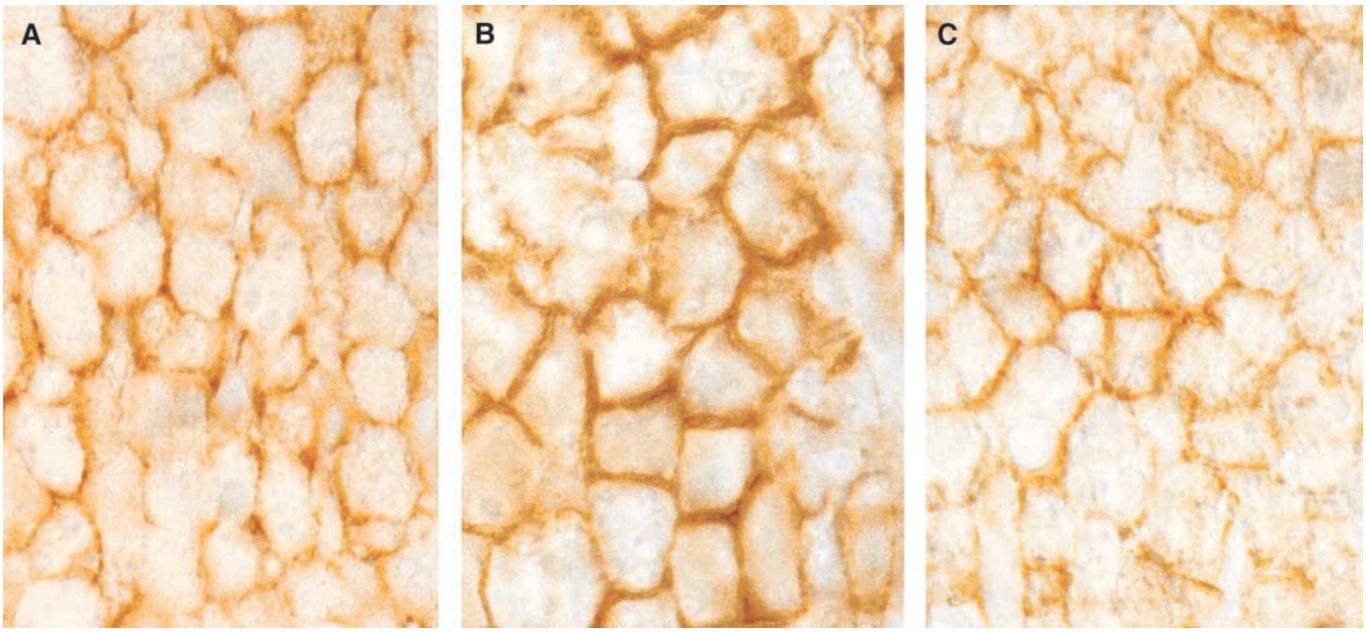

Fig. 4. Immunohistochemical localization of SR-BI in proximal regions of the z. fasciculata of adrenals from control (A), ACTH (B), or DEX (C) treated animals. All adrenocortical cells are outlined by SR-BI with ACTH cells showing the heaviest expression (B) and DEX cells (C) showing the lowest expression.

larger proportion of the z. fasciculata adrenocortical cell surface is recruited to the microvillar compartment [i.e., a doubling in microvillar area/unit length cell surface] in ACTH treated adrenals, and a 3-fold increase in microvillar area in 17α-E2 treated adrenals (see **Fig. 6A**). Within this larger microvillar compartment there is also a substantial ACTH and 17α-E2 induced increase in the amount of microvillar channels formed (total length of double membranes/unit microvillar area (Fig. 6B). Note: these calculations are corrected for the changing mass of the microvillar compartment itself. These changes translate into a very complex surface architecture for the adrenocortical cells with almost all the available microvilli associated with adjacent microvilli (forming channels), and many stacks and loops of these double membraned channels appear to be embedded within the peripheral cytoplasm of the cells as seen in Fig. 5. Indeed, the surfaces of both ACTH and 17α-E2 treated z. fasciculata cells appear to be covered with an intricately folded membrane sheet.

In stark contrast, microvillar compartments of DEX-treated animals show a dramatic loss of microvilli (Fig. 5D). The few remaining microvilli are solitary structures rarely connected to each other or invaginated into the body of the cell. As a result, few microvillar channels are found (Fig. 6B).

Lipoproteins and microvillar channels. In addition to the hormonally induced changes in the mass and configuration of microvilli and microvillar channels present in adrenocortical cells, the amount of lipoprotein trapped within the channels also varies. The microvillar channels of tissues from control, ACTH, and 17α-E2 treated animals are often filled with particles corresponding in size to rat HDL (**Fig. 7A**, arrowheads). Immunogold staining with antibodies to rHDL-apoA-I (Fig. 7B) confirms the notion that rHDL remnant particles are, in fact, associated with microvillar channels; however, in these immunostained preparations, the particles themselves appear only as poorly defined striations within the channel space (arrowheads). In contrast to the ACTH and 17α-E2 samples where microvillar channels are common, few lipoproteins can be identified in DEX treated samples where microvillar channels are rarely found (data not shown).

Use of 17α-E2 is known to induce the formation of clathrin coated pits and vesicles (38, 39). In the current study, an increase in the number of these structures is observed in tissues of both ACTH and 17α-E2 treated animals (data not shown), but in addition, many inverted microvilli embedded in the cell cytoplasm also show patchy clathrin-like coats on the outer leaflets of the newly formed double membraned channel structures (see arrow, Fig. 5C).

Ultrastructural SR-BI expression in response to hormonal changes

At the electron microscope level, SR-BI immunogold labeling is essentially limited to the microvillar compartment of adrenocortical cells. In this region, staining is associated with microvilli and microvillar channels. **Figures 8** and **9** indicate that SR-BI expression in the microvillar compartment increases dramatically with ACTH (Figs. 8B, 9) or 17α-E2 treatment (Figs. 8C, 9) when quantified as gold particles/microvillar area [i.e., corrected for the increase in microvillar area and for the number of gold particles/non-microvillar (cytoplasmic) mass].

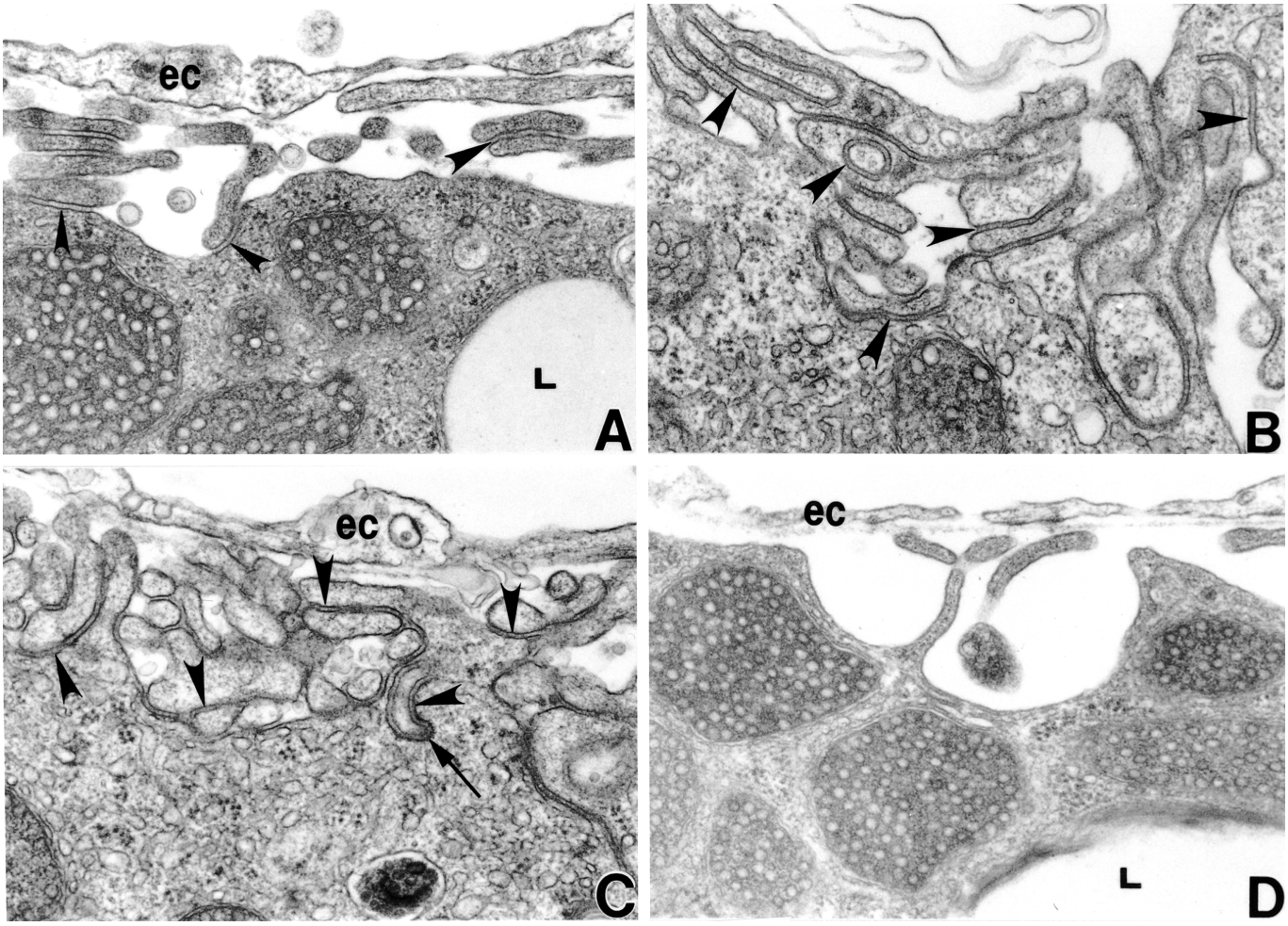

Fig. 5. Ultrastructural changes in the microvillar compartment of z.fasciculata cells from adrenals of control (A), ACTH (B), 17 α-E2 (C), and DEX (D) treated animals. Photos show typical changes in this compartment emphasizing increased microvillar complexity (interdigitating microvilli, increased microvillar mass, and double membrane formation) (arrowheads) with ACTH and 17α-E2 treatment (B, C), and decreased microvillar mass and complexity with DEX treatment (D). ec, endothelial cells; L, lipid. Arrow shows a patchy clathrin-like coat on the outer leaflet of a double membraned channel.

Conversely, after DEX treatment, there is less SR-BI expression than in control cells, as determined by the number of gold particles per corrected unit microvillar area (Figs. 8D, 9).

DISCUSSION

The current study illustrates the exceptional flexibility and responsiveness to hormonal stimuli of a structural compartment of the cell surface of rat adrenocortical cells. In control rats, the entire surface of adrenocortical cells is covered by limp and disorganized appearing microvilli, regardless of whether the exposed cell surface underlies sinusoidal endothelial lining cells or exists on other surfaces in direct contact with adjacent adrenocortical cells. Occasionally these microvilli are upright, and occasionally they lie sideways or are thrust into the cytoplasm of the same cell or adjacent cells forming a double membraned connection with the other cell surface or invaginated cytoplasm. The space found between these double membranes has been termed a microvillar channel (28, 29), and it is in this channel where circulating lipoproteins (HDL, LDL) appear to be trapped in vivo (2, 3, 28, 29), and where even small VLDL (<500 Å in diameter) can be found under certain in vitro conditions (30). The microvillar compartment is also the exclusive site of expression of the HDL receptor SR-BI in rat steroidogenic tissues (13, 15). In contrast, the LDL (B/E) receptor is usually found in separate coated pits and vesicles in these tissues, but is also found in dead-end bulbous single or double membraned structures at the deep end of the microvillar channel if a microvillus is embedded within the cell cytoplasm (3). The number of cell surface microvilli are most abundant in adrenocortical cells of the proximal z. fasciculata of the rat, and it is this region that exhibits the most SR-BI staining at the light microscope level.

Such microvillar compartments expressing SR-BI can be seen in other steroidogenic tissues [e.g., stimulated Leydig cells (15)]. In rat Leydig cells, the double mem-

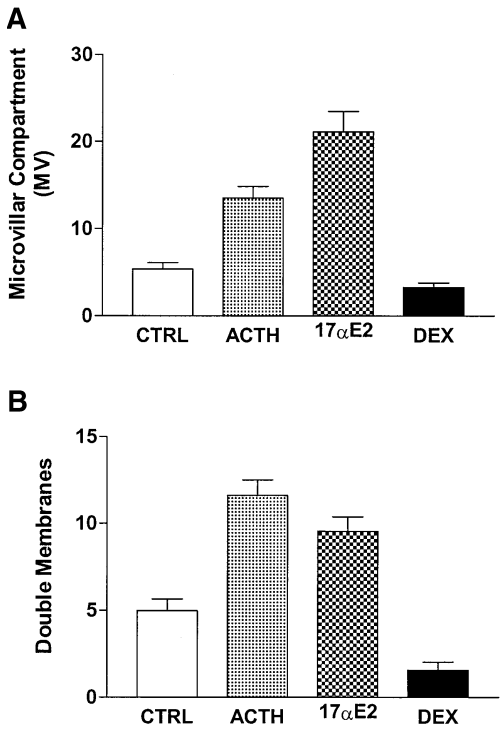

Fig. 6. Quantitation of ultrastructural changes in the microvillar compartment of adrenocortical cells as shown in the previous figure. A: Represents alterations in microvillar area as a result of ACTH, 17α-E2, and DEX treatment. B: Represents changes in microvillar channel (double membrane) formation with the above treatments. In A, microvillar area is expressed as 100 mm^2. In B, the total length of microvillar channels is measured in mm per unit (100 mm^2) microvillar area. As such, values are corrected for treatment-induced changes in microvillar area.

braned channels loop throughout the peripheral cytoplasm of the cells. A similar situation exists in insect ovary (*Sf*9) cells made to express SR-BI (31), and it is this cell system which was taken as a model for the current study. In the insect cell study, it became clear for the first time that non-steroidogenic cells, even a primitive insect cell type, could be instructed to form double membraned channels if the cell was engineered to express SR-BI. Thus, it was not pre-existing microvillar structures, which acquired SR-BI after a stimulus, but the presence of SR-BI itself that led ultimately to the new formation of double membraned structures within the cell. The plan for the current study was to further explore this effect in a physiologically responsive tissue; e.g., to hormonally alter the adrenal cell's need for cholesterol and thus SR-BI levels, and observe microvillar compartmental structural changes which might occur.

When compared with cells of control adrenals, microvillar compartmental changes of hormonally induced cells fall into two categories. In the first category, ACTH or 17α-E2 treatment stimulates SR-BI production as previously observed in adrenals of rats (9, 25) and mice (10). In the current study, ACTH and 17α-E2 treatment also leads to a significant increase in the number of microvilli, the formation of microvillar channels, and the general complexity of the microvillar compartment. In tissues from ACTH treated animals, there is a doubling of SR-BI immunogold particles per unit microvillar area over that observed in control adrenocortical cells. A similarly increased expression of SR-BI occurs also in the microvillar compartment of 17α-E2 treated rats, i.e., a large increment in SR-BI (adjusted for microvillar area) is seen after either treatment. Indeed, the extent of overall quantifiable structural changes of the microvillar compartment during both the ACTH and 17α-E2 treated adrenals is quite remarkable. Microvilli, which in control tissues appear randomly arranged with occasional connections (channels) formed with adjacent microvilli, can in 24 h form a complex surface of interdigitating fingers and loops greatly increasing the total quantifiable length of microvillar channels per microvillar area. What is not yet clear is whether the original cell surface merely rearranges to form microvillar structures (as a way of increasing cell surface), or whether new microvillar mass actually forms. What is certain, however, is that lipoproteins and/or lipoprotein remnants become trapped in the newly formed microvillar channels as seen by intramembrane striations at low magnification, globular appearing structures (presumptive HDL) at high magnifications, and specific immunostaining with antibodies to rat HDL apoA-I receptor proteins. The trapping of rat HDL occurs in adrenals of 17α-E2 treated animals when plasma cholesterol levels are <20% that of control animals and little cholesterol is found in the adrenal. Plasma corticosterone levels in such animals are more than 2× normal, however, suggesting that 17α-E2 treatment stimulates steroidogenesis despite low circulating cholesterol levels, and adrenocortical cells attempt to increase cholesterol uptake by whatever means possible. Indeed, in adrenocortical cells isolated from this tissue, even incubation with exogenous lipoproteins does not downregulate SR-BI expression.

The second category of experiments involves the use of dexamethasone to block endogenous ACTH secretion (via the CRF) and, in turn, to downregulate steroidogenesis in rats. After a 24-h treatment, plasma cholesterol levels and tissue cholesteryl ester levels are normal, but corticosterone secretion is very low (10% of control levels). Western blots indicate that adrenal SR-BI expression is also low, and following this trend, tissue expression of SR-BI at both light and ultrastructural levels is dramatically reduced. In the microvillar compartment of dexamethasone treated cells, microvillar area-corrected estimates of immunogold particles per unit microvillar area is ~20% of control levels, and only 10% of ACTH-induced levels. Most surprising is the rapidity by which the microvillar compartment of dexamethasone-treated adrenocortical cells is altered. By 24 h, total microvillar area is decreased by more than 50%, and channel length is only 25% of control levels. Indeed, the adrenocortical cell surface of dexamethasone treated rats barely resembles the cell surface of control or stimulated animals; the compartment is uncomplicated, microvilli are scarce, and double membraned channel structures are essentially gone. The rapid restructuring of adrenocortical cell surface in response to

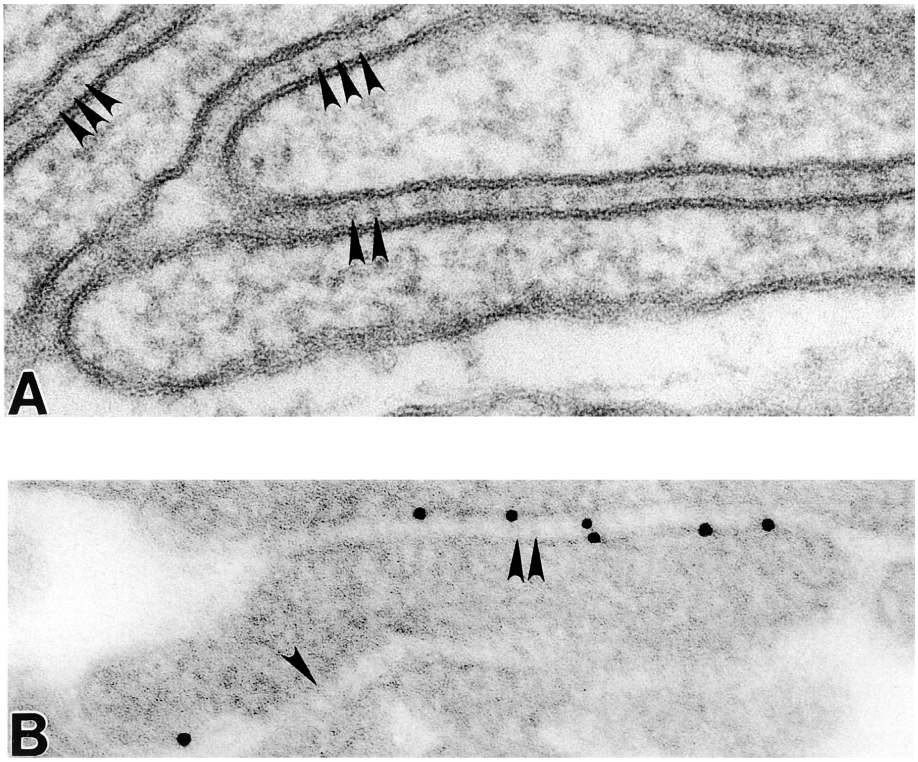

Fig. 7. High magnification view of typical double membraned channels observed in adrenal microvillar compartment of ACTH treated animals. A: Shows microvillar channels are filled with globular appearing structures (arrowheads) presumed to be exogenously acquired rat HDL. B: Shows tissue from the same rat fixed for ultrastructural immunochemistry to identify exogenous rHDL using rat antibodies to apoA-I. The immunogold particles indicate rHDL apoA-I association with the channels, and the poorly defined striations within the channels (see arrowheads) suggest the localization of the rHDL particles themselves.

hormonal signals and/or the need for exogenously supplied cholesterol has not previously been demonstrated in vivo. In the current study it seems likely that the regulating signal described in all the protocols is ACTH dependent, i.e., changing levels of adreno-corticotrophic hormone, whether delivered directly in response to stress or need, or indirectly through 17α-E2 induction (9), or down-regulated by the use of a synthetic corticosteroid such as dexamethasone has the capacity to define SR-BI levels and simultaneously re-configure the structural carriers of SR-BI for maximal efficiency. In steroidogenic tissues, it seems likely that an increased or decreased microvillar surface allows for changing numbers of binding sites for SR-BI-captured cholesterol-rich lipoproteins, while microvillar channel formations are needed to trap lipoproteins for substantial periods of time, and possibly for membrane domain (raft-like) specialization associated with cholesterol/phospholipid/protein changes.

In the previously described insect cell model system (31), it seems clear that the introduction of SR-BI into the system is the factor responsible for the creation of a complicated network of double membraned channels within the cells. We suggest that the same sequence of events may apply in the current experiments carried out in a physiologically intact mammalian system. In this case, hormone-driven alterations of SR-BI expression in adrenocortical cell microvillar plasma membranes may be ultimately responsible for changing the configuration of those membranes. Precisely how this occurs is not yet known, although Williams et al. (40) and more recently Silver and Tall (41) have suggested that SR-BI may alter the composition of lipid domains of plasma membranes facilitating free cholesterol flux, altered membrane cholesterol content, and associated changes in membrane physical/chemical properties. Such altered plasma membrane properties may permit architectural changes in microvilli compatible with the changing functional needs of the tissue. In addition, hormonal activation/de-activation of SR-BI dimerization may be another critical event in this process. In Western blots of the current study, we show that adrenal SR-BI exists primarily in the monomeric form with some (minor) dimer formation in control animals, but that dimerization of SR-BI increases dramatically after ACTH treatment or after 17α-E2 treatment. In contrast, dexamethasone treatment results in barely detectable dimeric SR-BI expression. These results suggest that dimerization of SR-BI is a function of direct or indirect ACTH action, and that SR-BI dimers are, in some way, associated with the structural/functional changes attributed to ACTH levels in the adrenal. It is now well established that a variety of cell surface receptors interact with each other, or other receptors to form homo-, or hetero-

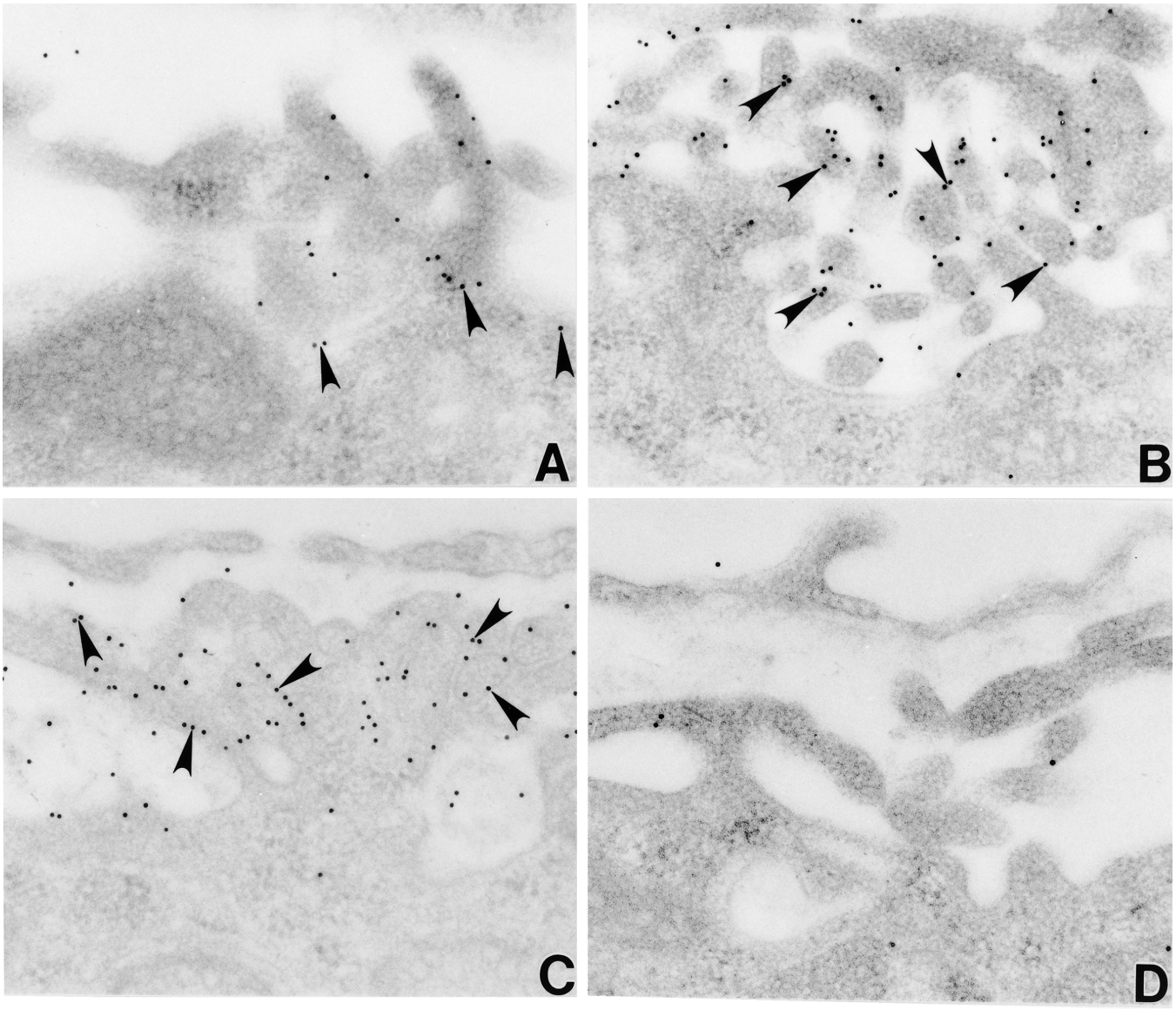

Fig. 8. Ultrastructural immunocytochemical localization of SR-BI in the microvillar compartment of adrenocortical cells of control animals (A) or animals treated with ACTH (B), 17α-E2 (C), or DEX (D). SR-BI immunogold labeling (arrowheads) shows large differences in SR-BI expression depending on hormonal treatment.

dimers, and that this event is often essential for receptor signaling (42–51). We suggest that in the rat adrenal microvillar compartment stimulated with ACTH or 17α-E2 treatment, SR-BI monomers on adjacent microvilli may interact with each other (or other plasma membrane outer leaflet sites) forming the tight membrane connections or zipper effect. It is these tight membrane connections that we recognize as microvillar channels, the site of lipoprotein trapping and selective cholesteryl ester uptake. Dexamethasone treated tissues, on the other hand, do not produce SR-BI dimers, do not form microvillar channels, and do not function successfully in lipoprotein trapping or selective cholesteryl ester uptake. Whether other proteins are also involved as constituents in this process remains to be seen.

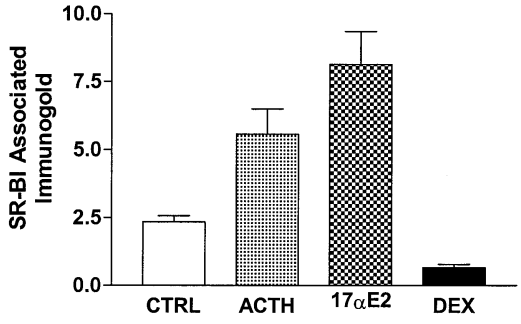

Fig. 9. Quantitative data derived from microvillar SR-BI immunocytochemistry seen in Fig. 8. The figure shows the number of SR-BI associated gold particles per unit area (100 mm^2) microvillar compartment. As such, values are corrected for changes in microvillar area, which occur as a result of hormonal treatment.

In summary, in vivo hormonal manipulation of a steroidogenic tissue, the adrenal cortex, leads rapidly to changes in the expression of the HDL receptor protein SR-BI to extensive ultrastructural changes in the adrenocortical cell microvillar compartment involving microvillar channel formation and, consequently, to changes in the ability of the cells to function in selective cholesteryl ester uptake.

ADDENDUM

During the review of this manuscript, it came to our attention that a complementary study of the adrenal microvillar compartment using the SR-BI knockout mouse model (Williams et al.) was in press (52). Data documenting the disorganization and disappearance of adrenal microvillar channels in such SR-BI null mice are remarkably similar to the adrenal data presented here with the use of dexamethasone in an otherwise normal rat. Together, the studies add heightened confidence in the importance of SR-BI to the microvillar compartment and to the selective cholesteryl ester uptake process.

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