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# Angiotensin II Promotes Selective Uptake of High Density Lipoprotein Cholesterol Esters in Bovine Adrenal Glomerulosa and Human Adrenocortical Carcinoma Cells Through Induction of Scavenger Receptor Class B Type I

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Angiotensin II is one of the main physiological regulators of aldosterone biosynthesis in the zona glomerulosa of the adrenal cortex. The hormone stimulates intracellular cholesterol mobilization to the mitochondrion for steroid biosynthesis. Here we have examined whether angiotensin II also modulates exogenous lipoprotein cholesterol ester supply to the steroidogenic machinery and whether this control is exerted on the selective transport of high density lipoprotein-derived cholesterol ester to intracellular lipid droplets through the scavenger receptor class B type I. In bovine adrenal glomerulosa and human NCI H295R adrenocortical carcinoma cells, high density lipoprotein stimulated steroid production. Angiotensin II pretreatment for 24 h potentiated this response. Fluorescence microscopy of cellular uptake of reconstituted high density lipoprotein containing a fluorescent cholesterol ester revealed an initial, time-dependent narrow labeling of the cell membrane followed by an intense accumulation of the fluorescent cholesterol ester within lipid droplets. At all time points, labeling was more pronounced in cells that had been treated for 24 h with angiotensin II. Fluorescence incorporation into cells was prevented by a mono-

clonal antibody directed against apolipoprotein A-I. Upon quantitative fluorometric determination, cholesterol ester uptake in angiotensin II-treated bovine cells was increased to  $175 \pm 15\%$  of controls after 2 h and to  $260 \pm 10\%$  after 4 h of exposure to fluorescent high density lipoprotein. The amount of scavenger receptor class B type I protein detected in cells treated with angiotensin II for 24 h reached  $203 \pm 12\%$  of that measured in control cells ( $n = 3$ ,  $P < 0.01$ ). In contrast, low density lipoprotein receptors were only minimally affected by angiotensin II treatment. This increase in scavenger receptor class B type I protein was associated with a 3-fold induction of scavenger receptor class B type I mRNA, which could be prevented by actinomycin D but not by cycloheximide. Similar results were obtained in the human adenocarcinoma cell line H295R. These observations show that angiotensin II regulates the scavenger receptor class B type I-mediated selective transport of lipoprotein cholesterol ester across the cell membrane as a major source of precursor for mineralocorticoid biosynthesis in both human and bovine adrenal cells. (*Endocrinology* 142: 4540–4549, 2001)

PLASMA LOW AND high density lipoproteins are the major source of cholesterol for most steroid hormone-producing tissues (1). The uptake of low density lipoprotein (LDL)-derived cholesterol esters (CE) involves binding of the LDL particles to specific cell surface receptors followed by internalization of the lipoprotein-receptor complex and lysosomal hydrolysis leading to the release of CE (2). In contrast, the delivery of CE from high density lipoproteins (HDL) takes place through a distinct mechanism termed the "selective pathway," and it involves binding of HDL to the cell surface followed by delivery of CE into the cells without internalization and degradation of the lipoprotein particle (3, 4). The lipid-poor HDL then dissociate from the cells and

reenter the circulation. In adrenocortical cells, lipoprotein-derived CE are stored within lipid droplets. After acute hormonal stimulation, these CE are hydrolyzed to free cholesterol by the cytosolic cholesterol ester hydrolase (5). Cholesterol is then transported to the mitochondrion for its conversion to cortisol and aldosterone.

Until a few years ago, a key missing element in the study of HDL metabolism was a well defined HDL receptor. Recent studies have identified a physiologically relevant membrane receptor for HDL that mediates cholesterol uptake through a selective pathway (6–8). This receptor has been termed scavenger receptor class B type I (SR-BI). SR-BI is believed to bind to the  $\alpha$  helical repeats of apolipoprotein A-I (ApoA-I) (7). It has been suggested that this interaction leads to the formation of a nonaqueous channel through which CE move down their concentration gradient to the plasma membrane (7). Because SR-BI has been shown to bind not only HDL but also LDL and modified LDL, it is likely that this receptor may also affect LDL metabolism *in vivo* (9–12). Tissue distribution studies revealed that SR-BI is expressed in the liver, where

Abbreviations: AngII, Angiotensin II; ApoA-I, apolipoprotein A-I; BODIPY FL C<sub>12</sub>, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-S-indacene-3-dodecanoate; CE, cholesterol ester; h, human; HDL, high density lipoprotein; HDL<sub>3</sub>, apolipoprotein E-poor high density lipoproteins; LDL, low density lipoprotein; rec-hHDL, cholesteryl BODIPY-high density lipoprotein; SR-BI, scavenger receptor class B type I; SSC, sodium chloride/sodium citrate buffer.

it contributes to the clearance of plasma CE in reverse cholesterol transport (4), and in steroidogenic tissues, where it is particularly abundant in the adrenal gland (13). This expression is induced by trophic hormones such as human CG (13), ACTH (14, 15), and LH (13). Also, HDL have been shown to be significantly more effective than LDL in supplying cholesterol for corticosterone production in adrenal glands of the rat and mouse (16, 17). HDL has also been reported to be a source of cholesterol for steroidogenesis in human ovarian cells (18). However, the potential role of HDL in mineralocorticoid biosynthesis and its regulation by hormones of the cardiovascular system, particularly in the human adrenal gland, have not been addressed adequately.

The aim of the present studies was to examine the ability of bovine adrenal glomerulosa cells and human NCI H295R adrenocortical cells to selectively take up CE from HDL when challenged with angiotensin II (AngII). In addition, we reasoned that if SR-BI is a physiologically relevant receptor for HDL-derived CE uptake and provides cholesterol for steroidogenesis, its expression might be the target of the same stimuli that enhance cholesterol uptake and aldosterone production. We provide here evidence that this is indeed the case: bovine adrenal glomerulosa cells and human NCI H295R cells efficiently internalize HDL-derived CE for aldosterone biosynthesis, both in the resting state and under AngII challenge. We also show that SR-BI is expressed in both cell models and that the increase in HDL-derived CE uptake observed upon AngII stimulation is accompanied by an increase in SR-BI protein and mRNA levels, demonstrating a coordinated regulation of SR-BI expression and of the intracellular steps leading to adrenal aldosterone biosynthesis.

## Materials and Methods

### Materials

[Ile<sup>5</sup>]AngII was purchased from Bachem (Bubendorf, Switzerland). Phosphatidylcholine, sphingomyelin, unesterified cholesterol, and triolein were obtained from Sigma (St. Louis, MO). Cholesteryl BODIPY FL C<sub>12</sub> (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-S-indacene-3-dodecanoate) was purchased from Molecular Probes, Inc. (Eugene, OR). Anti-SR-BI polyclonal antibody and SR-BI cDNA were generated by one of us (19). Antihuman LDL receptor was supplied by Research Diagnostic Inc. (Flanders, NJ). Antipregnenolone antiserum was purchased from Biogenesis Ltd. (Poole, UK). Antihuman ApoA-I monoclonal antibody was obtained after propagation of the hybridoma cell line H135D3 (ATCC, Manassas, VA). Hybond-N<sup>+</sup> membranes, rapid hybridization buffer, and the Rediprime random primer labeling kit were supplied by Amersham Pharmacia Biotech (Zurich, Switzerland).

### Methods

**Bovine adrenal zona glomerulosa cell preparation.** Bovine adrenal glands were obtained from a local slaughterhouse. Primary cultures of glomerulosa cells were prepared by enzymatic dispersion with dispase and purified on Percoll density gradients as described elsewhere (20). Cells were kept in serum-free medium in the absence or the presence of AngII (10 nM) for 24 h before the experiments, which were performed on the 4th day of culture.

**Human adrenocortical carcinoma cell culture (NCI H295R).** NCI H295R cells were kindly provided by Dr. W.E. Rainey (University of Texas Southwestern Medical Center, Dallas, TX) and maintained in a 1:1 mixture of DMEM and Ham's F12 medium containing pyridoxine, L-glutamine, and 15 mM HEPES (Life Technologies, Inc., Basel, Switzerland). The culture medium was supplemented with insulin, transferrin, selenium (ITS<sup>+</sup>, Becton Dickinson and Co. Labware, Bedford, MA), and Ultrosor

(Ligacon, Tagelswangen, Switzerland) as well as with antibiotics as described elsewhere (21).

**Human lipoprotein preparation.** LDL (density = 1.006–1.063 g/ml) and apolipoprotein E-poor HDL (HDL<sub>3</sub>; density = 1.125–1.21 g/ml) were isolated from human plasma by sequential ultracentrifugation as described previously (22). We used exclusively HDL<sub>3</sub> to avoid lipoprotein-derived CE uptake via the classic LDL-receptor pathway. Cholesteryl BODIPY-human HDL (rec-hHDL) were reconstituted as described previously (23). In brief, a mixture of egg phosphatidylcholine (2.3 μmol), sphingomyelin (0.6 μmol), fluorescent cholesteryl BODIPY FL C<sub>12</sub> as CE (2.24 μmol), unesterified cholesterol (0.6 μmol), and triolein (0.34 μmol) was dissolved in chloroform and dried under N<sub>2</sub>, then resuspended in a 10 mM Tris-HCl buffer (pH 8) containing 150 mM NaCl, 0.25 mM EDTA, and sonicated at 52°C using a Labsonic L sonicator (B. Braun, Melsungen, Germany) equipped with a microtip at a power setting of 30 W. After sonication for 40 min, the temperature was decreased to 42°C and 5 mg of delipidated hHDL<sub>3</sub> apolipoprotein in 2 ml of 2.5 M urea was added dropwise. Sonication was continued for an additional 10 min. The sonicated sample was centrifuged for 20 min at 4000 × g and dialyzed against PBS for 6 h at 4°C. Finally, the reconstituted cholesteryl BODIPY-human HDL<sub>3</sub> particles were isolated sequentially by ultracentrifugation (16 h, 150,000 × g at 20°C, then 25 h, 150 × g at 20°C, d = 1.21 g/ml) and dialyzed against PBS for 24 h at 4°C. Immediately before use, the preparation was dialyzed against serum-free culture medium, and the protein concentration of rec-hHDL<sub>3</sub> was determined using the Bio-Rad Laboratories, Inc. AG reagent (Glattbrugg, Switzerland) and BSA as a standard. BODIPY-CE are substrates for acid CE hydrolase (lysosomal) but are not hydrolyzed by the neutral CE hydrolase (cytosolic) (23). Consequently, intracellular fluorescence is considered to be caused by intact CE internalized through a nonendocytic pathway (*i.e.* a selective pathway).

**Fluorescence microscopy.** For the study of the uptake and accumulation of rec-hHDL-derived CE, glomerulosa cells were grown on round (25 mm diameter) glass coverslips (0.35 × 10<sup>6</sup> cells/coverslip) and pretreated with or without AngII (10 nM) for 24 h before incubation with rec-hHDL (30 μg/ml) for periods varying from 5 min to 4 h. After incubation, each coverslip was washed four to five times in Krebs buffer and subsequently immersed in a thermostatic chamber (Harvard Apparatus, Holliston, MA). Cells were immediately imaged on an Axiovert S100TV microscope equipped for epifluorescence microscopy using a 100× (or 40×) oil-immersion objective (numerical aperture, 1.3) (Carl Zeiss, Feldbach, Switzerland), 488 ± 10 nm excitation (DeltaRam, Photon Technology International, Inc., Monmouth Junction, NJ), a 505DRLP dichroic mirror and a 535RDF40 emission filter (Omega Optical, Brattleboro, VT). Fluorescence emission from the rec-hHDL was captured using a cooled back-illuminated 16-bit charge-coupled device frame transfer camera (Princeton Instruments, Roper Scientific, Trenton, NJ). All of the equipment was controlled for image acquisition and analysis with MetaMorph/Metafluor 4.1.2 software (Universal Imaging, West Chester, PA). The fluorescent images were processed with MetaMorph in black and white or in pseudocolors.

**Fluorescence quantification.** Glomerulosa cells were grown on six-well plates (2 × 10<sup>6</sup> cells/well). After starvation, cells were pretreated with or without AngII (10 nM) for 24 h before incubation with rec-hHDL (30 μg/ml) for periods varying from 5 min to 4 h. After incubation, cells were washed five times in cold PBS containing 0.1% BSA, and then lipids were extracted with hexane-isopropanol (3:2, vol/vol) as described previously (24). Each sample was dried under N<sub>2</sub> and then reconstituted with hexane/isopropanol before being transferred to a glass cuvette. Fluorescence was measured at excitation and emission wavelengths 503 and 540 nm, respectively, using a Jasco CAF-110 spectrofluorometer (Jasco Corporation, Tokyo, Japan).

**Effect of anti-ApoA-I antibody on rec-hHDL uptake.** To test the ability of the anti-ApoA-I antibody to prevent SR-BI-mediated CE uptake, rec-hHDL (30 μg/ml) were preincubated with a monoclonal anti-ApoA-I antibody (120 μg/ml; a generous gift from Dr. Jean-Michel Dayer, Division of Immunology and Allergology, University Hospital, Geneva, Switzerland) for 30 min at 4°C before being added to bovine glomerulosa cells for 60 min. Fluorescence microscopy and quantification of rec-hHDL uptake were then performed as described above.

**Steroid measurement.** For steroid production, adrenal glomerulosa cells were grown on 24-well plates ( $0.5 \times 10^6$  cells/well) and preincubated in the absence or the presence of 10 nM AngII for 24 h. Subsequently, cells were exposed or not to AngII and to increasing concentrations of hHDL<sub>3</sub> (125 to 500  $\mu$ g protein/ml) or hLDL (25 to 100  $\mu$ g protein/ml) for 3 h in serum-free medium. Equivalent masses of CE available to cells in the supplied hHDL<sub>3</sub> or hLDL were calculated on the basis of a 5:1 ratio. Pregnenolone was measured by RIA using a commercial antiserum. WIN 19758 (5  $\mu$ M), an inhibitor of  $3\beta$ -hydroxysteroid dehydrogenase, was included in the incubation medium to prevent further metabolism of pregnenolone into progesterone. The aldosterone content of the incubation medium was measured by direct RIA using a commercially available kit (DSL Inc., Webster, TX). Pregnenolone and aldosterone production were normalized to milligram cellular proteins.

**SDS-PAGE analysis and immunoblotting.** SDS-PAGE was performed according to Laemmli (25). Aliquots from either bovine glomerulosa or NCI H295R cell lysates were resolved by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore Corp., Volketswil, Switzerland) that was incubated in blocking buffer (PBS, 0.2% Tween 20, 5% nonfat dry milk) for 2 h and then exposed either to rabbit polyclonal anti-SR-BI antibodies (1:1000 dilution) at 4 C for 2 h or to antihuman LDL receptor antibodies (1:250 dilution) at 4 C overnight. The membrane was thoroughly washed with PBS/Tween buffer ( $3 \times 10$  min) and then incubated for 1 h with horseradish peroxidase-labeled goat antirabbit IgG (CovalAb, Oullins, France). The polyvinylidene fluoride sheet was then washed ( $4 \times 15$  min), and the antigen-antibody complex was revealed by enhanced chemiluminescence using the Western blotting detection kit from Amersham Pharmacia Biotech and Kodak Biomax film (Rochester, NY).

**RNA isolation and Northern blot analysis.** Glomerulosa cell total RNA was extracted using the RNeasy kit (Promega Corp., Zurich, Switzerland) according to the instructions of the manufacturer. This system consistently yields 50–80  $\mu$ g of total RNA per  $10^7$  cells. For Northern blot analysis, 20–30  $\mu$ g of RNA was size fractionated on a 1% formaldehyde agarose gel, vacuum transferred onto a Hybond-N<sup>+</sup> membrane, and fixed by UV cross-linking. The integrity of the 18s and 28s RNA was checked by ethidium bromide staining of the gel. Hybridization was performed using rat SR-BI cDNA (1.6 kb) generated by RT-PCR of RNA isolated from cAMP-treated rat granulosa cells (19). The cDNA was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using the Rediprime random primer labeling kit. Northern blots were prehybridized in Rapid Hybridization Buffer at 65 C for 30 min. The  $\alpha$ -<sup>32</sup>P-labeled probe (specific activity,  $2 \times 10^6$  cpm/ng DNA) was then added, and the incubation was continued for 2 h at 65 C. Blots were washed for 5 and 15 min successively at room temperature in  $2\times$  sodium chloride/sodium citrate buffer (SSC), 0.1% SDS, then for 15 min in  $1\times$  SSC, 0.1% SDS. The final wash was performed at 65 C for 15 min in  $1\times$  SSC, 0.1% SDS. RNA-cDNA hybrids were visualized on Kodak Biomax film after a 12- to 24-h exposure period. Blots were stripped and reprobed with mouse glyceraldehyde-3-phosphate dehydrogenase cDNA (Ambion, Inc. Lugano, Switzerland) to assess RNA loading.

**Analysis of data.** Results are expressed as means  $\pm$  SEM. The mean values were compared by ANOVA using Fisher's test or two-way ANOVA when appropriate.  $P < 0.05$  was considered as statistically significant. Quantitation of autoradiograms was performed using a Molecular Dynamics, Inc. (Sunnyvale, CA) Computing Densitometer and ImageQuant software.

## Results

### Steroidogenic response of bovine glomerulosa cells and NCI H295R cells to lipoproteins

To examine whether bovine adrenal glomerulosa cells can use HDL-derived CE for steroid production, we incubated cells that had been pretreated with or without 10 nM AngII for 24 h with increasing concentrations of hHDL<sub>3</sub> for 3 h, and the production of pregnenolone and aldosterone was measured in the incubation medium. As shown in Fig. 1A, non-stimulated cells responded to hHDL<sub>3</sub> with a concentration-

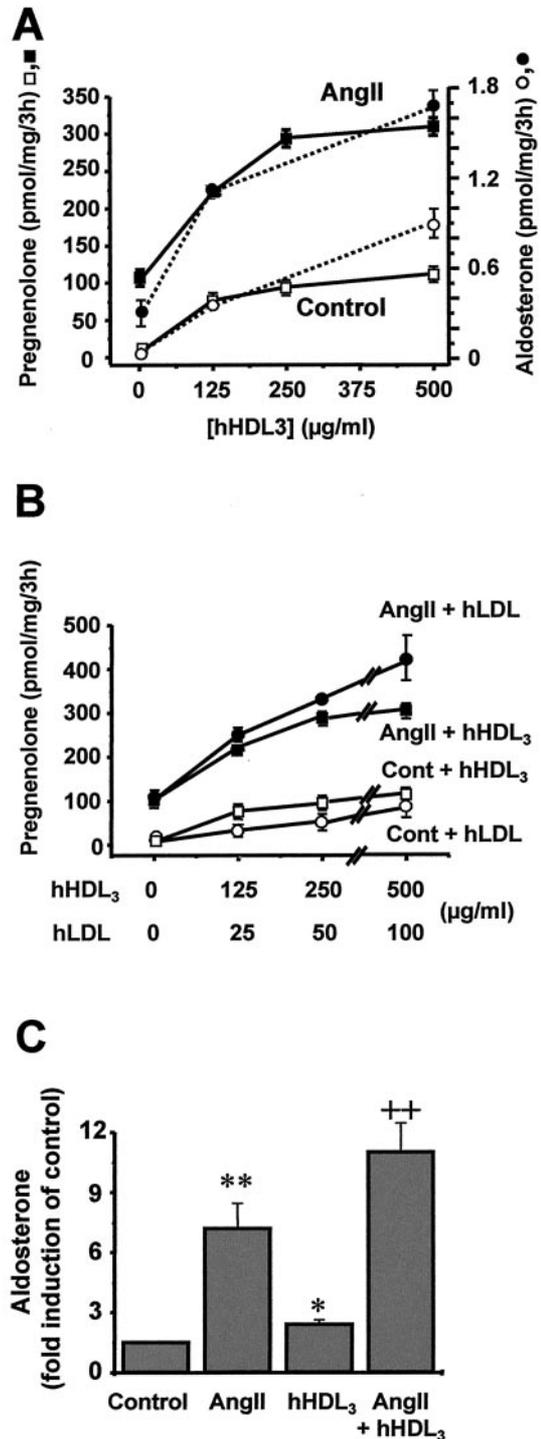


FIG. 1. Effect of lipoproteins on basal and AngII-stimulated steroidogenesis in bovine adrenal glomerulosa cells and NCI H295R cells. Glomerulosa cells and NCI H295R cells were cultured as described in *Materials and Methods*. A and B, Effect of hHDL<sub>3</sub> and hLDL on pregnenolone (squares) and aldosterone (circles) production in control (open symbols) and AngII-treated (closed symbols) cells. C, Effect of hHDL<sub>3</sub> (250  $\mu$ g/ml) on aldosterone production in NCI H295R cells treated or not with 10 nM AngII. The scales for hHDL and hLDL concentrations are adjusted to reflect equivalent amounts of CE. The results represent the mean  $\pm$  SEM of three to four and six independent experiments for bovine and human cells, respectively. \*\*\*, Significantly different from control,  $P < 0.05$  and  $0.01$ , respectively; ++, significantly different from AngII,  $P < 0.01$ .

dependent increase in pregnenolone production, which reached approximately 15-fold the basal values. Similarly, aldosterone production was stimulated 16-fold at 500  $\mu\text{g}/\text{ml}$  hHDL<sub>3</sub>. This suggested that HDL can deliver substantial amounts of cholesterol esters to glomerulosa cells to support mineralocorticoid synthesis. Furthermore, AngII-pretreated cells showed a concentration-dependent increase in pregnenolone and aldosterone formation that was approximately 3- and 6-fold greater than that observed in nonpretreated cells, respectively (Fig. 1A). We then compared the steroidogenic response of glomerulosa cells supplied with hHDL<sub>3</sub> to that of cells exposed to hLDL. As shown in Fig. 1B, no significant differences were observed in the steroidogenic response to both lipoprotein species, either in the basal state or after AngII pretreatment, a result suggesting that hHDL<sub>3</sub> and hLDL were equally effective.

We next examined the effect of hHDL<sub>3</sub> (250  $\mu\text{g}/\text{ml}$ ) on aldosterone biosynthesis in control and AngII-stimulated human NCI H295R cells during a 24-h period of incubation (Fig. 1C). hHDL<sub>3</sub> enhanced aldosterone production by 1.6-fold in both control and AngII-stimulated cells ( $n = 6$ ).

#### *Uptake and intracellular accumulation of fluorescent CE in bovine glomerulosa cells and human NCI H295R cells*

We next examined the uptake and storage of HDL<sub>3</sub>-derived CE within these cells, using hHDL<sub>3</sub> reconstituted with the nonhydrolyzable fluorescent CE, rec-hHDL<sub>3</sub>. Figure 2 shows fluorescence images of time-dependent CE uptake in intact bovine adrenal glomerulosa cells that had been pretreated in the absence (panels b–d) or in the presence (panels f–h) of AngII for 24 h. In the basal state and in the absence of rec-HDL<sub>3</sub> (Fig. 2, a and e), glomerulosa cells display a faint fluorescence, presumably attributable to intrinsic lipid droplets. In control cells, a narrow labeling of the plasma membrane appeared within 30–60 min of exposure to rec-HDL<sub>3</sub>. By 1–2 h of exposure to rec-HDL<sub>3</sub>, control cells had accumulated massive amounts of fluorescent CE within lipid droplets and increased membrane, cytosolic, and perinuclear signals were observed. Importantly, at all time points, the labeling in AngII-pretreated cells was more intense compared with that in the corresponding control cells (panels f–h *vs.* b–d). In Fig. 2i, AngII-pretreated glomerulosa cells are shown at a higher magnification after 4 h of exposure to rec-HDL<sub>3</sub>. The numerous highly fluorescent spots correspond in size, location, and number to lipid droplets, as confirmed by a Nomarski image of the same cells (Fig. 2j).

Images from similar experiments conducted in human NCI H295R cells are presented in Fig. 2, panels k to r. These cells internalized substantial amounts of hHDL<sub>3</sub>-derived CE in the basal state in a time-dependent manner (Fig. 2, l–n), and this process was markedly enhanced after a 24-h pretreatment with AngII (Fig. 2, p–r).

The major apolipoprotein component of HDL<sub>3</sub> is ApoA-I. Recent studies in ApoA-I knockout mice suggested that ApoA-I may play a crucial role in the delivery of HDL cholesterol to steroidogenic tissues (26). We preincubated rec-HDL<sub>3</sub> with a monoclonal antibody against ApoA-I before adding it to glomerulosa cells for 60 min. Fluorescence mi-

croscopy analysis revealed that HDL<sub>3</sub>-derived CE uptake was markedly reduced in control cells in the presence of the anti-ApoA-I antibody (Fig. 2, s and t). Similar results were obtained in AngII-pretreated cells (Fig. 2, u and v), suggesting that a crucial event in HDL<sub>3</sub>-derived CE uptake is mediated by ApoA-I.

To further substantiate these qualitative results, we next quantified the amounts of CE taken up by glomerulosa cells using fluorometry. As shown in Fig. 3, a time-dependent increase of CE uptake was observed in control cells. In agreement with the fluorescence imaging data, AngII pretreatment significantly increased CE uptake, reaching  $175 \pm 15\%$  of controls after 2 h of exposure to rec-HDL (n = 3,  $P < 0.01$ ) and  $271 \pm 8\%$  after 4 h (n = 3; data not shown). Qualitatively similar results were obtained in human NCI H295R cells: AngII pretreatment enhanced CE uptake to  $165 \pm 8\%$  of controls (n = 3,  $P < 0.01$ ) after a 4-h exposure to rec-hHDL<sub>3</sub>. When rec-hHDL<sub>3</sub> were first incubated with the anti-ApoA-I antibody, fluorometric measurements revealed that CE uptake was reduced by  $41 \pm 3\%$  (n = 3,  $P < 0.05$  *vs.* controls with no antiserum) in both control and AngII-pretreated bovine glomerulosa cells. In contrast, when we used a non-relevant monoclonal antibody directed against apolipoprotein E instead of the anti-ApoA-I antibody, AngII-induced BODIPY-CE uptake was not significantly affected ( $86 \pm 6\%$  of controls with no antibody, n = 3, NS).

#### *Effect of AngII on SR-BI protein expression*

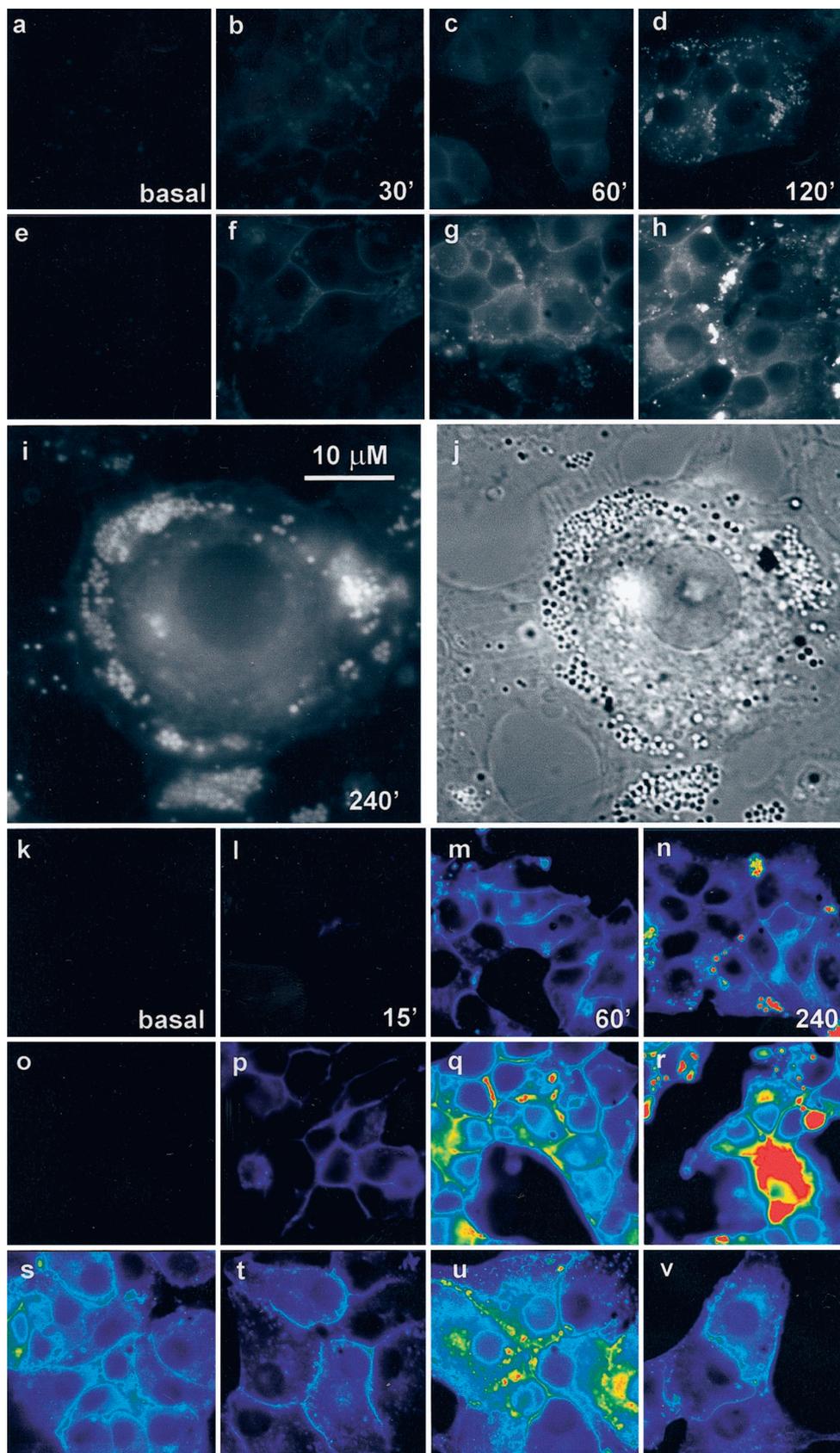
We next examined whether the expression of the HDL receptor SR-BI was modulated by AngII. The Western blots shown in Fig. 4A demonstrate the kinetics of expression of SR-BI protein in bovine glomerulosa cells incubated in the absence (basal) or in the presence of AngII. An immunoreactive band of approximately 86 kDa, a molecular mass similar to that described for murine SR-BI (6), was detected. In control cells, steady state levels of SR-BI expression were maintained for at least 24 h. AngII treatment caused a time-dependent increase in SR-BI, reaching  $203 \pm 12\%$  of control values (n = 4) after 24 h of stimulation (Fig. 4B). This effect of AngII was concentration dependent between  $10^{-10}$  and  $10^{-8}$  M (Fig. 4C).

To examine the specificity of the effect of AngII on SR-BI expression, we compared SR-BI and LDL receptor expression in human NCI H295R cells after AngII treatment. Western blot analysis revealed that both receptors were coexpressed in this cell line (Fig. 5). AngII increased SR-BI protein expression by  $230 \pm 15\%$  of control values after 24 h (Fig. 5A; n = 3,  $P < 0.001$ ), whereas LDL receptor expression was only modestly affected by AngII ( $122 \pm 3\%$  of control values; n = 3, NS; Fig. 5B). In contrast, stimulation of NCI H295R cells with dibutyryl cAMP for 24 h resulted in a more pronounced induction of LDL receptor expression ( $180 \pm 4\%$  of control values; n = 3,  $P < 0.001$ ; Fig. 5B).

#### *Effect of AngII on SR-BI mRNA steady state levels*

To determine whether the AngII-induced increase in SR-BI protein expression reflects changes in SR-BI mRNA levels, total RNA was analyzed by Northern blotting. Fig. 6A shows that a transcript of 2.8 kb was detected. Densitometric anal-

FIG. 2. Uptake and intracellular accumulation of rec-hHDL<sub>3</sub>-BODIPY-CE in bovine adrenal glomerulosa cells and human NCI H295R cells. a and e, Intrinsic fluorescence of bovine glomerulosa cells pretreated in the absence (a, control) or in the presence (e) of AngII for 24 h. Control (b–d) and AngII-pretreated cells (f–h) were exposed for 30, 60, and 120 min to rec-hHDL<sub>3</sub> and analyzed by fluorescence microscopy as described in *Materials and Methods*. Note the narrow labeling of the plasma membrane at early times and the large lipid aggregates appearing after long periods of exposure to fluorescent HDL<sub>3</sub>. i, AngII-pretreated glomerulosa cells incubated for 4 h with rec-hHDL<sub>3</sub>, at a higher magnification. j, Nomarski image of the same cell. k–r, rec-hHDL<sub>3</sub> uptake in human NCI H295R cells. The fluorescent images were processed in pseudocolor (black-purple, low level fluorescence; green-yellow, medium level fluorescence; red, high level fluorescence). k and o, Intrinsic fluorescence of NCI H295R cells incubated in the absence (k, control) or in the presence (o) of AngII for 24 h. Control (l–n) and AngII-pretreated cells (p–r) were exposed for 15, 60, and 240 min to rec-hHDL<sub>3</sub>. s–v, The effect of the anti-ApoA-I antibody on rec-hHDL<sub>3</sub> uptake in bovine glomerulosa cells. rec-hHDL<sub>3</sub> were preincubated with or without the antibody before being added to control cells (s and t) or AngII-pretreated cells (u and v).



### HDL<sub>3</sub>-Bodipy-CE uptake (% of control at 120 min)

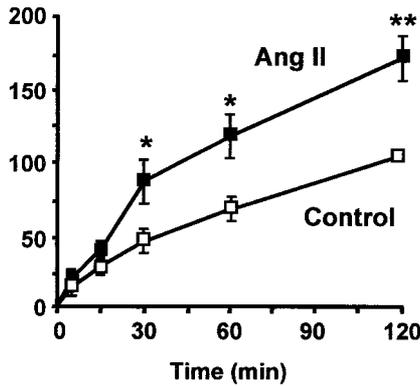


FIG. 3. Fluorometric quantification of rec-hHDL<sub>3</sub>-BODIPY-CE uptake in bovine adrenal glomerulosa cells. Control and AngII-pretreated cells (24 h) were exposed for 5, 15, 30, 60, and 120 min to rec-hHDL<sub>3</sub>. BODIPY-CE were extracted and analyzed fluorometrically as described in *Materials and Methods*. Values shown represent the mean  $\pm$  SEM of BODIPY-CE fluorescence, expressed as a percentage of the fluorescence recorded in control cells after a 120-min exposure to rec-hHDL<sub>3</sub>-BODIPY-CE in three independent experiments. \* and \*\*, Significantly different from control,  $P < 0.05$  and  $P < 0.01$ , respectively.

ysis indicates that AngII induced a 3-fold increase in SR-BI mRNA levels compared with control values ( $n = 4$ ; Fig. 6B). Cycloheximide did not affect the AngII-elicited increase in SR-BI mRNA, suggesting that transactivating factors are already present within the cell. However, SR-BI mRNA induction by AngII was abolished by actinomycin D, suggesting a transcriptional control of SR-BI expression by AngII. When glomerulosa cells were incubated for 24 h in the presence of hHDL<sub>3</sub>, a significant increase ( $152 \pm 4\%$ ;  $P < 0.05$ ) in SR-BI mRNA was observed. Finally, after a combined treatment with both AngII and hHDL<sub>3</sub>, the transcriptional effect of AngII on SR-BI mRNA levels was maintained. Very similar results were obtained in human NCI H295R cells. SR-BI mRNA levels were increased to  $188 \pm 13\%$  over controls after a 24-h exposure to AngII ( $n = 3$ ,  $P < 0.01$ ). This increase was not significantly altered by cycloheximide but was completely prevented by actinomycin D. The addition of HDL<sub>3</sub> did not affect AngII-elicited induction of SR-BI mRNA in human NCI H295R cells.

### Discussion

LDL are the major cholesterol-carrying circulating lipoproteins in humans, and the LDL receptor-mediated pathway is highly developed in this species. As a consequence, the role of HDL as a potential source of cholesterol to human cells has received less attention for several years and has remained elusive. The recent discovery that SR-BI mediates both HDL binding and HDL-derived CE selective uptake has provided an important link between the selective uptake pathway and a specific cell surface receptor (6, 27). These initial observations have led to extensive studies on the regulation of both SR-BI expression and HDL-derived CE selective uptake by trophic hormones and activators of adenylyl cyclase in steroidogenic tissues, particularly in the

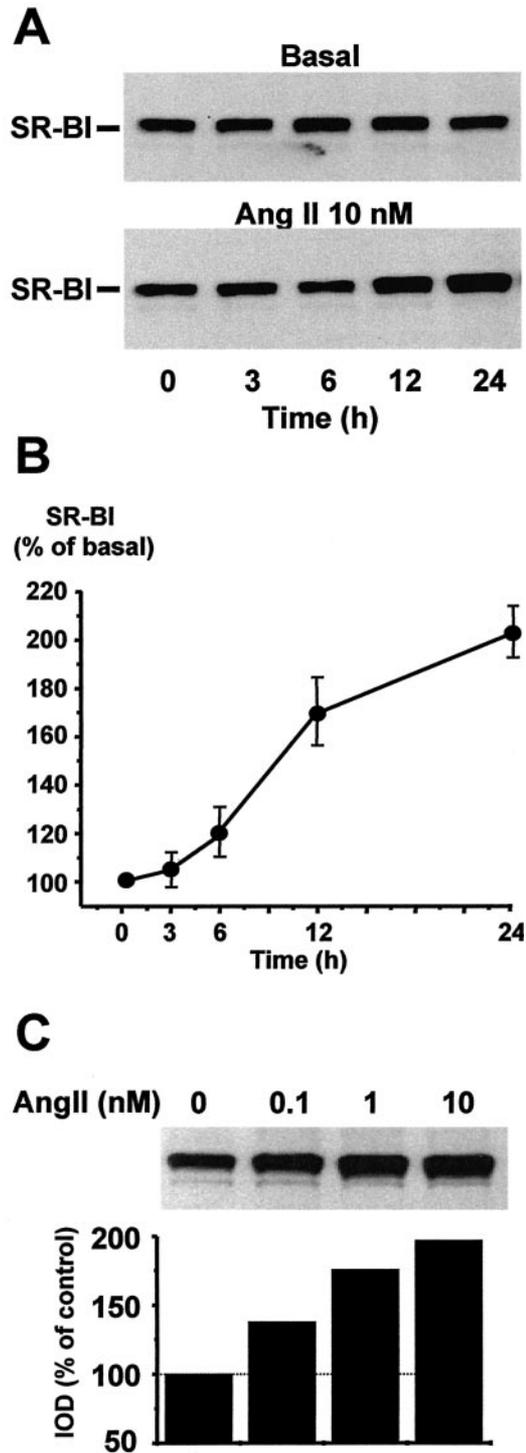


FIG. 4. Kinetics and concentration dependence of SR-BI expression in bovine adrenal glomerulosa cells stimulated with AngII. A, Total cell lysates from glomerulosa cells incubated in the absence (basal) or in the presence of 10 nM AngII for the time periods indicated were analyzed by Western blotting as described in *Materials and Methods*. B, Densitometric analysis of SR-BI protein expression from four independent experiments. C, Concentration-dependent effect of AngII treatment (24 h) on SR-BI protein expression. *Top*, Western blot from a typical experiment; *bottom*, mean densitometric analysis from two separate experiments. IOD, Integrated optical density.

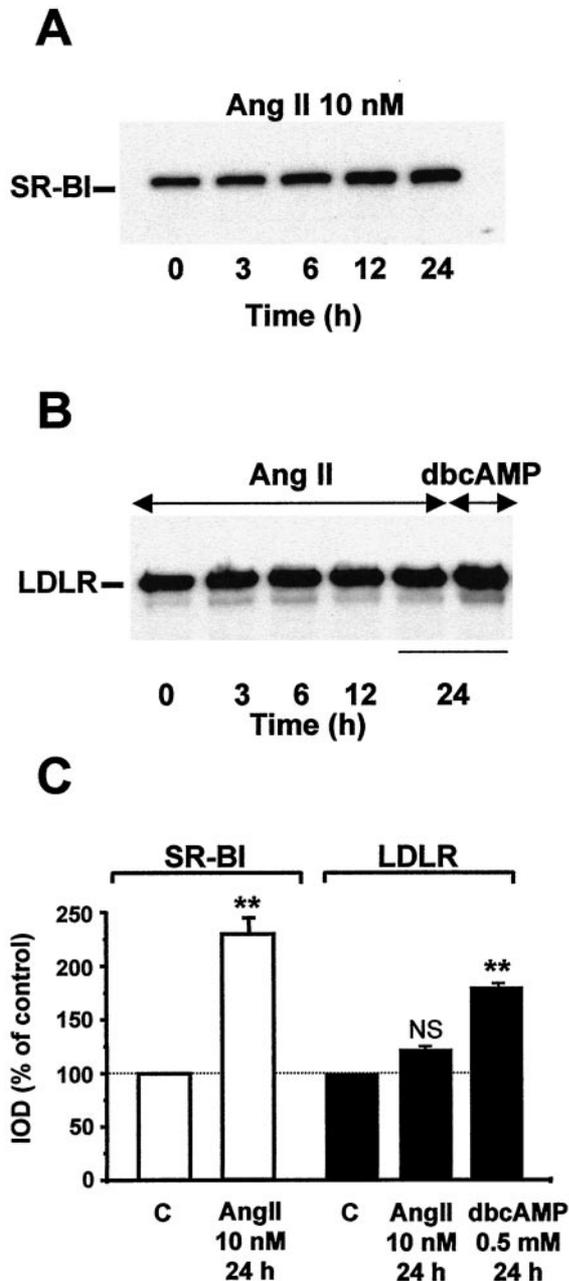


FIG. 5. Kinetics of SR-BI and LDL receptor (LDLR) expression in human adrenal NCI H295R cells stimulated with AngII. Total cell lysates from NCI H295R cells incubated in the absence (time 0) or in the presence of 10 nM AngII for the time periods indicated were analyzed by Western blotting for their SR-BI (A) or LDLR (B) protein content as described in *Materials and Methods*. Dibutyl cAMP (0.5 mM) was used as positive control of LDLR induction in NCI H295R cells (34). C, Densitometric analysis of SR-BI and LDLR protein expression after 24 h of treatment with the indicated agonists ( $n = 3$ ). \*\*, Significantly different from control,  $P < 0.01$ ; NS, not significantly different from control (C).

adrenal gland and the gonads (13, 18, 19, 28–35). In the present study, we have investigated whether HDL could also supply cholesterol to the human adrenocortical carcinoma cell line NCI H295R and to bovine adrenal glomerulosa cells challenged with the major physiological regulator of aldosterone biosynthesis, the  $Ca^{2+}$ -mobilizing hormone AngII.

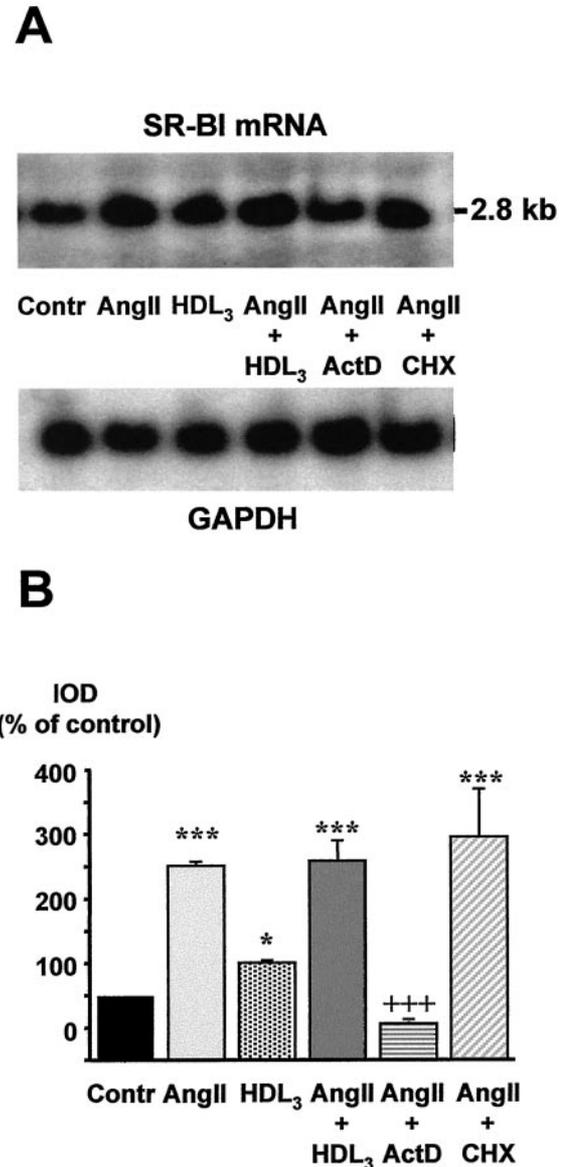


FIG. 6. Regulation of SR-BI mRNA expression in bovine adrenal glomerulosa cells. A, Total RNA from glomerulosa cells incubated for 24 h in the absence (Contr) or in the presence of 10 nM AngII and the agents indicated (HDL<sub>3</sub>, 250  $\mu$ g/ml; ActD, actinomycin D, 1  $\mu$ g/ml; CHX, cycloheximide, 10  $\mu$ g/ml) was prepared and analyzed by Northern blotting for SR-BI and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs as described in *Materials and Methods*. B, Densitometric analysis of SR-BI mRNA expression in four independent experiments. \* and \*\*\*, Significantly different from control,  $P < 0.05$  and  $P < 0.001$ , respectively; + + +, significantly different from AngII alone,  $P < 0.001$ . Values were corrected using GAPDH mRNA as an internal control.

Three major conclusions can be drawn from the present studies. 1) Human NCI H295R cells and bovine adrenal glomerulosa cells do indeed “selectively” internalize and process HDL-derived CE as a significant source of precursor for steroid hormone biosynthesis. 2) AngII enhances HDL-derived CE uptake as well as SR-BI expression (protein and mRNA) in both cell types, confirming a tight link between the selective uptake, the HDL receptor SR-BI, and steroidogenesis. 3) In contrast to cAMP, which has been shown to exert

a more pronounced induction on LDL receptors than on SR-BI protein in human adrenal cells (34), AngII behaves in the opposite manner, potentially increasing SR-BI expression while barely affecting LDL receptors. This finding suggests the possibility that the cells may switch between HDL and LDL utilization depending on the type of agonist that challenges them.

The demonstration of efficient HDL-derived CE uptake supporting steroidogenesis in glomerulosa cells is based on functional and fluorescence data. Using HDL as CE donors, we show that a substantial increase in pregnenolone and aldosterone production occurs in bovine adrenal glomerulosa cells and human NCI H295R cells. Of importance here is the finding that utilization of HDL<sub>3</sub>-derived CE occurs even in the resting state. This result is in contrast to observations made in human and rat ovarian granulosa cells, in which selective CE uptake is dependent on gonadotropin stimulation (18, 23, 36). Furthermore, AngII and lipoproteins act synergistically in supporting steroidogenesis, as indicated by the fact that the combined addition of AngII and HDL<sub>3</sub> is clearly more effective than either agent alone. We have previously demonstrated that AngII increases the mobilization of cholesterol to the inner mitochondrial membrane (37). In bovine adrenal fasciculata cells, ACTH-stimulated cortisol production was significantly greater in the presence of HDL compared with LDL (29). We report here that HDL and LDL are equally effective in supporting steroidogenesis in bovine glomerulosa cells, indicating that both the endocytic and selective pathways are functional in these cells. However, the contribution of SR-BI to LDL-derived CE selective uptake in bovine and human adrenal cells remains to be determined. Indeed, SR-BI has been shown to mediate the efficient uptake of LDL-derived CE via a selective uptake mechanism in Y1 adrenocortical cells and in SR-BI-transfected COS-7 cells (12) as well as in Chinese hamster ovary cells (10, 11). Furthermore, ovarian granulosa cells from LDL receptor knockout mice selectively internalize LDL, suggesting that SR-BI may also affect the metabolism of LDL *in vivo* (38). Most importantly, Leitersdorf *et al.* (39) have reported in earlier studies that AngII induces preferential uptake of the CE moiety over the protein moiety of LDL in bovine adrenal glomerulosa cells.

The BODIPY-CE fluorescence microscopy experiments allowed us to directly follow the fate of labeled CE within the cells. HDL<sub>3</sub>-derived CE uptake is a rapid process, visible within 5 min, in both bovine glomerulosa cells and human NCI H295R cells. In contrast to ovarian granulosa cells, in which the plasma membrane labeling has been reported to be minimal (40), the labeling of the cell membrane in both adrenal glomerulosa and human NCI H295R cells is pronounced and persistent, a finding consistent with the observation that the receptor concentrates in plasma membrane microdomains called caveolae (41, 42). Interestingly, most of the fluorescent CE accumulates within lipid droplets in areas essentially situated under the plasma membrane, where large numbers of mitochondria can be found (43, 44).

The fluorometric experiments allowed us to precisely quantitate total CE uptake while overcoming intrinsic difficulties of the microscopic approach, such as photobleaching of the probe (40). AngII pretreatment doubled HDL-derived

CE uptake, both in glomerulosa cells and in human NCI H295R cells. These results are of the same order of magnitude as those obtained with [<sup>3</sup>H]CE-labeled HDL in human NCI H295R cells prestimulated with 8-bromo-cAMP (34) and in Leydig cells treated with human CG (35). In contrast, the selective uptake was found to be dramatically increased in rat ovarian granulosa cells stimulated with dibutyryl cAMP, reaching 10- to 15-fold the value measured in nonstimulated cells (23).

The specificity of the SR-BI-mediated CE uptake in adrenal cells was further demonstrated with an anti-ApoA-I antibody. Recent studies of CE selective uptake in steroidogenic tissues of ApoA-I knockout mice stress that ApoA-I plays a critical role in this process (26, 45). Using an antibody against ApoA-I, we demonstrate a significant reduction (41%) of HDL<sub>3</sub>-derived CE uptake in bovine glomerulosa cells stimulated or not with AngII. This finding is consistent with data showing that 40% of the ApoA-I bound to SR-BI can be chemically cross-linked to the receptor (7).

Our study demonstrates a tight association between the function of the selective pathway and SR-BI expression, both processes being increased to the same extent by AngII. SR-BI has been shown to be up-regulated by ACTH in the murine adrenal gland *in vivo* and in Y1BS1 adrenal cells *in vitro*, as well as by dibutyryl cAMP in ovarian granulosa cells and by 8-bromo-cAMP in human adrenal cells (14, 19, 32–34, 46). We report here, for the first time, that SR-BI is induced to a comparable level in bovine and human adrenocortical cells after challenge with AngII in a concentration-dependent manner. Importantly, although LDL-derived CE may be taken up by the cell through both the endocytic and selective pathways, the major impact of AngII stimulation is to increase preferentially the expression of SR-BI and not that of the LDL receptor and thereby to promote the selective uptake of CE. Our data indicate that LDL receptor expression is more sensitive to the cAMP signaling pathway than to the Ca<sup>2+</sup>-messenger system. This finding is in line with previous data showing that the level of induction of SR-BI expression by cAMP was consistently lower than that of the LDL receptor in human NCI H295R cells (34).

The increase in SR-BI protein content after a 24-h exposure to AngII was accompanied by a similar induction of SR-BI mRNA levels. This concomitant increase in SR-BI protein and mRNA has been reported in various steroidogenic cell types from different species after stimulation with activators of the cAMP pathway, suggesting a conservation between species of SR-BI regulation (13, 15, 28, 30, 34). The induction of SR-BI mRNA by AngII does not require *de novo* protein synthesis, similar to what has been observed in NCI H295R cells exposed to ACTH (34), indicating that the effects of AngII are independent of short-lived proteins. Moreover, actinomycin D abolished the increase in SR-BI mRNA triggered by AngII, suggesting a transcriptional control of the *SR-BI* gene by the hormone, as has been reported for ACTH in human adrenal cells (34). The transcription factor steroidogenic factor 1 (SF-1) has been reported to mediate the transcription of human and murine *SR-BI* genes by cAMP (47, 48). The potential role of SF-1 in AngII-induced transcription of the *SR-BI* gene will require further analysis.

An important question is whether the AngII-induced in-

crease in SR-BI expression in both bovine glomerulosa cells and human NCI H295R cells is secondary to hormone-mediated changes in cellular cholesterol homeostasis or is the result of direct effects of AngII on *SR-BI* gene expression. Two sets of data support the latter hypothesis. First, in separate experiments, we have observed that AngII still increased SR-BI mRNA levels when glomerulosa cells were treated with aminogluthetimide to prevent cholesterol side chain cleavage and thereby cholesterol depletion (data not shown). This result speaks in favor of a direct effect of AngII on the *SR-BI* gene independent of cholesterol status. Consequently, an involvement of transcription factors such as sterol regulatory element binding proteins in AngII stimulation of SR-BI expression in bovine and human adrenocortical cells may be ruled out, although sterol regulatory elements have been described in the SR-BI promoter (49). It is worth mentioning that a sterol-independent regulatory element that binds the C/EBP transcription factor has been identified recently in the human LDL receptor promoter (50), in spite of the well documented regulation of this receptor by cellular cholesterol status and sterol regulatory element binding proteins (51, 52). Whether such a response element is also present in the SR-BI promoter is not known. Second, we found that the up-regulation of SR-BI by AngII is insensitive to HDL<sub>3</sub> loading. This result is similar to that obtained in luteinized granulosa cells incubated with HDL (19) but is in contrast to what has been observed in other cell systems, in which LDL induce a down-regulation of the LDL receptor and other cholesterol-sensitive genes (53–55).

In conclusion, the present study shows that bovine and, more importantly, human adrenocortical cells take up massive amounts of HDL-derived CE as a substrate for steroid hormone production in both the basal state and under AngII stimulation. Although blood lipoprotein profiles in human and cow are in favor of LDL (56), and although several earlier reports have concluded that HDL are not effective cholesterol donors for human and bovine steroidogenic cells (17, 57, 58), our data clearly indicate that adrenocortical cells from these species efficiently take up and metabolize HDL-derived CE to support AngII-induced aldosterone biosynthesis. This finding is in keeping with *in vivo* studies of adrenal function in familial hypercholesterolemia and hypobeta-lipoproteinemia, which have suggested a potential role for HDL in human adrenocortical cholesterol metabolism (59, 60).

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