Failure of adrenal corticosterone production in POMC-deficient mice results from lack of integrated effects of POMC peptides on multiple factors

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SYNTHESIS OF CORTICOSTEROIDS in the adrenal gland requires cholesterol as the precursor, which is then metabolized in the steroidogenic pathway. Numerous steps, from the import of cholesterol from plasma into adrenal cells, the conversion of cholesterol esters to free cholesterol, movement of free cholesterol into the inner mitochondrial membrane, to the enzymatic conversion in the mitochondria of cholesterol to pregnenolone, progesterone, deoxycorticosterone, and, finally, corticosterone have to function to produce baseline levels of corticosterone. Importantly, they need to be highly regulatable in order to respond to acute stress with high output of corticosterone.

Adrenocorticotropic hormone (ACTH), a 39-amino acid peptide processed in the pituitary from the proopiomelanocortin (POMC) prohormone, is the key mediator of adrenal corticosteroid production (5, 32). It is positioned centrally within the hypothalamic-pituitary-adrenal (HPA) axis: stress leads to release of corticotropin-releasing factor (CRF) from the hypothalamus, which in turn stimulates corticotroph cells of the anterior pituitary to release ACTH, which stimulates the adrenal to produce corticosterone. Production of corticosterone then negatively regulates the release of CRF in a feedback loop to the hypothalamus.

At the molecular level, ACTH has been shown to regulate many, if not all, of the factors involved in adrenal corticosteroidogenesis, among them the receptors which mediate uptake of cholesterol into the cell, the enzymes which mediate conversion of cholesterol esters to free cholesterol and translocation into mitochondria, as well as the mitochondrial enzymes converting cholesterol to corticosterone (7, 8, 17, 18, 33). Regulation of these factors was assessed at the gene or protein level either by adding ACTH to in vitro systems such as primary cell cultures from human, bovine, rat, and mouse adrenals, as well as the Y1 tumor adrenal cell line (10, 23, 27, 28, 30, 31), or by removing ACTH in vivo through hypophysectomy of rats (16, 18, 29). The resulting effects demonstrate either the potential of ACTH to regulate a particular factor (in vitro experiments) or the reaction of intact adrenals to the sudden depletion of ACTH (in vivo experiments).

We set out to further contribute to defining the role of ACTH in adrenal steroidogenesis by analyzing - in vivo - cholesterol metabolism, and gene transcription and protein levels of key factors of steroidogenesis, in the adrenals of mice genetically lacking POMC (POMC\(^{-/-}\)). POMC\(^{-/-}\) mice carry a deletion of the entire coding region of the POMC gene, thus lacking all POMC-derived peptides, including ACTH (38). These mice are born with morphologically normal adrenal glands, but the glands slowly regress postnatally. While present, mutant adrenals fail to make corticosterone, either basally or in response to acute stimulation with exogenous ACTH (12). Transplantation experiments showed that mutant adrenals are capable of normal corticosteroidogenesis when exposed to physiological levels of all POMC peptides by transplantation to wild-type recipients. As is the case with adrenals from hypophysectomized animals, pharmacological amounts of ACTH supplied
continuously to POMC−/− mice, by using either long-acting forms of ACTH (4) or osmotic minipumps to drive continuous ACTH stimulation at a constant rate (this report), result in corticosterone production.

At 3–6 wk postnatally, adrenals from POMC−/− mice are smaller than those of wild-type littermates, but they still show clear zonation of cortex (zona glomerulosa and fasciculata) and medulla. Importantly, zonal ratios and cell numbers at that age in POMC−/− mice are comparable to those of wild-type mice. We compared adrenals from wild-type and homozygous mutant mice at this time point with respect to parameters of cholesterol metabolism and steroidogenesis in an attempt to define relative effects of ACTH on individual factors involved in these processes.

METHODS

Animals. POMC wild-type, heterozygous, and mutant mice in the 129SvEv background (38) were housed under a 12:12-h light-dark cycle with a standard laboratory diet (PMI 5055; Purina Mills, Richmond, IN) and water provided ad libitum. Male and female heterozygous (POMC+/−) mice were mated, and wild-type (POMC+/+) and mutant (POMC−/−) offspring were used in the experiments. Mice were genotyped by PCR analysis of tail DNA (38). All procedures described below followed NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation.

Serum and plasma. Blood was collected from tail veins or by retroorbital phlebotomy into microfuge tubes using heparinized or nonheparinized capillaries. The blood was allowed to clot at room temperature for 15–20 min (for serum) and was then put on ice or was immediately put on ice (for plasma). Samples were spun at 4°C, 3,500 rpm, for 15 min, and serum was transferred to new microfuge tubes. Plasma was flash-frozen in dry ice-ethanol and stored at −80°C until analyzed for total cholesterol content. For extraction, −100 μl of plasma was diluted to 2 ml with 1 mM DTPA(aq).

Blood was collected from nonfasting animals through retroorbital bleeding into microfuge tubes (containing 1 ml 0.5 M EDTA/100 μl blood) using nonheparinized capillary tubes, and immediately put on ice. Samples were spun at 4°C, 3,500 rpm, for 15 min, and plasma was transferred to new microfuge tubes. Plasma was flash-frozen in dry ice-ethanol and stored at −80°C until analyzed for total cholesterol content. For extraction, −100 μl of plasma was diluted to 2 ml with 1 mM DTPA(aq).

For acute stimulation, ACTH (1-24), synthesized by Invitrogen (Carlsbad, CA) was used. Mice were injected intraperitoneally with ACTH (1 μg/0.1 ml PBS-0.5% BSA per mouse) subcutaneously (sc) between the shoulder blades. Control animals received saline (PBS-0.5% BSA) only. Tail blood was collected for corticosterone RIA 1 h after ACTH stimulation. Wild-type mice were given dexamethasone (0.4 mg/kg) 2 h before exogenous ACTH stimulation to block endogenous ACTH release from the pituitary.

For continuous stimulation, the same ACTH as described above was used. Alzet miniosmotic pumps (model 1007D; Durect, Cupertino, CA), placed subcutaneously between the shoulder blades, were used to supply mice with continuous ACTH stimulation. The mean pumping rate of the pumps was 0.5 μl/h, for a period of 7 days. The concentration of ACTH (250 μg-0.1 ml PBS-0.5% BSA per mouse) added to the pumps provided a constant release of 30 μg/day throughout the duration of the experiment. Sham-treated animals received the miniosmotic pump with carrier (0.1 ml PBS-0.5% BSA) only.

Tissue preparation and microscopy. For light microscopy, adrenal glands from POMC wild-type and mutant mice (either treated or untreated) were removed, dissected free of fat, and stored in RNAlater (Qiagen). Total RNA (10 μg) was DNase treated using the Omniscript RT kit (Qiagen), Ambion DNA-free (Ambion, Austin, TX). DNase-treated RNA (1 μg) was reverse transcribed (RT) using the Omniscript RT kit (Qiagen), utilizing oligo(dT)20 primers (Invitrogen) and RNaseOUT (RNase inhibitor, Invitrogen), to yield cDNA. A list of primer sequences for each gene is provided in Table 1. cDNAs were amplified using a Bio-Rad (Hercules, CA) iCycler iQ employing a standard qRT-PCR reaction using SYBR Green. Data are presented as relative expression of each gene in both wild-type and POMC−/− samples when normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blot analysis. POMC wild-type and mutant adrenals (from treated or untreated mice) were removed, dissected of fat, and homogenized in lysis buffer (50 mM HEPES, pH 7.6, 250 mM NaCl, 0.5 mM EDTA, 0.5% Triton X, and protease inhibitor cocktail: Roche,
Indianapolis, IN) using a glass rod mortar and pestle. The homogenate was allowed to shake at 4°C for 1 h, and protein contents of the high-speed supernatant (15,000 rpm/30 min) samples were measured using a BCA protein assay kit (Pierce, Rockford, IL). Protein samples (12 μg) from total cell lysates were separated by 10% SDS-PAGE minigel (Bio-Rad) and transferred to immunoblot polyvinylidene difluoride (PVDF) membrane (Bio-Rad) for immunoblots. After blocking of nonspecific sites, membranes were incubated overnight at 4°C in blocking buffer [Tris-buffered saline (TBS) containing 0.5% (wt/vol) nonfat milk powder and 0.05% Tween 20] with primary antibodies to SR-BI (1:1,000, rabbit polyclonal; Novus Biologicals, Littleton, CO), steroidogenic acute regulatory protein (StAR; 1:1,000, rabbit polyclonal, Novus Biologicals), P450 side-chain cleavage (CYP11A1; 1:1,000, rabbit polyclonal, Novus Biologicals), P450C21 (CYP21A1; 1:5,000, rabbit polyclonal; Corgen, Guilford, CT) or β-actin (1:1,000; rabbit polyclonal, Novus Biologicals). The washed blots were then incubated with ECL rabbit secondary antibodies conjugated to horseradish peroxidase (1:7,000; Amersham UK). Antibody binding to the membrane was visualized using the ECL plus (Amersham) chemiluminescent detection system. Gel bands were photographed on a Kodak Imaging Station 4000r and relative densities assayed using Kodak Imaging software. For quantification, three independent adrenal protein samples (wild type and mutant) were used. Relative densities were plotted as a percentage of wild type ±SE.

RESULTS

Adrenal cholesterol content. Free cholesterol (FC) is required for the synthesis of corticosteroids. The majority of adrenal FCs are derived from hydrolysis of cholesteryl esters (CE) stored in lipid droplets (20). There is evidence indicating that lipid droplets are a morphological characteristic that can reflect the rate of steroidogenesis (35).

Staining with Oil-red-O demonstrated abundant lipid staining, normally associated with CE storage, of the adrenal cortex in wild-type mice (Fig. 1, A and C) and no lipid staining in POMC−/− mice (Fig. 1, B and D). Tissue lipid analysis revealed that CE content of adrenal glands was markedly reduced in POMC−/− mice (4.93 ± 0.44 μg/mg wet wt) compared with wild-type mice (37.6 ± 2.3 μg/mg wet wt), with no change in free cholesterol (Fig. 1E).

Plasma cholesterol content. To test whether the supply of cholesterol from the blood stream to the adrenal is affected, we determined the content of FC and CE in plasma. Plasma cholesterol analysis revealed a slight decrease in both CE and FC in POMC−/− mice compared with wild-type mice (1.10 ± 0.07 μg/μl plasma (CE wild type) vs. 0.86 ± 0.02 μg/μl plasma (CE mutant) and 0.42 ± 0.01 μg/μl plasma (FC wild type) vs. 0.35 μg/μl ± 0.01 plasma (FC mutant)) (Fig. 1F).

Adrenal gene expression. Adrenal cholesterol metabolism requires a complex regulation of three pathways that lead to free cholesterol substrate for steroidogenesis. These include selective uptake of HDL-CE, nonselective uptake of LDL-CE, and de novo synthesis. The genes involved in these processes include SR-BI (selective CE uptake), LDLR (LDL receptor; nonselective CE uptake), apoE (apolipoprotein E; cholesterol storage/transport), ACAT (acetyl-coA:cholesterol acyltransferase; FE-to-CE conversion), HSL (hormone-sensitive lipase; CE-to-FC conversion), HMG-CoAR (hydroxymethylglutaryl-coenzyme A reductase; de novo synthesis), StAR (FC transport to mitochondria), PBR (peripheral benzodiazepine receptor; FC uptake in mitochondria), and ABCA1 (ATP-binding cassette type A1; cholesterol export). Despite the lack of cholesterol storage in the POMC−/− adrenal, qRT-PCR revealed the presence of transcripts of SR-BI, LDLR, StAR, HMG-CoAR, and HSL in the mutant adrenal (Fig. 2). Transcript levels of StAR were decreased approximately twofold. Also, HMG-CoAR and LDLR transcript levels were significantly increased; however, the increase in transcript did not lead to compensation of adrenal cholesterol storage as shown in Fig. 1. No obvious change in expression was seen for PBR, apoE, ABCA1, and ACAT in semiquantitative RT-PCR experiments (data not shown).

In addition to genes involved in cholesterol metabolism, we analyzed expression levels of key genes required for steroidogenesis: CYP11A1 (the rate-limiting enzyme in the steroidogenic pathway required for the conversion of cholesterol into pregnenolone), 3β-hydroxysteroid dehydrogenase Δ4–5-isomerase (3β-HSD; conversion of pregnenolone to progesterone), CYP21 (conversion of progesterone to deoxy corticosterone), and CYP11B1 (P450 11β-hydroxylase; conversion of deoxycorticosterone to corticosterone). As with the genes required for cholesterol metabolism, qRT-PCR revealed the presence of transcripts of CYP11A1 and CYP11B1 in the mutant adrenal (Fig. 2), with no significant change in transcript between genotypes.

Adrenal protein levels. Protein levels of genes involved in cholesterol uptake, cholesterol utilization, and steroidogenesis were all decreased, to different extents, in POMC−/− adrenals compared with wild-type controls, as demonstrated by Western blot analyses. SR-BI and StAR were both found at lower levels in mutant adrenals (Fig. 3). CYP11A1 is thought to be the rate-limiting enzyme in corticosteroid biogenesis, and CYP21 converts pregnenolone to progesterone in the steroidogenic pathway, leading to corticosterone production. Both of these

Table 1. Primers used for quantitative and semiquantitative RT-PCR

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<th>Gene</th>
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<th>Reverse Primer</th>
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<td>CCTATTCTGGAACCCTTTTATCTA</td>
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<tr>
<td>LDLR</td>
<td>TAAACTCTGAGCTCTCCGGTATGA</td>
<td>AGCAATCTGCTGAGAAGTTCCAGA</td>
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<tr>
<td>PBR</td>
<td>TCTCTCATTAGTCGGGAAGAAGCTG</td>
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<td>HMG-CoAR</td>
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<td>apoE</td>
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<td>CCCTTGCAGATCCTGCGGAT</td>
</tr>
<tr>
<td>HSL</td>
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<td>CATAGCTTCTACACCTTGGCCGT</td>
</tr>
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See text for definitions.
enzymes were also found at lower levels in POMC<sup>−/−</sup> adrenals, with only very subtle changes in CYP21 (Fig. 3). Acute stimulation of POMC<sup>−/−</sup> mice with supraphysiological doses of exogenous ACTH did not produce serum corticosterone in blood assayed 1 h later, even with doses of 30 μg (12 and data not shown). This same dose given to wild-type littermates stimulated maximal serum corticosterone levels. Injection of 30 μg of ACTH daily for 10 days was also without effect on corticosterone secretion (data not shown).

Continuous ACTH stimulation via osmotic minipumps supplied both POMC<sup>−/−</sup> mice and wild-type littermates with a constant flow rate of hormone release (30 μg/day) through the duration of the experiment. Table 2 shows the levels of peripheral blood ACTH achieved in treated mice. This treatment induced corticosterone production after 24 h in POMC<sup>−/−</sup> mice, with serum corticosterone levels of 123 ± 6.63 ng/ml (Fig. 4A). After 48 h, corticosterone levels further increased nearly sevenfold to plateau at 876 ± 138 ng/ml. Corticosterone levels on days 3–10 did not differ significantly from those reached at day 2 (Fig. 4A and data not shown). Wild-type littermates undergoing the same treatment produced serum corticosterone levels above the maximal limit of detection for the assay (Fig. 4A).

We then asked whether POMC<sup>−/−</sup> mice retained their ability to produce corticosterone after continuous ACTH supply had stopped. Accordingly, POMC<sup>−/−</sup> mice and wild-type littermates carried osmotic minipumps for 5 days; pumps were removed on day 5, and mice were assayed for serum corticosterone 2 days after removal of the pumps. As shown in Fig. 4B, basal, i.e., unstimulated, levels of corticosterone were 18 ± 3 ng/ml in wild-type and undetectable in mutant mice. Corticosterone levels measured 1 h after injection of 1 μg of ACTH were 497 ± 30 ng/ml in wild-type mice and 150 ± 15 ng/ml in POMC<sup>−/−</sup> mice. However, when acutely POMC<sup>−/−</sup> mice were stimulated 10 days after removal of the continuous ACTH supply, the corticosterone levels were reduced to barely measurable levels (data not shown).

Adrenal cholesterol content after ACTH stimulation. We then wanted to know whether the functional changes in the mutant adrenals correlated with changes in adrenal cholesterol content. Staining adrenal tissue with Oil-red-O after 24 h, 48 h, 3 days (POMC<sup>+/+</sup>), and 5 days (POMC<sup>−/−</sup>) of ACTH stimulation revealed a lack of lipid droplets in both wild-type and POMC<sup>−/−</sup> mice. Values represent means ± SE for 4–6 adrenals per group. *P < 0.01, **P < 0.008. Mice for all experiments were females 30–40 days old.

Fig. 1. Cholesterol content of adrenal glands in wild-type (WT) and proopiomelanocortin-null mutant (POMC<sup>−/−</sup>) mice. A–D: cross sections of adrenal glands were stained with Oil-red-O (red staining) and were counterstained with hematoxylin. A and C: WT (×4 and ×20 magnification); B and D: POMC<sup>−/−</sup> mice (×4 and ×20 magnification). E: adrenal gland cholesteryl ester (CE) and free cholesterol (FC) content (μg/mg wet wt) in WT and POMC<sup>−/−</sup> mice. Values represent means ± SE for 4–6 adrenals per group. *P < 0.01. **P < 0.008. Mice for all experiments were females 30–40 days old.

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mutant tissues (Fig. 5A and data not shown). This lack of lipid droplets in both tissue types is due most likely to the rapid conversion of cholesterol ester into free cholesterol to supply the high rate of steroidogenesis induced by the supra-physiological doses of ACTH.

Figure 5B gives further support to this conclusion. Shown are two individual adrenals of POMC−/− mice after 10 days of ACTH stimulation via osmotic minipumps. Although similar in levels of adrenal weight and plasma ACTH, the two mice differ in serum corticosterone levels measured at the time of serum and tissue collection. Correlating reversely with the levels of corticosterone are the adrenal cholesterol content and lipid staining: at high levels of corticosterone production mutant adrenals are almost depleted of cholesterol, whereas at lower levels of corticosterone production POMC−/− adrenals are clearly capable of storing cholesterol.

Strong support for a causative role of cholesterol storage in the POMC−/− adrenals in failing or succeeding in corticosterone production is derived from experiments utilizing the ability to experimentally regulate corticosterone production in mutant adrenals. POMC−/− mice did not produce corticosterone at basal levels, they did not respond with corticosterone production to acute stimulation with ACTH, and they lacked adrenal cholesterol (see above). When POMC−/− mice underwent 5 days of continuous ACTH stimulation and were assayed 2 days after discontinuation of ACTH treatment, they still did not produce corticosterone at basal levels, but they did respond with corticosterone production to acute stimulation with ACTH (see Fig. 4B), and they had adrenal cholesterol stores (Fig. 5C).

**Adrenal protein levels after ACTH stimulation.** To assess the molecular changes after ACTH stimulation, we determined adrenal levels of key proteins; a Western blot representative of the results is shown in Fig. 6A. Continuous ACTH stimulation led to a large increase of adrenal protein levels of SR-BI within the first 24 h in POMC−/− mice (Fig. 6A) and in wild-type controls (not shown). No significant changes in protein levels were detected after ACTH treatment in both wild-type and mutant tissues (Fig. 5A and data not shown). This lack of lipid droplets in both tissue types is due most likely to the rapid conversion of cholesterol ester into free cholesterol to supply the high rate of steroidogenesis induced by the supra-physiological doses of ACTH.
mutant adrenals for CYP11A1 (P450SCC) and for StAR (Fig. 6A).

In addition to the monomeric form, SR-BI can also exist in dimeric and oligomeric/heteromeric forms, and these forms are induced following stimulation with ACTH as shown previously by Williams et al. in rats (37). Although no forms other than the monomeric were detected in adrenals from untreated wild-type and POMC−/−/− mice, ACTH stimulation induced oligomer formation in both wild-type (not shown) and mutant adrenals (Fig. 6A).

Comparison of protein expression in adrenals from POMC−/−/− mice 24 and 48 h after ACTH treatment showed no significant further change in the protein levels of SR-BI, StAR, or CYP11A1 (Fig. 6A), despite the marked increase in serum corticosterone levels during this interval (190 ± 20 ng/ml at 24 h vs. 780 ± 23 ng/ml at 48 h; Fig. 6B).

DISCUSSION

The first requirement for steroidogenesis is the availability of the precursor, cholesterol. The majority of adrenal free cholesterol (FC) is derived from hydrolysis of cholesteryl esters (CE) stored in lipid droplets (20). POMC−/−/− adrenals are depleted of stored CE. The reasons for this could be decreased availability of CE in plasma, impaired import of cholesterol into adrenal cells, and/or inability to store CE in adrenal cells. Total and free cholesterol in plasma are slightly, Table 2. Plasma levels of ACTH after implantation of ACTH pumps

<table>
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<tr>
<th>ACTH Treatment</th>
<th>None</th>
<th>1 day</th>
<th>3 days</th>
<th>5 days</th>
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<tr>
<td>+/-</td>
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<td>773±122</td>
<td>710±188</td>
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<td>843±103</td>
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Values are plasma ACTH (pg/ml) ± SE. Wild-type (+/+ ) and POMC mutant (−/−) mice (40-day-old females) were implanted with miniosmotic pumps delivering either ACTH (30 µg/day) or carrier alone (SHAM: PBS-0.5% BSA) continuously with a constant flow rate. Blood was collected for corticosterone analysis every 24 h. POMC−/− mice and WT littermates (30- to 50-day-old males) carried pumps (30 µg ACTH/day) for 5 days. Two days after removal of pumps, mice were assayed for basal (unstimulated) levels of serum corticosterone and in response to acute stimulation (1 µg) of ACTH. For acute stimulation, mice were injected sc with ACTH, and blood was collected 1 h later for analysis of corticosterone by RIA. Values represent means ± SE for 4–6 animals per group. *Levels were undetectable.

Fig. 4. Corticosterone production in WT and POMC−/− mice after ACTH stimulation. A: serum corticosterone levels in WT and POMC−/− (MT) mice in response to continuously delivered ACTH. WT and POMC−/− mice (40-day-old females) were implanted with miniosmotic pumps delivering either ACTH (30 µg/day) or carrier alone (SHAM; PBS-0.5% BSA) continuously with a constant flow rate. Blood was collected for corticosterone analysis every 24 h. B: POMC−/− mice and WT littermates (30- to 50-day-old males) carried pumps (30 µg ACTH/day) for 5 days. Two days after removal of pumps, mice were assayed for basal (unstimulated) levels of serum corticosterone and in response to acute stimulation (1 µg) of ACTH. For acute stimulation, mice were injected sc with ACTH, and blood was collected 1 h later for analysis of corticosterone by RIA. Values represent means ± SE for 4–6 animals per group. *Levels were undetectable.
but significantly, decreased in POMC\(^{-/-}\) mice compared with wild-type littermates. However, it is highly unlikely that this small decrease of plasma cholesterol results in absence of adrenal CE. In comparison, mouse models with much more severely reduced plasma cholesterol, induced pharmacologically or genetically, show no reduction in adrenal CE storage (13, 36).

It is unclear at present how POMC deficiency mediates a change in plasma cholesterol and whether POMC deficiency has effects on cholesterol synthesis and/or metabolism in other organs. Our findings so far in plasma and adrenal suggest that analyses of other organs might be of interest.

The main mechanism, in mice, of cholesterol delivery into adrenal cells is selective uptake of HDL-CE through SR-BI. Although expression of the SR-BI gene was only slightly, but significantly, decreased in POMC\(^{-/-}\) adrenals, the presence of SR-BI protein was markedly reduced by over 50%. Supplementation with ACTH increased SR-BI protein in POMC\(^{-/-}\) adrenals to levels of those in unstimulated wild-type adrenals and led to presence of dimeric forms in addition to the monomer; it has been suggested that SR-BI dimers are an integral step in selective CE uptake (25).

It has previously been shown that SR-BI expression can be regulated by ACTH (1). It has also been suggested that depletion of adrenal cholesterol stores can act independently from ACTH to increase SR-BI gene expression and protein. However, our data demonstrate that decreased adrenal cholesterol storage by itself is not a sufficient signal to increase SR-BI protein but that this upregulation is dependent on ACTH. This correlates with the inverse situation in the StAR\(^{-/-}\) mouse, where SR-BI is upregulated despite high levels of CE storage due to high circulating levels of ACTH (3). Our findings in the POMC\(^{-/-}\) mice allow generalizing the observation made in the StAR\(^{-/-}\) mice, i.e., that ACTH overrides the feedback regulation of SR-BI in response to changes of cellular cholesterol stores.
However, the decrease in SR-BI protein alone cannot explain the lack of cholesterol stores in the POMC−/− adrenal. While SR-BI knockout mice have severely reduced adrenal cholesterol concentrations (decrease by ~72%), SR-BI mice heterozygous for the null mutation display reduced protein levels comparable to those in POMC-null mutant adrenals, but only modest reductions (~42%) in adrenal cholesterol concentrations (26). Despite the presence of SR-BI in POMC−/− adrenals comparable to those in SR-BI heterozygotes, POMC−/− adrenals have dramatically reduced cholesterol storage (~87% reduction), suggesting that ACTH might act synergistically with SR-BI to promote cholesterol uptake.

Selective cholesterol uptake through SR-BI accounts for 97% of adrenal CE; the endocytic pathway, using nonselective uptake of LDL-CE and involving LDLR, and endogenous de novo cholesterol synthesis, involving HMG-CoAR, are negligible under normal conditions (14, 21). However, under conditions of failing adrenal CE accumulation, as in the apoA-1−/− mouse, increased expression of these compensatory pathways provides enough cholesterol substrate for corticosterone production, albeit diminished (22). There is no functional compensation through alternate pathways in POMC−/− mice.

Different from CE, amounts of FC in POMC−/− adrenals are similar to those of wild-type mice. As is known from previous experiments, mere availability of FC does not lead to steroidogenesis in the absence of a concurrent ACTH stimulus (6). However, even acute ACTH stimulation of POMC−/− mice does not lead to corticosterone production, indicating that either the FC available is not enough for corticosterone production or it cannot be utilized due to lack of activity of downstream factors that cannot be sufficiently activated during an acute ACTH stimulus.

Pharmacological supplementation of POMC−/− mice with ACTH leads to CE storage in POMC-null mutant adrenals. The stored CE does not permit corticosterone production in the absence of ACTH; however, acute stimulation with physiological doses of ACTH now elicits corticosterone production. Once the supply of cholesterol is secured, in the absence of ACTH corticosterone production fails at the next ACTH-dependent steps, the StAR-mediated import of FC into the

**Fig. 6.** Western blot analysis of SR-BI, StAR, and P450scc (CYP11A1) proteins in adrenal gland of POMC−/− mice after ACTH stimulation. A. Right: representative Western blots showing SR-BI, StAR, and P450scc protein levels in adrenal glands of POMC−/− mice after 24 and 48 h of continuous ACTH stimulation (30 μg/day ACTH provided via miniosmotic pumps). For comparison, Western blot results from untreated POMC−/− mice depicted in Fig. 3 are displayed on left. Note 2 forms of SR-BI after ACTH stimulation: monomer (solid arrow) and dimer (dotted arrow; only present after ACTH stimulation). Actin protein was used as internal standard. B: serum was collected from the same POMC−/− mice used to determine protein levels after continuous ACTH stimulation (24 and 48 h), and levels of corticosterone were determined by RIA. Values represent means ± SE for 3 mice per group. Mice were females 40 days old.
inner mitochondrial membrane and the multistep synthesis of corticosterone from cholesterol.

Experiments adding or depleting ACTH in tissue culture cells or in hypophysectomized animals, respectively, have shown pronounced effects on the expression of adrenal genes involved in multiple steps of steroidogenesis. For example, ACTH stimulation of steroidogenic enzyme activity in primary bovine adrenocortical cells correlates with increased steroidogenic gene transcription (11, 39). And depletion of pituitary homones, including ACTH, in hypophysectomized rats showed marked decreases in transcript levels of \( \text{P450}\text{scC} \) and, to a lesser degree, \( \text{CYP21} \) (9). In our gene expression studies we found no major, log-fold decrease in gene expression in POMC null mutant adrenals of any of the key factors tested. We used adrenals from young mice (3–6 wk), i.e., at a stage when adrenals from POMC\(^{-/-}\) mice are significantly smaller than those of wild-type littermates but still have the distinct cortical zonation. We measured numerous adrenal glands (serial sections) of this age group and found no significant differences in the ratio of medulla to cortex in POMC mutant mice compared with wild-type littermates (unpublished results). And although the adrenals are significantly smaller, the cell numbers in both zona glomerulosa and zona fasciculata in POMC mutant adrenals at this age are comparable to those in wild-type adrenals.

ACTH stimulation increases the size of POMC mutant adrenals but not the number of cells. Taken together, at this stage the lack of dramatic differences in gene expression between POMC\(^{-/-}\) and wild-type adrenals cannot be explained by a shift in numbers of steroidogenic cells from zona fasciculata to glomerulosa or in total cells from cortex to medulla. A likely explanation for the differences in our results vs. the data on ACTH-mediated transcription of several of the genes we tested lies in the analysis of a steady-state absence of ACTH in POMC\(^{-/-}\) mice vs. the analysis of a change from presence of normal levels of ACTH to its addition or depletion.

Differences were more pronounced at the protein level: there was a 50% decrease in protein levels of \( \text{SR-BI} \) in POMC\(^{-/-}\) adrenals, less for \( \text{StAR} \) and \( \text{P450}\text{scC} \), and almost no change in levels of \( \text{CYP21} \). This is consistent with previously reported data showing that levels of \( \text{StAR} \) and \( \text{CYP11A1} \) are responsive to ACTH in vitro while \( \text{CYP21} \) appears unresponsive (24).

It is possible that gene and/or protein levels undergo transient changes not captured at the time points measured here. This is supported by in vitro studies reporting changes in transcript and protein levels within a 24-h period of ACTH stimulation (15, 24). The various target genes whose expression is activated by ACTH may differ in their kinetics of induction. Thus, the same single time point used in our studies for all genes may mask important differences in how the induction occurs temporally. Further studies in vivo are necessary to arrive at a more accurate picture of gene and protein regulation.

Taken together, the results from determining transcript and/or protein levels of key steroidogenic factors in POMC\(^{-/-}\) adrenals demonstrate that genes are expressed constitutively in the absence of POMC peptides. Although protein levels can be reduced by almost one-half, this hardly explains the complete absence of steroidogenesis in POMC\(^{-/-}\) adrenals. And even after POMC mutant adrenals have taken up cholesterol upon ACTH stimulation, this does not result in corticosterone production unless ACTH is present. The crucial effect of ACTH might be on posttranslational modification, translocation, and/or enzyme activity of these proteins. A point-in-case is the \( \text{StAR} \) protein, which helps mediate cholesterol delivery from CE to the steroidogenic pathway. In our studies, \( \text{StAR} \) does not show major changes at the protein level. A reasonable conclusion might be that \( \text{StAR} \) protein, for full activity, requires an ACTH-driven posttranslational modification that stimulates its activity. It is known that \( \text{StAR} \)’s phosphorylation status is directly stimulated by ACTH treatment in a manner that correlates with de novo steroidogenesis. Showing a difference in the phosphorylated state of \( \text{StAR} \) between untreated and ACTH-treated POMC mutant adrenals would further support the hypothesis of a crucial role of ACTH in posttranscriptional effects and might provide novel insights into the basis for the steroidogenic defect and subsequent rescue in POMC\(^{-/-}\) adrenals.

POMC\(^{-/-}\) adrenals start to produce corticosterone after 24 h of continuous ACTH stimulation and are functioning at their maximum by 48 h; wild-type adrenals have reached maximal corticosterone production after the first 24 h. It is not clear at this point which particular process or combination of processes requires this extended period of time in POMC\(^{-/-}\) adrenals. Whatever the changes that need to occur in the mutant adrenal to allow corticosterone production, whether they are based on protein level thresholds and/or morphological changes, it is apparent that the changes cannot be achieved during acute stimulation with high pharmacological amounts of ACTH but need the continuous presence of the hormone for an extended period of time.

ACTH secretion is normally highly pulsatile and has diurnal cyclicality, different from the continuous levels provided by the minipumps in our experiments. We previously showed (12) that adrenal function is completely rescued by physiological levels of ACTH only in the context of all POMC-derived peptides in transplantation experiments. Although the pharmacological rescue used in the studies here does limit the interpretations that can be drawn, at the same time it allows the dissection of individual aspects of the ACTH-mediated regulation of corticosterone production.

Our studies on the status of adrenal glands in POMC\(^{-/-}\) mice reveal that the absence of POMC peptides affects all aspects of corticosterone production, from import of the precursor cholesterol to its conversion to corticosterone. Impairment of corticosteroidogenesis and its pharmacological reconstitution by ACTH are not mediated by any one individual protein, but rather as an integrated effect on multiple factors. This integrated regulation effectively shuts down organ function in the absence of POMC peptides; inversely, it allows optimal production of corticosterone as demanded by the organism.

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