Group X Secretory Phospholipase A2 Regulates the Expression of Steroidogenic Acute Regulatory Protein (StAR) in Mouse Adrenal Glands*§

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We developed C57BL/6 mice with targeted deletion of group X secretory phospholipase A2 (GX KO). These mice have ~80% higher plasma corticosterone concentrations compared with wild-type (WT) mice under both basal and adrenocorticotropic hormone (ACTH)-induced stress conditions. This increased corticosterone level was not associated with increased circulating ACTH or a defect in the hypothalamic-pituitary axis as evidenced by a normal response to dexamethasone challenge. Primary cultures of adrenal cells from GX KO mice exhibited significantly increased corticosterone secretion compared with WT cells. Conversely, overexpression of GX secretory phospholipase A2 (sPLA2), but not a catalytically inactive mutant form of GX sPLA2, significantly reduced steroid production 30–40% in Y1 mouse adrenal cell line. This effect was reversed by the sPLA2 inhibitor, indoxam. Silencing of endogenous M-type receptor expression did not restore steroid production in GX sPLA2-overexpressing Y1 cells, ruling out a role for this sPLA2 receptor in this regulatory process. Expression of steroidogenic acute regulatory protein (StAR), the rate-limiting protein in corticosteroid production, was ~2-fold higher in adrenal glands of GX KO mice compared with WT mice, whereas StAR expression was suppressed in Y1 cells overexpressing GX sPLA2. Results from StAR-promoter luciferase reporter gene assays indicated that GX sPLA2 antagonizes StAR promoter activity and liver X receptor-mediated StAR promoter activation. In summary, GX sPLA2 is expressed in mouse adrenal glands and functions to negatively regulate corticosteroid synthesis, most likely by negatively regulating StAR expression.

The secretory phospholipase A2 (sPLA2) family represents a group of structurally related calcium-dependent enzymes that hydrolyze glycerophospholipids at the sn-2 position to liberate lysophospholipids and free fatty acids. Ten enzymatically active sPLA2s have been identified in mammals (group IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XIIA) (1). Individual sPLA2s exhibit unique tissue and cellular localizations and enzymatic properties suggesting their distinct roles in various physiological and pathophysiological events. Although sPLA2s have been implicated in a number of important biological processes based on in vitro studies, their true physiological functions are largely subject to debate.

Among these enzymes, group X sPLA2 (GX sPLA2) has the highest binding affinity to zwitterionic phospholipids, including phosphatidylcholine, and thus potently hydrolyzes the outer leaflet of intact mammalian membranes, which are rich in phosphatidylcholine. GX sPLA2 is reportedly expressed in spleen, thymus, blood leukocytes, lung, colon, brain, heart, placentia, prostate, small intestine, testis, uterus, and pancreas (2–4). The enzyme has an N-terminal propeptide and is believed to be synthesized as an inactive zymogen that requires proteolytic cleavage for activation (2). Almost all of the insights into GX sPLA2 function have been gleaned from in vitro studies, whereby cultured cells were either transfected with GX sPLA2 cDNA or antisense or were incubated with recombinant enzyme or pharmacological inhibitors. When added to intact cells, the mature enzyme effectively hydrolyzes plasma membranes to generate free fatty acids and lysophospholipids, most notably arachidonic acid (AA) and lysophosphatidylcholine (LPC). Based on its ability to generate bioactive lipid mediators, GX sPLA2 has been implicated in diverse biological functions, including prostaglandin-mediated inflammatory responses in airway epithelial cells, mast cells, and macrophages, and LPC-induced neutre outgrowth and melanocyte pigmentation (5–8). GX sPLA2 hydrolyzes phospholipids on lipoproteins, and evidence suggests that hydrolysis of low density lipoprotein by GX sPLA2 results in a modified particle that induces lipid accumulation in human monocyte-derived macrophages (9, 10). Its presence in atherosclerotic lesions suggests this enzyme could play a role in pro-atherogenic processes (9). The hydrolytic activity of GX sPLA2 has also been impli-
cated in innate immunity through its antiviral (11) and bacterioidal (12) effects.

Although many of GX sPLA₂'s biological effects have been attributed to its potent catalytic activity, this molecule may also induce intracellular signaling events through processes that are independent of phospholipid hydrolysis (13). GX sPLA₂ is a high affinity ligand for the M-type sPLA₂ receptor, a member of the C-type lectin family that is structurally similar to the macrophage mannose receptor (1, 14). Membranes prepared from lungs of mice deficient in the M-type receptor are defective in GX sPLA₂ binding (15). Whether the noncatalytic activities described for GX sPLA₂ are mediated through M-type receptor-dependent signaling remains to be determined. In transfected Chinese hamster ovary cells, the M-type receptor mediates GX sPLA₂ internalization and subsequent lysosomal degradation (16) and significantly reduces COX-2-dependent prostaglandin E₂ formation induced by GX sPLA₂ (15, 17). Thus, the M-type receptor may play a role in the inactivation of this potent sPLA₂.

The recent development of genetically manipulated mice has provided new insights into GX sPLA₂ functions in vivo. According to one report, lungs from mice deficient in GX sPLA₂ (GX KO mice) have significantly reduced ovalbumin-induced inflammatory responses, suggesting a role for GX sPLA₂ in asthma (5). GX KO mice exhibit significantly reduced myocardial infarct size in response to ischemia/reperfusion injury, which has been attributed to attenuated neutrophil cytotoxic activities (18). Transgenic macrophage-specific expression of the mature form of human GX sPLA₂ results in fatal pulmonary defects in mice suggesting an involvement of GX sPLA₂ in inflammatory lung diseases (19).

In this study, we present evidence for a novel role for GX sPLA₂ in adrenal physiology. We report for the first time that GX sPLA₂ is expressed in mouse adrenal glands and cultured murine adrenal cells. Based on immunohistochemical staining, the protein is primarily localized in the zona fasciculata cells of the adrenal cortex. Our data indicate that GX sPLA₂ negatively regulates corticosterone production by altering the expression of steroidogenic acute regulatory protein (StAR), likely by suppressing LXR-mediated StAR promoter activation.

**EXPERIMENTAL PROCEDURES**

**Biochemical Reagents and Assay—**Adrenocorticotropic hormone (ACTH) for cell culture treatments was purchased from Sigma. Plasma corticosterone and ACTH levels were determined using specific radioimmunoassay kits (MP Biomedicals, Solon, OH). Progesterone quantification was carried out using an EIA kit following the manufacturer’s protocol (Cayman Chemical Co., Ann Arbor, MI). Phospholipase activity in conditioned media was measured using a colorimetric assay as described by Wooton-Kee et al. (20) with 1-palmitoyl-2-oleoyl-phosphatidylglycerol (Matreya LLC, Pleasant Gap, PA) as substrate. Essentially, mixed micelles were prepared by warming 7 mg of 1-palmitoyl-2-oleoyl-phosphatidylglycerol with a 0.2 ml mixture of 4.0% (w/v) Nonidet P-40 and 2.0% sodium deoxycholate to 37 °C, and then adding 1.8 ml of warm assay buffer (0.12 mol/liter Tris–HCl, pH 8, 12 mmol/liter CaCl₂, 0.1 mmol/liter EDTA). For enzyme assays, 10 µl of conditioned media was added to 40 µl of substrate solution. After incubating at 37 °C for 20 min, the amount of free fatty acids released was quantified using a NEFA-C kit (Wako Chemicals).

**Animals and Experimental Treatments—**Targeted deletion of the GX sPLA₂ gene was performed by InGenious Targeting Laboratory, Inc. (Stony Brook, NY), using embryonic stem cells derived from C57BL/6 mice. The targeting vector contained a Neo cassette that was trapped inside exon 1, upstream of the translation start signal, replacing 564 bp of the GX sPLA₂ gene. Heterozygous GX sPLA₂ +/– mice were bred to produce GX sPLA₂ +/– (WT) and GX sPLA₂ −/– (GX KO) mice that were used for the experiments. Male and female mice (3–5 months old) were maintained on a 10-h light/14-h dark cycle and received standard mouse chow and water ad libitum. For experiments with ACTH, mice received subcutaneous injections of 0.1 ml of saline or saline containing 4 units of ACTH (ACTHAR-GEL, Questor Pharmaceuticals, Union City, CA) at time 0, 12, and 24 h (21). Three hours after the last injection, the mice were anesthetized, and blood was collected by cardiac puncture in EDTA-containing tubes. Plasma was stored at –80 °C until analysis. For dexamethasone treatments, animals were injected intraperitoneally three times, 12 h apart with either 0.1 ml of saline (control) or dexamethasone (Sigma, 40 µg) in 0.1 ml of saline (21). Animals were euthanized 3 h after the last injection, and plasma samples were collected as described above. All procedures were approved by the Lexington Veterans Affairs Institutional Animal Care and Use Committee.

**Expression of GX sPLA₂ in Y1 Cells—**Murine Y1 BS1 adrenal cells were obtained from the American Type Culture Collection and maintained in Ham’s F-12 medium supplemented with 12.5% heat-inactivated horse serum and 2.5% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin (complete medium). C-terminal FLAG-tagged cDNA was constructed by PCR using forward (F) and reverse (R) primers containing HindIII and EcoRI restriction sites, respectively, 5’-CTGAAAGCTTATGCTGCTGCTACTG-3’ (F) and 5’-GCTGAAATCTCATTGCTACGTCGTCGTGTCCCTTTAGTCTGCTGGCAGTGGTGA-3’ (R), and mouse GX sPLA₂ cDNA as a template. The PCR product was cloned into the mammalian expression vector pcDNA 3.1 (Invitrogen) to yield a coding sequence that expressed GX sPLA₂ with a C-terminal FLAG epitope tag. cDNA for catalytically inactive mutant of GX sPLA₂ (H46Q) was generated using the QuikChange kit (Stratagene, La Jolla, CA) using C-terminal FLAG-tagged cDNA for mouse GX sPLA₂ as template and the following forward (F) and reverse (R) primers: 5’-CTGGTGCTGCTGCTCACCGAAGACTGCTGCTACCCCTCC-3’ (F) and 5’-GGATTACGAGTCTCGTGGTCTAGCAGACCGAGAG-3’ (R). DNA sequencing was performed to confirm all expression constructions. Y1 cells were plated in 100-mm dishes and transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Stable transfecants were selected with 500 µg/ml G418 (Invitrogen). For inhibitor studies, Y1 cells were incubated for 12 h with the indicated concentration of indoixam (14).

**Cell Treatments—**Where indicated, Y1 cells were incubated with 10 µM LPC (Sigma), 10 µM AA (Sigma), or vehicle in Ham’s

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Primary Adrenal Cell Culture—Primary adrenal cell cultures were established as described by Cummins et al. (22). Briefly, adrenal glands were cleaned of surrounding fat tissues, minced, and incubated for 20 min at 37 °C with rotation in complete medium 199 (10 mM HEPES, 100 units/ml penicillin, 100 units/ml streptomycin, 4 mg/ml bovine serum albumin) that was supplemented with 3.7 mg/ml collagen type I and 1 μg/ml DNase I. The cell suspension was then passed through a 70-μm nylon mesh and rinsed with complete media 199. Cells were washed once with basal culture medium (Dulbecco’s modified Eagle’s medium/F-12 (1:1), 15 mM HEPES, 1 mg/ml bovine serum albumin, 2 μg/ml insulin, 5 μg/ml transferrin, 13.7 μg/ml fibronectin, 100 units/ml penicillin, 100 units/ml streptomycin), resuspended in the same medium, and distributed into poly-l-lysine-coated 6-well plates. The cells were cultured for 3 days prior to treatments.

Real Time RT-PCR—Total RNA was isolated from mouse adrenal glands and cultured cells using the TriReagent™ (Molecular Research Center, Inc., Cincinnati, OH). RNA was further processed with DNase I (Roche Applied Science). RNA (1–2 μg) was reverse-transcribed into cDNA using a reverse transcription system (Promega, Madison, WI). After a 4-fold dilution, 5 μl was used as a template for real time RT-PCR. Amplification was done for 40 cycles using Power SYBR Green PCR master mix kit (Applied Biosystems, Foster City, CA) and DNA Engine Optical 2 System (MJ Research Inc., Waltham, MA). Quantification was performed in duplicate using the standard curve method and normalizing to 18 S. The primers used for amplification reactions are described in supplemental Table 1.

Gene Silencing with Small Interfering RNA (siRNA)—A set of pre-designed synthetic oligonucleotides directed to the mouse M-type receptor with the sequence (5′-GGUAACUCUGUACUGACCCCT-3′) (sense) and 5′-GGGUACGUACAGAUUUA-CCTG-3′ (antisense) was purchased from Ambion (Austin, TX). The oligonucleotides were transfected into Y1 cells stably expressing GX sPLA₂ by nucleofector using the TX). The oligonucleotides were transfected into Y1 cells stably expressing GX sPLA₂ and incubated for 20 min at 37 °C with rotation in complete medium 199 (10 mM HEPES, 100 units/ml penicillin, 100 units/ml streptomycin, 4 mg/ml bovine serum albumin) that was supplemented with 3.7 mg/ml collagen type I and 1 μg/ml DNase I. The cell suspension was then passed through a 70-μm nylon mesh and rinsed with complete media 199. Cells were washed once with basal culture medium (Dulbecco’s modified Eagle’s medium/F-12 (1:1), 15 mM HEPES, 1 mg/ml bovine serum albumin, 2 μg/ml insulin, 5 μg/ml transferrin, 13.7 μg/ml fibronectin, 100 units/ml penicillin, 100 units/ml streptomycin), resuspended in the same medium, and distributed into poly-l-lysine-coated 6-well plates. The cells were cultured for 3 days prior to treatments.

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corticosterone levels in GX KO mice were not due to trophic effects of ACTH. Plasma corticosterone and ACTH levels were suppressed to 6 and 11%, respectively, of base-line levels in GX KO mice following dexamethasone treatment, indicating that central negative feedback through the hypothalamic-pituitary-adrenal axis was intact in these mice (Fig. 2, A and B). For assessment of corticosterone production *ex vivo*, primary adrenal cells isolated from GX KO and WT mice were cultured in the presence or absence of 100 nM ACTH for 20 h. The amount of corticosterone secreted by the two cell types under basal conditions was not significantly different, although there was a trend for increased production by cells from GX KO mice (p = 0.07). Notably, ACTH induced significantly higher levels of corticosterone secretion in cells derived from GX KO mice compared with WT mice (Fig. 2C). Taken together, our data provide strong evidence that GX sPLA₂ regulates adrenal corticosterone synthesis through a mechanism that is at least partially independent of a systemic effect.

**GX sPLA₂ Negatively Regulates Steroid Production in Y1 Cells**—To further investigate the regulation of corticosterone production by GX sPLA₂, we established a mouse adrenocortical cell line (Y1 cells) overexpressing mouse GX sPLA₂. Stable transfection of GX sPLA₂ in Y1 cells (Y1-GX cells) resulted in a 4–5-fold increase in phospholipase activity in the culture medium (Fig. 3A) compared with cells transfected with control vector (Y1-C cells). Y1 cells do not express 21-hydroxylase enzyme required for the conversion of progesterone to corticosterone (24), and hence, progesterone secretion was analyzed in these cells as an index of steroid production. The increase in GX sPLA₂ expression resulted in a significant (p < 0.01) reduction in steroid production under basal as well as ACTH-treated conditions (Fig. 3B). Thus, consistent with our finding that deficiency of GX sPLA₂ in vivo results in an increase in corticosterone production, overexpression of GX sPLA₂ in vitro results in decreased steroid production. We have confirmed these results using at least two stably transfected cell lines and several transiently transfected lines that had variable GX sPLA₂ expression.

**GX sPLA₂ Regulation of Steroid Synthesis Is Independent of the M-type Receptor**—Published studies indicate that mouse GX sPLA₂ is a high affinity ligand for the mouse M-type receptor, and it has been proposed that binding and activation of this receptor may mediate some of the biological effects of GX sPLA₂ (14). Thus, it was of interest to determine whether the decreased glucocorticoid production in Y1-GX cells involves GX sPLA₂ binding to this receptor. Accordingly, we used an siRNA-directed gene silencing approach to investigate whether suppressing M-type receptor expression increases progesterone production in Y1-GX cells. Analysis by RT-PCR readily detected M-type receptor mRNA in both mouse adrenal glands and Y1-C cells (data not shown) as well as Y1-GX cells treated with a control siRNA (Fig. 4A). Transfection with a gene-specific siRNA significantly reduced M-type receptor mRNA expression in Y1-GX cells by 70–80% (Fig. 4A). However, this decrease in receptor expression was not accompanied by an increase in steroid production; on the contrary, progesterone production was significantly reduced (Fig. 4B). These
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FIGURE 3. Y1 cells stably overexpressing GX sPLA₂ (Y1-GX) secrete significantly less progesterone compared with control Y1 cells (Y1-C). A, phospholipase activity in 20-h conditioned media from Y1-C or Y1-GX cell cultures was determined and normalized to cell protein. B, Y1-C or Y1-GX cells were incubated for 20 h in culture media containing either 0 or 100 nM ACTH, and progesterone levels in the media, normalized to cell protein, were determined. The data shown are means ± S.E. and are representative of three independent experiments. ***, p < 0.001; **, p < 0.01; *, p < 0.05. For both cell types, ACTH treatment resulted in a significant increase in progesterone secretion (p < 0.001).

results indicate that the negative regulation of adrenal steroid production by GX sPLA₂ is not directly mediated by M-type receptor signaling.

GX sPLA₂ Regulation of Steroid Synthesis Is Dependent on Its Catalytic Activity—We investigated whether indoxam, an inhibitor of sPLA₂ enzymatic activity (25) alters adrenal steroid production. In the case of untransfected Y1 cells, treatment with 20 μM indoxam resulted in a 37% reduction in secreted phospholipase activity, which was accompanied by a significant increase in progesterone production (Fig. 5, A and B). This result suggests a role for endogenous sPLA₂ in regulating steroid production in Y1 cells. Treatment of Y1-GX cells with increasing concentrations of indoxam resulted in a significant reduction in phospholipase activity and a dose-dependent increase in progesterone secreted into the media (Fig. 5, C and D). A structural analogue of indoxam devoid of inhibitory properties failed to alter the amount of phospholipase activity or progesterone secreted by either Y1 or Y1-GX cells (data not shown). Taken together, these results provide evidence that GX sPLA₂ enzymatic activity plays a role in the regulation of adrenal steroidogenesis.

As another approach to investigate whether GX sPLA₂ negatively regulates steroidogenesis through a mechanism that requires phospholipid hydrolysis, we developed Y1 cells stably expressing an active site mutant of GX sPLA₂ whereby a histidine residue in the active site was replaced by glutamine (Y1-H46Q cells) (26). Both WT and H46Q were expressed as fusion proteins containing a FLAG epitope at the C terminus. Y1-GX and Y1-H46Q cells produced comparable levels of recombinant protein as assessed by Western blotting using an anti-FLAG antibody (Fig. 6A). As expected, an increase in phospholipase activity was only detected in conditioned media from Y1-GX cells (data not shown). In contrast to Y1-GX cells, there was no statistically significant difference in the amount of progesterone secreted by Y1-H46Q cells compared with Y1-C cells either in the absence (Fig. 6B) or presence (Fig. 6C) of ACTH, indicating that the effect of GX sPLA₂ on steroid production was dependent on its catalytic activity. We next investigated whether lipolytic products generated by GX sPLA₂ recapitulated the effect of the enzyme on progesterone production. Whereas incubations with 10 μM LPC did not significantly alter progesterone production in Y1 cells, the production was significantly reduced in cells treated with 10 μM AA (Fig. 6D). The ability of AA to inhibit progesterone production was not reversed by indoxam (data not shown).

GX sPLA₂ Regulates StAR Expression—To further understand the underlying mechanisms for GX sPLA₂ regulation of adrenal corticosteroid synthesis, the expression of genes known to be involved in steroidogenesis and/or cholesterol homeostasis was analyzed by semi-quantitative real time RT-PCR. The expression of a number of genes, namely scavenger receptor-BI, cytochrome P450 side chain cleavage enzyme (P450scc), apoE, hormone-sensitive lipase, and hydroxymethylglutaryl-CoA reductase, was not significantly altered in Y1 cells when GX sPLA₂ was overexpressed (supplemental Fig. S1A). In contrast, the expression of StAR mRNA was significantly reduced in Y1-GX cells compared with Y1-C cells in the absence (p < 0.05) and presence (p < 0.001) of ACTH (Fig. 7A). Consistent with these findings, StAR mRNA expression in adrenal glands of GX KO mice was significantly increased compared with WT mice.
under both basal ($p < 0.05$) and ACTH-treated ($p < 0.01$) conditions (Fig. 7B), whereas the expression of SR-B1 and P450scc was not different in the two strains (supplemental Fig. S1B). StAR protein levels were also inversely related to GX sPLA₂ expression in mouse adrenal glands (data not shown) and Y1 cells (Fig. 7C). Treatment of Y1 cells with 10 μM AA mimicked the effects of GX sPLA₂ on StAR mRNA expression (supplemental Fig. S1C). Interestingly, our analysis also showed that the expression of LXR target genes ABCA1, ABCG1 (Fig. 7D), and SREBP-1c (data not shown) were significantly reduced in Y1-GX cells compared with Y1-C cells.

**GX sPLA₂ Regulates StAR Promoter Activity**—Previous studies by Cummins et al. (22) established that StAR is an LXR target gene. We confirmed that StAR mRNA expression is significantly increased when Y1-C cells were treated with LXR and RXR agonists T0901317 and 9-cis-retinoic acid, respectively (Fig. 8A). Interestingly, agonist-induced StAR expression was significantly reduced in Y1-GX cells, suggesting that GX sPLA₂ modulates LXR activation of StAR expression. To provide additional evidence for this effect, HEK293 cells were co-transfected with mLXRx, mRXRx, and a luciferase reporter construct containing a portion of the StAR promoter (StARp254-luc) (22) along with either the GX sPLA₂ or control expression vector. Reporter expression was significantly enhanced in Y1-C cells treated with LXR and RXR agonists, consistent with the presence of a functional LXRE in the StAR promoter (Fig. 8B) (22). Interestingly, both basal and ligand-induced luciferase activity was significantly reduced in cells overexpressing GX sPLA₂ (Fig. 8B) indicating that GX sPLA₂ regulates LXR-mediated StAR promoter activation. To confirm that the LXRE-like region of the StAR promoter is important in GX sPLA₂-mediated regulation of StAR, a luciferase reporter construct containing the StAR promoter with a mutation in the LXRE half-sites (StAR-m) (22) was also assessed. LXR and RXR agonist T0901317 and 9-cis-retinoic acid failed to cause enhancement in luciferase activity in cells co-transfected with StAR-m; interestingly, GX sPLA₂ also failed to suppress promoter activity in the mutant construct (Fig. 8B). Finally, to directly determine whether GX sPLA₂ regulates LXR activation in adrenal cells, we carried out LXRE promoter reporter assays in Y1 cells. As expected, incubations with T090317 resulted in a dose-dependent increase in luciferase activity in Y1 cells transfected with a control expression vector (Fig. 8C). Notably, overexpression of GX sPLA₂ resulted in a significant reduction in reporter activity for all concentrations of LXR agonist tested (Fig. 8C).
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StAR is a nuclearly encoded mitochondrial protein that mediates the rate-limiting step of steroid synthesis by delivering cholesterol to the inner mitochondrial membrane, where steriodogenic enzymes reside. Many steriodogenic stimuli that increase steriod hormone biosynthesis increase StAR protein production, whereas factors responsible for the negative regulation of steriodogenesis cause disruption of StAR expression (27). In this study, we report the novel findings that GX sPLA$_2$, is expressed in mouse adrenal glands and that deficiency of this protein in mice leads to increased levels of circulating corticosterone and a more robust response to ACTH. GX sPLA$_2$ protein appears to be localized mainly in zona fasciculata and zona reticularis and not in zona glomerulosa regions of the adrenal cortex, which is consistent with the observation that GX sPLA$_2$ influences glucocorticoid production but not mineralocorticoid production. Our data indicate that GX sPLA$_2$ exerts a direct effect in the adrenal gland to negatively regulate StAR expression via transcriptional suppression of the gene, most likely by reducing the activation of LXR.

Because GX sPLA$_2$ is reportedly expressed in rat brain and neuronal cells (28), we considered the possibility that this molecule acts centrally to modulate adrenal function. However, altered steriod hormone production in GX KO mice appears to be primarily due to a direct effect of this molecule on adrenal cells and not a defect in the hypothalamic-pituitary-adrenal axis based on the following findings. 1) GX KO mice respond normally to dexamethasone by markedly reducing corticosterone levels. 2) Plasma ACTH levels in GX KO mice are not altered compared with WT mice. 3) Secretion of corticosterone by primary adrenal cells from GX KO mice in response to ACTH is increased compared with cells from WT mice. 4) Y1 adrenal cells overexpressing GX sPLA$_2$ secrete significantly less progesterone compared with control Y1 cells.

Results from gene expression analyses indicated that StAR expression was significantly down-regulated in Y1 cells overexpressing GX sPLA$_2$ compared with control Y1 cells, whereas StAR was up-regulated in adrenal glands from GX KO mice compared with WT mice. Thus, GX sPLA$_2$ appears to negatively regulate StAR, the rate-limiting protein in steriodogenesis. Results from luciferase reporter assays support the conclusion that increased GX sPLA$_2$ expression leads to reduced StAR promoter activity in Y1 cells. Given its key role in steriod production, StAR mRNA expression is under both positive and negative control by a variety of transcription factors, including AP-1, SF-1, C/EBP, Sp-1, GATA, DAX-1, and SREBP1α (29). A role for LXR in the transcriptional regulation of StAR expression has also been described. Administration of the synthetic LXR agonist T090317 increases plasma corticosterone levels (22, 30) and adrenal StAR mRNA expression in mice (22), although other studies with a different LXR agonist reported a suppressive effect on StAR expression (31). An LXR-response element (LXRE)-like sequence has been identified in the StAR promoter and shown to be functional (22). In this study, we confirmed that LXR and RXR agonists activate StAR promoter activity in adrenal Y1 cells. Basal and LXR-induced promoter activity was blunted in cells overexpressing catalytically active GX sPLA$_2$. The ability of GX sPLA$_2$ to suppress reporter activity was abolished when LXRE-like sequences were mutated in...
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the StAR promoter reporter construct. Taken together, our data indicate that hydrolytic products generated by the enzyme may suppress LXR-mediated trans-activation. This possibility was supported by results from luciferase reporter assays using a synthetic LXRE promoter construct and also the finding that other known LXR target genes (ABCA1, ABCG1, and SREBP1c) are down-regulated in adrenal cells when GX sPLA₂ is overexpressed.

Given the known potent ability of GX sPLA₂ to hydrolyze membrane phospholipids (32–34), it seems likely that AA, or other polyunsaturated fatty acids generated by GX sPLA₂, mediates the suppressive effect on LXR activation. An abundance of data has established that polyunsaturated fatty acids, including AA, serve as endogenous LXR antagonists in various cell lines (35, 36). Perhaps counter-intuitively, LXRαβ⁻/⁻ mice exhibit increased StAR expression and glucocorticoid production, leading to the suggestion that activated LXR not only up-regulates the expression of steroidogenic genes but may also repress their expression when in the unliganded state (22, 30). Thus, basal StAR expression may be increased in LXRαβ⁻/⁻ mice due to the removal of the repressive effect of LXR. It would be interesting to investigate whether GX sPLA₂ plays a role in LXR-mediated repression of steroidogenesis under basal conditions. We cannot rule out the possibility that GX sPLA₂ exerts an effect on StAR promoter activity through alternative pathways in addition to suppression of LXR-mediated StAR activation. Earlier studies have shown that AA metabolites generated by COX-2 inhibit steroidogenesis (37), and prostaglandin F2α negatively regulates corticosteroid production by inhibiting StAR gene expression through the AP-1 family member c-Fos (38). Contrary to these reports, certain other studies indicate AA can activate steroidogenesis (39) through the generation of 15-lipoxygenase-derived metabolite(s) (40).

The effect of GX sPLA₂ overexpression to reduce progesterone secretion in Y1 cells was reversible by indoxam, an active site-directed competitive inhibitor of sPLA₂s (25). Importantly, control Y1 cells showed increased steroid production when incubated with indoxam, suggesting that endogenously expressed GX sPLA₂ exerts a negative effect on steroidogenesis. Indoxam not only inhibits enzymatic activity but also blocks the binding of sPLA₂s to the M-type receptor (25). To our knowledge, we provide the first demonstration that mouse adrenal glands and a murine adrenal cell line express the M-type receptor. However, the finding that the catalytically inactive H46Q mutant did not alter steroid production in Y1 cells sheds doubt on the role of M-type receptor binding, because it would be expected that this mutant would bind the receptor similarly to WT GX sPLA₂. Furthermore, silencing of M-type receptor expression in Y1 cells overexpressing GX sPLA₂ did not restore steroid production to the level of control Y1 cells, indicating that M-type receptor binding is not directly involved in the negative regulation of steroidogenesis mediated by GX sPLA₂. Indeed, reduced M-type receptor expression was associated with a significant decrease in steroid production in Y1-GX cells. Future studies will investigate whether this reduction is due to the ability of the receptor to internalize and/or inactivate GX sPLA₂, as has been suggested by other researchers (16, 41).

Alternatively, the M-type receptor may play another role in regulating steroidogenesis that is independent of GX sPLA₂.

Another intriguing observation from this study is that ACTH significantly increased the amount of enzyme mass (Fig. 6A) and activity (not shown) secreted by GX sPLA₂-overexpressing Y1 cells. It is likely that this increase reflects post-transcriptional regulation, because the SV40 promoter driving recombinant GX sPLA₂ expression would not be expected to be regulated by ACTH. Phospholipase activity secreted by control Y1 cells was also increased by ACTH treatment, suggesting an effect on endogenously expressed GX sPLA₂. These data suggest a cross-talk in adrenal cells whereby ACTH up-regulates GX sPLA₂ secretion, which in turn negatively regulates ACTH-mediated steroid production during stress. The mechanism by which ACTH post-transcriptionally regulates GX sPLA₂ is not known. Unlike most members of the sPLA₂ family, GX sPLA₂ is expressed in an inactive form and requires removal of 11 amino acid residues at the N terminus for catalytic activity (3).

The identity of the protease(s) responsible for the proteolytic processing and activation of GX sPLA₂ has not been elucidated, and it is tempting to speculate that ACTH may promote the proteolytic cleavage of GX sPLA₂ through an unknown mechanism. However, Western blot analysis suggests that the bulk of GX sPLA₂ in conditioned media from transfected Y1 cells in the presence and absence of ACTH corresponds to the mature form of the protein.

Interestingly, plasma ACTH levels were not altered in GX KO mice despite significantly increased corticosteroid levels, as might be expected if the hypothalamic-pituitary-adrenal axis was functioning normally in these mice. Our findings are reminiscent of LXRαβ⁻/⁻ mice, where similar increases in plasma corticosteroid levels were accompanied by no differences in plasma ACTH (22). It seems possible that in both mouse models, the negative feedback control of the hypothalamic-pituitary-adrenal axis may be somewhat disrupted, given the recently described role of LXR in regulating the hypothalamic-pituitary-adrenal axis at the pituitary level (31, 42). Glucocorticoids exert pleiotrophic effects on whole body metabolism and energy partitioning. Centrally, they increase food intake and reduce energy expenditure, although peripherally they promote insulin resistance and fat accumulation (43). A preliminary analysis indicates that GX KO mice have a modest increase in body weight and adiposity compared with WT littermates, but no change in food intake. Studies are currently ongoing to investigate whether alterations in adiposity in GX KO mice are secondary to hypercorticosteronemia or perhaps a direct consequence of suppressed LXR activation in adipose tissues of these mice, because LXR is known to play a role in modulating adipogenesis by regulating adipocyte-specific gene expression (44).

In conclusion, our data clearly demonstrate that GX sPLA₂ is expressed in adrenal glands where it plays a direct role in negatively regulating corticosteroid production. GX sPLA₂ down-regulates adrenal StAR mRNA expression, at least partly by suppressing LXRE-dependent promoter activation. It remains

³ X. Li, P. Shridas, K. Forrest, W. Bailey, and N. R. Webb, unpublished data.
to be determined whether GX sPLA2 plays a similar functional role in other steroidogenic tissues, including ovaries, testis, and brain where this gene is expressed in abundance (3, 45).

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REFERENCES

1. Lambau, G., and Gelb, M. H. (2008) Annu. Rev. Biochem. 77, 495–520
2. Cupillard, L., Koumanov, K., Mattei, M. G., Lazdunski, M., and Lambeau, G. (1997) J. Biol. Chem. 272, 15745–15752
3. Morioka, Y., Saiga, A., Yokota, Y., Suzuki, N., Ikeda, M., Ono, T., Nakano, K., Fujii, N., Ishizaki, A., Irita, H., and Hanasaki, K. (2000) Arch. Biochem. Biophys. 381, 31–42
4. Suzuki, N., Ishizaki, J., Yokota, Y., Higashino, K., Ono, T., Ikeda, M., Fuji, N., Kawamoto, K., and Hanasaki, K. (2000) J. Biol. Chem. 275, 5785–5793
5. Henderson, W. R., Jr., Chi, E. Y., Bollinger, J. G., Tien, Y. T., Ye, X., Castelli, L., Rubtsov, Y. P., Singer, A. G., Chiang, G. K., Nevalainen, T., Rudensky, A. Y., and Gelb, M. H. (2007) J. Exp. Med. 204, 865–877
6. Mitsuishi, M., Masuda, S., Kudo, I., and Murakami, M. (2006) Biochim. Biophys. Acta 1761, 1289–1300
7. Hanasaki, K., Yamada, K., Yamamoto, S., Ishimoto, Y., Saiga, A., Ono, T., Ikeda, M., Notoya, M., Kamitani, S., and Irita, H. (2002) J. Biol. Chem. 277, 29116–29124
8. Karabina, S. A., Brochériou, I., Le Naour, G., Agrapart, M., Durand, H., Gelb, M., Lambau, G., and Ninio, E. (2006) FASEB J. 20, 2547–2549
9. Mitsushi, M., Masuda, S., Kudo, I., and Murakami, M. (2006) Biochem. J. 393, 97–106
10. Koduri, R. S., Grönnroos, J. O., Laine, V. J., Le Calvez, C., Lambau, G., Nevalainen, T. J., and Gelb, M. H. (2002) J. Biol. Chem. 277, 5849–5857
11. Granata, F., Petroiri, A., Boilard, E., Bezzine, S., Bollinger, J. D., Del Vecchio, L., Gelb, M. H., Lambau, G., Marone, G., and Triggiani, M. (2005) J. Immunol. 174, 464–474
12. Rouault, M., Le Calvez, C., Boilard, E., Surrel, F., Singer, A., Ghomashchi, F., Bezzine, S., Scarchistol, S., Bollinger, J. D., and Lambau, G. (2007) Biochemistry 46, 1647–1662
13. Hanasaki, K., and Arita, H. (2002) Prostaglandins Other Lipid Mediat. 68, 71–82
14. Yokota, Y., Notoya, M., Higashino, K., Ishimoto, Y., Nakano, K., Arita, H., and Hanasaki, K. (2001) FEBs Lett. 509, 250–254
15. Saiga, A., Uozumi, N., Ono, T., Seno, K., Ishimoto, A., Irita, H., Shimizu, T., and Hanasaki, K. (2005) Prostaglandins Other Lipid Mediat. 75, 79–89
16. Fujioka, D., Saito, Y., Kobayashi, T., Yano, T., Tetsu, H., Ishimoto, Y., Suzuki, N., Yokota, Y., Nakamura, T., Ohta, J. E., Kanazawa, M., Kawabata, K., Hanasaki, K., and Kugiyama, K. (2008) Circulation 117, 2977–2985
17. Curfs, D. M., Ghesquiere, S. A., Vergouwe, M. N., van der Made, I., Gijbels, M. J., Greaves, D. R., Verbeek, J. S., Holker, M. H., and de Winther, M. P. (2008) J. Biol. Chem. 283, 21640–21648