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Ectopically Expressed Pro-group X Secretory Phospholipase A_2 Is Proteolytically Activated in Mouse Adrenal Cells by Furin-like Proprotein Convertases

*IMPLICATIONS FOR THE REGULATION OF ADRENAL STEROIDOGENESIS**

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Joseph D. Layne[‡], Preetha Shridas[§], and Nancy R. Webb[§1]

From the [‡]Department of Pharmacology and Nutritional Sciences, Division of Nutritional Sciences, the Saha Cardiovascular Research Center, and the [§]Department of Internal Medicine, University of Kentucky Medical Center, Lexington, Kentucky 40536

Background: GX $sPLA_2$ is expressed as an inactive pro-enzyme; the protease(s) responsible for pro-GX $sPLA_2$ processing are unknown.
Results: Epitope-tagged pro-GX $sPLA_2$ is proteolytically activated by furin and PCSK6 in adrenal cells.
Conclusion: Furin-like proprotein convertases regulate GX $sPLA_2$ activity.
Significance: Identifying the factors involved in activating GX $sPLA_2$ provides novel insight into the mechanisms surrounding its regulation.

Group X secretory phospholipase A_2 (GX $sPLA_2$) hydrolyzes mammalian cell membranes, liberating free fatty acids and lysophospholipids. GX $sPLA_2$ is produced as a pro-enzyme (pro-GX $sPLA_2$) that contains an N-terminal 11-amino acid propeptide ending in a dibasic motif, suggesting cleavage by a furin-like proprotein convertase (PC). Although propeptide cleavage is clearly required for enzymatic activity, the protease(s) responsible for pro-GX $sPLA_2$ activation have not been identified. We previously reported that GX $sPLA_2$ negatively regulates adrenal glucocorticoid production, likely by suppressing liver X receptor-mediated activation of steroidogenic acute regulatory protein expression. In this study, using a FLAG epitope-tagged pro-GX $sPLA_2$ expression construct (FLAG-pro-GX $sPLA_2$), we determined that adrenocorticotropic hormone (ACTH) enhanced FLAG-pro-GX $sPLA_2$ processing and phospholipase activity secreted by Y1 adrenal cells. ACTH increased the expression of furin and PCSK6, but not other members of the PC family, in Y1 cells. Overexpression of furin and PCSK6 in HEK 293 cells significantly enhanced FLAG-pro-GX $sPLA_2$ processing, whereas siRNA-mediated knockdown of both PCs almost completely abolished FLAG-pro-GX $sPLA_2$ processing in Y1 cells. Expression of either furin or PCSK6 enhanced the ability of GX $sPLA_2$ to suppress liver X receptor reporter activity. The PC inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone significantly suppressed FLAG-pro-GX $sPLA_2$ processing and $sPLA_2$ activity in Y1 cells, and it significantly attenuated GX $sPLA_2$-dependent inhibition of steroidogenic acute regulatory protein expression and progesterone production. These findings provide strong evidence that pro-GX $sPLA_2$ is a substrate for furin and PCSK6 proteolytic processing and define a novel mechanism for regulating corticosteroid production in adrenal cells.

The secreted phospholipase A_2 ($sPLA_2$)[2] enzymes hydrolyze membrane phospholipids at the *sn*-2 position to liberate free fatty acids and lysophospholipids. The $sPLA_2$ family is characterized by their low molecular mass (~14–18 kDa), requirement for millimolar concentrations of Ca^{2+}, and utilization of a highly conserved catalytic histidine within their active sites. Eleven $sPLA_2$ members have been identified (group IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA, and XIIB PLA_2-like protein that lacks catalytic activity) and placed into different groups based on the number and position of conserved cysteine residues that form disulfide bridges, generating a semi-rigid three-dimensional structure (1). Members of the $sPLA_2$ family exhibit unique tissue distributions and disparate substrate specificities, reflecting their distinct roles in a range of physiological processes.

Among the $sPLA_2$s, group X (GX) $sPLA_2$ has the most potent hydrolytic activity toward zwitterionic phospholipids, including phosphatidylcholine (2), the most abundant phospholipid in mammalian plasma membranes and lipoprotein particles. GX $sPLA_2$ has a wide tissue distribution, including the small intestine, testes, brain, pancreas, lung, thymus, spleen, peripheral blood leukocytes, among others (3, 4). Notably, GX $sPLA_2$ appears to preferentially hydrolyze arachidonate and linoleate at the *sn*-2 position of phosphatidylcholine-containing lipoproteins (3, 5). The predilection of GX $sPLA_2$ for arachidonate has important consequences with respect to the generation of bioactive lipids. For example, *in vitro* studies suggest that hydrolysis of phosphatidylcholine by GX $sPLA_2$ results in cyclo-

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[1] To whom correspondence should be addressed: Dept. of Pharmacology and Nutritional Sciences, 535 CT Wethington Bldg., 900 S. Limestone St., Lexington, KY 40536. Tel.: 859-218-1385; Fax: 859-257-3646; E-mail: nrwebb1@uky.edu.

[2] The abbreviations used are: $sPLA_2$, secreted phospholipase A_2; GX, group X; pro-GX $sPLA_2$, GX $sPLA_2$ produced as pro-enzyme; PC, proprotein convertase; StAR, steroidogenic acute regulatory protein; LXR, liver X receptor; PG, prostaglandin; qRT, quantitative RT; $sPLA_2$-R, $sPLA_2$ receptor; mLXR, mouse liver X receptor; mRXR, mouse retinoid X receptor.

oxygenase-2 (COX-2)-dependent prostaglandin E_2 (PGE_2) production (6). In lipopolysaccharide (LPS)-treated mouse peritoneal macrophages, the addition of recombinant GX sPLA$_2$, but not GIB or GIIA, results in a robust increase in the production of PGE_2 and thromboxane A_4 (7). In a Th2 cytokine-driven mouse model of asthma, GX sPLA$_2$ has been implicated in the production of eicosanoids, including PGE_2, PGD_2, leukotriene B_4, and cysteinyl leukotrienes (8).

The generation of C57BL/6 mice with targeted deletion of GX sPLA$_2$ (GX KO mice) has led to new insights into novel mechanisms by which GX sPLA$_2$ modulates physiological processes. Our laboratory reported that GX KO mice fed a normal rodent diet gain more weight and exhibit increased adiposity compared with wild-type mice (9). We also determined that stromal vascular cells isolated from adipose tissue of GX KO mice accumulate significantly more triglyceride when induced to differentiate into adipocytes compared with cells from wild-type mice. Conversely, overexpression of GX sPLA$_2$ in OP9 pre-adipocytes results in a significant 50% reduction in triglyceride accumulation during differentiation into mature adipocytes, an effect that was associated with significantly reduced induction of adipogenic genes, including *Pparγ*, *Srebf1*, *Scd1*, and *Fasn*. Activation of the liver X receptor (LXR), a nuclear receptor known to up-regulate adipogenic gene expression, was suppressed in OP9 cells when GX sPLA$_2$ was overexpressed, leading us to conclude that GX sPLA$_2$ negatively regulates adipogenesis, possibly by suppressing LXR activation. We also determined that GX sPLA$_2$ suppresses LXR activation in macrophages, resulting in reduced expression of ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1) (10). Consequently, macrophages from GX KO mice exhibit increased cellular cholesterol efflux and decreased cellular cholesterol content (10). GX sPLA$_2$ is expressed in adrenal cells, where it suppresses corticosteroid production through a mechanism that also appears to involve LXR. Compared with wild-type mice, GX KO mice have significantly increased plasma corticosterone levels under both basal and adrenocorticotropic hormone (ACTH)-induced conditions. The expression of steroidogenic acute regulatory protein (StAR), the rate-limiting protein in corticosteroid production, is significantly increased in adrenal glands from GX KO mice compared with wild-type adrenal glands. Conversely, in the mouse adrenal Y1 cell line, overexpression of GX sPLA$_2$ suppresses StAR expression. Results from luciferase reporter assays indicated that GX sPLA$_2$ antagonizes StAR promoter activity and LXR-mediated StAR promoter activation in adrenal cells. In summary, results from gain-of-function and loss-of-function studies in multiple tissues indicate that GX sPLA$_2$ modulates cellular metabolism by negatively regulating LXR target gene expression through a mechanism that is dependent on GX sPLA$_2$ hydrolytic activity.

Given the potent ability of GX sPLA$_2$ to hydrolyze cell membranes and generate bioactive lipid mediators, its hydrolytic activity is likely under tight regulation. GX sPLA$_2$ is one of only three sPLA$_2$s produced as an inactive pro-enzyme, such that cleavage of an N-terminal pro-segment is necessary for its enzymatic activity (3). Studies in transgenic mice with constitutive GX sPLA$_2$ expression indicate that the inactive precursor is the predominant form expressed in most tissues under normal conditions (11). However, enzymatically active GX sPLA$_2$ is detected in the transgenic mice in tissues with inflammatory granulation, suggesting that proteolytic activation may occur during inflammation. The N-terminal pro-segment of GX sPLA$_2$ includes 11 amino acids ending in a dibasic motif, suggesting cleavage by member(s) of the furin-like proprotein convertase (PC) family. Recently, Jemel *et al.* (12) showed that in transfected HEK 293 cells, the second residue within the dibasic doublet is necessary and sufficient for GX sPLA$_2$ processing and hydrolytic activity. Furthermore, using a panel of nonspecific protease inhibitors, the involvement of PCs in GX sPLA$_2$ maturation and activation in transfected 293 cells was confirmed. However, the identity of the individual PCs involved in GX sPLA$_2$ processing in physiologically relevant tissues remains to be investigated.

During the course of studying GX sPLA$_2$ in adrenal cells, we noted significantly increased phospholipase activity secreted by Y1 cells stably transfected with a GX sPLA$_2$ expression construct and, to a lesser extent, control-transfected Y1 cells, in response to ACTH treatment (13). We reasoned that this increase in secretion reflected post-transcriptional regulation of GX sPLA$_2$, because the promoter driving recombinant GX sPLA$_2$ expression in our cell system would not be expected to be regulated by ACTH. Thus, mouse Y1 cells provide us a physiologically relevant model for understanding GX sPLA$_2$ regulation. In this study, we establish that an epitope-tagged form of pro-GX sPLA$_2$ is proteolytically activated in Y1 adrenal cells by furin and PCSK6, two members of the PC family. We also provide evidence that PC-dependent proteolytic activation of pro-GX sPLA$_2$ is enhanced under ACTH-stimulated conditions, suggesting a novel mechanism for regulating adrenal steroidogenesis.

EXPERIMENTAL PROCEDURES

Biochemical Reagents and Assays—Adrenocorticotropic hormone (ACTH) was purchased from Sigma. The quantification of progesterone levels in cell media was achieved using a progesterone EIA kit (Cayman Chemical); sPLA$_2$ activity was measured using our previously published method with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylglycerol (Matreya) as substrate (14) or a commercially available sPLA$_2$ assay kit (Cayman Chemical).

GX sPLA$_2$ Expression in Y1 Adrenal Cells—Murine Y1 adrenal cells were purchased from American Type Culture Collection (ATCC) and maintained in F-12K media (ATCC) supplemented with 2.5% nonheat-inactivated fetal bovine serum (Invitrogen), 15% nonheat-inactivated horse serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin. A C-terminal 3× FLAG-tagged mouse GX sPLA$_2$ cDNA was developed by PCR using forward (5′-CTGAAGCTTATGCTGCTGCTACTG-3′) and reverse (5′-ATGAATTCTCACTTGTCATCGTCGTC-CTTGTAGTCGATATCGTGGTCCTTGTAGTCTCCATCG-TGGTCCTTGTAGTAGTCATT-3′) primers containing HindIII and EcoRI restriction sites, respectively, and a previously generated pcDNA3.0 (Invitrogen) expression construct encoding GX sPLA$_2$ fused to a single C-terminal FLAG tag as template (13). Thus, the amplified fragment containing the GX sPLA$_2$ cDNA did not include any 5′- or 3′-noncoding sequences derived from the GX sPLA$_2$ gene. Y1 cells were cultured on 100-mm dishes and

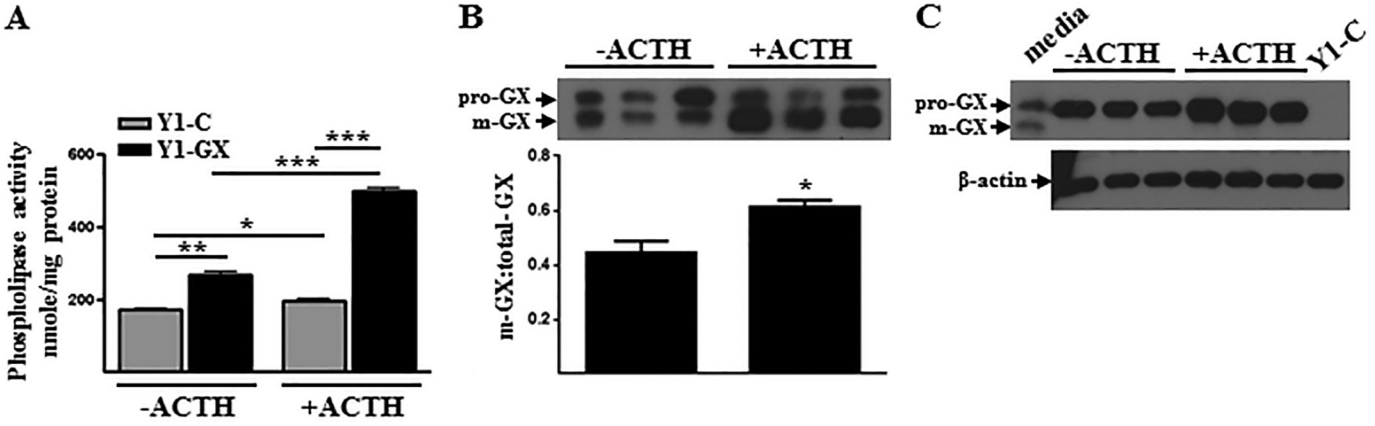

FIGURE 1. **ACTH increases FLAG-pro-GX sPLA$_2$ processing and phospholipase activity secreted by Y1 adrenal cells.** Y1 adrenal cells were stably transfected with either a control expression vector (*Y1-C*) or a vector expressing 3× FLAG-tagged GX sPLA$_2$ (*Y1-GX*) and then incubated with 0 or 100 nM ACTH for 20 h. *A,* phospholipase activity in conditioned media was measured ($n = 6$). *B,* conditioned media (20 μl) was immunoblotted using an anti-FLAG antibody (*top*); results from densitometric analyses are shown below ($n = 3$). Data are expressed as the ratio of m-GX sPLA$_2$/total-GX sPLA$_2$ in the media. *C,* whole-cell lysates (10 μg of protein) were immunoblotted using anti-FLAG antibody (*top*) or β-actin (*bottom*). For comparison, conditioned media from vehicle-treated Y1-GX cells (*media*) and lysates from Y1-C cells (*Y1-C*) were also analyzed. Data are means ± S.E. and are representative of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

transfected with either 3× FLAG-tagged GX sPLA$_2$ or the corresponding pcDNA3.0 using Lipofectamine 2000 according to the manufacturer's instructions. G418 was then used to select for stable transfectants.

Gene Silencing with Small Interfering RNA (siRNA)—A set of ON-TARGET plus SMART pool siRNA synthetic oligonucleotides directed toward the mouse PCSK6 target sequences (5′-UAAACAAGCUUUCGAGUAU-3′, 5′-GGUCAGAGAUGAACGUCCA-3′, 5′-CGAGAUGCCUGGCGUCACA-3′, and 5′-GAUGAGACCUUCUGCGCGA-3′) and toward the mouse furin target sequences (5′-CGACAUCGGCAAACGGCUA-3′, 5′-GAAGAAUCAUCCCGACCUA-3′, 5′-GAAAGUGAGCCAUUCGUAU-3′, and 5′-GCGCCACACAGUUCGGCAA-3′) were purchased from Thermo Scientific. The ON-TARGET plus nontargeting pool was used as a control. Cells were transfected with siRNAs using Dharmafect 1 transfection reagent (Thermo Scientific) according to the manufacturer's instructions.

Quantitative RT-PCR—Total RNA was isolated from mouse Y1 adrenal cells using the RNeasy mini kit (Qiagen). RNA (1 μg) was reverse-transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystems). cDNA was diluted 4-fold at which point quantitative PCR was performed with Power SYBR Green PCR master mix kit (Applied Biosystems) and the iQ5 multicolor RT-PCR Detection System (Bio-Rad) through 40 cycles of amplification. Primer sequences used in qRT-PCR are available upon request.

Immunoblotting—Proteins were resolved using SDS-PAGE. The 3× FLAG-tagged GX sPLA$_2$ chimeric protein was detected by Western blot analysis using anti-FLAG M2 primary antibody (Stratagene). StAR was detected using anti-StAR primary antibody (Santa Cruz Biotechnology). The secondary HRP-conjugated antibody was from Abcam.

Reporter Assays—HEK 293 cells were grown to ~75% confluence and then transfected with mouse 3× FLAG-tagged GX sPLA$_2$ expression vector or a control vector encoding green fluorescent protein (GFP) (0.4 μg), along with pTK-3-LXRE-Luc reporter construct (0.4 μg), mouse liver X receptor (mLXRα) (0.1 μg), mouse retinoid X receptor (mRXR) (0.1 μg), *Renilla* luciferase (Promega, 0.02 μg), and either furin, PCSK6 (Origene), or GFP expression constructs (0.4 μg) using Lipofectamine 2000 according to the manufacturer's protocol. The mLXR and mRXR expression constructs and the 3× LXR element reporter plasmid were gifts from Dr. Peter Tontonoz, UCLA. After 18 h, cells were incubated in fresh media (high glucose DMEM (HyClone), supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin), and either 0 or 1 μM T0901317 for 24 h. Cells were washed with DPBS and harvested in 1× PLB buffer (Promega). Luminescence was measured using the dual-luciferase reporter assay system (Promega).

Statistics—Statistical significance is reported in the figures and/or figure legends. Statistical analysis of data sets was performed using GraphPad Prism. For experiments in which only two groups were compared, a two-tailed Student's *t* test was performed. For comparison of means between more than two groups, a one- or two-way analysis of variance was used where appropriate. Post hoc analysis was done using Bonferroni's test. All data sets conformed to the constraints of the parametric analysis.

RESULTS

ACTH Increases Ectopically Expressed GX sPLA$_2$ Proteolytic Activation in Y1 Adrenal Cells—To investigate post-translational mechanisms involved in regulating GX sPLA$_2$ activity in adrenal cells, we generated mouse Y1 cells stably expressing GX sPLA$_2$ fused to a C-terminal 3× FLAG sequence (Y1-GX) under the control of a CMV-driven promoter. This chimeric construct provided us with a model for studying the post-translational processing of GX sPLA$_2$ based on differences in the molecular mass of the inactive precursor, pro-GX (~15.2 kDa) and the mature form of the enzyme, m-GX (~13.9 kDa). Phospholipase activity secreted by Y1-GX cells was increased ~1.5-fold compared with Y1-C cells (Fig. 1*A*) indicating that GX sPLA$_2$ expression was only modestly increased over endogenous levels in Y1-GX cells. In accordance with our previous findings (13), ACTH treatment resulted in a significant increase

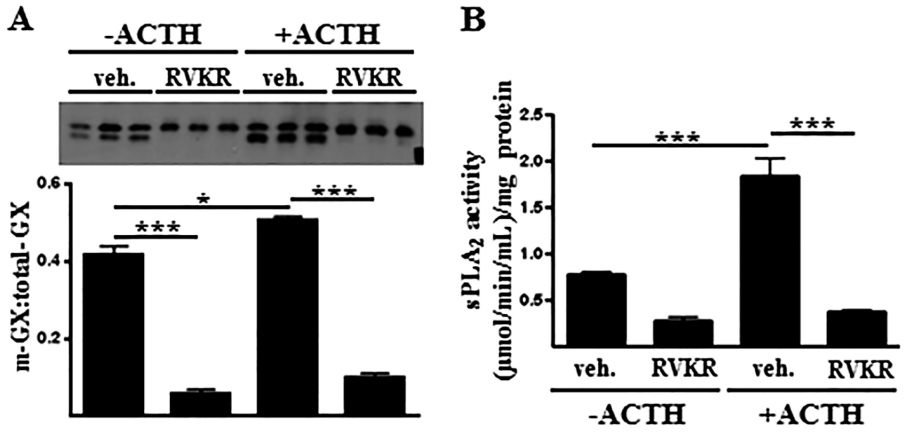

FIGURE 2. **FLAG-pro-GX sPLA$_2$ processing and activity are blocked by the furin-like proprotein convertase inhibitor RVKR.** *A,* Y1-GX cells were incubated with 0 or 100 nM ACTH in the presence or absence of 25 μM RVKR for 20 h, at which time media were collected for immunoblotting with anti-FLAG (*top*); results from densitometric analyses are shown below. Data are expressed as the ratio of m-GX sPLA$_2$/total-GX sPLA$_2$ in the media. *B,* sPLA$_2$ activity in the media was quantified. Data are means ± S.E. and are representative of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *veh,* vehicle.

in the phospholipase activity secreted by control-transfected Y1 cells (Y1-C) (Fig. 1*A*). The increase in phospholipase activity in response to ACTH was even more robust in Y1-GX cells (Fig. 1*A*). Associated with the increase in phospholipase activity in ACTH-treated Y1-GX cells was an increase in FLAG-pro-GX sPLA$_2$ processing, expressed as the ratio of m-GX/total-GX sPLA$_2$, in response to ACTH stimulation (Fig. 1*B*). In the case of untreated Y1-GX cells, ~44% of total FLAG-GX sPLA$_2$ present in the media was processed to the mature form. This contrasts to ACTH-treated cells, where ~61% of secreted FLAG-GX sPLA$_2$ had undergone proteolytic processing. m-GX sPLA$_2$ was not detected in cell lysates of Y1-GX cells in either the absence or presence of ACTH (Fig. 1*C*). Taken together, these results suggest that ACTH enhances the phospholipase activity secreted by Y1-GX cells, at least partly by increasing the proteolytic processing of pro-GX sPLA$_2$, and that pro-GX sPLA$_2$ is the major, if not exclusive, form detected intracellularly.

FLAG-Pro-GX sPLA$_2$ Processing and Activity Are Blocked by the Furin-like Proprotein Convertase Inhibitor RVKR—Based on results from studies using a panel of protease inhibitors, the family of furin-like PCs has recently been implicated in the proteolytic activation of GX sPLA$_2$ in transfected HEK 293 cells (12). To investigate the role of PCs in regulating GX sPLA$_2$ proteolytic activation in mouse Y1 adrenal cells, sPLA$_2$ activity and FLAG-pro-GX sPLA$_2$ processing was assessed in Y1-GX cells treated with the PC inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone. After overnight incubation, both the precursor and mature forms of FLAG-GX sPLA$_2$ were detected in the media of vehicle-treated Y1-GX cells, and ACTH treatment significantly increased the ratio of m-GX sPLA$_2$/total-GX sPLA$_2$ (Fig. 2*A*). However, the processing of FLAG-pro-GX-sPLA$_2$ was almost completely abolished in RVKR-treated cells, both in the absence and presence of ACTH. This decrease in processing was accompanied by a significant reduction in sPLA$_2$ activity secreted by the cells (Fig. 2*B*). These results demonstrate a role for furin-like PCs in the proteolytic activation of GX sPLA$_2$ in Y1 adrenal cells under both basal and ACTH-stimulated conditions.

Furin and PCSK6 Expression Is Increased in Y1 Cells Treated with ACTH—To identify candidate PCs that may be responsible for regulating GX sPLA$_2$ activity in adrenal cells, we quantified mRNA abundance for each member of the PC family in Y1 cells under basal and ACTH-stimulated conditions. Among the six candidate convertases, only four were expressed at appreciable levels (*Ct* value <35) as follows: furin, PCSK5, PCSK6, and PCSK7 (Fig. 3). Interestingly, both furin and PCSK6 mRNA abundance were significantly increased in response to overnight incubation with ACTH, consistent with the enhanced pro-GX sPLA$_2$ processing. Therefore, we further investigated the role of furin and PCSK6 in regulating GX sPLA$_2$ processing in Y1 cells.

FLAG-Pro-GX sPLA$_2$ Is Proteolytically Cleaved by Furin and PCSK6—Previous reports characterizing the substrate specificity of PCs suggest some redundancy in functionality for some, but not all, targets (15). To assess whether pro-GX sPLA$_2$ is a substrate for furin and/or PCSK6 proteolytic activation, FLAG-pro-GX sPLA$_2$ was co-expressed with either furin or PCSK6 in HEK 293 cells. The ratio of m-GX sPLA$_2$/total-GX sPLA$_2$ in media from HEK 293 cells expressing GX sPLA$_2$ alone was ~1:1 (Fig. 4*A*). Expression of either furin or PCSK6 resulted in an almost complete conversion of FLAG-pro-GX sPLA$_2$ to the mature form, indicating that pro-GX sPLA$_2$ is a substrate for both PCs. m-GX sPLA$_2$ was not detected in whole-cell lysates, indicating that the active form of the enzyme does not accumulate intracellularly after pro-GX sPLA$_2$ is processed by furin or PCSK6 (Fig. 4*B*).

Both Furin and PCSK6 Contribute to FLAG-Pro-GX sPLA$_2$ Processing in Y1 Cells—To determine whether furin and/or PCSK6 mediate GX sPLA$_2$ proteolytic activation in adrenal cells, we employed small interfering RNAs (siRNAs) to suppress the expression of furin mRNA, PCSK6 mRNA, or both mRNAs. Both furin and PCSK6 mRNA expression was effectively reduced ~75% using this approach (Fig. 5, *A* and *B*). Neither knockdown of furin nor PCSK6 alone was able to significantly inhibit processing of FLAG-pro-GX sPLA$_2$ secreted by Y1 cells (Fig. 5*C*). However, knockdown of both furin and PCSK6 together resulted in a significant reduction in FLAG-pro-GX sPLA$_2$ cleavage. As expected, ACTH treatment signif-

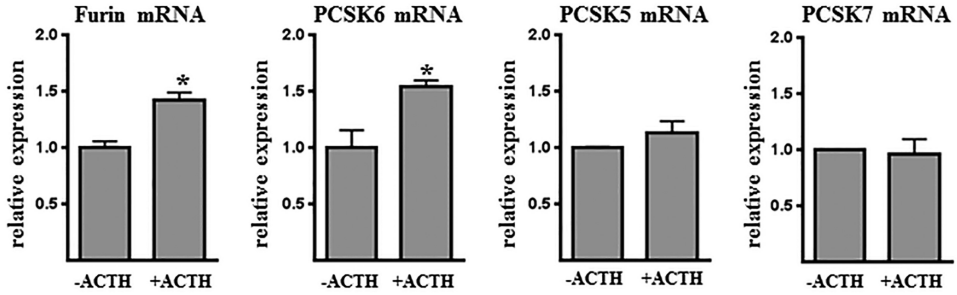

FIGURE 3. **ACTH increases furin and PCSK6 gene expression in adrenal cells.** Y1 cells were incubated for 16 h with either 0 or 100 nM ACTH, and furin-like proprotein convertase gene expression was quantified by qRT-PCR ($n = 3$). Data are means ± S.E. and are representative of two independent experiments. *, $p < 0.05$.

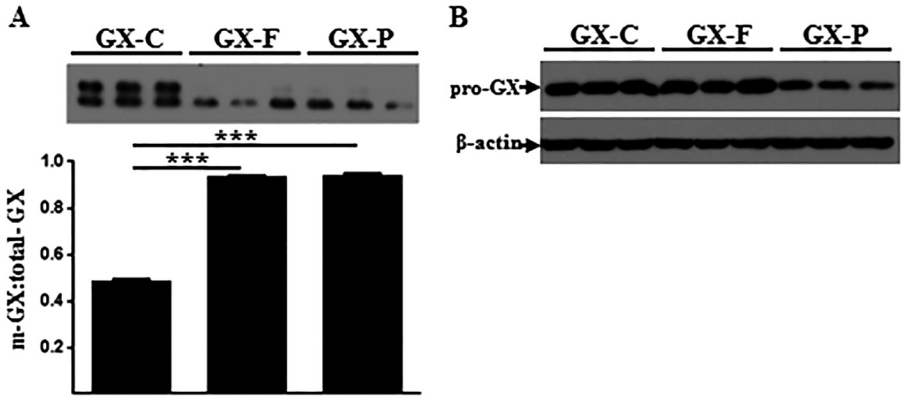

FIGURE 4. **FLAG-pro-GX sPLA$_2$ is proteolytically cleaved by furin and PCSK6.** HEK 293 cells were transiently co-transfected with 3× FLAG-tagged GX sPLA$_2$ and either a control vector (*GX*) or a vector encoding furin (*GX+F*) or PCSK6 (*GX+P*). Cells were then incubated in fresh media for 24 h. *A,* conditioned media were immunoblotted using anti-FLAG antibody (*top*); results from densitometric analyses are shown below. Data are expressed as the ratio of m-GX sPLA$_2$/total-GX sPLA$_2$ in the media. *B,* whole-cell lysates (10 μg of protein) were immunoblotted using anti-FLAG antibody (*top*) and anti-β-actin (*bottom*). Data are means ± S.E. and are representative of two independent experiments. ***, $p < 0.001$.

icantly increased the ratio of m-GX sPLA$_2$/total-GX sPLA$_2$ in the media compared with vehicle-treated cells (Fig. 5*D*). However, enhanced FLAG-pro-GX sPLA$_2$ processing in response to ACTH was significantly decreased when furin and PCSK6 expression was suppressed. These findings strongly suggest that both furin and PCSK6 mediate GX sPLA$_2$ processing in Y1 adrenal cells, and ACTH-induced increases in GX sPLA$_2$ activity is at least partly due to up-regulation of these convertases.

Suppression of LXR Activation by GX sPLA$_2$ Is Enhanced When Furin or PCSK6 Are Overexpressed—We previously reported that GX sPLA$_2$ inhibits LXR-mediated target gene activation through a mechanism that is dependent on its enzymatic activity (10). Therefore, we investigated whether proteolytic activation of GX sPLA$_2$ by furin or PCSK6 enhances GX sPLA$_2$-dependent inhibition of LXR activation. As expected, GX sPLA$_2$ overexpression in HEK 293 cells suppressed the transcriptional activation of an LXR reporter construct under both basal and T0901317-treated conditions (Fig. 6, *A* and *B*). The inhibitory effect of GX sPLA$_2$ on LXR activation was augmented when either furin or PCSK6 was co-expressed, indicating that GX sPLA$_2$ processing by furin-like proprotein convertases may represent an important mechanism for regulating GX sPLA$_2$-mediated inhibition of LXR transcriptional activation.

GX sPLA$_2$-mediated Suppression of Steroidogenesis Requires Furin-like Proprotein Convertase Activity—StAR plays a critical function in adrenal corticoid synthesis by delivering cholesterol to steroidogenic enzymes located in the inner mitochondrial membrane. Given its key role in corticosteroid production, StAR mRNA expression is under both positive and negative control by a variety of transcription factors, including LXR (16). Given our previous finding that GX sPLA$_2$ suppresses StAR expression in an LXR-dependent manner (13), it was of interest to investigate whether inhibiting PC activity impacted the ability of GX sPLA$_2$ to regulate StAR, and hence steroid production in adrenal cells. As we reported previously, GX sPLA$_2$ overexpression inhibited StAR protein expression in Y1 cells under both basal (Fig. 7*A*) and ACTH-stimulated conditions (Fig. 7*B*). This inhibitory effect was abolished when cells were treated with RVKR. In a subsequent study, we determined that RVKR increased StAR protein abundance in control Y1 cells, but this effect was not significant ($p = 0.08$). The trend for increased StAR in the absence of GX sPLA$_2$ overexpression could be due to the effect of the PC inhibitor on endogenous GX sPLA$_2$ processing. Collectively, our results indicate that furin-like proprotein convertases are required for GX sPLA$_2$-mediated StAR regulation.

We next assessed the effect of inhibiting PCs on steroid production in Y1-GX cells. Because Y1 cells do not express 21-hydroxylase, the enzyme required for conversion of progesterone to corticosterone (17), progesterone levels in conditioned media were measured as an indicator of steroid production by these cells. As expected, treatment with increasing concentrations of RVKR resulted in a dose-dependent decrease in

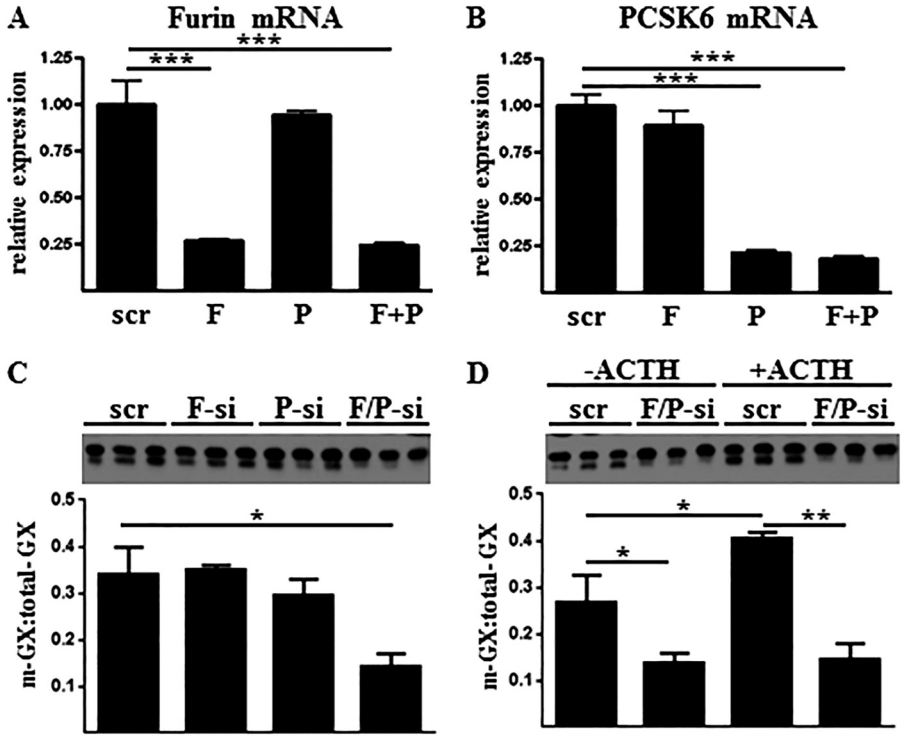

FIGURE 5. **FLAG-pro-GX sPLA$_2$ processing in mouse Y1 adrenal cells is dependent on furin and PCSK6 gene expression.** Y1-GX cells were transiently transfected with either control siRNA (*scr*) or siRNA targeting furin (*F-si*), PCSK6 (*P-si*), or both siRNAs (*F/+P-si*). *A* and *B,* Furin (*A*) and PCSK6 (*B*) mRNAs were quantified by qRT-PCR. *C,* conditioned media were immunoblotted using anti-FLAG antibody (*top*); results from densitometric analyses are shown *below*. *D,* Y1-GX cells transiently transfected with either control siRNA (*scr*) or siRNA targeting both furin and PCSK6 (*F/P-si*) were then incubated in fresh media containing either 0 or 100 nM ACTH for 20 h. Conditioned media were immunoblotted using anti-FLAG antibody (*top*); and densitometric analysis is shown *below*. Data (means ± S.E.) are expressed as the ratio of m-GX sPLA$_2$/total-GX sPLA$_2$ in the media and are representative of two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

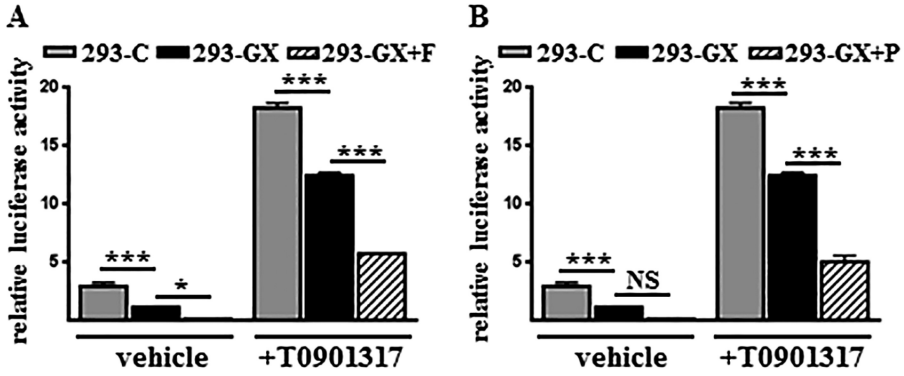

FIGURE 6. **GX sPLA$_2$ processing by furin or PCSK6 enhances GX sPLA$_2$-dependent inhibition of LXR-mediated gene activation.** HEK 293 cells were transiently co-transfected with ptk-3× LXRE-luc reporter construct and vectors encoding mLXRα, mRXR, *Renilla* luciferase, and either a control vector encoding GFP (*293-C*) or a vector encoding 3× FLAG-tagged GX sPLA$_2$ in the absence (*293-GX*) or presence of furin (*293-GX+F*) (*A*) or PCSK6 (*293-GX+P*) (*B*). Cells were then incubated in media containing either 0 or 1 μM T0901317 for 24 h prior to measurements of luciferase activity. Data are means ± S.E. ($n = 4$) and are representative of two independent experiments. *, $p < 0.05$; ***, $p < 0.001$; *NS,* not significant.

phospholipase activity secreted by Y1-GX cells (Fig. 8*A*). Notably, progesterone production was reciprocally increased in response to increasing concentrations of RVKR (Fig. 8*B*). In accordance with previous findings, GX sPLA$_2$ overexpression significantly decreased progesterone production by Y1 cells under both basal and ACTH-stimulated conditions (Fig. 8*C*). The inhibition was ablated when cells were treated with RVKR at a dose that significantly reduced sPLA$_2$ activity secreted by these cells. This result is consistent with the conclusion that pro-GX sPLA$_2$ processing by PCs is necessary for GX sPLA$_2$-mediated suppression of adrenal steroidogenesis.

DISCUSSION

Furin-like PCs are calcium-dependent serine proteases that mediate the post-translational processing and activation of numerous molecules important for tissue and whole-body homeostasis, such as cell surface receptors, pro-hormones, growth factors, matrix metalloproteinases, and adhesion molecules (15). Perturbations in PC activity have been implicated in multiple pathological conditions, including various endocrinopathies, infectious diseases, cancer, and Alzheimer disease, reflecting their fundamental role in diverse physiological pro-

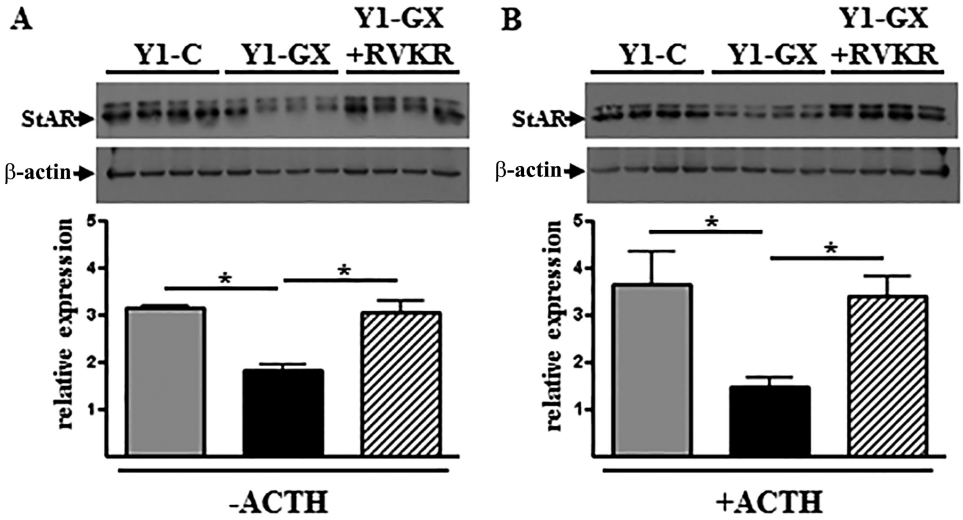

FIGURE 7. **Inhibition of StAR protein expression by GX-sPLA$_2$ is abolished by RVKR.** Y1 cells were transiently transfected with either a control vector (*Y1-C*) or a vector encoding 3×-FLAG tagged GX sPLA$_2$ (*Y1-GX*). Cells were then incubated in media containing 0 or 100 nM ACTH and either DMSO vehicle or 25 μM RVKR for 18 h. Immunoblot analysis of total cell lysates was performed using antibodies specific for StAR and β-actin. *A,* StAR and β-actin expression in Y1-C cells (*1st* to *4th lanes*), Y1-GX cells (*5th* to *8th lanes*), and Y1-GX cells treated with RVKR (*9th* to *12th lanes*) in the absence of ACTH (*top*). Results from densitometric analyses (*bottom*) are expressed as the ratio of StAR/β-actin. *B,* StAR and β-actin expression in Y1-C cells (*1st* to *4th lanes*), Y1-GX cells (*5th* to *8th lanes*), and Y1-GX cells treated with RVKR (*9th* to *12th lanes*) in the presence of 100 nM ACTH (*top*). Results from densitometric analyses (*bottom*) are expressed as the ratio of StAR/β-actin. Data are means ± S.E. ($n = 3$). *, $p < 0.05$.

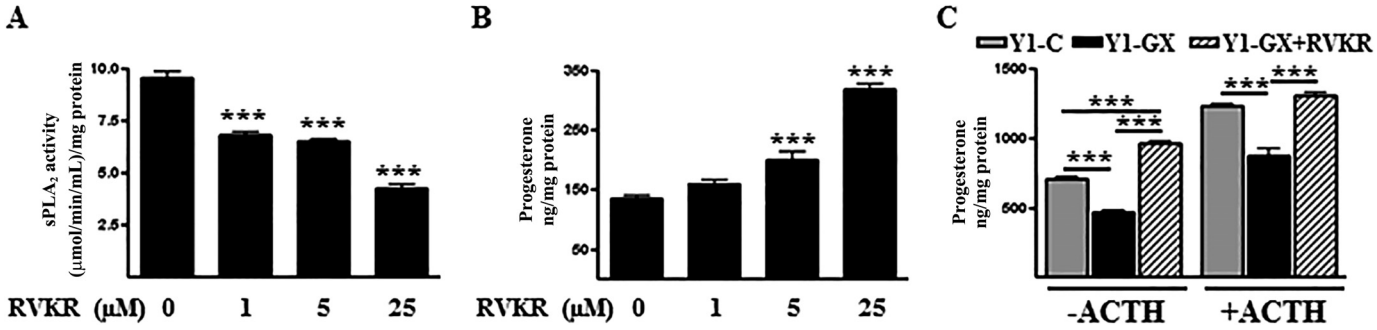

FIGURE 8. **GX sPLA$_2$-mediated inhibition of progesterone production by adrenal cells requires furin-like proprotein convertase activity.** *A* and *B,* Y1 cells were transiently transfected with 3× FLAG-tagged GX sPLA$_2$. After 16 h, cells were incubated with the indicated concentrations of RVKR for 20 h. sPLA$_2$ activity (*A*) and progesterone concentrations (*B*) were assayed in the media and expressed relative to total cellular protein ($n = 6$). Data are means ± S.E. ***, $p < 0.001$ compared with control-treated cells. *C,* Y1 cells were transiently transfected with either a control vector (*Y1-C*) or a vector encoding 3× FLAG-tagged GX sPLA$_2$ (*Y1-GX*). Cells were then incubated in media containing 0 or 100 nM ACTH and either DMSO vehicle or 25 μM RVKR for 20 h. The concentration of progesterone in the media was measured and expressed relative to total cellular protein ($n = 6$). Data are means ± S.E. ***, $p < 0.001$.

cesses. Here, we provide strong evidence that pro-GX sPLA$_2$ is a previously unrecognized substrate for two members of the PC family, furin and PCSK6, and we identify proteolytic activation of pro-GX sPLA$_2$ by PCs as a novel mechanism for regulating glucocorticoid production in adrenal cells.

The initial observation that prompted our study was the finding that phospholipase activity secreted by Y1-GX cells, and to a lesser extent Y1-C cells, was significantly increased when cells were stimulated by ACTH (13). We speculated that the increase in sPLA$_2$ activity was due to enhanced conversion of both endogenous and ectopically expressed pro-GX sPLA$_2$ to m-GX sPLA$_2$ in ACTH-treated cells. A recent study by Jemel *et al.* (12) suggested that pro-GX sPLA$_2$ is proteolytically activated by a furin-like PC, although the identity of the specific PC(s) was not determined. In an initial screening by qRT-PCR, we determined that four members of the PC family are expressed in Y1 adrenal cells, furin, PCSK5, PCSK6, and PCSK7. We conclude that both furin and PCSK6 play a major role in pro-GX sPLA$_2$ processing in adrenal cells based on the following finding. 1) Furin and PCSK6 mRNA abundance were both significantly induced by ACTH in Y1 adrenal cells. 2) FLAG-pro-GX sPLA$_2$ processing and activation were blocked in ACTH-treated Y1 cells by the PC inhibitor RVKR. 3) Co-transfection of FLAG-pro-GX sPLA$_2$ with either furin or PCSK6 significantly enhanced FLAG-pro-GX sPLA$_2$ processing in HEK 293 cells. 4) FLAG-pro-GX sPLA$_2$ processing in adrenal cells was effectively blocked when the expression of both furin and PCSK6 was suppressed. Analysis of PC cleavage preferences and substrate specificities reveals considerable overlap among several members of the PC family (18). However, caution should be taken when attempting to draw conclusions from studies using short unfolded peptides as substrates. Redundancy in furin substrate cleavage specificity has been described in the liver using an interferon-inducible Mx-Cre/loxP furin-deficient mouse model (19). Both soluble furin and PCSK6 are able to cleave pro-Nodal while bound to its co-receptor at the cell surface (20, 21). However, unique furin substrates, including the iron regulatory protein, pro-hepcidin, and pro-bone morphogenic pro-

tein 10 (pro-BMP10) in the developing heart, have also been described (22, 23). Our data indicate that suppression of both furin and PCSK6 by siRNA-mediated gene silencing is required to effectively block FLAG-pro-GX sPLA$_2$ processing and provide strong evidence for redundancy, at least in transfected Y1-GX cells.

In this study, using a FLAG-tagged pro-GX sPLA$_2$, we could detect m-GX sPLA$_2$ only in the media and not in the cell lysates, although both pro-GX and m-GX sPLA$_2$ were found in conditioned media. Because Western blotting would detect steady state levels of the precursor and processed forms of GX sPLA$_2$, we carried out radioimmunoprecipitation experiments to determine whether the mature form could be detected in cells metabolically labeled for a relatively brief period (4 h). However, only pro-GX sPLA$_2$ was detected in cell lysates (data not shown), indicating that if intracellular processing does occur, m-GX sPLA$_2$ must be either rapidly degraded or secreted. Furin is thought to process substrates in the *trans*-Golgi network, at the cell surface, and in endosomes, although PCSK6-dependent processing is believed to take place predominantly in the extracellular matrix (15). Notably, there appear to be distinct mechanisms underlying the compartment-specific regulation of substrate processing by furin. Pro-ADAMTS4 co-localizes with furin in the *trans*-Golgi network, where its pro-segment is proteolytically removed (24). However, pro-ADAMTS9 processing by furin takes place at the cell surface before being secreted into the media such that no evidence for mature- ADAMTS9 is detected in the cell lysates (25). Our conclusion that m-GX sPLA$_2$ does not accumulate intracellularly is in contrast to a previous report that utilized cell-permeable and cell-impermeable inhibitors to define the cellular location of pro-GX sPLA$_2$ processing. In this study, the authors concluded that pro-GX sPLA$_2$ processing takes place both before and after secretion (12). This conclusion was supported by the finding that exogenously added pro-GX sPLA$_2$ does not result in significant cellular membrane hydrolysis. The reason for the discrepancy between these two findings is unclear. One possibility is that PC-dependent processing of pro-GX sPLA$_2$ occurs coincident with secretion in a cellular compartment near the cell surface that is inaccessible to cell-impermeable PC inhibitors.

This study confirms previous findings from our laboratory documenting the role of GX sPLA$_2$ in modulating adrenal steroidogenesis (13). GX KO mice have increased plasma corticosterone levels under both basal and ACTH-induced stress conditions (13). This phenotype is due at least partly to a direct effect of GX sPLA$_2$ in the adrenal gland, because primary adrenal cells isolated from GX KO mice produce higher levels of glucocorticoids in response to ACTH compared with cells from wild-type mice (13). We determined that GX sPLA$_2$-mediated suppression of progesterone production in Y1 adrenal cells is dependent on its hydrolytic activity, as evidenced by the fact that GX sPLA$_2$, but not a catalytically inactive mutant lacking the active-site histidine residue, suppressed basal and ACTH-induced progesterone production in Y1 cells. Our finding that treatment with the PC inhibitor, RVKR, abolished GX sPLA$_2$-dependent suppression of progesterone production under both basal and ACTH-stimulated conditions is consistent with previous observations that removal of the N-terminal pro-segment is necessary for GX sPLA$_2$ hydrolytic activity (4). Importantly, the derepression of progesterone production in Y1-GX cells treated with RVKR was associated with both decreased FLAG-pro-GX sPLA$_2$ processing and sPLA$_2$ activity in conditioned media from these cells. Furthermore, RVKR resulted in significantly reduced sPLA$_2$ activity secreted by Y1-C cells (data not shown), suggesting that upon processing endogenous GX sPLA$_2$ contributes significantly to the phospholipase activity secreted by Y1 adrenal cells. Taken together, these data indicate that PCs proteolytically activate pro-GX sPLA$_2$, which in turn acts to suppress glucocorticoid production in adrenal cells. Although the mechanism has not been completely delineated, GX sPLA$_2$ appears to negatively regulate adrenal glucocorticoid production through transcriptional suppression of StAR, most likely by reducing the activation of LXR (13). StAR represents the rate-limiting protein in steroid hormone production, and many of the factors known to regulate steroidogenesis, including LXR, have their effect by targeting StAR (16, 25, 26). Results from this study demonstrate that suppression of LXR reporter activation by GX sPLA$_2$ is enhanced in the presence of either furin or PCSK6 and that inhibition of pro-GX sPLA$_2$ processing by RVKR restores StAR protein expression in Y1-GX cells to levels comparable with Y1-C cells. Together, these findings suggest that processing of pro-GX sPLA$_2$ by PCs is necessary for GX sPLA$_2$-dependent suppression of LXR target gene activation.

Mammals have evolved a complex regulatory network for fine-tuning adrenal steroid production. Pituitary-derived ACTH stimulates the adrenals to produce glucocorticoids, which feedback on both the anterior pituitary and the hypothalamus to inhibit ACTH and corticotropin-releasing hormone, respectively. Here, we provide evidence for a negative feedback loop in adrenal cells whereby ACTH increases PC expression, resulting in conversion of pro-GX sPLA$_2$ to m-GX sPLA$_2$, which in turn acts to suppress glucocorticoid production. What remains to be determined are the mechanism(s) responsible for turning off the activity of GX sPLA$_2$. One potential pathway is the M-type sPLA$_2$ receptor (sPLA$_2$-R), which has been implicated in the lysosomal degradation of GX sPLA$_2$. Chinese hamster ovary (CHO) cells overexpressing the sPLA$_2$-R rapidly degrade GX sPLA$_2$, resulting in a marked reduction in PGE$_2$ production compared with non-sPLA$_2$ receptor-expressing cells (27). Mice deficient in sPLA$_2$-R have higher levels of GX sPLA$_2$ present in the bronchoalveolar lavage fluid in response to ovalbumin-induced airway inflammation, and this is associated with the increased production of eicosanoids, Th2 cytokines, and infiltration of inflammatory neutrophils and eosinophils (28). A soluble form of the sPLA$_2$-R has been identified in mouse plasma that binds and inactivates GX sPLA$_2$. The *in vitro* incubation of GX sPLA$_2$ with plasma from wild-type mice but not sPLA$_2$-R-deficient mice decreases phospholipase activity (29). We determined that the sPLA$_2$-R is expressed in mouse adrenal glands and Y1 cells (13). Interestingly, silencing sPLA$_2$-R expression in YI-GX cells results in significantly reduced progesterone production, consistent with the possibility that the sPLA$_2$-R internalizes and/or inactivates GX sPLA$_2$, thereby reducing the magnitude of GX sPLA$_2$'s suppressive effect (13).

Understanding the tissue-specific regulation of pro-GX sPLA$_2$ processing will provide novel insights into the regulatory mechanisms governing both the physiological and pathophysiological processes in which GX sPLA$_2$ is thought to play a role, including asthma, ischemia-reperfusion injury, and atherosclerosis to name a few (30–32). In addition to adrenal cells, studies in our laboratory document that GX sPLA$_2$ negatively regulates LXR target gene expression in macrophages and adipocytes, with significant consequences with respect to macrophage cholesterol efflux capacity (10), macrophage-mediated inflammatory responses (33), and adipocyte lipogenesis (9). Given that both furin and PCSK6 are ubiquitously expressed (15), it is tempting to speculate that these PCs are the predominant proteases involved in the conversion of pro-GX sPLA$_2$ to m-GX sPLA$_2$ in multiple cell types. Interestingly, furin mRNA expression and sPLA$_2$ activity are significantly increased in J774 macrophages stably expressing GX sPLA$_2$ when treated with lipopolysaccharide.[3] Clearly, further studies are needed to solidify the roles of individual PCs in modulating GX sPLA$_2$ activity in different tissues.

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