Metformin Inhibits Follicle-Stimulating Hormone (FSH) Action in Human Granulosa Cells: Relevance to Polycystic Ovary Syndrome

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Background: Women with anovulatory polycystic ovary syndrome (PCOS) are generally insulin-resistant and as a consequence are often treated with the biguanide metformin. Results with metformin have, however, been variable with some studies demonstrating induction of regular cycles and an increase in ovulation, whereas others do not. Hence more understanding is needed regarding the mechanism of metformin's actions in ovarian granulosa cells especially in light of previous demonstrations of direct actions.

Objective: The aim of this study was to investigate metformin's interaction with the FSH/cAMP/protein kinase A pathway, which is the primary signaling pathway controlling *CYP19A1* (aromatase) expression in the ovary.

Methods: The effect of metformin on FSH and forskolin-stimulated aromatase expression in human granulosa cells was measured by quantitative real-time PCR. Activity was assessed after transfection with a promoter II-luciferase construct, and by an RIA measuring conversion of androgen to estrogens. The effect on FSH receptor (*FSHR*) mRNA was assessed by quantitative PCR. Levels of phosphorylated cAMP response element binding protein (CREB) and CREB-regulated transcription coactivator 2 (CRTC2) were measured by Western blotting and cAMP by a bioluminescent assay.

Results: Metformin markedly reduced FSH but not forskolin-stimulated aromatase expression and activity. This effect was exerted by inhibition of basal and ligand-induced up-regulation of *FSHR* expression. Metformin also reduced FSH-induced phosphorylation of CREB and hence CRE activity, which could potentially disrupt the CREB–CREB-binding protein–CRTC2 coactivator complex that binds to CRE in promoter II of the aromatase gene. This is mediated in an AMP-activated protein kinase-independent manner, and does not involve alteration of cAMP levels.

Conclusion: These finding have implications for the use of metformin in the treatment of anovulation in women with PCOS. (*J Clin Endocrinol Metab* 98: E1491–E1500, 2013)

Women with anovulatory polycystic ovary syndrome (PCOS) are generally insulin-resistant and as a consequence are often treated with the biguanide metformin. Results with metformin have, however, been variable with some studies demonstrating induction of regular menstrual cycles and increases in both ovulation and live birth rates (in women with PCOS) (1, 2), whereas others

have shown no effect on ovulation (3, 4). The patients who will most benefit remain to be identified, and this is due in part to a lack of understanding of the mechanism by which metformin exerts its effects. The systemic antihyperglycemic and insulin-sensitizing effects of metformin are well documented (5), but metformin also exerts direct effects on insulin-mediated steroidogenesis and glucose uptake in

ovarian cells. Metformin exerted its effects on the expression and activity of aromatase (encoded by *CYP19A1*) via the ERK pathway (6, 7) and was also able to directly enhance the insulin-mediated translocation of glucose transporters to the granulosa cell plasma membrane (8). However, many questions regarding the mechanisms by which metformin exerts its effects on the ovary remain to be elucidated, for example, how metformin interacts with the FSH-stimulated cAMP/protein kinase A (PKA)/cAMP response element binding protein (CREB) pathway, the primary signaling pathway for the regulation of *CYP19A1* in the ovary (9). Interestingly, treatment of women with PCOS with metformin was associated with a reduction in aromatase activity in response to FSH, supporting a direct effect (10, 11).

FSH binding activates cAMP and PKA to affect aromatase gene expression via the activation of cAMP-responsive transcriptional regulatory proteins. The best known is CREB, which when phosphorylated binds to a cAMP response element (CRE) in the gonad-specific promoter II (PII) of *CYP19A1* (12, 13). Downstream of CRE is another important regulatory binding site for the orphan nuclear receptor steroidogenic factor 1 (SF-1, official symbol NR5A1). Activation of both leads to recruitment of a cohort of multiple coregulators and transcription factors such as CREB-binding protein (CBP), which then activate PII-specific expression to induce aromatase expression (12).

In 2003, a new family of CREB coactivators were identified, known either as CREB-regulated transcription coactivators (CRTCs) or as transducers of regulated CREB activity (TORC) (14, 15). The 3 members of the CRTC/TORC family have a common N-terminal domain that associates with CREB DNA binding/dimerization domain (bZIP) to mediate transcriptional activity of CREB in response to both cAMP and calcium influx (16). Studies have demonstrated the involvement of CRTCs (principally CRTC2) in the regulation of cAMP-responsive genes, primarily *CYP19A1* promoter activity in MCF-7 cells (17) and *StAR*, *P450*scc and *3β-HSD* in rat granulosa cells (18). Under basal conditions, CRTC2 is sequestered in the cytoplasm via phosphorylation, and nuclear entry is brought about by dephosphorylation by calcium and cAMP (19).

Regulation of *FSHR* (FSH receptor) transcription is not well understood, but an E-box (element that binds transcription factors) in the proximal promoter, along with SF-1 and the upstream proteins stimulatory factor-1 and -2, appear important (20). In addition, transgenic mice studies have indicated that *FSHR* transcription is also highly dependent on regulatory elements that lie distal to the promoter region (21). The aim of this study was to investigate the interaction of metformin with the FSH-stimulated cAMP/PKA pathway in human granulosa cells to further elucidate the mechanism of action of metformin in women with PCOS. We hypothesized that metformin is able to directly regulate *FSHR* in addition to *CYP19A1* expression, thereby influencing follicle development in the ovary.

Materials and Methods

KGN cell culture, real-time PCR, and activity assays

All experiments used the KGN human granulosa-like tumor cell line. Cells were cultured overnight at 3×10^5 cells per well in DMEM-F12 (Life Technologies) plus 1% fetal calf serum before addition of medium containing aromatase substrate testosterone (5×10^{-7}M) and 10^{-7}M metformin (16 ng/mL); FSH at 1, 5, and 10 ng/mL with or without metformin; or forskolin (Fsk) at $10\,\mu$M and $25\,\mu$M with or without metformin (all drugs from Sigma-Aldrich Co Ltd except hFSH from Endocrine Services). After another 48 hours in culture, the medium was removed and stored to measure estradiol (E_2) as an indicator of aromatase activity using an RIA (22). The cells were lysed, and RNA was extracted and reverse transcribed to cDNA (6). In additional experiments, cells were treated as above, with the addition of 10 and 100 ng/mL activin to up-regulate *FSHR* mRNA expression.

In vivo luteinization is associated with a cAMP-driven up-regulation of *LHR* and corresponding decrease in expression of many FSH-driven genes. To determine whether Fsk treatment was able to induce mRNA expression of LH receptor (*LHR*), cells were treated with $25\,\mu$M Fsk with or without metformin for 24 to 72 hours before lysis for RNA extraction.

Real-time quantitative PCR (qPCR) was performed using the Bio-Rad CFX real-time cycler (Bio-Rad Laboratories Ltd). Duplicate PCRs were performed with SYBR Green I master mix (Agilent Technologies) (22) with *L19* (mitochondrial ribosomal protein L19) as the reference gene. Primer sequences are listed in Supplemental Table 1 (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org) (Sigma-Aldrich). All primer pairs annealed at 60°C except *FSHR* primers, which annealed at 50°C. Data are the mean of gene expression ratio ± SEM.

KGN cell culture, transfection, and reporter gene assays

KGN cells were plated at 2×10^4 cells per well and incubated overnight. To investigate PII activity, the cells were transfected with a *CYP19A1* PII-516 reporter construct expressing firefly luciferase (kindly donated by Dr Brown and Professor Simpson, Prince Henry's Institute, Melbourne, Australia) (23). To investigate CRE activity, cells were transfected with a *CRE*-pADneo2 C6-BL reporter construct also expressing firefly luciferase (courtesy of Dr Axel Themmen) (24). In addition, 5 ng/μL of a control plasmid expressing Renilla luciferase from a constitutive promoter, and 6 ng/μL of a transcription enhancing element, PVAi, was added to each well (courtesy of Dr Axel Nohturrft, St George's University of London) and incubated for another 24 hours (25). Cells were serum-starved for 2 hours, and then triplicate wells were treated as described in the PCR assay. After

another 24 hours in culture, luciferase reporter assays were carried out using the Dual-Glo Luciferase Assay System (Promega) (25). Data are presented as the mean of the ratio of luciferase to renilla luminescence normalized to the control ± SEM.

KGN cell culture and cAMP assay

KGN cells were plated at 2×10^4 cells per well and incubated overnight in DMEM plus 1% fetal calf serum before treatment with metformin from 10^{-7}M to 10^{-4}M for 24 hours followed by exposure to serum-free media containing FSH (5 and 10 ng/mL) or Fsk (25μM) with or without metformin for 1 hour, after which cAMP was measured using the cAMP-Glo Max assay (Promega). This is a bioluminescent assay that measures cAMP levels in cells using a coupled luciferase reaction: as cAMP increases, it binds to PKA and the regulatory subunits undergo conformational change releasing the catalytic subunits. These then catalyze transfer of the terminal phosphate of ATP to a PKA substrate, consuming ATP in the process. Remaining ATP is measured by luciferase-based Kinase-Glo reagent where luminescence is inversely proportional to cAMP levels. Data in the table are mean of cAMP (nanomolar) ± SEM. The results are presented graphically after normalization to the control (no treatment).

KGN cell culture, protein fractionation, and Western blot analysis

KGN cells were plated at 1×10^6 cells per well, incubated overnight, treated with 10^{-7}M metformin (16 ng/mL) for 24 hours and subsequently exposed to 5 ng/mL FSH with or without metformin; 25μM Fsk with or without metformin and 2mM 5′-aminoimidazole-4-carboxamide ribonucleoside (AICAR) with or without FSH/Fsk. AICAR activates the AMP-activated protein kinase (AMPK) pathway and was added to determine the involvement of this pathway in the mechanism. After 1 hour, cells were scraped into ice-cold PBS and centrifuged, and the pellets were resuspended into RIPA buffer (Cell Signaling Technologies, New England Biolabs [UK] Ltd) with protease and phosphatase inhibitors and stored at $-80°$C. For the time-course experiments, cells were exposed to FSH or Fsk for 30 or 60 minutes or 3 or 24 hours before lysing for protein extraction.

To determine the effect of treatment on expression of CRTC2 (TORC2) and phosphorylated CREB (pCREB), Western blotting was performed using the Odyssey Imaging System (Li-Cor Biosciences) (8). Primary antibodies were used at the following concentrations: anti-CRTC2 at 1:500 (sc-46272; Santa Cruz Biotechnology), anti-pCREB at 1:500 (no. 9198) and anti-CREB at 1:1000 (no. 9104) (both from Cell Signaling), and anti–α-tubulin at 1:2000 (Abcam). The former 2 were detected with goat antirabbit IR680 (1:5000) and the latter two with goat anti-mouse IR800 (1:5000) (from Li-Cor Biosciences).

Statistical evaluation

All data represent the mean ± SEM of triplicate observations from a minimum of 3 or more independent experiments. Statistical significance was determined by ANOVA followed by post hoc tests: unpaired Student's or paired t test when 2 groups were compared (depending on the design of the experiment) or a one-sample t test when comparing with normalized control values. If the data were not normally distributed, a Wilcoxon matched-pairs signed rank test was used to compare 2 groups. Significance

was set at $P \leq .05$. Quantitative real-time PCR data were analyzed using the $\Delta\Delta$Ct method if the amplification efficiencies of the reactions were 100% ± 5%. For reactions (usually the case with the FSHR qPCR) where the amplification efficiency fell outside this range, then the Pfaffl method was used to calculate the fold change in expression ratios between the means of the 2 treatment groups (26). Data from the Western blots represent the mean densitometry measurements taken from all individual experiments and normalized to α-tubulin and then the control, except for pCREB, which was normalized to total CREB first. For the transfection experiments, the ratio of luciferase to renilla luminescence was calculated for each well and the mean ratio for each treatment group then determined and normalized to the untreated control within each experiment to give the fold change in CRE activity.

Results

The effect of metformin on FSH- and Fsk-stimulated aromatase expression and activity

FSH had a dose-related stimulatory effect on aromatase mRNA expression (ANOVA $P < .0001$), which was significantly attenuated by addition of metformin at 1 and 5 ng/mL ($P < .05$), with no inhibition at 10 ng/mL FSH (Figure 1A). This was via a direct significant inhibition of both basal ($P = .006$) and FSH-stimulated ($P < .05$) PII expression and activity (Figure 1B). The substantial increase in PII activity at 1 ng/mL FSH (Figure 1B) was not reflected in total aromatase mRNA (Figure 1A) or E_2 levels (Figure 1E).

Consistent with the mRNA and transfection data, metformin significantly reduced both basal ($P = .002$) and 5 ng/mL FSH-stimulated E_2 levels ($P = .009$), as measured by testosterone conversion to E_2 (Figure 1E). Fsk increased aromatase mRNA dose-dependently (ANOVA $P < .0001$) and also PII activity (Figure 1, C and D). Fsk treatment resulted in a 28-fold higher PII activity than FSH (Figure 1D), and metformin was unable to reduce this increase in aromatase (Figure 1C), PII activity (Figure 1D), or E_2 levels (Figure 1E).

Metformin attenuates *FSHR* expression without altering cAMP levels

Because metformin reduced FSH- but not Fsk-mediated increases in aromatase expression and activity, we conjectured that the effect might be upstream of cAMP, ie, on the FSH receptor itself. Interestingly, metformin reduced basal *FSHR* mRNA expression ($P < .005$, 1-sample t test) and the ligand-mediated increase in FSHR induced by 1 and 10 ng/mL FSH ($P < .05$, Wilcoxon matched-pairs t test) (Figure 2A). Fsk reduced basal *FSHR* mRNA levels (ANOVA $P = .05$), particularly at 5μM ($P = .01$, 1-sam-
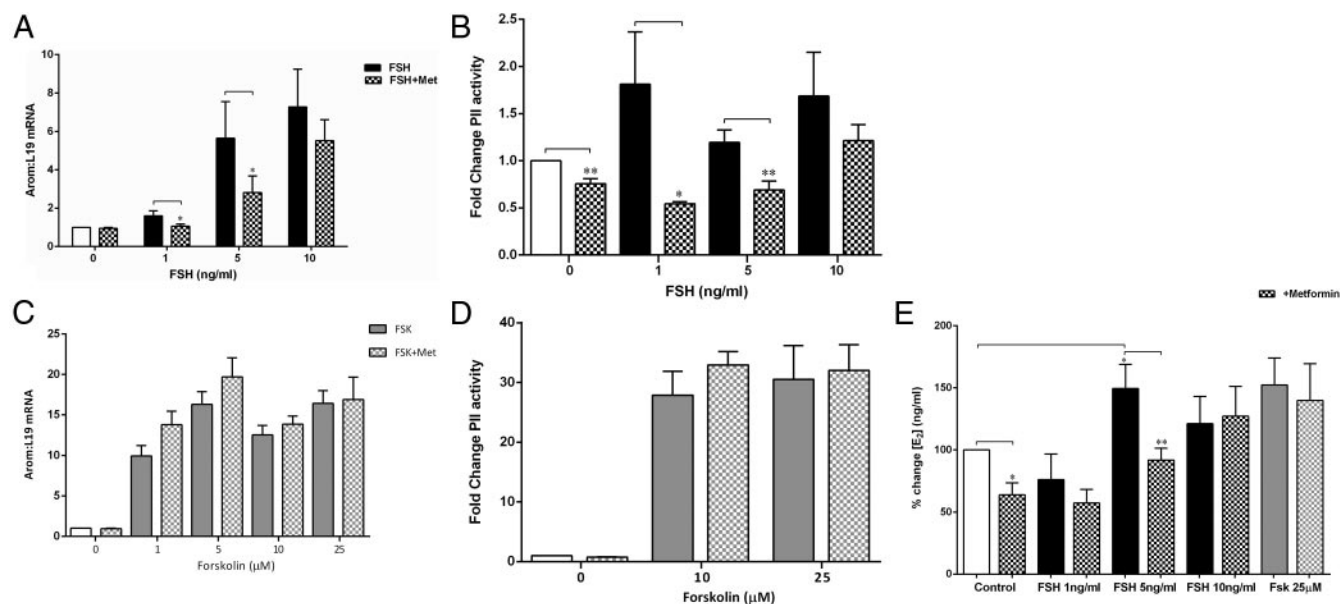
Figure 1. A, Effect of metformin and FSH on aromatase mRNA. Cells were treated for 48 hours with testosterone (as an aromatase substrate) and increasing doses of FSH (black solid bars) with or without metformin (Met) (10^{-7}M) (checked bars). Aromatase mRNA was measured by real-time qPCR and expressed as a fold change in expression relative to control values (no treatment) and normalized to *L19* mRNA expression. Metformin reduced the FSH-mediated increase in *CYP19* mRNA levels at 1 and 5 ng/mL FSH (mean ± SEM, n = 5–7; *, $P < .05$, paired t test). B, Effect of metformin on FSH-stimulated PII activity. Cells were transfected with the CYP19-PII-516 reporter construct and incubated for 24 hours before serum starvation for 2 hours. The cells were then treated with increasing doses of FSH (black solid bars) with or without metformin (10^{-7}M) (checked bars). Luciferase reporter assays demonstrated that metformin reduced basal and FSH-stimulated PII activity at 1 and 5 ng/mL FSH (data expressed as the fold change in the ratio of luciferase: renilla activity and normalized to the control) (n = 5–7, mean ± SEM; **, $P < .005$, paired t test). C, Effect of metformin and Fsk on aromatase mRNA. Cells were treated for 48 hours with increasing doses of Fsk (gray solid bars) (doses shown on graph) with or without metformin (checked bars) (10^{-7}M) and testosterone as an aromatase substrate. Aromatase mRNA was measured as described in A. Metformin had no effect on the Fsk-mediated increase in *CYP19* mRNA levels (mean ± SEM, n = 5–7). D, Effect of metformin on Fsk-stimulated PII activity. Cells were transfected with the *CYP19A1* reporter construct as described for B. Metformin had no effect on Fsk-stimulated increase in PII activity (data expressed as fold change activity of luciferase compared with renilla) (n = 5–7, mean ± SEM). E, Effect of metformin on FSH- and Fsk-stimulated E_2 levels. The medium from cells treated as described in A was removed, and the conversion of testosterone to E_2 was measured as an indicator of aromatase activity using an RIA. Metformin (checked bars) significantly reduced basal (white bar) E_2 levels (*, $P < .05$, 1-sample t test) as well as FSH-stimulated E_2 (black bars) at 5 ng/mL (mean ± SEM, n = 5–8; *, $P < .05$; **, $P < .005$, unpaired t test). It had no effect on the Fsk-stimulated (gray bars) increase in E_2. E_2 levels are expressed as a percent change normalized to basal.

ple t test) and 25μM ($P = .005$, 1-sample t test), and unexpectedly, the addition of metformin to the latter dose of Fsk further attenuated *FSHR* mRNA expression ($P = .003$, paired t test) (Figure 2B).

To further up-regulate *FSHR* expression, cells were cultured with activin (10 and 100 ng/mL), and qPCR analysis for aromatase, *FSHR*, and *SF-1* mRNA was performed. Although activin did not alter the expression of any of these genes with or without FSH, the addition of metformin to activin and FSH (both at 10 ng/mL) significantly down-regulated both aromatase and *FSHR* mRNA levels (data not shown).

FSH significantly increased intracellular cAMP levels as the dose increased from 5 to 10 ng/mL (Figure 2C). However, metformin had no effect on FSH- or Fsk-induced cAMP levels (Supplemental Table 2), confirming that metformin's attenuation of FSH-induced aromatase is via a reduction in *FSHR* expression and not a decrease in cAMP levels.

Mechanism of action of metformin via pCREB, CRTC2, and CRE

FSH dose-dependently increased CRE activity, but metformin attenuated this at 1 and 5 ng/mL FSH ($P < .05$) but had no effect on 10 ng/mL (Figure 3A) or on Fsk-mediated up-regulation of CRE (Figure 3B).

We determined that the optimal time for detection of pCREB after stimulation with either FSH or Fsk was 1 hour. Densitometric analysis of the immunoblots for pCREB/CREB showed that, as expected, FSH (5 ng/mL) increased pCREB compared with control ($P = .016$), and this was reduced to basal levels by metformin ($P = .03$) (Figure 4, A and B). Fsk (25μM) significantly increased pCREB, and this was not attenuated by metformin (Figure 4, A and B). AICAR had no effect on basal or FSH/Fsk-up-regulated pCREB levels (Figure 4C).

Anti-CRTC2 antibody detected both the phosphorylated (cytosolic, open arrowhead) and nonphosphorylated (nuclear, filled arrowhead) forms (Figure 5A), re-
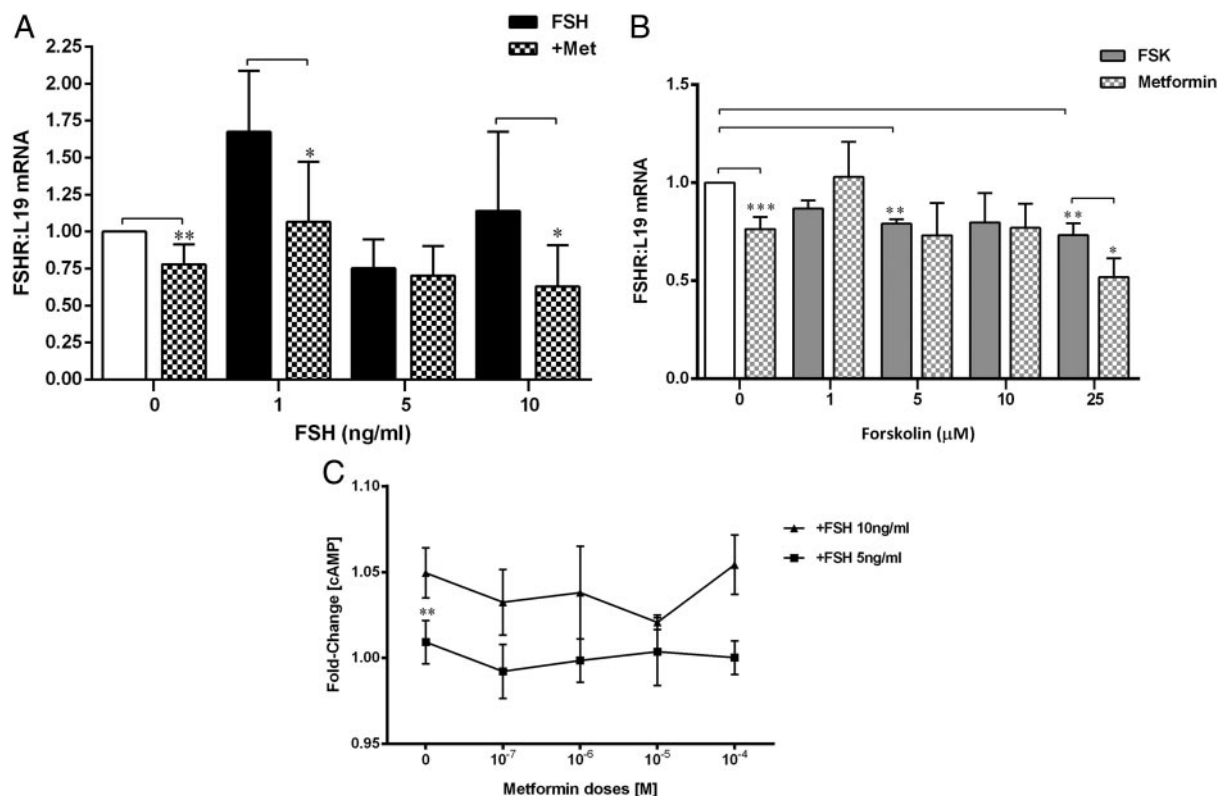
Figure 2. A, Effect of metformin (Met) and FSH on *FSHR* mRNA. Cells were treated as described in Figure 1A, and *FSHR* mRNA was measured by real-time qPCR and expressed as described previously. Metformin (checked bars) reduced basal mRNA levels of FSHR (mean ± SEM, n = 5–7; **, $P < .005$, 1-sample t test) as well as FSH (black bars) stimulation of its own receptor at 1 and 10 ng/mL FSH (*, $P < .05$, Wilcoxon matched-pairs signed rank t test). B, Effect of metformin and Fsk on FSHR mRNA. Cells were treated with Fsk (gray solid bars) as described in Figure 1C, which significantly decreased basal levels of *FSHR* mRNA with 5μM and 25μM (**, $P < .005$, ***, $P < .0005$, 1-sample t test). This decrease in *FSHR* mRNA was further attenuated by metformin (checked gray bars) (*, $P < .05$, paired t test) (mean ± SEM, n = 5–7). C, Effect of metformin on intracellular cAMP levels. Cells were treated with metformin at a range of doses from 10^{-7}M to 10^{-4}M for 24 hours and then exposed to FSH at 5 or 10 ng/mL for 1 hour. Intracellular cAMP levels were measured using a bioluminescent assay and normalized to the control (no treatment). Increasing FSH concentration from 5 to 10 ng/mL produced a significant increase in the fold change of cAMP levels (**, $P = .003$, paired t test), and metformin did not alter this.

vealing a clear increase in the nonphosphorylated form (nuclear) at 1 hour with both Fsk and FSH treatment, the time used in subsequent experiments. Densitometric analysis of the nonphosphorylated form (which would be translocated into the nucleus to bind to the CREB-CBP complex) showed that metformin keeps the basal levels low ($P = .02$), ie, retains CRTC2 in the cytosol, but is unable to prevent the FSH- or Fsk-stimulated dephosphorylation and nuclear translocation of CRTC2 (Figure 5, B and C). Interestingly, AICAR had no effect on CRTC2 either basally or in the presence of FSH/Fsk (Figure 5, B and C).

LHR expression induction

There was no induction of *LHR* mRNA expression by Fsk at 24, 48, or 72 hours and hence no additional effect of metformin (data not shown).

Discussion

This is the first demonstration that metformin reduces FSH-stimulated aromatase expression and activity in granulosa cells. It does so by reducing *FSHR* mRNA and consequently the activity of FSH as measured by aromatase expression and E_2, without altering cAMP levels. This involves blocking activation of CRE on PII of *CYP19* via inhibition of pCREB and hence possible disruption of the formation of the CREB-CRTC2 coactivator complex. This is via an AMPK-independent mechanism. This is especially relevant for our understanding of the mechanism of action of metformin in the ovaries of women with PCOS.

It is well established that FSH is the major inducer of aromatase activity in granulosa cells and that this occurs primarily via activation of the cAMP/PKA signaling pathway (9) as demonstrated in our study. Interestingly, metformin down-regulated aromatase mRNA via an abrogation of PII activity, which translated into reduced FSH-stimulated E_2. It is noteworthy that this was seen only at 1 and 5 ng/mL FSH. Previous dose-finding experiments in cultured primary granulosa cells from follicles <12 mm diameter from normal and polycystic ovaries revealed that the peak E_2 response to FSH was elicited by a dose of between 2.5 and 5 ng/mL (27), which is the range in which
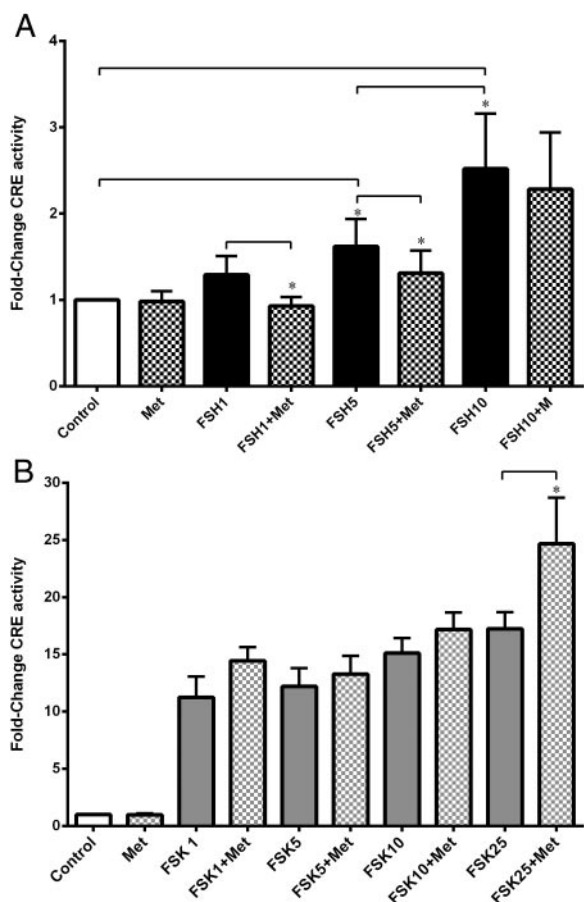
Figure 3. Effect of metformin on FSH- and Fsk-stimulated CRE activity. Cells were transfected with the *CRE-C6-BL* reporter construct and treated as described previously. Luciferase reporter assays demonstrated that metformin (checked bars) significantly reduced *CRE* activity in the presence of 1 and 5 ng/mL FSH (black bars) (*, $P < .05$, ratio of paired *t* test) (A) but had no effect on Fsk-stimulated *CRE* (gray solid bars), apart from in the presence of 25 μM Fsk where metformin significantly up-regulated CRE activity (B) (data expressed as fold change in ratio of luciferase to renilla luminescence, normalized to the control) (n = 5–7, mean ± SEM; *, $P = .039$, ratio of paired *t* test).

Figure 4. Effect of metformin on pCREB. A, Representative Western blot using total protein lysates from cells treated with metformin (10^{-7}M) for 24 hours and then exposed to either FSH (5 ng/mL) or Fsk (25 μM) with or without metformin for 1 hour. Cells were also treated with AICAR (2 mM) with or without FSH/Fsk for 1 hour before lysis and then blotted with anti–α-tubulin (1:2500), anti-pCREB (1:500), and anti-CREB (1:1000). The anti-pCREB antibody also detects the phosphorylated form of the CREB-related protein, ATF-1 (activating transcription factor). The effect of all treatments on pCREB levels is described in B. Lanes L, ladder; lane 1, control; lane 2, metformin; lane 3, FSH; lane 4, FSH plus metformin; lane 5, Fsk; lane 6, Fsk with or without metformin; lane 7, AICAR; lane 8, AICAR plus FSH; lane 9, AICAR plus Fsk. B, Graph of densitometry analysis of Western blots (mean ± SEM, n = 4). FSH (black bar) significantly increased levels of pCREB (**, $P = .016$, 1-sample *t* test), which was significantly reduced by metformin (checked bar) (*, $P = .03$, unpaired *t* test, n = 4–7 mean ± SEM). Fsk (gray bar) up-regulated pCREB (*, $P = .048$, 1-sample *t* test), and this was not affected by the addition of metformin. AICAR (spotted bar) did not alter basal levels of pCREB, but neither did it reduce the FSH- or Fsk-stimulated increase in pCREB. Abbreviations: Con, control; Met, metformin.

metformin exerted its inhibitory effect on FSH action in this study.

As with the highest FSH dose, metformin had no effect on Fsk-induced aromatase expression or activity, possibly due to induction of high cAMP levels. Our findings differ from those of Brown et al (28) who showed a reduction in Fsk-mediated PII activity by metformin in primary human breast adipose stromal cells. However, they used significantly higher doses of metformin (10 μM and 50 μM) compared with 100nM used in this study and a different cell type. High metformin doses are known to produce different effects (29). Because metformin did not alter intracellular cAMP in the presence of 5 or 10 ng/mL FSH, an alternative explanation for our results is that it acts upstream of cAMP on the FSHR itself. Indeed, to our surprise, metformin significantly reduced basal levels of *FSHR* mRNA. In addition, it also reduced ligand-induced
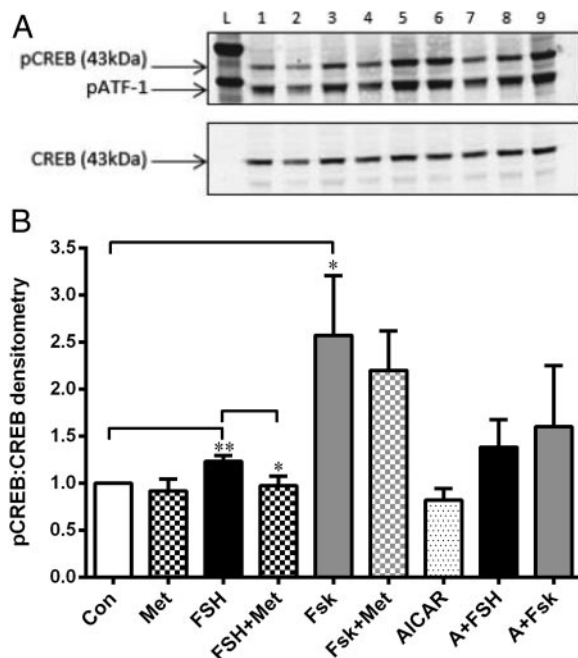
FHSR up-regulation. FSH itself was unable to stimulate the expression of its receptor further, and it is known that there is considerable species variation and also dose and time dependence (reviewed in Ref. 30). Unfortunately, we were also unable to increase FSHR by treating cells with activin. The activin signaling pathway is present in KGN cells, but there is extremely low expression of type 1B activin receptor, which is the main activin receptor for these cells (31). Despite this, metformin still reduced aromatase and *FSHR* mRNA expression in the presence of both activin and FSH. This is an extremely important finding because *FSHR* mRNA levels have been shown to parallel those of FSHR protein in the human ovary (32). Moreover, genetic models of FSH and FSHR mutations have indicated that FSH signaling is quite sensitive to the quantity of functional FSHR (33). This also explains our
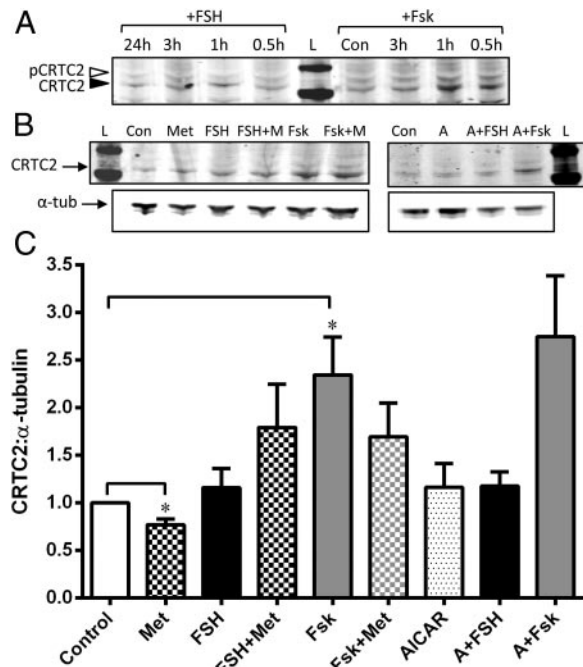
Figure 5. Effect of metformin on CRTC2. Panel A, Representative Western blot showing the effect of FSH or Fsk with time on phosphorylated (cytosolic) and nonphosphorylated (nuclear) CRTC2 levels. Cells were treated with 5 ng/mL FSH for 24 hours, 3 hours, 1 hour, and 30 minutes or $25\mu M$ Fsk for 3 hours, 1 hour, and 30 minutes before extraction of total protein lysates. Western blotting was performed with anti-CRTC2 antibody (1:2500), which detects both the phosphorylated (pCRTC2) (open arrowhead) and nonphosphorylated (CRTC2) (solid arrowhead) forms. Exposure to Fsk for 30 minutes and 1 hour significantly increased the expression of nonphosphorylated CRTC2 compared with pCRTC2 and after 1 hour FSH also increased CRTC2 levels. Panel B, Cells were treated as described for Figure 4A and lysates blotted with anti-CRTC2 and α-tubulin. The levels of CRTC2 (nuclear) were related to α-tubulin, and the effect of all treatments is analyzed and described in panel C. Panel C, Graph of densitometry analysis of Western blot experiments described in panel B. Metformin (checked bars) decreased levels of CRTC2 from basal (*, $P = .02$, 1-sample t test), indicating increased retention of pCRTC2 in the cytoplasm. Fsk (gray bars) significantly increased levels of CRTC2 (*, $P = .03$, 1-sample t test) as did FSH (black bars), indicating increased movement of dephosphorylated CRTC2 into the nucleus. Addition of metformin (checked bars) to either FSH/Fsk was unable to alter this. Unlike metformin, AICAR (spotted bars) did not alter basal CRTC2 levels, nor was it able to affect the FSH/Fsk-induced increase in nuclear localization of CRTC2. Abbreviations: A, AICAR; Con, control; L, ladder; Met or M, metformin; α-tub, α-tubulin.

Figure 6. Hypothetical mechanism of metformin's action on FSH activity in granulosa cells. The FSHR is a G protein-coupled receptor, which upon binding by FSH activates adenylyl cyclase (Ad Cy) to produce cAMP from ATP, which then acts to activate PKA downstream. Fsk directly stimulates adenylyl cyclase activity to greatly increase intracellular cAMP levels. Activation of PKA results in phosphorylation of CREB (pCREB), which allows it to bind to a *CRE* on PII of the aromatase gene (*CYP19*). In addition, activated PKA dephosphorylates CRTC2, allowing it to enter into the nucleus and bind to *CRE* in a coactivator complex with CBP and pCREB, resulting in *CYP19* transcription. We have shown that metformin reduces the levels of FSHR, which then reduces FSH activity as measured by aromatase expression and E_2 production. It does this without altering intracellular cAMP levels. Metformin decreases levels of pCREB, which may inhibit *CYP19* transcription by disrupting the pCREB-CRTC2-CBP coactivator complex that stimulates *CRE* activation on PII. This is supported by the reduction in FSH-stimulated CRE activity brought about by metformin. This occurs independently of AMPK activation. Metformin is also able to prevent the dephosphorylation of basal CRTC2, thus retaining it in the cytosol. It is, however, unable to prevent the cAMP-driven dephosphorylation and nuclear translocation of CRTC2 induced by exposure to either FSH or Fsk.

previous observation of a reduction in basal aromatase expression by metformin that was not via activation of the ERK pathway (6). Interestingly a number of clinical studies showed that addition of metformin to FSH during induction of ovulation lowered serum androgens and E_2 and also produced significantly more monofollicular cycles (11, 34). Our results may explain these findings.

Fsk treatment reduced *FSHR* mRNA, which is consistent with observations that high doses of cAMP analog and Fsk attenuate both the binding and mRNA steady-state levels of FSHR in the ovary and testis (35, 36). Un-
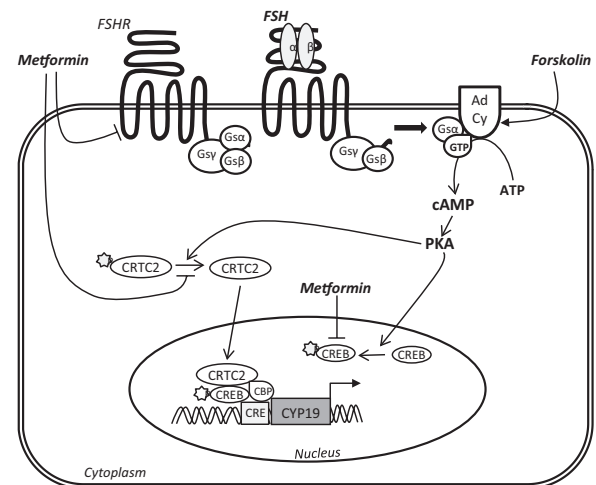
expectedly, this attenuation was enhanced further in the presence of metformin. In vivo, luteinization is associated with an E_2- and cAMP-driven increase in *LHR* mRNA and a rapid and dramatic decrease in mRNA levels of many FSH-stimulated genes including aromatase (37). However, in our experiments, the decrease in *FSHR* mRNA induced by Fsk was not accompanied by an induction in *LHR* mRNA, even after 72 hours exposure. Moreover, there was no accompanying reduction in aromatase, although this may be accounted for by the fact that CRE activity remains high in the presence of Fsk, and in fact, the addition of metformin to the highest dose of Fsk used further enhanced CRE activity. The KGN cells' physiology is closer to that of immature granulosa cells than preovulatory cells, and so LH receptors are not present, but presumably the pathway for their induction would be; hence, this requires further investigation.

The mechanism of FSHR transcriptional regulation is not well known, particularly in the human ovary. SF-1 has been shown to be involved in both FSHR and aromatase

regulation (38, 39), but none of our treatment conditions altered SF-1 expression. As stated earlier, it has been shown that FSH-induced transcription occurs via phosphorylation and activation of CREB, which then binds to CRE to promote transcription of aromatase via PII (12, 13). Interestingly metformin was able to significantly reduce CRE activity in the presence of FSH, but not the Fsk-mediated effect. Investigation of CREB phosphorylation, demonstrated a rapid (1 hour) increase in phosphorylation by both FSH and Fsk, with the effect of Fsk being double that of FSH. Although metformin was able to reduce the FSH-stimulated increase in pCREB, AICAR had no effect on the FSH- or Fsk-mediated increases in pCREB, suggesting that, in the ovary at least (17), inhibition of cAMP-driven aromatase by metformin may be AMPK-independent (Figure 6).

In addition to CREB phosphorylation, what is of importance is the corecruitment of other transcriptional regulators to the promoter (40). In rat granulosa cells, FSH was able to stimulate progesterone secretion via recruitment of the CRTC2, CREB, and CBP complex to up-regulate key steroidogenic genes (*Star*, *CYP11a1*, and *HSD3b*) (18). Metformin enhanced the basal retention of CRTC2 in the cytoplasm, but because metformin (at physiologically relevant doses) does not alter cAMP levels, it was ineffective in preventing the FSH/Fsk-mediated dephosphorylation and translocation of CRTC2 into the nucleus, as was AICAR. Despite this, metformin still reduced FSH-mediated increases in pCREB and CRE activity and hence aromatase expression (Figure 6).

In women with PCOS, the response to metformin treatment could depend on the underlying disturbance; if it is primarily related to insulin resistance and defective insulin signaling, then metformin could help systemically (41) and locally in the ovary (6), but if it is related to a defect in steroidogenesis, then the effectiveness of metformin treatment may be more variable. Metformin has been shown to have the beneficial effect of reducing androgen levels, but it is clear that it also reduces aromatase and hence E_2. We can speculate that if there is initial accelerated growth/proliferation of granulosa cells due to augmentation of insulin and FSH in women with PCOS (42, 43), leading to hyperresponsiveness and premature luteinization, then metformin could slow this down by decreasing both the FSH- and insulin-mediated increase in aromatase directly and indirectly via a reduction in *FSHR* expression, which could be beneficial. It has been shown that granulosa cells from women with PCOS have higher levels of *FSHR* expression compared with those from normal ovaries (44, 45). In effect, metformin could be acting to reset the follicular growth system, and because it takes at least 3 months to grow a cohort of antral follicles that can enter

the menstrual cycle, this would take time. This hypothesis is supported by the recent findings that 3 months pretreatment of obese (2) and nonobese (1) women with metformin improved pregnancy and live-birth rates. In addition, a recent Cochrane review on metformin concluded that ovulation and pregnancy rates were higher in women with PCOS taking metformin (46). However, the effect of metformin on potentiating the down-regulation of *FSHR* expression brought about by sustained cAMP exposure could be detrimental to hyperresponsive follicles from women with PCOS, likewise its reduction of aromatase activity (47). Although as a single treatment metformin has failed to fulfill its promise in women with PCOS, it has proven to have utility when given in conjunction with ovulation-inducing agents where, interestingly, it appears to significantly reduce the incidence of ovarian hyperstimulation syndrome (48, 49). Our results may point to a possible mechanism for this beneficial action.

To summarize, metformin was able to reduce FSH-stimulated aromatase expression and activity by reducing *FSHR* expression. Metformin also reduced FSH-induced phosphorylation of CREB and hence CRE activity, which could result in disruption of the CREB-CBP-CRTC2 coactivator complex that binds to CRE in PII of the aromatase gene. This is mediated in an AMPK-independent manner. This has implications for the use of metformin in treating women with anovulatory PCOS.

Acknowledgments

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