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Adiponectin Induces Periovulatory Changes in Ovarian Follicular Cells

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Adiponectin, the most abundantly synthesized protein in adipose tissue, has plieotropic effects on liver, muscle, endothelium, placenta, and other tissues. We examined direct effects of recombinant porcine adiponectin on porcine ovarian granulosa cells *in vitro*. We demonstrate that adiponectin, at physiologically relevant levels (10–25 μg/ml), provokes expression of genes associated with periovulatory remodeling of the ovarian follicle over a time frame of 6–24 h. These include cyclooxygenase-2, prostaglandin E synthase, and vascular endothelial growth factor. Adiponectin modulates steroid synthetic protein gene expression, increasing steroidogenic acute regulatory protein transcript abundance and reducing cytochrome P450aromatase. Adiponectin has antidiabetic properties and sensitizes tissues to insulin. We show that it interacts with both LH and insulin in inducing expression of cyclooxygenase-2 transcripts in granulosa cells. We determined that the MAPK pathway, via phosphorylation of ERK1/2, is involved in mediation of the adiponectin signal in ovarian granulosa cells, rather than protein kinase A or the classic adiponectin transducer, AMP-activated protein kinase. Adiponectin synthesis is reduced in obesity, and our findings suggest that this reduction plays a role in obesity-related ovarian dysfunction. (*Endocrinology* 147: 5178–5186, 2006)

ADIPOSE TISSUE HAS emerged as a source of endocrine regulators that act on numerous tissues and processes. Adiponectin, the most abundant secretory product of white adipose tissue, is a protein that, in its most basic form, is a homotrimer of three subunits of 25–30 kDa (1). Its synthesis and secretion are related to the differentiation and lipid depletion status of fat; it is highly expressed during adipogenesis, whereas secretion is significantly reduced in obesity (2). Adiponectin circulates as a multimer in plasma, at concentrations from 8 to 25 μg/ml in humans, with sexual dimorphism in the form of higher levels in females (3). Hypoadiponectinemia associates with insulin resistance in both humans and experimental animals (1) and development of type 2 diabetes (4). Three putative receptors have been identified, based on their capability to bind adiponectin. Adiponectin receptors R1 and R2 are proteins with seven-transmembrane domains, products of different genes and are differentially distributed across mammalian tissues (2, 5). They differ from G-coupled receptors in topology: the N terminus is cytoplasmic, whereas the C terminus is extracellular (2). Full-length adiponectin binds to R2, whereas the globular domain of adiponectin preferentially binds the R1 receptor isoform (2). A new study provides evidence that these receptors are biologically active because they bind to adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain, and leucine zipper motif (APPL1) and provoke intracellular signaling (6). The glycolipid-anchored extracellular protein, T-cadherin, has been identified as a receptor for hexameric and higher molecular weight forms of adiponectin (7).

Although the major intracellular signaling transducer for full-length and globular adiponectin is believed to be the AMP-activated protein kinase (AMPK) pathway (2), adiponectin appears to signal through multiple intracellular routes, depending on the cellular context. In human aortic endothelial cells, the cAMP or protein kinase A (PKA) pathway was evoked by adiponectin (8, 9), whereas in other endothelial cell models, the protein kinase B or Akt system transduces the adiponectin signal (10). There is evidence for adiponectin activation of the peripheral peroxisome-activated receptor-γ in placental tissue (11), and MAPK pathways in vascular tissue (12, 13), placenta (11), and osteoblasts (14).

Adiponectin induces multiple effects on target tissues, including increased sensitivity to insulin, vasodilation, antiatherogenic activity, abrogation of adhesion molecule expression, and inhibition of cellular responses to epidermal and platelet-derived growth factors (2). Circulating adiponectin is significantly decreased in women with polycystic ovarian syndrome (PCOS), independent of obesity (15, 16). Furthermore, variants of the adiponectin gene (single nucle-

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Abbreviations: AICAR, 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; APPL1, adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain, and leucine zipper motif; COX, cyclooxygenase; PCOS, polycystic ovarian syndrome; PG, prostaglandin; PKA, protein kinase A; StAR, steroid acute regulatory protein; VEGF, vascular endothelial growth factor.

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otide polymorphisms) are more prevalent in women with PCOS (17). PCOS is characterized by insulin resistance at the ovarian level, elevated androgen synthesis, insufficient FSH to induce aromatization of androgens, and consequent arrest in follicular growth and anovulation (18).

We previously reported the occurrence of adiponectin receptors R1 and R2 in the ovary (5), but there are no reports of direct effects of adiponectin on ovarian cells. Recent studies identified cyclooxygenase (COX)-2 and prostaglandin (PG) E synthesis as downstream targets of adiponectin in myocytes and fibroblasts (19), placenta (11), and hematopoietic cells (20). In addition, adiponectin up-regulates angiogenesis (10, 13). This cluster of physiological phenomena resulting in prostaglandin synthesis (21) and vasculogenesis (22) also characterizes the events provoked by the gonadotropin/insulin interactions that induce mammalian ovulation. Herein we establish that adiponectin, alone and in concert with insulin and gonadotropins, induces gene expression, resulting in prostaglandin and vascular endothelial growth factor (VEGF) synthesis in ovarian granulosa cells. Moreover, adiponectin modulates steroid synthetic protein expression. This pattern of induction of gene expression is consistent with the changes occurring during the periovulatory remodeling of the ovarian follicle.

Materials and Methods

Recombinant porcine adiponectin production

The complete adiponectin coding sequence was obtained by amplification of 5′ and 3′ cDNA ends [5′- and 3′-RACE system (rapid amplification of cDNA ends); Invitrogen, Carlsbad, CA] using primers (Table 1) designed from porcine adiponectin sequences (5). All cloning, expression and purification steps were performed using the QIAexpressionist kit (QIAGEN, Mississauga, Ontario, Canada) with vector pQE-30 (N-terminal 6×HIS tag) according to the manufacturer's instructions. For cloning, *Bam*HI (5′) and *Hin*dIII (3′) restriction sites were added by PCR (primers *Bam* and *Hind*) to a porcine adiponectin sequence from which the signal sequence (nucleotides 1–45) was excluded. *Escherichia coli* strain M15[pREP4] (QIAGEN) was used for transformation. An aliquot of the final product was subjected to SDS-PAGE (12%) separation and Coomassie Blue staining. Purified protein was resuspended in granulosa cell culture medium (MEM; Invitrogen). Protein concentration was determined the Bradford technique (Bio-Rad, Mississauga, Ontario, Canada) and aliquots were frozen at −86 C. Parallel to production of recombinant adiponectin, the *E. coli* strain was transformed by the vector pQE30 lacking the adiponectin sequence. The bacterial extract (6×HIS tag without adiponectin) served as control for nonspecific effects of bacterial products.

Cell culture and treatments

Granulosa cells were aspirated from medium-sized (3–5 mm) follicles of ovaries collected from prepubertal gilts at the slaughterhouse and cultured as previously described (23). Briefly, 25×10^6 viable cells/well were pooled in Opti-MEM (Invitrogen) containing, 5×10^4 IU/liter penicillin (Invitrogen), 50 μg/liter streptomycin (Invitrogen), 0.5 mg/liter Fungizone (Invitrogen), and 4% fetal bovine serum (Invitrogen) and plated at 37 C in 95% humidified air with 5% CO_2 for 24 h. Medium was replaced with serum-free medium and treatments were added after 18 h. Cells were incubated with 0, 2.5, 10, or 25 μg/ml of recombinant porcine adiponectin for 2, 6, 12, and 24 h. Initial trials demonstrated that the latter two dose induced expression of candidate genes in the cell model and were selected for further investigation. The agonist of the AMPK pathway, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; 1 and 2.5 mM; Sigma, St. Louis, MO), was added to the cells for period from 5 min to 12 h. To test interactions among adiponectin, LH, and insulin, granulosa cells were treated with combinations of adiponectin (25 μg/

TABLE 1. The sequences of oligonucleotide primer pairs used in this study for amplification of porcine adiponectin, adiponectin receptors R1 and R2, COX-2, PGE synthase, VEGF, StAR, cytochrome P450 aromatase (CYP19), LH receptor, and cyclophilin

Gene	Oligonucleotide primers	Sequence (5′–3′)
Adiponectin	Forward (A)	YCCVGGAACCCCWGGCAGGAAA
	Reverse (1)	CCRTACACC TGGAGCCAGACTT
	Forward (B)	TCAGCRTTCAGTGTGGGGYTGGAGA
	Reverse (2)	GCCTGGTCCACATTATTTTCCT
	Forward (C)	TGCTGGGAGCTGTTCTACTG
	Reverse (3)	AGTGGAATTTGCCAGTGGTGACA
	5′R-1	ATACCCGCCATCCAGCCT
	5′R-2	GTAGTGCTCCAGGCTTCT
	5′R-3	GGTTTCCTGGCCGAGACT
	3′R-1	GAAGGTCAGCCTCTACAA
	3′R-2	GAAGGACAAGGCTGTACT
	3′R-3	CACCTACGACCAGTACCA
Adiponectin R1	Forward	AACCCACCCAAAGCTGAAGA
	Reverse	CTGAGCATGGTCAAGATTCC
Adiponectin R2	Forward	GCCTGGGGATCTTTTATATGTTTC
	Reverse	GCCGATCATGAAGCGAA
COX-2	Forward	ATGGGTGTGAAAGGGAGGAAAGAG
	Reverse	ATCATCAGACCAGGCACCAGACCA
PGE synthase	Forward	GCTGCGGAAGAAGGCTTTTG
	Reverse	AGGTAGGCCACGGTGTGTAC
VEGF	Forward	CTCCGAAACCATGAACTTTCTG
	Reverse	CTCGCTCTATCTTTCTTTGGTCT
StAR	Forward	AACATGAAGGGGCTGAGGCAC
	Reverse	TCCACCACCACCTCCAGC
CYP19	Forward	TGCTGCTCACTGGCTTTCTTCTCT
	Reverse	AGAGGTTGTTAGAGGTGTCCAGCA
LH receptor	Forward	AGAGTGAACTGAGTGACTGG
	Reverse	TGATGACTGTGAGGGTGTAG
Cyclophilin	Forward	ACCGTCTTCTTCGACATCGC
	Reverse	CTTGCTGGTCTTGCCATTCC

ml), LH (10 ng/ml National Institutes of Health, U.S. Department of Agriculture-Reproduction Laboratory, Beltsville, MD), and insulin (10 ng/ml; Sigma) for 12 h. To examine the role of the PKA pathway, cultures were treated the PKA inhibitor H89 (10 μM; Sigma) beginning 1 h before adiponectin treatment. The MAPK/ERK pathway in the adiponectin-induced responses was probed by treatment of the granulosa cells with 10 μM of the specific ERK 1/2 inhibitor, U0126 (Sigma), 1 h before the addition of adiponectin.

Extraction of RNA and purification and reverse transcription of granulosa cells

Cells were homogenized in guanidine isothiocyanate (Invitrogen) with 0.12 M β-mercaptoethanol (Sigma) and the QIAshredder kit (QIAGEN). RNA was purified by RNeasy Protect Mini kit (QIAGEN), following the manufacturer's recommendations. A 1-μg sample of total RNA was reverse transcribed with the SuperScript kit (Invitrogen) according to the manufacturer's instructions.

Semiquantitative RT-PCR

COX-2, VEGF, steroid acute regulatory protein (StAR), CYP19, adiponectin receptors 1 and 2, LH receptor, and cyclophilin primers (Table 1) were obtained from published porcine sequences (GenBank AY028583, AF318502, NM 213755, U92245, AY452710, AY452711, M29525, AY266299, respectively). PGE synthase primers were designed based on homologous sequences in the rat (AF280967) and cow (NM_174443). PCR products of expected size were excised and purified, and their authenticity confirmed by sequencing. Semiquantitative PCR for each target gene used the number of cycles (21–35) that ensured exponential phase amplification. PCR products were separated in a 1.5% agarose gel, stained in ethidium bromide, and densities of amplified fragments analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Results were expressed as density ratio of target gene to cyclophilin.

Assays for PGE_2 and VEGF

PGE_2 concentration was evaluated by enzyme immunoassay (Oxford Biomedical Research, Oxford, MI) and VEGF by the Quantikine human VEGF immunoassay (R&D Systems, Minneapolis, MN) kits, according to the manufacturers' instructions. All samples were tested as duplicates within a single assay for each protein. The within-assay coefficient of variation, calculated between duplicates, varied from 1.1 to 4.3% for PGE_2 and 3.5 to 6.5% for VEGF.

Immunoblotting procedures

Serum from prepubertal gilts and fluid from porcine small (1–3 mm diameter), medium (3–5 mm), and large (>8 mm) follicles was collected, and an amount of 10 μg total protein was subjected to denaturing by treatment with 200 mM β-mercaptoethanol and boiling (5 min) followed by immunoblotting to establish presence and concentration of adiponectin in these biological sources. For culture experiments, three wells per treatment were pooled for Western blotting. Total proteins (30 μl) were resolved by one-dimensional 12% SDS-PAGE minigel at 100 V and then electrophoretically transblotted to nitrocellulose membranes (Trans-Blot; Bio-Rad). Membranes were washed in 0.1% (vol/vol) Tween 20 in Tris-buffered saline [100 mM Tris, 0.9% NaCl (pH 7.5)] and incubated with primary antibodies: antimouse adiponectin (1:5,000; gift of T. S. Tsao, Whitehead Institute, Cambridge, MA); antimouse adiponectin (1:1,000; Cedarlane, Hornby, Ontario, Canada); antisheep COX-2 (1:10,000; gift of Stacia Kargman, Merck, Kirkland, Québec, Canada); anti-ERK1/2-CT (1:10,000; Upstate, Lake Placid, NY); antiphospho-ERK1/2 (1:12,000; Upstate); anti-AMPK and antiphospho-AMP kinase (Cedarlane, 1:1,000) anti-α-tubulin (1:5,000; Developmental Studies Hybridoma Bank, National Institute of Child Health and Human Development). After addition of second antibody, horseradish peroxidase antirabbit IgG (1:10,000), signal was detected by adding the peroxidase substrate (SuperSignal West Pico chemiluminescent substrate; Pierce, Rockford, IL) and exposure to photographic films (Kodak, Rochester, NY).

Statistical analysis

All experiments were performed in triplicate and repeated at least three times. Treatment means were analyzed using least square ANOVA and the general linear model procedures of SAS (Cary, NC). In the presence of a significant overall treatment effect, means were compared by Duncan's multiple range test. A probability level of $P < 0.05$ was considered significant.

Results

Adiponectin is present in follicular fluid

To confirm that adiponectin was present in follicular fluid, we performed a Western blot on aliquots of 10 μg total

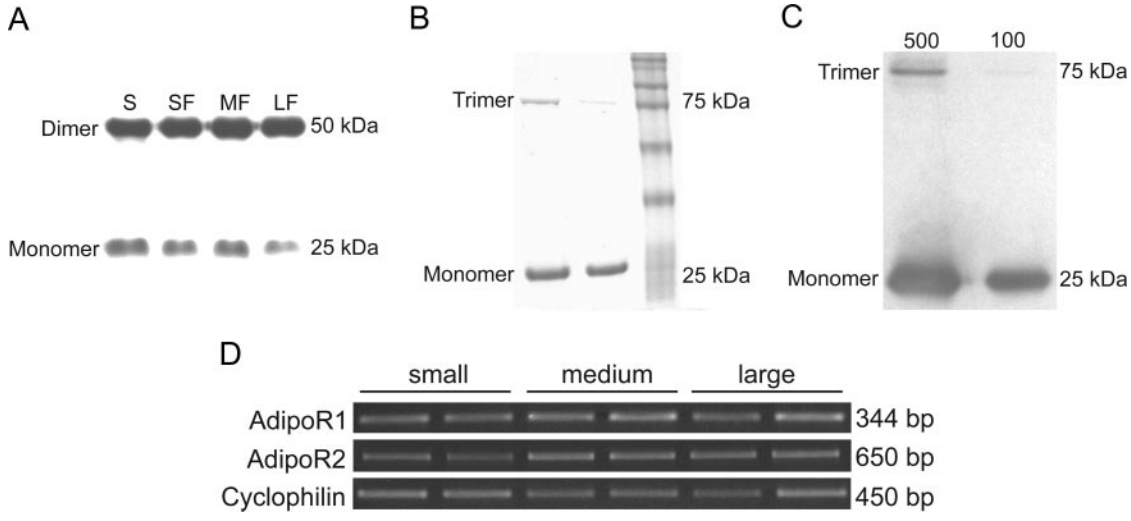

FIG. 1. Adiponectin and adiponectin receptors are present in antral follicles. A, Western analysis indicates that adiponectin is present in the antral fluid of small (SF), medium (MF), and large porcine (LF) follicles at concentrations similar to serum (S). B, Recombinant adiponectin was produced based on the porcine sequence. The final eluate from the purification steps was separated by SDS-PAGE (12% acrylamide gel), followed by Coomassie staining of gel. C, Confirmation of purification of adiponectin was performed by immunoblotting using an antibody raised against mouse adiponectin. D, RT-PCR analysis demonstrating the presence of transcripts for both the R1 and R2 isoforms of the adiponectin receptors in porcine follicles. Two follicles, each from a different animal, are represented in this image.

protein from small, medium, and large porcine follicles. An equivalent aliquot of total serum protein was run for comparison (Fig. 1A). Densitometric evaluation and summing of the two protein bands indicated that adiponectin concentrations in follicular fluid to be 80–90% of those present in serum.

Expression and purification of recombinant porcine adiponectin

Recombinant porcine adiponectin, based on the full-length porcine sequence (5), was produced the *E. coli* M15[pREP4] expression system. SDS-PAGE resolved monomeric and trimeric forms of porcine adiponectin from the final eluate (Fig. 1B). Western analysis confirmed the authenticity and the size distribution of recombinant porcine adiponectin (Fig. 1C). By RT-PCR, we showed both adiponectin receptor R1 and R2 transcripts to be present in granulosa cells of porcine follicles during development, with no apparent evolution of their abundance as follicles matured (Fig. 1D).

Adiponectin induces COX-2 transcription and protein production in porcine granulosa cells

To examine effects of adiponectin on ovarian cells, we used porcine granulosa cells exposed briefly to serum, followed by incubation in serum-free conditions. This model recapitulates estrogen synthetic conditions of antral follicles *in vivo*, as indicated by retention of expression cytochrome P450aromatase (CYP19), lost during luteal differentiation (24). Preliminary trials revealed that adiponectin at doses of 25 μg/ml (Fig. 2A) increased abundance of COX-2 mRNA in this cell model over 12 h of treatment. Augmentation of COX-2 transcript abundance was accompanied by similar induction of COX-2 protein expression after 12 h of incubation of cells with adiponectin (Fig. 2B). His-tag-purified recombinant extracts from bacterial transformation with the control vector (no adiponectin sequence) failed to induce either COX-2 mRNA (Fig. 2A) or protein (Fig. 2B), confirming adiponectin-specific effects of the recombinant protein. Time-course studies of COX-2 mRNA induction by 25 μg/ml adiponectin resulted in a significant time-dependent increase in transcript abundance from 2 to 12 h after treatment of granulosa cell cultures (Fig. 2C). We then examined whether adiponectin modulated either adiponectin receptor 1 and 2 expression in the granulosa cell model *in vitro* over 2, 6, 12, and 24 h of treatment, as indicated by increases in their mRNAs. There was no change in either receptor transcript population (Fig. 2D), suggesting that the receptors, clearly present in our cell model, did not vary in expression in response to their ligand.

Adiponectin increases PGE synthase mRNA and PGE_2 secretion in porcine granulosa cells

We examined whether PGE_2, the product of prostaglandin E synthase and the key prostanoid in ovulation (25) downstream of COX-2 (21), can be regulated by adiponectin in granulosa cells. We observed a significant increase in PGE synthase message abundance after 6, 12, and 24 h of treatment with adiponectin (25 μg/ml) (Fig. 3A). The expression

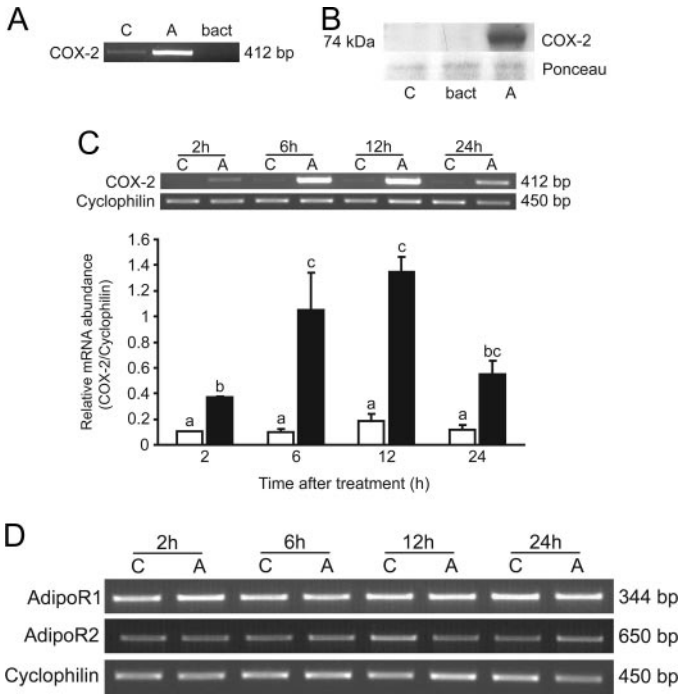

FIG. 2. Adiponectin stimulates COX-2 mRNA and protein expression in granulosa cells. A, RT-PCR analysis demonstrates increase in abundance of mRNA for COX-2 in porcine granulosa cells treated for 12 h with 25 μg adiponectin (A). Medium alone (C), or extract form of bacteria transformed with the vector devoid of the adiponectin sequence (bact) had no effect. B, Representative immunoblot demonstrating that adiponectin (A) at 25 μg/ml induces expression of COX-2 protein in porcine granulosa cells at 12 h of treatment, whereas medium alone (C) or bacterial extract (bact) failed to do so. Ponceau staining of the blot was used to demonstrate equivalent loading of sample lanes. C, Adiponectin at 25 μg/ml induces COX-2 transcript over 2, 6, 12, and 24 h. *Open bars* represent means of transcript abundance in control cultures, *black bars* are treated cultures. The quantification represents mean ± SEM of triplicate experiments. *Different superscripts* represent significant differences in means between treatments and across time ($P < 0.05$). D, RT-PCR analysis shows that treatment of porcine granulosa cells with adiponectin (25 μg/ml) does not alter the abundance of transcripts for either adiponectin receptor isoform, AdipoR1 or AdipoR2.

of the protein form of the enzyme and its activity was confirmed by the observation that adiponectin induced the accumulation of PGE_2 in cultures at 12 and 24 h after initiation of treatment (Fig. 3B). There was a nonsignificant trend toward increase at 6 h of incubation ($P = 0.08$).

Adiponectin induces VEGF expression in porcine granulosa cells

The granulosa cell compartment is the major site of extensive VEGF synthesis during the periovulatory transition of the follicle to the corpus luteum (26). Because there is evidence that PGE induces VEGF synthesis in the ovary (27), we examined VEGF mRNA and protein expression in granulosa cells in response to adiponectin. The ligand, at 25 μg/ml, induced a significant increase in VEGF mRNA at 6 h of treatment, peaking at 12 h after the initiation of the experiment (Fig. 3C). This was accompanied by a time-dependent response, comprising severalfold increase in VEGF pro-

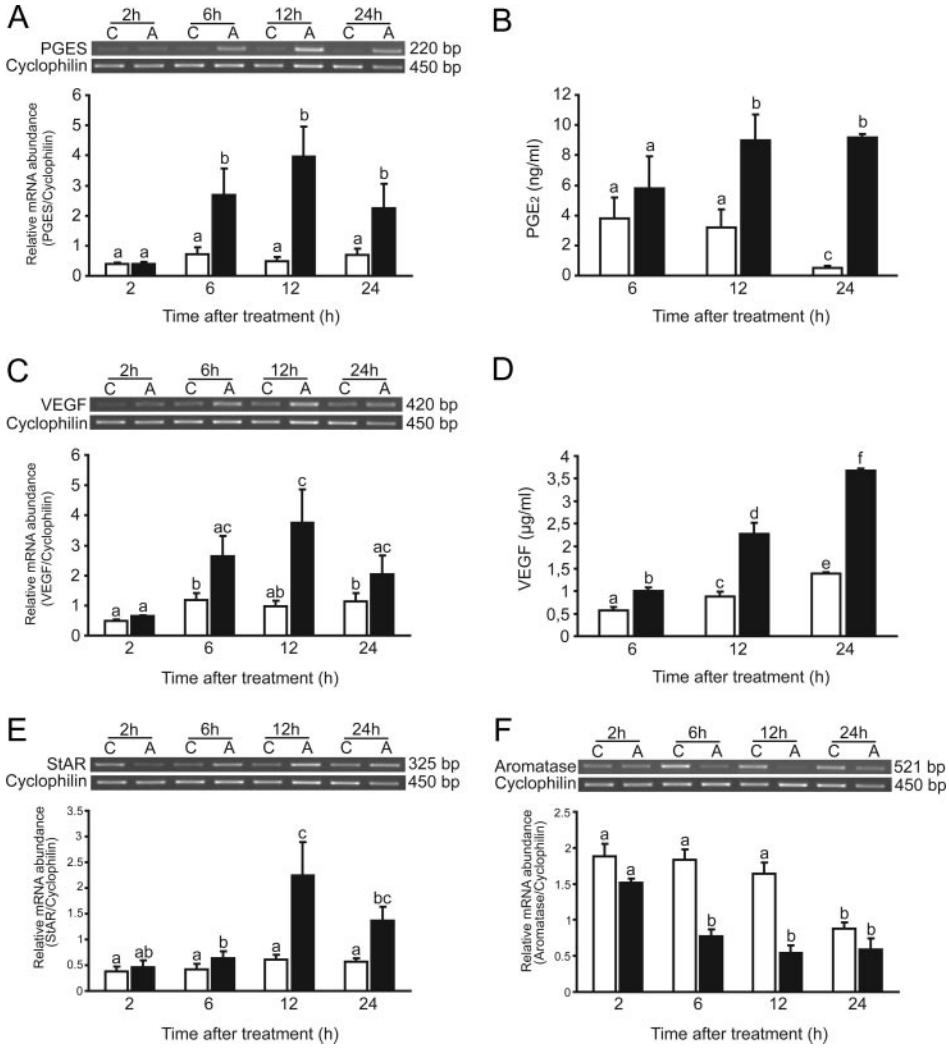

FIG. 3. Adiponectin stimulates expression of PGE synthase (PGES) and VEGF expression in cultured porcine granulosa cells. A, PGES transcript abundance is up-regulated at 6, 12, and 24 h in granulosa cells cultured with 25 μg/ml adiponectin. *Open bars* represent means of transcript abundance in control cultures; *black bars* are treated cultures in all panels of this figure. B, Confirmation of increased PGES activity by increased mean accumulation of PGE_2, the enzyme product in media of cultures treated with 25 μg/ml adiponectin at 12 and 24 h of treatment. C, Mean VEGF mRNA abundance increases in response to treatment of granulosa cells with 25 μg/ml adiponectin. D, VEGF protein accumulation in porcine granulosa cells, measured by ELISA, is increased at 6, 12, and 24 h of treatment with adiponectin. StAR message is up-regulated in porcine granulosa cell cultures from 6 to 24 h by 25 μg/ml adiponectin (E), whereas cytochrome P450aromatase (CYP19) mRNA declines in response to adiponectin treatment (F). Data are presented as mean ± SEM of triplicate experiments. *Different superscripts* represent significant differences in means between treatments and across time ($P < 0.05$).

tein secretion, beginning as early as 6 h and continuing over 24 h of adiponectin treatment (Fig. 3D).

Adiponectin modifies steroid synthetic enzyme expression

The periovulatory period in the granulosa cells of the porcine follicle is characterized by acquisition of steroidogenic synthetic capacity and decline in estrogen synthesis, as a result of extinction of CYP19 expression (28). We therefore investigated the effects of adiponectin on the rate-limiting step in steroidogenesis, synthesis of StAR, and the decline in CYP19 expression. An increase in StAR mRNA abundance (Fig. 3E) was seen as early as 6 h after initiation of treatment with 25 μg/ml adiponectin, mirrored by declines in CYP19 transcript abundance (Fig. 3F). StAR mRNA peaked at 12 h and remained elevated through 24 h, whereas the CYP19 message was suppressed through 24 h (Fig. 3, E and F).

Adiponectin interacts with LH and insulin

In mammals, ovulation is provoked by the preovulatory surge of LH from the anterior hypophysis. Gonadotropins, including LH, interact with insulin in induction of steroidogenesis (29). Adiponectin increases sensitivity of peripheral tissues to insulin, and there is evidence for cross talk between adiponectin and insulin signaling pathways (4). We therefore explored interactions among LH, insulin, and adiponectin in induction COX-2 expression in our granulosa cell model by incubation with each and their combinations for 12 h. Adiponectin provided the expected increase in COX-2 mRNA abundance, but there was little response to either LH or insulin alone, or to their combination (Fig. 4A). In contrast, combinations of insulin and adiponectin, LH and adiponectin and insulin, and LH and adiponectin substantially augmented the mRNA for COX-2 relative to adiponectin alone (Fig. 4A). We then examined whether these effects resulted from changes in LH receptor expression. The results indicate that adiponectin induced no increase in LH receptor transcript abundance through 24 h (Fig. 4B).

Intracellular pathways in adiponectin induction of prostaglandin and VEGF genes

It had previously been shown that adiponectin acts through the cAMP/PKA pathway in endothelial cells (9). We therefore used the PKA inhibitor, H89, at a dose (10 μM) expected to completely block activity of this kinase (30). H89

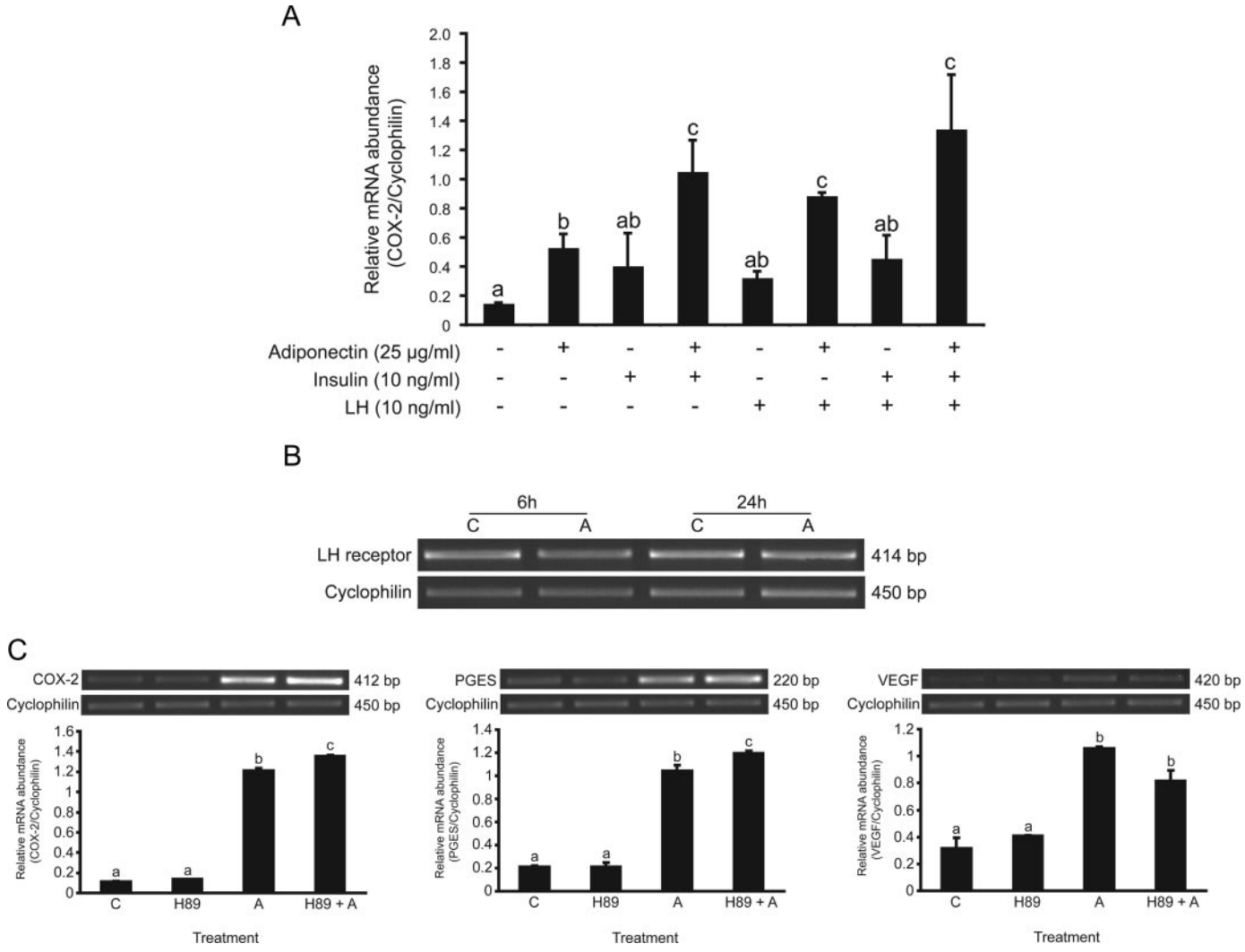

FIG. 4. Adiponectin interacts with LH and insulin in induction of COX-2 transcript accumulation but has no effect on LH receptor mRNA abundance. Adiponectin does not act via the PKA pathway. A, Porcine granulosa cells incubated for 12 h with adiponectin (25 μg/ml) displayed increase in mean ± SEM. COX-2 message. Insulin (10 ng/ml) and LH (10 ng/ml) alone had little effect, whereas the combination of adiponectin or insulin produced an additive elevation of COX-2 expression. There was no further additive effect of all three ligands in combination. PGES, PGE synthase. B, Adiponectin (A) at 25 μg/ml does not alter the abundance of LH receptor transcripts relative to control (C) cultures over 6 or 24 h of incubation. C–E, Mean ± SEM. COX-2, PGE synthase, and VEGF message abundance in cultures treated for 12 h with adiponectin or H89, an inhibitor of PKA activity, demonstrates no effect of this inhibitor on the cellular response to adiponectin. Means bearing *different superscripts* are significantly different at $P < 0.05$.

treatment had no effect on adiponectin induction of COX-2, PGE synthase, or VEGF transcript abundance at 12 (Fig. 4, C–E) and 24 h (data not shown), indicating that the PKA pathway is not involved in the response. These data further point to a direct effect of adiponectin on VEGF, rather than through PGE induction, as the principal mode PGE signal transduction is via PKA activation (31).

We then examined the AMPK pathway. Repeated trials demonstrated that adiponectin elevated AMPK phosphorylation (1.8- to 2.0-fold) over 1 h (Fig. 5A). If this mechanism is functional in granulosa cells, we expected that the effects of adiponectin could be recapitulated by the synthetic AMPK agonist, AICAR. We therefore treated granulosa cells over 6 and 12 h with two doses, bracketing the biological activity of this compound in other tissues (32). This induced only modest (40–60%) increases in AMPK phosphorylation in the short term (Fig. 5B). Furthermore, rather than increase COX-2 mRNA abundance, AICAR suppressed expression of the transcript in a dose- and time-dependent manner (Fig. 5C). AICAR further inhibited adiponectin-induced MAPK-ERK 1/2 phosphorylation by a mean of 30% in three independent trials (data not shown). There is evidence that H89, at the dose used, reduces AMPK activity (30), but, as can be seen from Fig. 4C, it had no effect on adiponectin induction of target genes. The sum of the information suggests the AMPK pathway does not subserve adiponectin action in the ovary.

Adiponectin has been shown to activate (14) and inhibit (12) the MAPK-ERK1/2 pathway, depending on cellular context. We next confirmed that adiponectin at the dose and in the cell model used induces phosphorylation of ERK1/2, more than 2-fold over a 1-h treatment (Fig. 6B). This effect is abrogated by U0126, a specific inhibitor of MAPK-ERK1/2

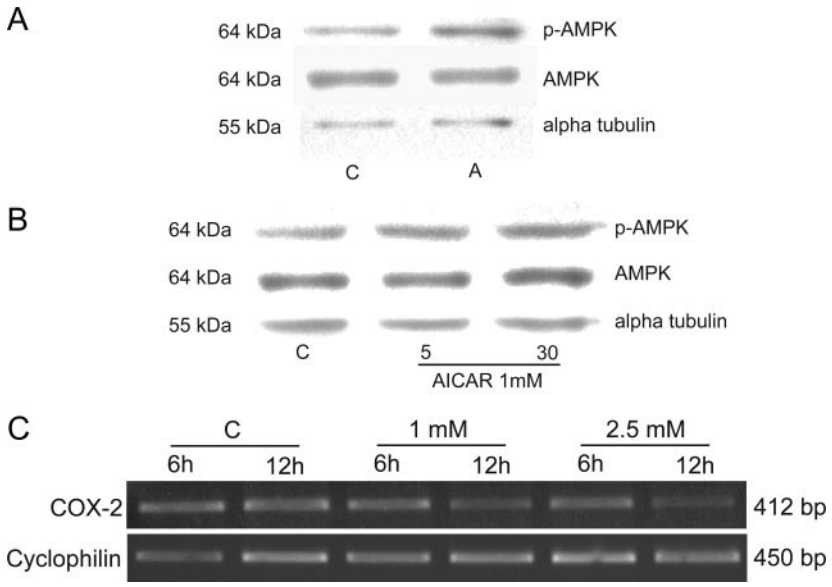

FIG. 5. Adiponectin and AICAR induce phosphorylation of AMP kinase, whereas AICAR decreases COX-2 expression in granulosa cell cultures. A, Immunoblot demonstrating the presence of total (AMPK) and phosphorylated (p-AMPK) forms of AMP kinase in control (C) cultures or those treated with and adiponectin (A) at 1 h of treatment. Adiponectin induced modest increases. B, Immunoblot demonstrating that AICAR at 1 mM mildly elevated phosphorylation of AMPK. C, Longer-term treatment of porcine granulosa cells with two doses of AICAR reduces COX-2 transcript accumulation in porcine granulosa cells, beginning as early as 12 h after treatment. Data depicted in A–C are representative of at least three replicate experiments.

(Fig. 6B). We therefore incubated porcine granulosa cells with U0126 in the presence and absence of adiponectin for 12 h. Adiponectin induced the expected increases in COX-2, PGE synthase, and VEGF mRNA expression, whereas U0126 treatment dramatically reduced the adiponectin-induced stimulation of abundance of all three transcripts (Fig. 6A). The inhibitor was used at a concentration previously shown to be effective in the porcine granulosa cell model (33). It had no apparent effect on basal expression of the three target genes. Together these results implicate the MAPK-ERK1/2 cascade in mediation of some of the actions of adiponectin in the ovary.

Discussion

Herein we show that adiponectin acts directly on granulosa cells, inducing expression of genes and proteins in a pattern that resembles the periovulatory remodeling of the ovarian follicle. Rapid and consistent induction of COX-2, along with concomitant provocation of the cytosolic form of the PGE synthase gene and activity downstream of COX-2, recapitulates events in the ovulatory process (21). Likewise, adiponectin treatment up-regulates StAR and VEGF, although down-regulating CYP19, all changes characteristic of the interval between initiation of the ovulatory process by gonadotropins and subsequent rupture of the ovarian follicle (28). Moreover, adiponectin interacts with insulin and LH, cogonadotropins in induction of granulosa cell gene expression. Adiponectin is present in porcine follicular fluid at concentrations similar to those found in serum and to concentrations used in these trials, demonstrating that this is a physiological phenomenon.

Mice bearing null mutations of the adiponectin gene are fertile, suggesting that this protein is not absolutely essential for ovarian function (34). It may nonetheless be required for ovulation in other species or play a complementary role in mammals, either acting directly on ovulation or by increasing sensitivity to insulin and gonadotropins, thereby influencing the ovulatory process. There is ample evidence that reduced insulin sensitivity plays a role in impairment of final maturation and ovulation of the follicles in PCOS ovaries. Women with PCOS have significantly lower circulating concentrations of adiponectin (35). Whereas it might be argued that their lowered adiponectin synthesis results from the elevated androgen profile in PCOS (3), women with polymorphisms in the adiponectin gene that reduce adiponectin are 5-fold more likely to be afflicted with PCOS (35). These correlations, along with our data, strongly implicate adiponectin as an authentic modulator of follicular function.

We used recombinant porcine adiponectin, produced in bacteria, similar to previous reports (36), comprised of full-length monomeric and trimeric forms. Although the trimeric form has greater bioactivity relative to multimeric forms in other systems (1), it appears not to interact with the putative t-cadherin receptor (7). Furthermore, multimeric, trimeric, and globular forms of recombinant adiponectin display differing biological activities. The multimeric form is highly active in liver (1) and endothelial cells (37). The trimeric and full-length forms are active in a wide range of tissues, acting through the AMPK cascade (2). Globular adiponectin, a proteolytic product devoid of the collagen domain, has similar effects to full-length adiponectin in some cellular contexts (38) and effects that differ from the full-length form in others (39). The role of multimeric and globular forms in the ovary awaits further investigation.

The accumulated literature implicates multiple intracellular signaling pathways in transduction of the adiponectin signal. Because the canonical route is the AMPK pathway (2), we were surprised when three lines of evidence indicated that species-specific, full-length adiponectin does not function through via AMPK in provoking periovulatory responses in porcine granulosa cells. Whereas adiponectin increased abundance of the phosphorylated form of the enzyme, treatment of primary granulosa cultures with AICAR only modestly increased AMPK phosphorylation. In fact, this compound suppressed MAPK-ERK 1/2 phosphorylation and COX-2 expression. These results are consistent with a previous report in which AICAR interfered with MAPK-ERK 1/2 phosphorylation and attenuated synthesis

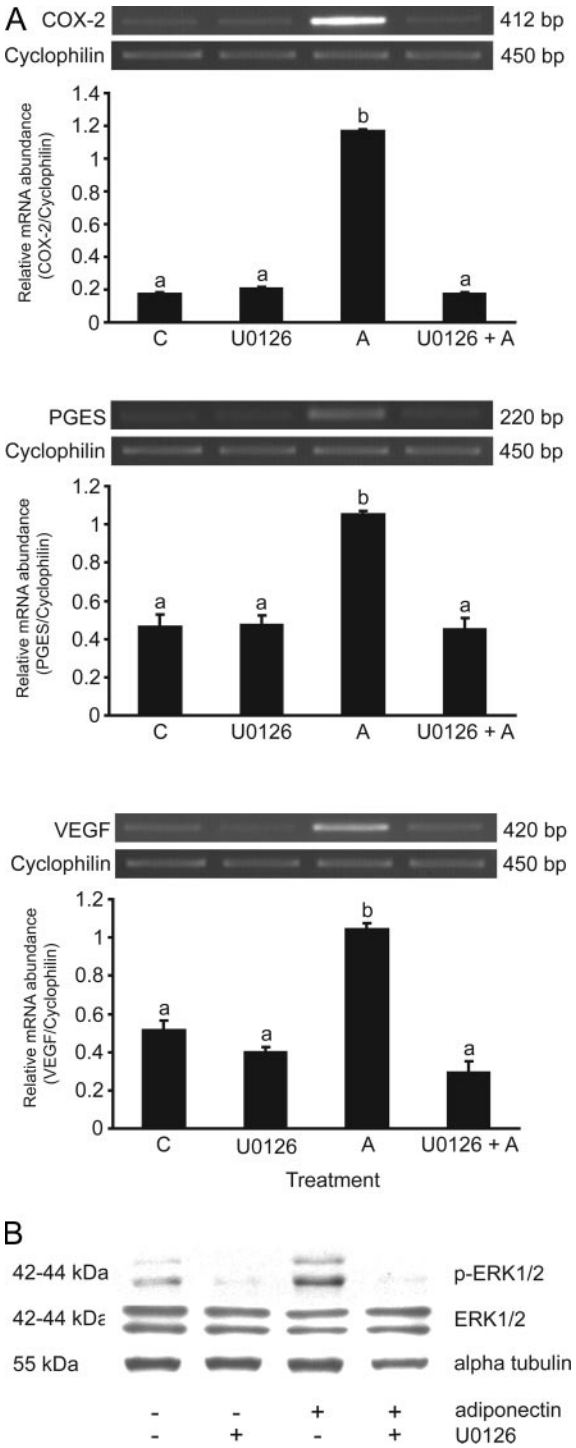

FIG. 6. Induction of COX-2 by adiponectin is mediated through phosphorylation of MAPK-ERK1/2. A, In cells pretreated for 1 h with U1026 (10 μM), followed by adiponectin (A) treatment (25 μg/ml), adiponectin alone, or dimethylsulfoxide for 12 h, adiponectin induction of expression of COX-2, PGE synthase (PGES), and VEGF was completely blocked. Relative mRNA abundance of COX-2, PGE synthase, and VEGF was assayed by RT-PCR. *Different superscripts* represent significant differences in means of three replicates of the experiment ($P < 0.05$). B, Western analysis demonstrates that adiponectin induces phosphorylation of ERK1/2 (p-ERK1/2) at 1 h of treatment, an effect completely abrogated by the specific ERK 1/2 inhibitor U1026 (10 μM) in porcine granulosa cells.

of progesterone in rat granulosa cells (40). In the present study, the AICAR-induced reductions in COX-2 are interpreted as further confirmation that this pathway does not regulate adiponectin-modulated periovulatory events. Use of H89, which, at the dose used, abolishes PKA activity (30), provides strong evidence to eliminate this intracellular route in adiponectin action on all three genes studied. Moreover, H89 blunts AMPK activation in other systems (30), whereas we show no effect on adiponectin induction of COX-2, PGE synthase, or VEGF.

Adiponectin appears to activate the MAPK pathway because our results indicate that it phosphorylates ERK 1/2. Moreover, U0126, a specific blocker of this activation (30), used at a dose known to be functional in the porcine granulosa cell model (33), abrogated the COX-2, PGE synthase, and VEGF responses to adiponectin in ovarian cells. This is consistent with observations that adiponectin modifies ERK1/2 phosphorylation in vascular tissue (12, 13) and upregulates specific gene expression via ERK1/2 in human placental explants (11). Further investigation is required to establish whether these are direct effects or mediated through cross talk between signaling pathways and whether other pathways subserve the multiple actions of adiponectin in the ovary.

Intriguing new evidence suggests that adiponectin-modulated increase in sensitivity to insulin are linked via APPL1, an adaptor protein that interacts with elements of both signaling pathways (6). Remarkably, APPL1 is implicated in gonadotropin signaling by association with the FSH receptor (41). These findings provide a potential mechanism for gonadotropin/insulin/adiponectin interactions in the ovary.

In summary, we provide the first evidence that adiponectin stimulates gene expression in ovarian cells. Its effects are consistent with gene expression changes during periovulatory remodeling of the ovarian follicle. This work extends previous observations that adiponectin can induce prostanoid synthesis and provides potential mechanisms to link adipose tissue abundance to ovarian function and fertility.

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