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Effect of Epidermal Growth Factor-Like Peptides on the Metabolism of In Vitro-Matured Mouse Oocytes and Cumulus Cells[1]

Dulama Richani, Melanie L. Sutton-McDowall, Laura A. Frank, Robert B. Gilchrist,[3] and Jeremy G. Thompson[2,3]

Research Centre for Reproductive Health, Discipline of Obstetrics and Gynaecology, The Robinson Institute, School of Paediatrics and Reproductive Health, The University of Adelaide, Adelaide, South Australia, Australia

ABSTRACT

Oocyte in vitro maturation (IVM) is an assisted reproductive technology that involves the maturation of cumulus-oocyte complexes (COCs) that are then capable of normal development. We have shown that epidermal growth factor (EGF)-like peptide signaling is perturbed in mouse COCs undergoing IVM when matured with follicle-stimulating hormone (FSH) and/or EGF, but supplementation of IVM with EGF-like peptides amphiregulin or epiregulin improves oocyte developmental competence. Here we aimed to determine whether EGF-like peptides regulate COC metabolism. Immature 129/Sv mouse COCs underwent IVM with FSH, EGF, amphiregulin, epiregulin, betacellulin, or no treatment (control). Epiregulin significantly increased intraoocyte flavin adenine dinucleotide (FAD) and REDOX (reduction and oxidation) ratio compared to FSH and control. Amphiregulin and epiregulin significantly increased the proportion of J aggregates (from JC-1) in oocyte mitochondria compared to control, FSH, or EGF, and this coupled with FAD and REDOX measures indicates greater mitochondrial activity. There were no differences in glucose consumption, lactate production, or glycolysis between COCs matured with FSH, EGF, and EGF-like peptides. COCs matured with EGF or EGF-like peptides exhibited significantly higher mRNA expression of the hexosamine biosynthesis pathway (HBP) rate-limiting enzyme gene *Gfpt2*, *Has2* expression, and global beta-*O*-linked glycosylation of proteins, compared to control or FSH, suggesting greater HBP activity. Our findings suggest that 1) EGF-like peptides, particularly epiregulin, induce more oocyte mitochondrial activity than EGF or FSH and 2) EGF-like peptides and EGF induce greater HBP activity, enabling more hyaluronic acid synthesis and protein beta-*O*-linked glycosylation. These metabolic alterations may be a mechanism by which EGF-like peptides increase oocyte developmental competence.

amphiregulin, β-O-linked glycosylation, EGF, epiregulin, FSH, glucose, IVM, metabolism, mitochondria, oocyte

INTRODUCTION

The luteinizing hormone (LH) surge in females triggers oocyte meiotic resumption and ovulation, as well as the expansion/mucification of the surrounding cumulus cell matrix, in ovarian antral follicles [1]. A key molecular cascade that propagates these LH-induced processes involves key members of the epidermal growth factor (EGF) family of proteins called EGF-like peptides [2]. The LH surge induces rapid and transient expression of three EGF-like peptides, amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC), in follicular granulosa cells [3]. These peptides are produced as transmembrane precursors that shed and bind to the membrane-bound EGF receptor (EGFR), which is expressed on mural and cumulus granulosa cells, to activate the key downstream effector extracellular signal-regulated kinases 1 and 2 complex (ERK1/2) [4]. Along with oocyte meiotic resumption, ERK1/2 activation via the EGF-like peptides/EGFR is crucial for cumulus matrix expansion, as it stimulates the expression of hyaluronan synthase 2 (*Has2*), pentraxin 3 (*Ptx3*), tumor necrosis factor alpha-induced 6 (*Tnfaip6*), and prostaglandin-endoperoxide synthase 2 (*Ptgs2*), all of which are required for cumulus matrix expansion and some of which are essential for ovulation [4–6].

We have previously shown that EGF-like peptide signaling is perturbed when cumulus-oocyte complexes (COCs) are removed from the antral follicle at the immature germinal vesicle stage and in vitro matured (IVM) [7]. IVM is a technique widely used in animal reproductive technologies and, less commonly, in human assisted reproduction [8]. The clinical use of IVM as an assisted reproductive technology has been limited because of its significantly lower efficiency compared with conventional in vitro fertilization [9]. This is because IVM oocytes are less capable of supporting embryo development than their in vivo-matured counterparts [10–12]. The decreased developmental competence is likely in part attributable to the use of suboptimal oocyte maturation conditions, which leads to a disruption of the finely balanced cytoplasmic and nuclear processes in the oocyte as it attempts to develop in an artificial in vitro environment [13]. COCs undergoing IVM are almost universally cultured in the presence of follicle-stimulating hormone (FSH), and less commonly with or without EGF (another member of the EGF family of proteins) [14]. FSH and EGF are inducers of cumulus cell EGF-like peptide expression and, consequently, oocyte meiotic resumption and cumulus matrix expansion in vitro [7, 15–17]. However, we have recently shown that using FSH or EGF as IVM additives does not maintain adequate activation of the EGF-like peptide signaling cascade in the cumulus cells of IVM-matured COCs when compared to COCs matured in vivo [7]. Furthermore we, and others, have shown that the addition of exogenous AREG or EREG during IVM increases oocyte developmental competence over FSH or EGF

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[2]Correspondence: Jeremy G. Thompson, School of Paediatrics and Reproductive Health, Faculty of Health Sciences, The University of Adelaide, Level 2, Medical School South, Frome Road, Adelaide, SA 5005, Australia. E-mail: jeremy.thompson@adelaide.edu.au
[3]These authors are joint senior authors.

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in the mouse and pig [7, 17]. We hypothesize that the improvement in oocyte developmental competence seen in COCs matured in the presence of AREG or EREG, over FSH or EGF, would be reflected in differences in oocyte and cumulus cell metabolism.

There is abundant evidence that COC metabolism is an important component of oocyte developmental competence. COCs acquire the large amount of energy required for meiotic and cytoplasmic maturation through the metabolism of various substrates including glucose, lipids, and amino acids [18], and imbalances in energy metabolism kinetics have been shown to compromise oocyte developmental competence [18, 19]. COCs preferentially use glucose as an energy substrate [20]. Cumulus cells have a higher capacity for glucose uptake than the oocyte and hence consume more glucose but provide the oocyte with many of the metabolic substrates it requires via gap junctions [21]. The majority of glucose consumed by COCs is metabolized via glycolysis; however, a small proportion is also metabolized via the hexosamine biosynthesis pathway (HBP), the pentose phosphate pathway, and the polyol pathway to provide substrates for essential cellular processes (reviewed by Sutton-McDowall et al. [22]). Glycolysis plays a role in energy production as it provides the oocyte with the essential metabolites such as pyruvate and lactate [20, 23]; pyruvate facilitates ATP production as it is metabolized via the tricarboxylic acid cycle, which is coupled with mitochondrial oxidative phosphorylation to generate ATP for energy [24]. Glucose has several other important roles in the COC. During COC maturation, the HBP is particularly up-regulated when the cumulus matrix is undergoing expansion, because glucose metabolized via this pathway can be used for the production of hyaluronic acid, an essential glycosaminoglycan for cumulus matrix formation [25, 26]. Glucose metabolized via the HBP is converted to UDP-N-acetyl glucosamine, which can then be converted to hyaluronic acid by HAS2 [22]. Alternatively, the UDP-N-acetyl glucosamine produced can be used for the β-*O*-linked glycosylation of proteins; β-*O*-linked glycosylation is an inducible and dynamic posttranslational regulatory modification of cytosolic and nuclear proteins that can modulate transcriptional and signal transduction events [27]. β-*O*-linked glycosylation is reversible and affects protein function in a similar manner to phosphorylation.

In this study, we sought to characterize the metabolic profiles of IVM COCs matured in the presence of FSH, EGF, and the EGF-like peptides. We hypothesized that these ligands will exert differential effects on COC metabolism that relate to oocyte developmental competence.

MATERIALS AND METHODS

COC Collection

Female 129/Sv ([in-house] inbred) mice were used in this study as they provide a reduced oocyte competence model in our hands [28, 29]. Mice were maintained in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and with the approval of the University of Adelaide Animal Ethics Committee. Immature COCs were collected from 21- to 26-day-old mice, 46 h after intraperitoneal injection of 5 IU of equine chorionic gonadotropin (Folligon; Intervet, Boxmeer, Holland) to stimulate follicular growth. COCs were isolated from preovulatory follicles using a 27-gauge needle and collected using flame-pulled borosilicate Pasteur pipettes in HEPES-buffered α minimum essential medium (αMEM; Gibco Life Technologies, Grand Island, NY) supplemented with 3 mg/ml bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO) and 100 μM 3-isobutyl-1-methylxanthine (Sigma Aldrich).

COC IVM

IVM COCs were cultured in bicarbonate buffered αMEM (Gibco) supplemented with 3 mg/ml BSA and either recombinant human FSH (50 mIU/ml; Puregon; Organon, Oss, The Netherlands), recombinant human EGF (10 ng/ml; R&D Systems, Minneapolis, MN), recombinant mouse AREG (50 ng/ml; R&D Systems), recombinant mouse EREG (50 ng/ml; R&D Systems), or recombinant mouse BTC (50 ng/ml; R&D Systems), at 37°C with 5% CO_2 in air. Doses of EGF-like peptides and EGF were chosen on the basis of doses used in previous studies [7, 15, 30, 31]. AREG and EREG had been previously shown to increase oocyte developmental competence relative to FSH and EGF using these culture conditions [7].

Glucose and Lactate Measurement

Groups of 10 COCs were cultured for 17 h in 100 μl media. Spent culture media drops were collected, snap frozen, and stored at −80°C. Glucose and lactate concentrations in spent media were measured using a Cobas Integra 400 Plus (Roche Diagnostics, Basel, Switzerland). Data are from six replicate experiments where each replicate measurement is the mean of three individual COC media sample readings. Glucose consumption and lactate production are expressed as pmol/COC/h.

Flavin Adenine Dinucleotide and Nicotinamide Adenine Dinucleotide (Phosphate) Autofluorescence

Oocyte autofluorescence intensities of reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) and oxidized flavin adenine dinucleotide (FAD) are commonly measured to examine cellular REDOX (reduction and oxidation) state [32]. Following 17 h IVM, COCs were transferred into HEPES-buffered Vitro-Wash medium, where cumulus cells were removed from oocytes by mechanical shearing using a P200 pipette. Denuded oocytes were immediately transferred to glass-bottom confocal dishes (Cell E&G, Houston, TX) in 5 μl Vitro-Wash medium and overlaid with mineral oil. Autofluorescence of NAD(P)H and FAD was measured using blue (excitation 405 nm, emission 420–520 nm) and green (excitation 473 nm, emission 490–590 nm) filters, respectively, using a FluoView FV10i confocal microscope (Olympus, Tokyo, Japan). The laser-power and photomultiplier settings were kept constant for all experiments. Fluorescent intensities were normalized to a fluorescence standard (Inspeck; Molecular Probes, Eugene, OR). A single optical scan through the center of the oocyte was used for the analysis, and mean fluorescent intensities were quantified using Image J software (NIH, Bethesda, MD). Forty-five COCs were imaged per treatment group over three replicate experiments.

JC-1 Staining

Mitochondrial activity was examined by staining oocytes with JC-1, a mitochondrial membrane potential ($\delta\Psi m$)-sensitive fluorescence dye [33]. JC-1 exhibits potential-dependent accumulation in mitochondria whereby it aggregates in high-polarized mitochondria ($\delta\Psi m \geq -140$ mV) as J aggregates that emit a red fluorescence [34]. In low-polarized mitochondria ($\delta\Psi m \leq -100$ mV), the dye accumulates as JC-1 multimers that emit a green fluorescence. Hence, an increase in the red:green fluorescence intensity ratio indicates increased mitochondrial polarization, and thus increased mitochondrial activity [34]. Following 17 h IVM in their respective treatments, oocytes were denuded of their cumulus vestment by mechanical shearing using a P200 pipette and cultured for 15 min in their treatments and 2 μg/ml JC-1 dye (Molecular Probes). Oocytes were then washed once in Vitro-Wash medium and fluorescence was measured using narrow green (excitation 490 nm, emission 540 nm) and narrow red (excitation 570 nm, emission 620 nm) filters using a FluoView FV10i confocal microscope. The laser-power and photomultiplier settings were kept constant for all replicate experiments. A single optical scan through the center of the oocyte was used for the analysis where red and green JC-1 fluorescence intensities were quantified using Image J software. The red:green fluorescence ratio was then calculated from ≥23 oocytes per treatment group over three replicate experiments. All values were corrected for background fluorescence.

RNA Isolation and RT-qPCR

Following 6 h IVM, COC samples (50 COCs per treatment) were resuspended in TRI reagent (Ambion, Life Technologies, Carlsbad, CA), vortexed for 1 min, and incubated for 5 min at room temperature to solubilize and homogenize samples. Chloroform (Sigma Aldrich) was then added and samples were centrifuged to cause phase separation. The aqueous phase was

TABLE 1. Sequences of PCR primers used for RT-qPCR.

Gene	GenBank accession no.	Forward primer*	Reverse primer*	Size (bp)
Has2	U52524	AAG ACC CTA TGG TTG GAG GTC TT	CAT TCC CAG AGG ACC GCT TAT	167
Ptgs2	NM_011198	CCTTCCTCCCGTAGCAGATG	ATGAACTCTCTCCGTAGAAGAACCTT	111
Ptx3	X83601	GGA CAA GCA AAT AGA CAA TGG ACT T	CGA GTT CTC CAG CAT GAT GAA C	109
Tnfaip6	NM_011198	CCT TCC TCC CGT ACG AGA TG	ATG AAC TCT CTC CGT AGA AGA ACC TT	111
Mrpl19	NM_026490	GAAAGGTGCTTCCGATTCCA	TGATCGCTTGATGCAAATCC	116
Ppia	NM_008907.1	TGGCAAATGCTGGACCAA	CCTTCTTTCACCTTCCCAAAGA	106

* All primers are given in the 5′ to 3′ orientation.

collected and RNA was extracted using the RNeasy Micro Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. DNA that may have been copurified was removed by addition of DNase (0.34 Kunitz units/μl, supplied with kit). RNA was eluted in 14 μl of RNAse-free water and stored at −80°C. The final RNA concentrations were determined by absorbance using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Scoresby, Australia).

An equal amount of total RNA from each sample was reverse transcribed using random hexamers (Invitrogen, Carlsbad, CA) and Superscript III reverse transcriptase (Invitrogen). Primers (10 pmol/reaction; Table 1) and cDNA were then added to 20 μl total reaction volume with SYBR Green (Applied Biosystems, Mulgrave, Australia). For *O*-linked β-N-acetylglucosaminyltransferase (*Ogt*) and glutamine:fructose-6-phosphate transaminase isoforms 1 and 2 (*Gfpt1* and *Gfpt2*), QuantiTect mouse primers (QuantiTect Primer Assay; Qiagen) were used. PCRs were performed using a Corbett Rotor-Gene 6000 (Qiagen). Thermal cycling conditions were set at 10 min at 95°C for denaturing, then 40 cycles at 95°C for 15 sec and 60°C for 60 sec for annealing and extension, followed by 95°C, 60°C, and 95°C for 15 sec each for dissociation. A seven-point serial dilution standard curve was produced for each transcript from cDNA generated from mural granulosa cells. The relative gene expression values were calculated using the standard curve method, presented relative to a calibrator, and normalized to the geometric mean of two housekeeping genes (*Mrpl19* and *Ppia*). To validate primer pairs, amplicons generated from mouse cumulus cell cDNA were run on 2% agarose gels, and primer pairs were considered valid when a single product at the correct size was observed and primer efficiency was above 90%. Data are means of six replicate experiments.

Cumulus Matrix Expansion Scoring

COCs were matured for 17 h IVM in media containing 5% heat-inactivated fetal bovine serum (FBS; Gibco) along with the indicated treatments. FBS-supplemented media was used specifically for assessment of cumulus matrix expansion because, in the presence of BSA alone, the cumulus matrix expansion is poor. Blinded scoring by an experienced assessor of the cumulus expansion index was then performed using the scoring system reported by Vanderhyden et al. [35].

CTD 110.6 Immunocytochemistry

Global β-*O*-linked glycosylation was examined in COCs using immunohistochemistry. IVM COCs were matured for 12 h and then immediately fixed in 4% paraformaldehyde diluted in PBS for 1.5 h at 4°C. This time point was chosen because a greater difference in global β-*O*-linked glycosylation between treatments was observed at 12 h than at 6 or 17 h IVM, as determined using Western blot analysis (data not shown). COCs were adhered to Cell-Tak (BD Biosciences, Franklin Lakes, NJ) coated glass slides and incubated with permeabilizing solution (0.25% Triton-X in PBS), followed by blocking solution (10% goat serum, 0.2% Tween-20 in PBS). COCs were then incubated with the primary antibody CTD110.6 (anti-O-GlcNAc; Covance, Princeton, NJ) diluted 1:250 in blocking solution overnight at 4°C, followed by the secondary antibody Alexa Fluor 488 goat anti-mouse immunoglobulin M (Invitrogen) diluted 1:250 in blocking solution for 1 h at room temperature. COCs were then incubated with 1.5 μM of the nuclear stain propidium iodide (PI) for 30 min at room temperature. Cells were covered with Dako Fluorescent Mounting Medium (Dako, Glostrup, Denmark), covered with a cover slide, and allowed to dry for ~4 h at 4°C. COC CTD110.6 (green; excitation 473 nm, emission 520 nm) and PI (red; excitation 559 nm, emission 619 nm) fluorescence was then visualized using a FluoView FV10i confocal microscope. The laser-power and photomultiplier settings were kept constant for all replicate experiments. A single optical scan at 60× magnification through the center of the oocyte was used for the analysis. Fluorescence intensities were quantified using Image J software and normalized to a fluorescence standard (Inspeck). A total of 30 COCs from three replicate experiments were imaged. For each replicate experiment, negative primary and secondary antibody control slides were prepared.

Statistical Analyses

Statistical analyses were conducted using SigmaPlot 11.0 software (Systat Software, San Jose, CA). Statistical significance was assessed by one-way ANOVA followed by Tukey multiple-comparison post hoc tests to identify individual differences between means. Where data were not normally distributed, statistical significance was assessed by nonparametric Kruskal-Wallis one-way ANOVA by ranks. All values are presented with their corresponding SEM. Probabilities of $P \leq 0.05$ were considered statistically significant.

RESULTS

EGF-Like Peptides, EGF, and FSH Exert No Differential Effects on COC Glycolysis

Glucose consumption and lactate production by COCs matured in the presence of EGF-like peptides, FSH, or EGF were measured after 17 h of IVM. The relative rate of glycolysis was determined by the ratio of lactate production to glucose consumption (lactate:glucose ratio); two lactate molecules are produced for every glucose molecule consumed via glycolysis. Control (no treatment) COCs exhibited significantly ($P < 0.001$) lower glucose consumption, lactate production, and glycolytic rate than those matured in the presence of FSH, EGF, or the EGF-like peptides (Fig. 1). There were no significant differences in glucose consumption, lactate production, or glycolytic rates between COCs matured with FSH, EGF, or the EGF-like peptides (Fig. 1).

EGF-Like Peptides and EGF Stimulate Greater HBP Activity than FSH

The relative mRNA expression of key genes (illustrated in Fig. 2A) known to be up-regulated during glucose metabolism via the HBP was measured, and cumulus matrix expansion was measured to relate gene expression data with morphological expansion. The control (no treatment) group did not stimulate cumulus matrix expansion following 17 h maturation; FSH induced significantly less expansion than all other treatments ($P < 0.001$; Fig. 2B). AREG and EGF induced the greatest expansion, followed by EREG and BTC, and then FSH ($P < 0.05$). In general, EGF and the EGF-like peptides induced robust cumulus cell expression of the matrix-associated genes compared to FSH. AREG, EREG, and EGF induced significantly higher *Has2*, *Ptgs2,* and *Tnfaip6* expression, and BTC induced significantly higher *Has2* and *Ptgs2* expression, than FSH or control (Fig. 2C). Induction of the HBP rate-limiting enzymes, *Gfpt1* and *Gfpt2*, and the cumulus matrix-associated transcripts, *Has2*, *Ptx3*, *Ptgs2*, and *Tnfaip6*, was measured to assess the relative flux of glucose down the HBP in COCs matured in the presence of EGF-like peptides, FSH, or EGF for 6 h (Fig. 2C). There were no significant differences in *Gfpt1* expression among treatment groups, with the

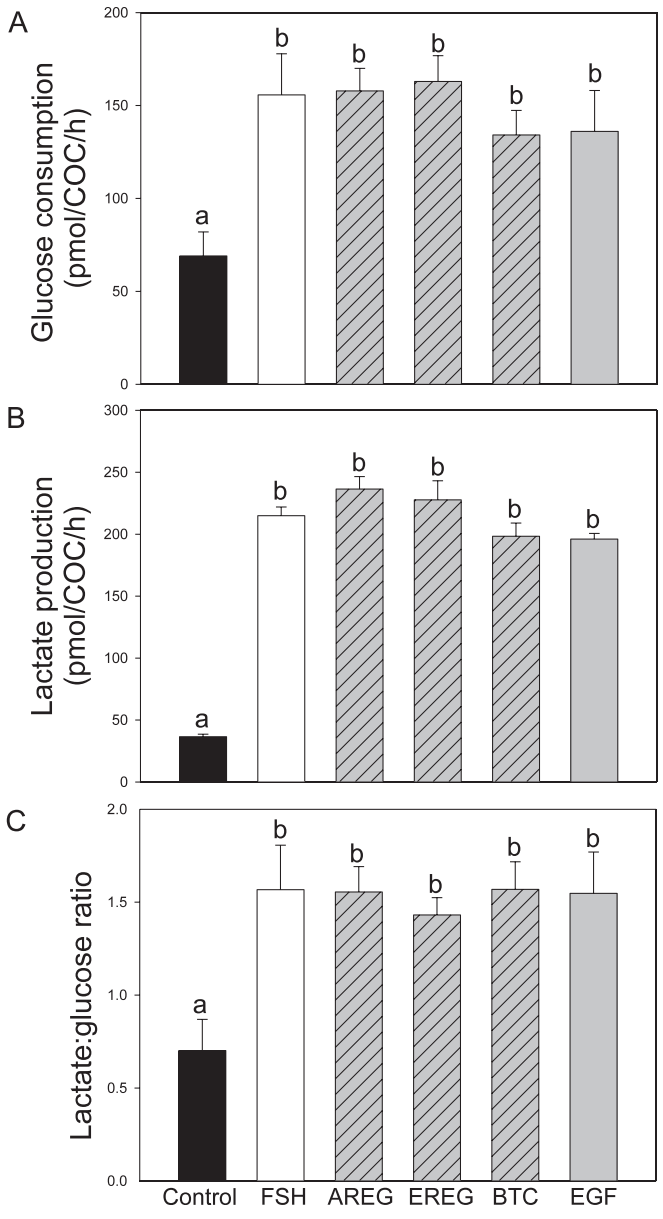

FIG. 1. Effect of FSH versus EGF-like peptides on COC glucose consumption and lactate production. COCs underwent 17 h IVM in the presence of control (no treatment), FSH, AREG, EREG, BTC, or EGF. Glucose consumption (**A**) and lactate production (**B**) were measured in IVM COC conditioned maturation media. The ratio of lactate to glucose (**C**) was quantified as an indicator of COC glycolytic activity. Bars not sharing a common letter are significantly different ($P < 0.05$). The data represent means ± SEM. Data are from six replicate experiments where each replicate is the mean of three individual media samples of 10 cultured COCs.

exception of BTC, which induced significantly lower *Gfpt1* expression than FSH, AREG, EREG, and EGF. Expression of *Gfpt2*, however, was significantly higher in the presence of EGF and all three EGF-like peptides than in the presence of FSH or control (Fig. 2C).

EGF-Like Peptides and EGF Stimulate Greater Cumulus Cell β-O-Linked Glycosylation than FSH

To assess the effect of treatment on COC protein β-*O*-linked glycosylation, immunocytochemical localization of β-*O*-linked glycosylated proteins, as well as mRNA expression of *Ogt*, was examined (Fig. 3). Cumulus cell β-*O*-linked glycosylation levels in the presence of FSH were significantly lower than in the presence of control, EGF, or the EGF-like peptides (Fig. 3A1 shows representative images of COCs stained for β-*O*-linked glycosylation, and Fig. 3A2 shows the quantified relative levels of this glycosylation). Although higher than FSH, the level of cumulus cell β-*O*-linked glycosylation stimulated by BTC was significantly lower than that stimulated by AREG, EREG, and control. EREG stimulated significantly higher β-*O*-linked glycosylation within the oocyte than FSH or control (Fig. 3A2). In support of the glycosylation pattern observed in the cumulus cells, COC *Ogt* mRNA expression was significantly lower in the presence of FSH than in the presence of control, EGF, or the EGF-like peptides (Fig. 3B).

EREG Alters Oocyte REDOX State

In order to compare the cytoplasmic and mitochondrial REDOX state of FSH versus EGF-like peptide-matured oocytes, the autofluorescent intensities of the endogenous fluorophores NAD(P)H and oxidized FAD were measured in denuded oocytes that were cultured as intact COCs for 17 h of IVM. FAD is exclusively localized to the mitochondria and NAD(P)H is localized to the cytoplasm and mitochondria [36]. Denuded oocytes matured as COCs in the presence of EREG exhibited significantly higher levels of FAD than control (Fig. 4). NAD(P)H levels were not significantly different between treatments with the exception of FSH, which led to significantly higher levels than AREG.

The ratio of fluorescence of these fluorophores (FAD: NAD(P)H) is indicative of oocyte REDOX state [37]. With the exception of BTC, the REDOX ratio was significantly increased by all treatments over control ($P < 0.05$, Fig. 4), which is suggestive of increased oxidative metabolism and mitochondrial activity. Moreover, the REDOX ratio was significantly higher ($P \leq 0.032$) in the presence of EREG than in the presence of FSH, BTC, or control.

EGF-Like Peptides Stimulate Greater Mitochondrial Activity than FSH or EGF

To investigate whether EGF-like peptides alter mitochondrial activity in oocytes, the relative levels of JC-1 monomers (green fluorescence) and J aggregates (red fluorescence) were measured (Fig. 5A). The red:green fluorescence ratio, which provides an index of mitochondrial activity [38], was then calculated (Fig. 5B). AREG, EREG, and BTC stimulated similar mitochondrial activity, and this was significantly higher than ratios seen with FSH, EGF, and control ($P < 0.05$).

DISCUSSION

EGF-like peptides are the natural intrafollicular signal transducers of the ovulatory cascade to the immature COC [2]. As IVM COCs are not exposed to the ovulatory signal in vivo, these COCs experience a deficiency in EGF-like peptide signaling [7]. Furthermore, although FSH treatment of COCs stimulates EGF-like peptide expression, levels are not sustained and are significantly lower than in vivo [7]. Exogenous EREG or AREG during IVM has been shown to increase subsequent embryo quality (increased proportion of ICM cells), and EREG has been shown to increase blastocyst rates, compared to FSH or EGF [7]. This study aimed to compare the metabolic consequences of FSH, EGF, and EGF-like peptide supplementation on IVM oocytes and cumulus cells. Here we have shown that these factors induce similar

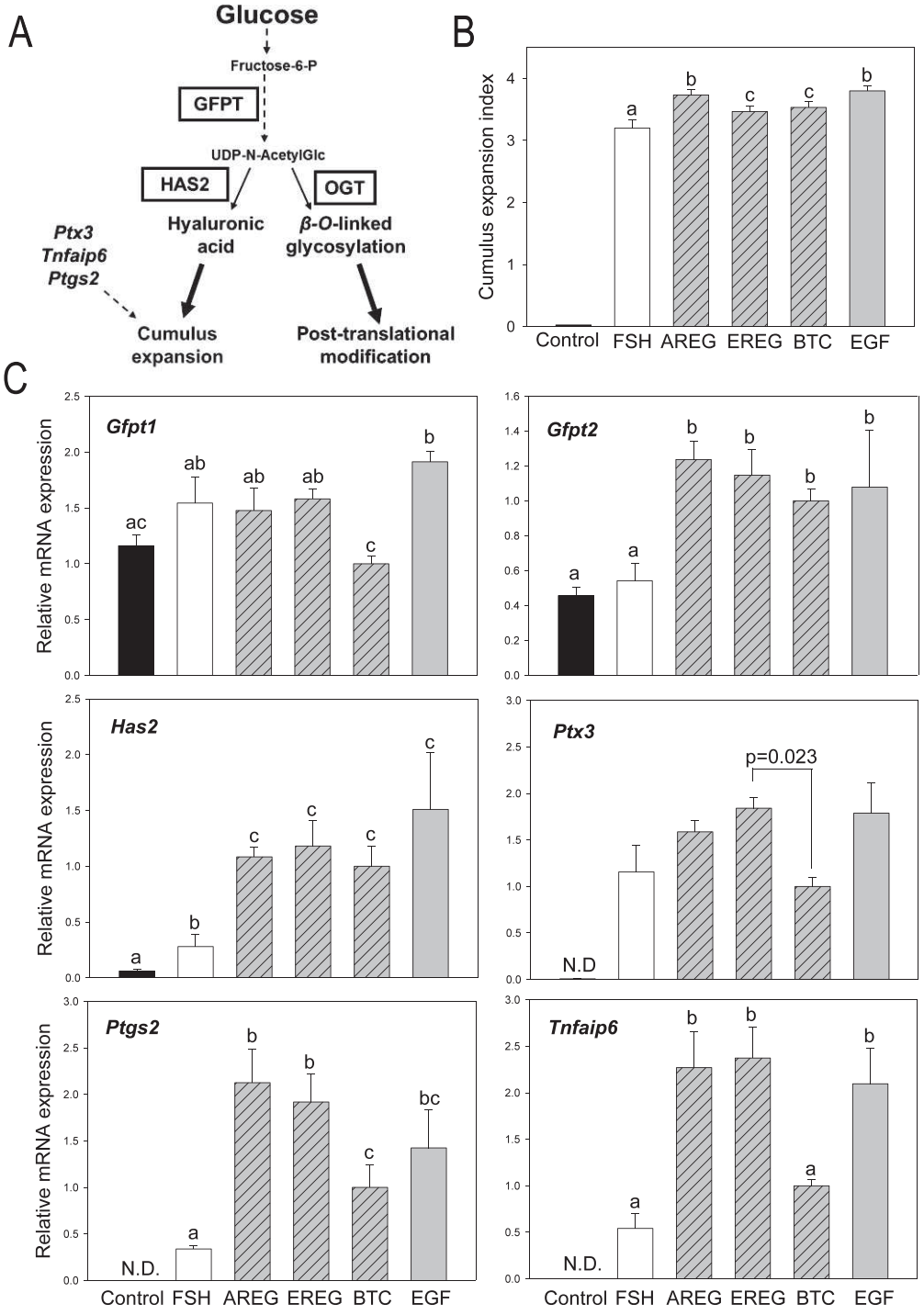

FIG. 2. Induction of cumulus matrix expansion and cumulus matrix and HBP mRNA expression by FSH, EGF, and EGF-like peptides. **A**) Schematic diagram of glucose metabolism via the HBP leading to cumulus matrix expansion or protein posttranslational modification via β-*O*-linked glycosylation. **B**) Cumulus matrix expansion was scored after 17 h IVM in which COCs were cultured in the presence of control (no treatment), FSH, AREG, EREG, BTC, or EGF (n = 30 COCs over three replicate experiments). **C**) Messenger RNA expression was quantified after 6 h IVM using RT-qPCR and normalized to the geometric mean of the *Ppia* and *Mrpl19* housekeeper genes (n = 6). Bars not sharing a common letter are significantly different ($P < 0.05$); N.D., below limit of detection. The data represent means ± SEM.

uptake of glucose and stimulate comparable glycolytic activity in IVM COCs. However, we have found that EGF-like peptides and EGF increase glucose metabolism via the HBP, and increase the level of global β-*O*-linked glycosylation of proteins, in comparison to COCs treated with FSH. We also report that EGF-like peptides promote significantly higher oocyte mitochondrial activity, and EREG induces an increased preference for oxidative phosphorylation, within the oocyte than EGF or FSH.

Mitochondria play a vital role in cellular metabolism. The quantity, localization, and activity of mitochondria in the oocyte have been shown to significantly influence the quality of oocytes matured both in vivo and in vitro [33, 39]. In mature oocytes, decreased mitochondrial reserves (as measured by

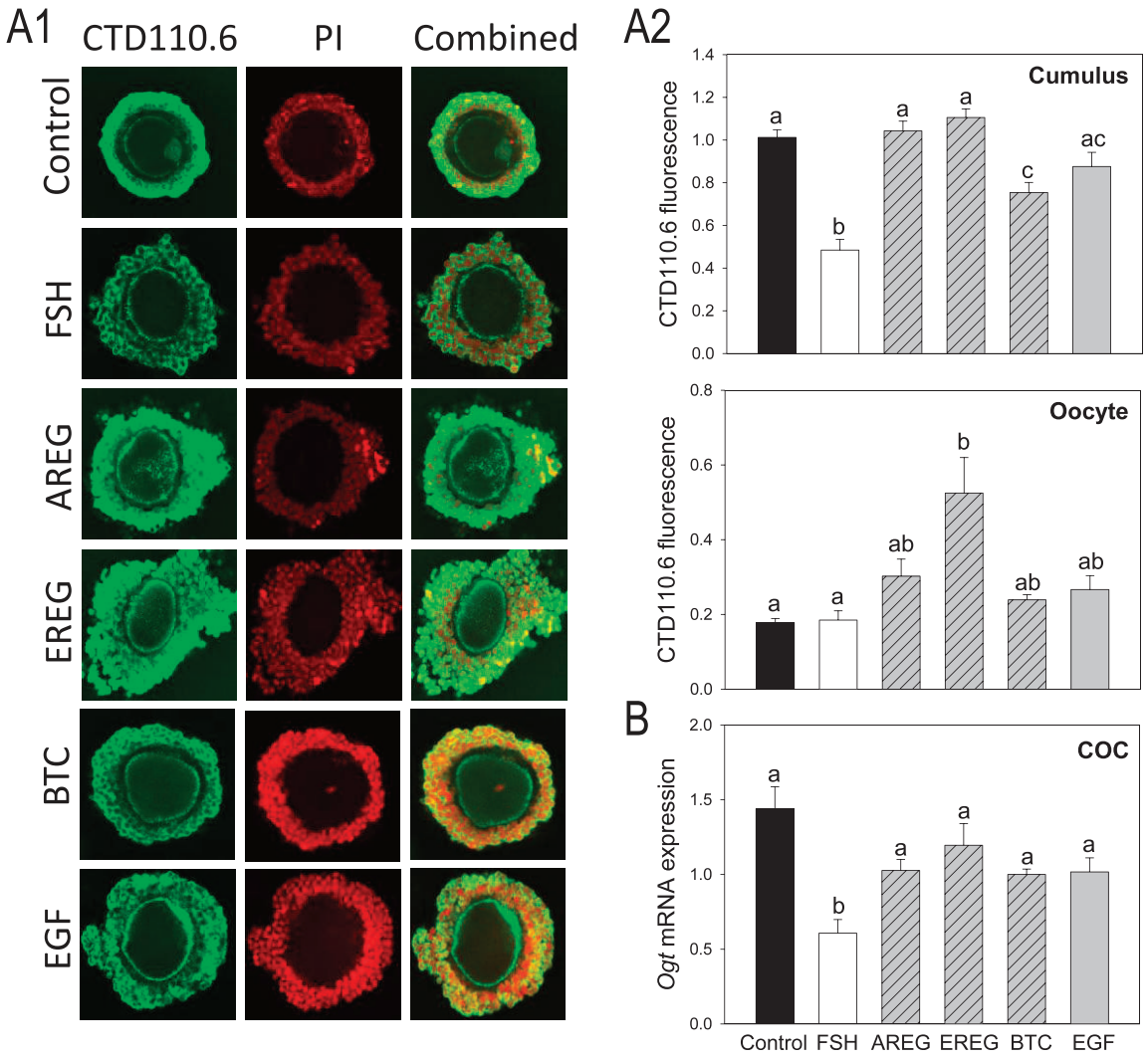

FIG. 3. Effect of FSH, EGF, and EGF-like peptides on COC β-*O*-linked glycosylation. **A1**) Protein β-*O*-linked glycosylation was examined at 12 h IVM in the presence of control (no treatment), FSH, AREG, EREG, BTC, or EGF and β-*O*-linked glycosylation (CTD110.6) and nuclear staining (PI) fluorescence were imaged. Images shown are representative of 30 COCs per treatment group over three replicate experiments. Original magnification ×60. **A2**) Quantification of relative CTD110.6 fluorescence in cumulus cells and oocytes. **B**) COC mRNA expression of *Ogt* was measured at 6 h IVM (n = 6). Bars not sharing a common letter are significantly different ($P < 0.02$). The data represent means ± SEM.

mitochondrial DNA content) have been associated with decreased fertilization and blastocyst rates [40]. The effect of EGF-like peptides on oocyte mitochondrial function has not been previously reported; however, the addition of another member of the EGF family of proteins and an EGFR ligand, TGFα, to IVM media has been shown to increase porcine oocyte mitochondrial colocalization with endoplasmic reticulum, cristae presence in subsequent blastocysts, and blastocyst development [41]. As for mitochondrial activity, human and animal studies have shown a positive correlation between oocyte mitochondrial activity and subsequent blastocyst development from both in vivo matured and IVM oocytes [33, 42, 43]. In this study, we have shown mitochondrial activity to be greatly increased in oocytes matured with all three EGF-like peptides in comparison to EGF, FSH, and control. This, coupled with findings from our previous IVM study showing EREG and AREG to increase blastocyst rates and/or quality above FSH and EGF [7], suggests that increased mitochondrial activity, due to a higher energy demand in the oocyte, may be a mechanism by which EGF-like peptides confer increased oocyte developmental competence. BTC is generally regarded as having a minor role in the mouse follicle, as it is less potent at stimulating meiotic maturation and cumulus cell EGF-like peptide mRNA expression [2, 7]. As such, the effect of BTC on developmental competence is yet to be examined. In light of its marked effect on mitochondrial activity, however, the effect of BTC on oocyte developmental requires further investigation.

Changes in mitochondrial function can also give rise to changes in cellular REDOX state [44]. The REDOX state refers to the ratio of reduced FAD to oxidized NAD(P)H, and increased REDOX ratios are indicative of increased oxidative metabolic activity [45]. We found that EREG significantly increased the oocyte REDOX ratio above all treatments except AREG by the end of IVM. EREG also significantly increased FAD levels compared to the control. FAD is found exclusively in mitochondria and acts as an electron acceptor in the electron transport chain during ATP synthesis. Hence, our data suggest that EREG induces a preference for oxidative phosphorylation in IVM oocytes. Interestingly, we have previously shown that EREG, and not AREG, increases blastocyst yield from mouse IVM oocytes compared to FSH and EGF [7].

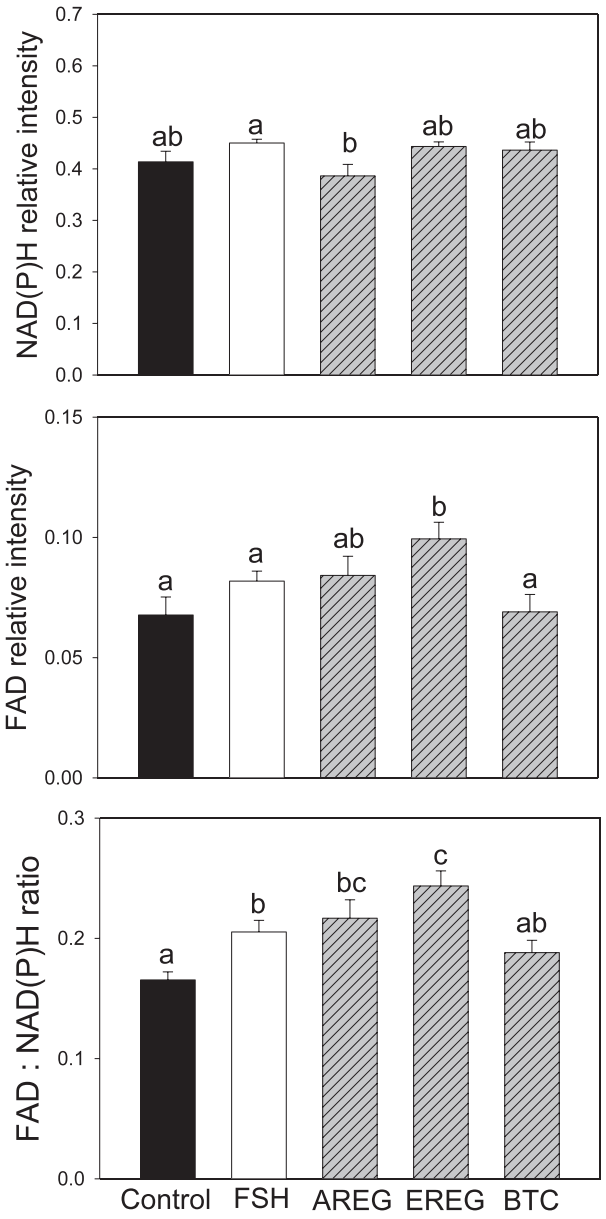

FIG. 4. Effect of FSH, EGF, and EGF-like peptides on intraoocyte NAD(P)H and FAD autofluorescence and REDOX ratio. COCs underwent 17 h IVM in the presence of control (no treatment), FSH, AREG, EREG, BTC, or EGF and oocyte autofluorescence was measured. n = 45 oocytes from three replicate experiments. Bars not sharing a common letter are significantly different ($P < 0.05$). The data represent means ± SEM.

Glucose is a preferred energy substrate for COCs and is predominantly metabolized via glycolysis in the cumulus cells to produce oxidizable pyruvate and/or lactate, which can be further metabolized in both cumulus cells and the oocyte via the tricarboxylic acid cycle to produce ATP [32, 44]. In this study, we observed no significant differences in the net amount of glucose consumed, or glycolytic activity, of whole IVM COCs matured with EGF-like peptides, FSH, and EGF. However, FSH is a well-known stimulator of cumulus cell glycolysis [22, 46], and this study is the first to show that EGF-like peptides are equally potent stimulators of this important metabolic process during IVM.

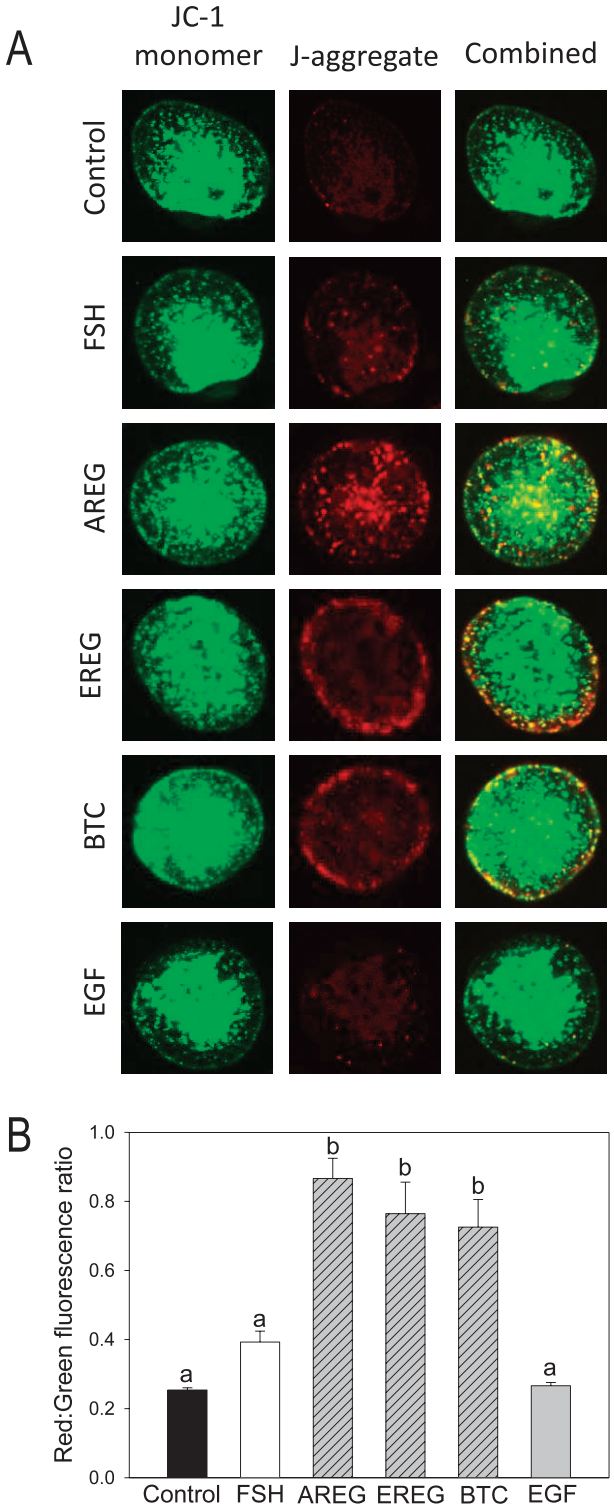

FIG. 5. Effect of FSH versus EGF-like peptides on oocyte mitochondrial activity. **A**) COCs underwent 17 h IVM in the presence of control (no treatment), FSH, AREG, EREG, BTC, or EGF before oocytes were denuded and stained with JC-1, a fluorescent dye that emits a green fluorescence as JC-1 monomers in low-polarized mitochondria and a red fluorescence as J aggregates in high-polarized mitochondria. Original magnification ×60. **B**) The ratio of green (JC-1 monomer) to red (J-aggregate) fluorescence was quantified as an indicator of mitochondrial activity. Bars not sharing a common letter are significantly different ($P < 0.05$); n = 30 oocytes from three replicate experiments. The data represent means ± SEM.

The metabolism of glucose via the HBP is particularly upregulated during the later stages of maturation, when the cumulus matrix is undergoing expansion, as a major fate of glucose via this pathway is hyaluronic acid, which is an essential constituent of the expanded cumulus matrix [47]. Here we report that gene expression of the HBP rate-limiting enzyme *Gfpt2* was significantly lower in COCs matured with FSH than with EGF or any of the EGF-like peptides, suggesting FSH leads to less metabolism of glucose via the HBP. Furthermore, FSH promoted significantly less expression of the cumulus matrix genes *Has2*, *Ptgs2*, and *Tnfaip6* than the EGF-like peptides or EGF, and promoted less cumulus matrix expansion. Because no difference in the level of glucose consumption or glycolytic activity was observed between these treatments, but lower HBP activity was seen with FSH, the fate of the remainder of glucose consumed in the presence of FSH remains unclear. FSH may promote an increase in the activity of one of the other glucose metabolic pathways, namely the pentose phosphate or polyol pathways; however, this requires further investigation.

The importance of the HBP in regulating oocyte developmental competence is becoming increasingly evident as the relatively new study of protein β-*O*-linked glycosylation in cell biology is further elucidated. Aside from hyaluronic acid synthesis, UDP-N-acetyl glucosamine produced via the HBP can be used for the β-*O*-linked glycosylation of proteins (reviewed by Wells et al. [27]). β-*O*-linked glycosylation is a reversible regulatory posttranslational modification of cytosolic and nuclear proteins, including many signaling and cytoskeletal proteins, and alters protein function in a similar manner to phosphorylation [27, 48]. β-*O*-linked glycosylation can alter the behavior of proteins in several ways, such as altering enzymatic activity, subcellular localization, DNA binding capacity, and protein-protein interaction [27]. Hence, alterations in cellular protein β-*O*-linked glycosylation can have profound and long-lasting effects on cell function. Here we observed significantly lower global β-*O*-linked glycosylation levels in the cumulus cells of IVM COCs matured with FSH compared with all other treatments, lending further evidence that FSH induces lower stimulation of the HBP. Perturbed COC HBP activity and, consequently, global β-*O*-linked glycosylation using glucosamine has been reported to negatively affect oocyte developmental competence of murine, porcine, and bovine IVM COCs [49, 50]. Although we observed increased cumulus cell global β-*O*-linked glycosylation in the presence of EGF-like peptides, we and others have previously shown AREG and EREG to increase IVM oocyte developmental competence over FSH or EGF [7, 17]. Our data also show cumulus cells cultured with EGF and control exhibit similar global β-*O*-linked glycosylation levels to those cultured with EGF-like peptides, even though they confer significantly lower oocyte developmental competence than EGF-like peptides [7]. Within the oocyte, global β-*O*-linked glycosylation levels between COCs matured spontaneously (control) and with FSH were not significantly different, even though FSH increases developmental competence in vitro [31, 51]. In early embryonic development, Pantaleon et al. have demonstrated that either too little or excessive β-*O*-linked glycosylation is associated with poor development, suggesting an optimal glycosylation level is required [52–54]. It appears likely that a similar homeostatic regulation of β-*O*-linked glycosylation might be required within the COC for optimal subsequent development. Characterization of the different β-*O*-linked glycosylation targets within COCs matured with these treatments may further elucidate differentially activated pathways during maturation, thus providing new insight into differential control of oocyte competence.

It is noteworthy that, even though EGF and the EGF-like peptides are closely related and activate the same EGFR, in general the effects on COC metabolism were different. A striking difference in the intraoocyte mitochondrial activity was observed, where EGF stimulated significantly lower activity than the EGF-like peptides. This observation is consistent with, and may be a contributing factor to, a previous observation showing EGF to confer decreased oocyte developmental competence compared to AREG and EREG [7]. The differential effects between these closely related proteins may be due to differential binding specificities and affinities with EGFR. AREG, EREG, and EGF have been shown to be functionally distinct in nonfollicular cells, as they are able to stimulate divergent biological responses both in vitro and in vivo [55]. The differential biological outcomes elicited by these growth factors are due to ligand-induced variations in EGFR conformation, and subsequent variances in tyrosine phosphorylation sites and EGFR coupling to signaling effectors [56].

In conclusion, findings from this study demonstrate that the EGF-like peptides, FSH, and EGF induce differential effects on IVM COC metabolism. We have shown that EREG, AREG, and BTC stimulate greater oocyte mitochondrial activity than FSH and EGF, consistent with the superior effects of EREG and AREG on oocyte developmental competence. Furthermore, these EGF-like peptides induce greater COC glucose metabolism via the HBP and, consequently, higher levels of cumulus cell β-*O*-linked glycosylation compared to FSH. Additionally, EREG also increases oxidative phosphorylation within the oocyte, indicating increased energy production. These important metabolic alterations may be a mechanism by which EGF-like peptides, particularly EREG in the mouse, confer increased oocyte developmental competence.

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