Adiponectin: A prosurvival and proliferation signal that increases bovine mammary epithelial cell numbers and protects them from endoplasmic reticulum stress responses¹

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ABSTRACT: Cell-cell interactions between epithelial and stromal cells are predominant in the mammary gland, and various stromal cell-derived factors can elicit mitogenic responses in adjacent epithelial cells. Adiponectin is a hormone secreted mainly by adipocytes that mediates stromal-epithelial interactions in a number of tissues. Adiponectin receptors are expressed by bovine mammary epithelial cells, but the regulatory effects of adiponectin on the development and function of the mammary gland remain unclear. We therefore sought to investigate the effects of adiponectin on bovine mammary epithelial (MAC-T) cells and the regulatory mechanisms that underlie these adiponectin-induced actions. Our results revealed an increase in MAC-T cell proliferation and cell cycle progression in response to adiponectin. The expression of nuclear proliferating cell nuclear antigen (PCNA) and cyclin D1 was induced in MAC-T cells, and intracellular signaling molecules such as serine/threonine protein kinase (AKT), 70 kDa ribosomal S6 kinase (P70S6K), ribosomal protein

S6 (S6), extracellular signal-regulated kinases 1 and 2 (ERK1/2), 90 kDa ribosomal S6 kinase (P90S6K), and cyclin D1 were activated in a dose-dependent manner. The abundance of adiponectin-induced signaling proteins was suppressed following inhibition of AKT or ERK1/2 mitogen-activated protein kinase (MAPK) signaling. In addition, inhibition of AKT or ERK1/2 signaling significantly reduced adiponectin-stimulated MAC-T cell proliferation. Furthermore, adiponectin reduced tunicamycin-induced expression and activation of endoplasmic reticulum stress-related proteins in MAC-T cells and attenuated the repressive effect of tunicamycin on proliferation of MAC-T cells. Collectively, these results suggest that adiponectin-mediated signaling may affect the development and function of the mammary gland in dairy cows by increasing mammary epithelial cell numbers. These findings may result in important implications for improving our fundamental understanding of lactation physiology in livestock species.

Key words: adiponectin, cell proliferation, ER stress, intracellular signaling, mammary epithelium, milk production

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J. Anim. Sci. 2017.95:5278–5289
doi:10.2527/jas2017.1885

INTRODUCTION

Interactions between epithelial and stromal cells occur through various stromal cell-derived factors that may affect mammary development and function. Adiponectin, one of the most abundant circulating adipokines, is a hormone derived mainly from adipocytes that plays many paracrine and autocrine roles in various cell types that express adiponectin receptors (ADIPOR; ADIPOR1 and ADIPOR2; Mitchell et al., 2005). In fact, adiponectin is involved in anti-inflammatory responses

¹This research was funded by the Basic Science Research Program (2016R1D1A1B03934186) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology. The authors declare no conflict of interest.

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Received July 4, 2017.

Accepted September 26, 2017.

and glucose and fatty acid metabolism and is well known to have a role in nutrient partitioning by increasing sensitivity to insulin in humans and mice (Yokota et al., 2000; Maeda et al., 2002). In the human mammary gland, adipocytes are 1 of the predominant stromal cell types, and adiponectin secreted from adipocytes could enter the milk (Bronsky et al., 2006). In bovine mammary tissue, low expression of adiponectin mRNA was observed in all areas, and *ADIPOR1* and *ADIPOR2* mRNA were also detected in mammary tissue, with prominent expression in the parenchyma, which consists of the alveoli and ducts (Lecchi et al., 2015). The secretion of adiponectin from the stroma and the expression of adiponectin and *ADIPOR* in bovine mammary epithelial cells suggest a possible complementary paracrine-autocrine role of adiponectin signaling in local regulation of the bovine mammary gland (Ohtani et al., 2011; Lecchi et al., 2015). However, at present there is a paucity of information about the function and mechanism of adiponectin as a local paracrine factor in mammary epithelial cells.

In the current study, we tested the hypothesis that bovine mammary epithelial cells respond to recombinant adiponectin by altering their proliferation and cellular function. Therefore, to gain insight into the potential role of adiponectin in mammary epithelial cells, we 1) investigated the functional effects of adiponectin on proliferation and cell cycle progression of bovine mammary alveolar (MAC-T) cells, 2) identified the adiponectin-induced intracellular signaling pathways in MAC-T cells, and 3) determined the effects of adiponectin on tunicamycin-mediated endoplasmic reticulum (ER) stress responses and decrease of cell proliferation.

MATERIALS AND METHODS

Reagents and Antibodies

Recombinant human adiponectin (catalog number 1065-AP) was purchased from R&D Systems (Minneapolis, MN). Tunicamycin (catalog number T7765) was purchased from Sigma (St. Louis, MO). Anti-proliferating cell nuclear antigen (PCNA) antibody (catalog number PC10) was purchased from Abcam (Cambridge, MA). Antibodies against phosphorylated (p)-serine/threonine protein kinase (AKT; Ser⁴⁷³, catalog number 4060), p-extracellular signal-regulated kinases 1 and 2 (ERK1/2; Thr²⁰²/Tyr²⁰⁴, catalog number 9101), p-70 kDa ribosomal S6 kinase (P70S6K; Thr⁴²¹/Ser⁴²⁴, catalog number 9204), p-90 kDa ribosomal S6 kinase (P90S6K; Thr⁵⁷³, catalog number 9346), p-ribosomal protein S6 (S6; Ser^{235/236}, catalog number 2211), p-cyclin D1 (catalog number 3300), phosphorylated eukaryotic translation initiator factor 2 α (p-eIF2 α ; Ser⁵¹, catalog number 3398), and

total AKT (catalog number 9272), ERK1/2 (catalog number 4695), P70S6K (catalog number 9202), P90S6K (catalog number 9335), S6 (catalog number 2217), cyclin D1 (catalog number 2922), eIF2 α (catalog number 5324), and inositol-requiring protein 1 α (IRE1 α ; catalog number 3294) were purchased from Cell Signaling Technologies (Beverly, MA). Antibodies against phosphorylated protein kinase RNA-like ER kinase (p-PERK; Thr⁹⁸¹, catalog number sc-32577) and total PERK (catalog number sc-13073), activating transcription factor 6 α (ATF6 α ; catalog number sc-166659), glucose-regulated protein 78 (GRP78; catalog number sc-13968), and growth arrest- and DNA damage-inducible gene 153 (GADD153; catalog number sc-7351) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phosphoinositide 3-kinase (PI3K)/AKT inhibitor (wortmannin, catalog number 9951) was from Cell Signaling Technologies, and the ERK1/2 inhibitor (U0126, catalog number E1282) was obtained from Enzo Life Sciences (Farmingdale, NY).

Cell Culture

Bovine mammary epithelial cells (MAC-T cells) were a gift from Dr. Hong Gu Lee (Konkuk University, Republic of Korea). The MAC-T cells were developed by immortalizing primary bovine mammary alveolar cells via stable transfection with replication-defective retrovirus (simian vacuolating virus 40 [SV40]) large T antigen, which rendered the cells immortal for more than 350 serial passages in culture without showing any signs of senescence (Huynh et al., 1991). The MAC-T cells display a cobblestone shape when grown on a plastic substratum and form a single monolayer at confluence. All analyses with MAC-T cells were performed between passages 25 and 30. Briefly, MAC-T cells (5×10^5 cells) were seeded to a 100-mm tissue culture dish and grown to 80% confluence in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μ g/mL streptomycin. The MAC-T cells were maintained at 37°C in an atmosphere of 5% CO₂ and air. Cells were serum starved for 24 h prior to treatment, then incubated with various reagents as appropriate. To identify adiponectin-activated signaling pathways, serum-starved MAC-T cells were treated with adiponectin (0, 1, 5, 10, and 20 ng/mL) for 30 min, and then the cells were lysed using cell lysis buffer. To further confirm the sequence of adiponectin-induced signaling cascades, MAC-T cells were pretreated with pharmacological inhibitors of PI3K (wortmannin, 1 μ M) or ERK1/2 (U0126, 20 μ M) for 2 h prior to treatment with 20 ng/mL adiponectin (30 min).

Proliferation Assay

Dose-dependent effects of adiponectin on MAC-T cell proliferation were determined using a Cell Proliferation ELISA, BrdU Kit (catalog number 11647229001, Roche, Basel, Switzerland) according to the manufacturer's recommendations. Briefly, MAC-T cells were seeded onto a 96-well plate (5×10^3 cells/well) and incubated in serum-free DMEM for 24 h. Cells were then treated with various concentrations (0, 1, 5, 10, 20, 50, 100, and 150 ng/mL) of recombinant human adiponectin (R&D Systems) in a final volume of 100 μ L/well. After 48 h of incubation, 10 μ M BrdU was added to the cells and incubated for an additional 2 h at 37°C.

Cell Cycle Analysis

Dose-dependent effects of adiponectin on MAC-T cell cycle progression were determined using flow cytometric analyses with propidium iodide (PI). Cells were seeded onto a 6-well plate (2×10^5 cells/well) and incubated in serum-free DMEM for 24 h. Cells were then treated with different doses (0, 1, 5, 10, and 20 ng/mL) of adiponectin for 48 h. After treatment of trypsin/EDTA solutions, cells were centrifuged, washed twice with cold 0.1% BSA in PBS, and fixed in 70% ethanol at 4°C for 24 h. Cells were then centrifuged at $500 \times g$ for 5 min at room temperature, and the supernatant was discarded. Pellets were washed twice with 0.1% BSA in PBS and stained with PI (BD Biosciences, Franklin Lakes, NJ) in 100 μ g/mL RNase A (Sigma-Aldrich) for 30 min in the dark. Fluorescence intensity was analyzed using a flow cytometer (BD FACSCalibur; BD Biosciences).

Immunofluorescence

The effects of adiponectin on PCNA and cyclin D1 expression were determined using immunofluorescence microscopy. The MAC-T cells (3×10^4 cells per 300 μ L) were seeded onto confocal dishes (catalog number 100350, SPL Life Science, Pocheon, Republic of Korea) and incubated in serum-free DMEM for 24 h. For detection of PCNA and cyclin D1, serum-starved cells were treated with 20 ng/mL adiponectin for 24 h, then fixed using methanol. Cells were probed with mouse anti-human monoclonal PCNA (Abcam) or rabbit anti-human polyclonal cyclin D1 (Cell Signaling Technologies), both at 1:100 dilution. Negative controls for background staining were performed by substituting the primary antibody with purified nonimmune mouse IgG or rabbit IgG. Cells were then incubated with goat anti-mouse IgG Alexa 488 (catalog number A11017, Invitrogen, Carlsbad, CA) or goat anti-rabbit IgG Alexa 488 (catalog number A-11008, Invitrogen)

at 1:200 dilution for 1 h at room temperature. Finally, cells were washed in 0.1% BSA in PBS and counter-stained with 4',6-diamidino-2-phenylindole (DAPI). Images were captured using a Zeiss LSM710 confocal microscope (Carl Zeiss, Thornwood, NY) and processed using ZEN imaging software (Carl Zeiss). Two different magnifications from the same images are presented in Fig. 1 and 2. Relative fluorescence signal intensity was calculated using mean fluorescent signal values for the 40 $\times$ objective in at least 3 fields of view randomly selected from each slide.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from in vitro cultured MAC-T cells using Trizol reagent (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The quantity and quality of total RNA were determined by spectrometry and denaturing agarose gel electrophoresis, respectively. Complementary DNA was synthesized from cellular RNA using AccuPower RT PreMix (Bioneer, Daejeon, Republic of Korea), random hexamer (Invitrogen), and oligo(dT) primers.

Reverse Transcription PCR Analysis

Specific primers for bovine adiponectin (forward: 5'-GGA ATG ACA GGA GCT GAA GG-3'; reverse: 5'-GTA GAG TCC CGG AAT GTT GC-3'), *ADIPOR1* (forward: 5'-GCT GGA CTA TTC AGG GAT CG-3'; reverse: 5'-CAC AGC CAT GAG GAA GAA CC-3'), and *ADIPOR2* (forward: 5'-TCT TGG GTT GTG TGT TCT TCC-3'; reverse: 5'-CGA GAT GAC GTA GTG CAA GG-3') were designed from sequences in the GenBank database using Primer 3 (version 4.0.0). All primers were synthesized by Bioneer Inc. (Daejeon, Republic of Korea). The expression of target transcripts in MAC-T cells was investigated using semi-quantitative reverse transcription PCR (RT-PCR). The PCR amplification was conducted as follows: 1) 95°C for 3 min; 2) 95°C for 20 s, 60°C for 40 s, and 72°C for 1 min for 33 cycles; and 3) 72°C for 5 min. After PCR, equal amounts of reaction product were analyzed using a 1% agarose gel, and PCR products were visualized using ethidium bromide staining. The amount of DNA present was quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using a Gel DocTM XR+ system with Image LabTM software (Bio-Rad, Hercules, CA). The *GAPDH* (forward: 5'-CAC AGT CAA GGC AGA GAA CG-3'; reverse: 5'-CAT AAG TCC CTC CAC GAT GC-3') gene was used as the endogenous control to standardize the amount of RNA in each reaction.

Figure 1. Adiponectin induces bovine mammary alveolar (MAC-T) cell proliferation. (A) Dose-dependent (0, 1, 5, 10, 20, 50, 100, or 150 ng/mL) effects of adiponectin on MAC-T cell proliferation determined using a BrdU cell proliferation assay. Adiponectin-induced cell proliferation rates are presented as relative percentage (%) changes relative to untreated (0 ng/mL) MAC-T cells (100%). Pooled data from 3 independent experiments ($n = 3$) performed in triplicate are shown as mean $\pm$ SEM. (B) Relative abundance and localization of proliferating cell nuclear antigen (PCNA) protein in adiponectin-treated (20 ng/mL) or untreated MAC-T cells analyzed using immunofluorescence microscopy. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue; second horizontal panels). Scale bars represent 40 μ m (first and third vertical panels) or 20 μ m (second and fourth vertical panels). The relative intensity of the immunoreactive PCNA protein is presented in a graph. Pooled data from 3 independent experiments ($n = 3$) performed in triplicate are shown as mean $\pm$ SEM. Asterisks indicate significant effect of treatment (*** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$).

Western Blot

Protein concentrations in whole-cell extracts were determined using the Bradford protein assay (Bio-Rad) with BSA as the standard. Proteins were denatured, separated using SDS-PAGE, and transferred to a nitrocellulose membrane. The membranes were incubated overnight at 4°C with primary antibodies at dilutions recommended by the manufacturers. Blots were developed using enhanced chemiluminescence detection (SuperSignal West Pico, Pierce, Rockford, IL) and quantified by measuring the intensity of light emitted from correct-sized bands under ultraviolet light using a ChemiDoc EQ system and Quantity One software (Bio-Rad). Immunoreactive phosphorylated and to-

tal proteins were detected using goat anti-rabbit polyclonal antibody (catalog number 474-1506; Kirkegaard & Perry Laboratories, Gaithersburg, MD) or goat anti-mouse polyclonal antibody (catalog number 474-1806; Kirkegaard & Perry Laboratories) at 1:1,000 dilution. As a loading control, total proteins or α -tubulin (TUBA) were used to normalize results for the target proteins. Multiple exposures of each Western blot were performed to ensure linearity of chemiluminescent signals.

Statistical Analyses

Differences in the variances between the untreated control and each treatment among the results

Figure 2. Adiponectin regulates bovine mammary alveolar (MAC-T) cell cycle progression. (A) Changes in cell cycle progression of MAC-T cells with increasing doses (0, 1, 5, 10, or 20 ng/mL) of adiponectin investigated using flow cytometry. Each independent experiment was replicated 3 times, and percentages of cell populations in G1, S, and G2/M phases are presented in a bar chart. (B) Histograms showing changes in DNA content of MAC-T cells positive for propidium iodide (PI). Pooled data from 3 independent experiments ($n = 3$) performed in triplicate are shown as mean $\pm$ SEM. (C) Abundance and localization of cyclin D1 protein in adiponectin-treated (20 ng/mL) or untreated MAC-T cells analyzed using immunofluorescence microscopy. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue; second horizontal panels). Scale bars represent 40 μ m (first and third vertical panels) or 20 μ m (second and fourth vertical panels). The relative intensity of the immunoreactive cyclin D1 protein is presented in a graph. Pooled data from 3 independent experiments ($n = 3$) performed in triplicate are shown as mean $\pm$ SEM. Asterisks indicate significant effect of treatment ($*P < 0.05$).

from the proliferation assay and Western blot were analyzed with a 1-way ANOVA based on the general linear model procedure of SAS (SAS Inst. Inc., Cary, NC); those were confirmed using the F test, and differences between means were subjected to Student's t test. Western blot data were corrected for differences in sample loading using total protein or TUBA data as a covariate. Multiple comparisons of groups were performed using a 1-way ANOVA with the post hoc Tukey's test. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. A P -value less

than or equal to 0.05 was considered significant. Data are presented as least squares means with SE.

RESULTS

Stimulatory Effects of Adiponectin on Proliferation of MAC-T Cells

Treatment with 5, 10, 20, 50, or 100 ng/mL adiponectin significantly increased MAC-T cell proliferation with a maximum increase (50%; $P < 0.001$) at 20 ng/mL compared to untreated MAC-T cells (Fig. 1A).

Figure 3. Adiponectin activates phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) mitogen-activated protein kinase (MAPK) ERK1/2 MAPK signaling proteins in MAC-T cells. (A) Phosphorylation of serine/threonine protein kinase (AKT), (B) 70 kDa ribosomal S6 kinase (P70S6K), (C) ribosomal protein S6 (S6), (D) ERK1/2, (E) 90 kDa ribosomal S6 kinase (P90S6K), and (F) cyclin D1 in response to increasing concentrations of adiponectin (0, 1, 5, 10, or 20 ng/mL) determined using Western blot. Images were captured to calculate normalized values for p-proteins relative to t-proteins. Bar graphs above blot images show abundance of p-proteins normalized to the abundance of t-proteins; values are presented as fold change relative to untreated (0 ng/mL) control MAC-T cells. Pooled data from 3 independent experiments ($n = 3$) performed in triplicate are shown as mean $\pm$ SEM of relative band intensities. Asterisks indicate significant differences compared to control cells (*** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$).

Consistent with these results, immunofluorescence microscopy revealed an increase ($P < 0.01$) in nuclear PCNA protein expression in MAC-T cells in response to 20 ng/mL adiponectin stimulation (Fig. 1B). The relative fluorescence intensity of immunoreactive PCNA proteins was 7.8-fold greater in adiponectin-treated cells than in control cells ($P < 0.01$).

Inducible Effects of Adiponectin on Cell Cycle Progression in MAC-T Cells

Compared to untreated control cells, the percentage of cells in the G2/M phase increased ($P < 0.05$), whereas the percentage of cells in G0/G1 decreased ($P < 0.05$) as the dose of adiponectin increased (Fig. 2A and 2B). From these results, a 20 ng/mL dose of recombinant adiponectin was used in all subsequent experiments. An adiponectin-induced change in cyclin D1 protein expression was observed in the cells using immunofluorescence microscopy (Fig. 2C). The amount of nuclear cyclin D1 protein was increased in MAC-T cells in response to adiponectin treatment compared to untreated

cells. Immunofluorescence analysis revealed that the relative fluorescence intensity of immunoreactive cyclin D1 proteins was increased 2.3-fold ($P < 0.05$) in MAC-T cells following adiponectin stimulation.

Activation of PI3K/AKT and ERK MAPK Signaling Pathways in MAC-T Cells in Response to Adiponectin

Treatment of cultured MAC-T cells with adiponectin significantly increased cell proliferation and cell cycle progression; therefore, we hypothesized that these adiponectin-induced effects may be mediated through activation of intracellular signaling cascades that are associated with cell proliferation. Semiquantitative RT-PCR analysis revealed that there was no expression of the adiponectin gene in MAC-T cells (Supplementary Fig. S1; see online version of the journal). We also found that both *ADIPOR1* and *ADIPOR2* transcripts were expressed in MAC-T cells. To identify which signaling pathways are activated by adiponectin, we assayed changes in the abundance of p-signaling molecules in MAC-T cells as the dose of adiponectin increased (0 to

Figure 4. Adiponectin-induced signaling pathways activate common downstream molecules. Effects of inhibition of phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) on activation of their downstream signaling proteins by adiponectin were determined using Western blot. Abundance of phosphorylated (A) AKTserine/threonine protein kinase (AKT) (B) 70 kDa ribosomal S6 kinase (P70S6K), (C) ribosomal protein S6 (S6), (D) ERK1/2, (E) 90 kDa ribosomal S6 kinase (P90S6K), and (F) cyclin D1 analyzed in MAC-T cells treated with adiponectin (20 ng/mL) plus wortmannin (1 μ M, PI3K inhibitor) or U0126 (20 μ M, ERK1/2 inhibitor). After preincubation with each pharmacological inhibitor for 2 h, cells were treated with 20 ng/mL adiponectin. Bar graphs above blot images show abundance of p-proteins normalized to abundance of t-proteins; values are presented as fold change relative to control (no adiponectin and no inhibitor), which was given a value of 1. Pooled data from 3 independent experiments ($n = 3$) performed in triplicate are shown as mean $\pm$ SEM. Asterisks indicate significant differences between 2 groups (** $P < 0.01$, and * $P < 0.05$).

20 ng/mL; Fig. 3). We found that adiponectin treatment increased the phosphorylation of AKT, P70S6K, S6, ERK1/2, P90S6K, and cyclin D1 proteins and that this was dose dependent. The maximum increase in p-AKT ($P < 0.01$), p-P70S6K ($P < 0.001$), p-S6 ($P < 0.01$), p-ERK1/2 ($P < 0.01$), p-P90S6K ($P < 0.05$), and p-cyclin D1 ($P < 0.01$) levels was reached in cells treated with 20 ng/mL adiponectin, compared to control (untreated) cells.

Interrelation of Adiponectin-Induced Signaling Pathways and Their Coinvolvement in the Proliferation-Stimulating Effects of Adiponectin

To further determine the sequence of activation of adiponectin-induced signaling molecules and how they are related, MAC-T cells were pretreated with pharmacological inhibitors against PI3K (wortmannin, 1 μ M) or ERK1/2 (U0126, 20 μ M) prior to treatment with 20 ng/mL adiponectin (Fig. 4A to 4F). The effect of adiponectin on AKT phosphorylation was blocked ($P <$

0.01) by pretreatment with the PI3K inhibitor (wortmannin), whereas AKT activation was maintained in the presence of the ERK1/2 inhibitor. The levels of adiponectin-induced p-P70S6K, p-S6, and p-P90S6K were reduced following PI3K ($P < 0.01$) or ERK1/2 ($P < 0.01$ or $P < 0.05$) inhibition. Adiponectin-induced ERK1/2 activation was completely inhibited ($P < 0.01$) only by the ERK1/2 inhibitor. In addition, inhibition of the PI3K or ERK1/2 pathway completely blocked ($P < 0.01$) the ability of adiponectin to activate cyclin D1. Next, we investigated the relationship between the adiponectin-induced signaling pathways and the effect of adiponectin on MAC-T cell proliferation. Consistent with the data in Fig. 5, 20 ng/mL adiponectin alone increased MAC-T cell proliferation by 65% compared to controls ($P < 0.001$), but the proliferation-stimulatory effect of adiponectin was completely inhibited ($P < 0.001$) by blocking either the PI3K or the ERK1/2 pathway. These results indicate that the inductive effect of adiponectin on MAC-T cell proliferation occurs

Figure 5. Adiponectin-induced signaling pathways control the functional effects of adiponectin to induce cell proliferation. Effect of phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) inhibition on adiponectin-induced MAC-T cell proliferation determined using a bromodeoxyuridine (BrdU) cell proliferation assay. Serum-starved MAC-T cells were incubated with adiponectin and either wortmannin or U0126 for 48 h. Cell proliferation rates are presented as relative percentage changes relative to untreated control cells (100%). Pooled data from 3 independent experiments ($n = 3$) performed in triplicate are shown as mean $\pm$ SEM. Asterisks indicate significant differences between 2 groups (*** $P < 0.001$ and ** $P < 0.01$).

through activation of the PI3K/AKT and mitogen-activated protein kinase (MAPK) pathways.

Effects of Adiponectin on Reducing ER Stress Responses in MAC-T Cells

To determine the effects of adiponectin on ER stress response, we analyzed changes in cell proliferation and ER stress regulatory protein levels following treatment with adiponectin alone (20 ng/mL), tunicamycin alone (0.25 μg/mL), or a combination of both. Compared to untreated control cells, tunicamycin, an inducer of ER stress, decreased ($P < 0.001$) proliferation of MAC-T cells (Fig. 6A). The suppressive effect of tunicamycin on proliferation of MAC-T cells was diminished ($P < 0.05$) in cells treated with both tunicamycin and adiponectin compared to cells treated with only tunicamycin. Tunicamycin also increased the abundance of major stress sensor proteins, IRE1 α ($P < 0.01$) and ATF6 α ($P < 0.01$), compared to untreated control cells (Fig. 6B and 6C). However, these effects of tunicamycin were attenuated ($P < 0.05$, respectively) by cotreatment with adiponectin compared to cells treated with only tunicamycin. Similarly, as shown in Fig. 6D to 6G, tunicamycin

stimulated the phosphorylation of PERK ($P < 0.01$) and eIF2 α ($P < 0.01$) and induced the expression of GRP78 ($P < 0.01$) and GADD153 ($P < 0.01$) compared to untreated control cells. Again, these effects were reduced (p-eIF2 α and GADD153; $P < 0.01$ or p-PERK and GRP78; $P < 0.05$) when the cells were cotreated with tunicamycin and adiponectin.

DISCUSSION

The mammary gland is the functional foundation for the synthesis and secretion of milk in all mammals. Understanding the molecular mechanisms involved in the development and function of the mammary gland is therefore critical to improving the efficiency of milk production in livestock mammals and humans, as well as for advancing our knowledge of lactation biology and diseases of the mammary gland. Prior to the current study, the functional role(s) and regulatory mechanism(s) of adiponectin in MAC-T cells had not been defined. The major findings of the present study were that 1) adiponectin controls cell cycle progression and proliferation in MAC-T cells and attenuates the effect of tunicamycin on ER stress responses and cell proliferation and 2) the proliferation-stimulating effect of adiponectin in MAC-T cells occurs through the activation of interrelated PI3K and MAPK signal transduction pathways. These results suggest that greater circulating concentrations of adiponectin or greater local concentrations of adiponectin may contribute to increasing proliferation of mammary epithelial cells and, in turn, promote mammarygenesis.

Mammarygenesis and lactation are controlled by steroid hormones and locally produced factors (e.g., stromal cell derived) that can induce a mitogenic response in adjacent epithelial cells in a variety of mammals, including cows (Topper and Freeman, 1980; Capuco et al., 2001; Ormandy et al., 2003; Joshi et al., 2010; Inman et al., 2015). Adiponectin is the most predominant adipokine derived mainly from adipocytes. Interestingly, adiponectin has been reported to be involved in breast cancer cell survival, proliferation, and tumor development and is thought to be a key mediator of stromal-epithelial interactions that influence the growth and proliferation of malignant cells in the mammary gland (Mantzoros et al., 2004; Barb et al., 2007; Körner et al., 2007). Adipocytes are one of the predominant stromal cell types in the mammary gland; these cells secrete adiponectin, whereas the epithelial cells express adiponectin receptors in the bovine mammary gland (Ohtani et al., 2011). Expression of adiponectin mRNA is greater in adipose tissue than in other bovine tissues, and its transcript levels decrease significantly in bovine mammary tissues during lactation (Ohtani et al., 2011). Concentrations of adiponectin

Figure 6. Adiponectin attenuates tunicamycin-induced endoplasmic reticulum (ER) stress and cell proliferation inhibition. (A) MAC-T cells were treated with tunicamycin (0.25 µg/mL) to induce ER stress. Inhibitory effects of tunicamycin and adiponectin alone or in combination on MAC-T cell proliferation analyzed using a bromodeoxyuridine (BrdU) cell proliferation assay. Cell proliferation rates are presented as relative percentage changes relative to control cells (100%). Pooled data from 3 independent experiments ($n = 3$) performed in triplicate are shown as mean $\pm$ SEM. (B)–(G) Expression and activation of ER stress-related proteins in response to tunicamycin and adiponectin alone or in combination were analyzed by Western blot. The tunicamycin-induced increase in inositol-requiring protein 1 α (IRE1 α), activating transcription factor 6 α (ATF6 α), p-phosphorylated (p)-protein kinase RNA-like ER kinase (PERK), p-p-eukaryotic translation initiator factor 2 α (eIF2 α) glucose-regulated protein 78 (GRP78), and growth arrest- and DNA damage-inducible gene 153 (GADD153) was reduced in the presence of adiponectin. Bar graphs above blot images show the abundance of proteins of interest normalized to t-proteins or α -tubulin (TUBA); values are presented as fold change relative to control MAC-T cells, which were given a value of 1. Pooled data from 3 independent experiments ($n = 3$) performed in triplicate are shown as mean $\pm$ SEM of relative band intensities. Asterisks indicate significant differences between 2 groups (*** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$).

in plasma vary during different physiological stages in cattle, with the highest levels found in late pregnancy and lowest concentrations on the day after parturition and in early lactation (Giesy et al., 2012).

There are discordant findings regarding expression of *ADIPOR* mRNA during lactation. Ohtani et al. (2011) reported higher expression of *ADIPOR1* transcript at peak lactation than in nonlactating cows, whereas Giesy et al. (2012) found that lactation had no effect on the expression of *ADIPOR* in the mammary gland (Ohtani et al., 2011; Giesy et al., 2012). Recently, Lecchi et al. (2015) reported that the *adiponectin*, *CIQ* and *collagen domain containing (ADIPOQ)* transcript was detectable in bovine mammary parenchymal tissue as well, which supports the capacity of parenchymal tissue to synthesize adiponectin in low abundance. They also detected low amounts of adiponectin protein in epithelial cells lining the secretory alveoli (Lecchi et al., 2015). In the present study, by using the RT-PCR analysis, we found that MAC-T cells express *ADIPOR1* and *ADIPOR2* transcripts, but there was no expression of the adiponectin gene. However, detailed information on the functional effects and paracrine actions of adiponectin in mammary epithelial cells has not been fully elucidated. In the present study, we found that treatment of in vitro cultured MAC-T cells with adiponectin (20 ng/mL) in-

creased nuclear PCNA and cyclin D1 expression and induced cell cycle progression and proliferation. However, there was no adiponectin-induced effect on cell proliferation with the highest concentration (e.g., 150 ng/mL). One possibility is that in vitro cultured cells adapt, mutate, and/or become resistant to molecules when given at high concentrations. Nevertheless, it is possible to conclude that the growth-stimulating effect of adiponectin in cultured MAC-T cells is dose dependent, with the most stimulatory effects observed at 20 ng/mL.

Because mammary epithelial cells are the functional unit of the mammary gland and are required for milk production in all mammals (Akers, 2002), the number and secretory activity of mammary epithelial cells greatly affect the capacity of the mammary gland to secrete milk (Capuco et al., 2003; Sorensen et al., 2006; Rezaei et al., 2016). The mammary gland is 1 of the few tissues that undergo repeated cycles of structural and functional development and regression. Regression of the mammary gland is characterized by massive apoptosis and a loss of mammary alveolar epithelial cells (Accorsi et al., 2002; Seeth et al., 2015). Between periods of lactation, the mammary gland of dairy cows undergoes extensive development and remodeling (De Vries et al., 2010), during which time cell proliferation drives mammary cell turnover

(Nørgaard et al., 2008). In contrast, during lactation, mammary cell proliferation is very low in dairy cows, with only 0.3% of epithelial cells proliferating in a 24-h period (Capuco et al., 2001). The number of mammary epithelial cells is greatest at the onset of lactation and declines with advancing lactation in cattle (Capuco et al., 2001). On the basis of results from in vitro studies, several hormones and growth factors, including IGF-I, epidermal growth factor, and hepatocyte growth factor, were found to play roles in the local regulation of mammary gland functions (Bauman and Vernon, 1993; Akers, 2002). For example, IGF-I is a potent mitogen for mammary epithelial cells that stimulate cell proliferation and inhibit apoptosis in dairy cows (Cohick, 1998). Results from the current study suggest that circulating or locally produced adiponectin may also contribute to the morphogenesis, development, remodeling, and function of the bovine mammary gland, where it would act as a positive factor that increases and maintains mammary epithelial cell number.

In a number of cell types, the effects of adiponectin on regulating cellular activities are mediated by 2 receptors, ADIPOR1 and ADIPOR2, and the receptor-conjugated intracellular signaling cascades (Kadowaki and Yamauchi, 2005). During adiponectin-mediated signaling, adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1 (**APPL1**) transduces signals from the activated adiponectin receptors to downstream intracellular signaling pathways (Mao et al., 2006). The intracellular signaling cascades induced by adiponectin vary according to the target cells and their functional effects. For example, adiponectin activates ERK1/2 MAPK signaling via activation of the APPL1 protein in human embryonic kidney cells (Lee et al., 2008), whereas in human and bovine endothelial cells adiponectin activates the AMPK and PI3K/AKT pathways to stimulate vascular development (Chen et al., 2003; Ouchi et al., 2004). A previous report demonstrated that ADIPOR gene expression can be detected in bovine epithelial cells and that this expression changes during different stages of lactation in the mammary gland (Ohtani et al., 2011). In the present study, we showed that adiponectin induces phosphorylation of PI3K and ERK1/2 MAPK signaling molecules in MAC-T cells and that these pathways are not completely independent or parallel, but rather transduce signals to common downstream target proteins (P70S6K, P90S6K, S6, and cyclin D1). In mouse mammary epithelial cells, activation of PI3K/AKT1 and MAPK signaling pathways is thought to mediate cell survival, proliferation, and apoptosis in response to extracellular signals (Fata et al., 2007; Maroulakou et al., 2008). We found that inhibition of these adiponectin-mediated PI3K and ERK1/2 signaling cascades blocked the effects of adiponectin on

MAC-T cell proliferation. Although we did not determine the precise mechanism of adiponectin receptors in transduction of the adiponectin signal into the cells, we propose that the effect of adiponectin on the activation of intracellular signaling pathways is via adiponectin receptor activation, as abundant expression of *ADIPOR* transcripts was found on MAC-T cells.

Intracellular (e.g., DNA damage and DNA replication stress) and extracellular (e.g., deprivation of nutrients or calcium ions, hyper-/hypo-osmosis, and hypoxia) stress can influence tissue homeostasis (Lockshin and Zakeri, 2007; Avivar-Valderas et al., 2014; Oakes and Papa, 2015). Regardless of the nature of the stress, the outcome is often the accumulation of misfolded proteins such as PERK in the ER, and persistent unresolved stress can activate ER stress signals that results in cell death (Park et al., 2008; Liu et al., 2012; Avivar-Valderas et al., 2014). At the onset of lactation, bovine mammary epithelial cells elicit proliferative and hypertrophic responses with dramatically increased biosynthetic activity to produce a large quantity of milk fat and protein (Invernizzi et al., 2012). Because the ER is the key organelle for protein and lipid biosynthesis, modification, and secretion (Baumann and Walz, 2001; Harding et al., 2002), the ER load is expected to increase in mammary epithelial cells. In highly metabolic cells, increases in unfolded proteins and consequent ER stress could trigger specific adaptive response known as the unfolded protein response (**UPR**), which intersects with various inflammatory and stress signaling pathways (Ron and Walter, 2007).

Changes in the ER stress pathway are therefore a normal method by which mammary tissue adapts to changing physiological states of cows, which are required for the functional and structural maturation of epithelial cells (Invernizzi et al., 2012). In the heart of rat, a lack of adiponectin can trigger ER stress and increase inflammation (Guo et al., 2013). Other studies have shown that adiponectin reduced ER stress in various tissues and cell types, including mouse adipocytes (Liu et al., 2016), human liver cells (Jung et al., 2015), and rat smooth muscle (Lu et al., 2015). The results from the current study demonstrate the inhibitory effects of adiponectin on ER stress in MAC-T cells. We found that treating MAC-T cells with tunicamycin activated the UPR signaling molecules (IRE1 α , ATF6 α , PERK, eIF2 α , GRP78, and GADD153) in response to ER stress and reduced cell viability but that these effects were alleviated in the presence of adiponectin. This indicates that adiponectin has a cell protective effect on MAC-T cells. Taken together, our results suggest that adiponectin could be used to enhance the development and remodeling of the mammary gland in

lactating cows by protecting the epithelial cells from ER stress responses and by inducing cell proliferation.

In summary, our findings provide evidence that adiponectin acts as a positive factor that can increase mammary epithelial cell number, which may impact mammary development and milk production. However, further research is needed to elucidate the more precise mechanisms of secretion, action, and hormonal regulation of adiponectin in the mammary gland. Such in-depth knowledge of the development and function of mammary tissue has important implications in improving our fundamental understanding of lactation physiology and helping to develop methods to increase the efficiency of milk production in livestock species.

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