Caffeine Inhibits EGF-Stimulated Trophoblast Cell Motility through the Inhibition of mTORC2 and Akt

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Impaired trophoblast invasion is associated with pregnancy disorders such as early pregnancy loss and preeclampsia. There is evidence to suggest that the consumption of caffeine during pregnancy may increase the risk of pregnancy loss; however, little is known about the direct effect of caffeine on normal trophoblast biology. Our objectives were to examine the effect of caffeine on trophoblast migration and motility after stimulation with epidermal growth factor (EGF) and to investigate the intracellular signaling pathways involved in this process. Primary first-trimester extravillous trophoblasts (EVT) and the EVT-derived cell line SGHPL-4 were used to study the effect of caffeine on EGF-stimulated cellular motility using time-lapse microscopy. SGHPL-4 cells were further used to study the effect of caffeine and cAMP on EGF-stimulated invasion of fibrin gels. The influence of caffeine and cAMP on EGF-stimulated intracellular signaling pathways leading to the activation of Akt were investigated by Western blot analysis. Caffeine inhibits both EGF-stimulated primary EVT and SGHPL-4 cell motility. EGF stimulation activates phosphatidylinositol 3-kinase, and Akt and caffeine inhibit this activation. Although cAMP inhibits both motility and invasion, it does not inhibit the activation of Akt, indicating that the effects of caffeine seen in this study are independent of cAMP. Further investigation indicated a role for mammalian target of rapamycin complex 2 (mTORC2) as a target for the inhibitory effect of caffeine. In conclusion, we demonstrate that caffeine inhibits EGF-stimulated trophoblast invasion and motility *in vitro* and so could adversely influence trophoblast biology *in vivo*. **(*Endocrinology* 153: 4502–4510, 2012)**

Trophoblasts are specialized cells of the placenta that play a central role in establishing and maintaining a successful pregnancy. Extravillous trophoblasts (EVT) invade through the decidua, migrating toward the uterine spiral arteries where they interact with the vessel wall, resulting in the loss of both endothelial and vascular smooth muscle cells. The resulting transformation ensures the enhanced delivery of blood to the developing fetus at a much reduced pressure (1, 2). Failure of EVT to adequately invade the decidua results in poor vessel remodeling and a reduction in placental perfusion, characteristic of common pregnancy complications such as preeclampsia, intrauterine growth restriction (IUGR), and even early pregnancy loss (3).

Caffeine is a methylxanthine found naturally in plants used to make coffee, tea, cocoa/chocolate, and cola drinks. It is also artificially added to many energy drinks as well as a range of over-the-counter medications and, as such, is the most commonly consumed psychostimulant in the world. The effects of caffeine on pregnancy outcome are controversial (4–8); however, several large epidemiological studies have established a correlation between increased caffeine intake and risk of miscarriage (4) and IUGR (5). In response to these latter studies, the United Kingdom Food Standards Agency changed the recommendation given to pregnant women advising that they restrict their caffeine intake during pregnancy (9). The effect of caffeine on the normal trophoblast functions essential for successful placentation, such as cellular invasion and motility, remains to be comprehensively studied.

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Abbreviations: DMSO, Dimethylsulfoxide; EGF, epidermal growth factor; EVT, extravillous trophoblasts; FBS, fetal bovine serum; IUGR, intrauterine growth restriction; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; PI_3, phosphatidylinositol 3; RICTOR, rapamycin-insensitive companion of mTOR.

Caffeine is lipid soluble and passes freely across biological membranes. It has been detected in the preimplantation blastocyst (10) and has been shown to cross the placenta (11). Caffeine can elicit a number of cellular responses; it is an inhibitor of cAMP phosphodiesterase (12), resulting in the elevation of intracellular cAMP, and it can stimulate calcium release from intracellular stores, regulate cellular proliferation, and can induce cell cycle arrest and apoptosis (13). Caffeine can also inhibit tumor cell invasion (14).

Epidermal growth factor (EGF) is a pluripotent growth factor that can regulate a number of fundamental cellular processes including growth, differentiation, invasion, and apoptosis in numerous cell types. It is secreted by human endometrium, decidua, and placenta, and both EGF and its receptors have been isolated in cytotrophoblasts (15). We and others have shown that EGF plays an important role in the regulation of trophoblast invasion (16) and motility and migration (17).

In the light of the controversy over the effects of caffeine on pregnancy outcome, the aim of our study was to examine whether caffeine had any detrimental effect on trophoblast biology, a crucial determinant of pregnancy success.

Materials and Methods

Unless otherwise stated, all reagents were obtained from Sigma (Dorset, UK) or VWR (Nottingham, UK).

Cells and reagents

Isolation of EVT

EVT were isolated from first-trimester placentas using the method of Chui *et al.* (18). Briefly, villi were dissected from the placental membrane, washed in Hanks' balanced salt solution (37 C, pH 7.5; Biosera, East Sussex, UK) and the wet weight of the villi recorded. Trypsin digestion buffer (5 ml/g tissue) was added (0.1% vol/vol) [trypsin (Difco, Scientific Laboratory Supplies, Yorkshire, UK), 4.2 mM $MgSO_4$, 1 M HEPES (pH 7.4)] with 5 U/ml deoxyribonuclease, and villi were incubated at 37 C for 15 min. Excess buffer was then removed and replaced by 8 ml warm Hanks' balanced salt solution at 37 C. Tissue was gently agitated, and the supernatant was examined for the presence of grape-like clusters of EVT. When EVT were present, the supernatant was collected through a 100-μm filter into DMEM/F12 containing 30% (vol/vol) fetal bovine serum (FBS). This washing process was repeated between 10 and 20 times until the numbers of EVT clusters in the placental washes began to decrease. The collected EVT were then centrifuged at 200 $\times$ *g* for 5 min, resuspended in 2 ml serum-free EVT medium, and passed through a 40-μm filter. The filtrate was layered onto a Percoll gradient (20–55%) and centrifuged at 1200 $\times$ *g* for 30 min with no brake. Two distinct layers of trophoblasts could be identified between 30 and 45% and were collected, diluted to 50 ml in EVT medium, and centrifuged at 500 $\times$ *g* for 7 min. Cells were resuspended in DMEM/F12 containing 10% (vol/vol) FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 2 mM L-glutamine and plated onto a 60-mm tissue culture plate for 10 min to remove adherent stromal cells. EVT were then plated onto a thin layer of Matrigel at a density of 2.5×10^5 cells per well. EVT isolated by this method had an average purity of 88 $\pm$ 1.9% (SEM) (n = 6) as determined by human leukocyte antigen-G and cytokeratin-7 double immunocytochemical staining.

The human EVT-derived cell line SGHPL-4 was used (19, 20). These cells are well characterized and share many characteristics with isolated primary cells, including the expression of cytokeratin-7 and human leukocyte antigen-G (19, 21). Cells were cultured in Ham's F10 supplemented with 10% (vol/vol) FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM L-glutamine at 37 C in 5% CO_2. Recombinant human EGF was purchased from Peprotech (London, UK). Modulators of intracellular cAMP and inhibitors were obtained from the following companies; 8-bromo-cAMP (Biolog, Bremen, Germany), rapamycin, and KU-0063794 (Calibiochem, Nottingham, UK).

Motility assays

Time-lapse digital image microscopy using an Olympus IX70 inverted microscope equipped with a Hamamatsu C4742-95 digital camera was performed to examine cell motility as previously described (19). The microscope and stage were enclosed within a heated (37 C) humidified chamber in an atmosphere of 5% CO_2 (Solent Scientific, Fareham, UK). Primary EVT were incubated overnight in DMEM/F12 containing 10% (vol/vol) FBS, transferred to serum-free medium for 6 h, and then incubated with or without EGF (10 ng/ml) in serum-free culture medium and motility assessed over 24 h. SGHPL-4 cells were incubated in medium containing 0.5% (vol/vol) FBS for 24 h, at which time they were transferred to the microscope in fresh medium containing vehicle (PBS) or 10 ng/ml EGF. Where appropriate, cells were pretreated with the indicated inhibitors for 30 min at 37 C or the vehicle [dimethylsulfoxide (DMSO)] and subsequently transferred to the microscope chamber in the presence or absence of EGF. In accordance with previous studies (20), 10 ng/ml was found to be the optimal EGF concentration to stimulate motility (data not shown) and was therefore used in this study. Images were captured every 15 min over a period of 24 h (primary EVT) and 6 h (SGHPL-4). Cells were randomly chosen at the beginning of the experimental sequence and their movement track manually using Image Pro-Plus software (Media Cybernetics, Bethesda, MD). Up to 60 cells were tracked per treatment, and the experiments were repeated on at least three separate occasions. Visualizing the cells by this way allows both proliferation and viability to be monitored throughout the experimental period (19, 20, 22).

Invasion assay

Invasion assays were carried out as a modification of the method detailed previously (19). In brief, SGHPL-4 cells were grown to confluence in Ham's F-10 medium supplemented with 10% (vol/vol) FBS. Approximately 10^6 cells were incubated with 120 μl of gelatin-coated micro-carrier beads prepared according to the manufacturer's recommendations in a flat-bottom container and incubated at 37 C for 30 min to allow the cells to adhere to the beads before being washed gently with PBS. Fibrin gels were prepared by dissolving bovine fibrinogen (>95% of clottable protein) in calcium- and magnesium-free PBS at 2.5

mg/ml with 200 U/ml aprotinin (Trasylol, Bayer, Germany) in a 35-mm plate. Individual bead-cell complexes were added to the fibrinogen and clotting induced by adding thrombin (0.625 U/ml). After the gel had set, 1.5 ml Ham's F-10 containing 0.5% (vol/vol) FBS was layered over the top. The medium was allowed to equilibrate with the gel for 2 h in a 37 C incubator with 5% CO_2 in air and then removed and replaced by fresh medium containing 0.5% (vol/vol) FBS.

After 16 h, the cells were stimulated with 10 ng/ml EGF in the presence or absence of cAMP at the doses indicated. After 48 h, images of 20 bead-cell complexes per plate were taken. The beads were visualized using an Olympus 1 × 70 inverted microscope at ×10 magnification, and images were captured using a Hamamatsu C4742-95 digital camera. Twenty beads were selected at random and the number and length of process determined using Image Pro-Plus software (Media Cybernetics) (19).

Western blot analysis

SGHPL-4 cells were serum starved for 24 h in Hams F10 containing 0.5% FBS (vol/vol) at 37 C. Primary EVT were culture overnight in DMEM/F12 containing 10% (vol/vol) FBS, and the cells were then serum starved for 6 h before experimentation. Cells were then treated in the absence of serum with 10 ng/ml EGF or vehicle control (PBS) for 0, 5, 15, 30, or 60 min as stated. Where indicated, cells were pretreated with DMSO or the appropriate inhibitor for 30 min at 37 C before the addition of EGF. Cells were harvested in 1× RIPA buffer containing a protease inhibitor cocktail of aprotinin (60 μg/ml), phenylmethylsulfonyl fluoride (1 mM), and sodium orthovanadate (1 mM). Lysates were sheared three times by passage through a 27-gauge needle, and cell debris was removed by centrifugation at 18,000 × *g* at 4 C for 15 min. Total protein concentrations were determined using Bradford protein assay reagent, and an equal amount of total protein in each well was resolved on 8–10% (wt/vol) SDS-PAGE gels before transfer to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, UK). Nonspecific reactivity was blocked with 5% (wt/vol) nonfat dried milk in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 for 1 h at room temperature. Blots were incubated overnight with the following antibodies obtained from Cell Signaling Technology (Danvers, MA) and used at 1:1000 dilution as per supplier's instructions: rabbit anti-mammalian target of rapamycin (mTOR) (CS-2972), rabbit anti-phospho-mTOR-Ser^{2481} (CS-2974), rabbit anti-phospho-rictor-Thr^{1135} (CS-3806), rabbit anti-phospho-Akt-Ser^{473} (CS-9271 or CS-4060), and rabbit anti-phospho-Akt-Ser^{308} (CS-9275). The blots were then washed in Tris-buffered saline/0.1% Tween 20 and incubated with goat antirabbit IgG conjugated to horseradish peroxidase (1:10000; A5420, Sigma, UK) for 1 h at room temperature, and antigen-antibody complexes were detected using an enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, UK). Blots were subsequently stripped in buffer containing 62.5 mM Tris (pH 6.7), 2% (wt/vol) sodium dodecyl sulfate, and 100 mM β-mercaptoethanol and probed with a rabbit polyclonal antibody to human β-actin or mouse monoclonal antibody to human tubulin (1:10000, Ab7291; Abcam Cambridge, UK). Where indicated, Western blots were scanned and the integrated intensity of each band determined using ImageJ (http://rsb.info.nih.gov/ij/). Results are expressed as a ratio to loading control within the same sample.

Statistical analysis

Data are presented as the mean + SEM. The data were analyzed using a repeated-measures ANOVA followed by a Bonferroni multiple-comparison *post hoc* test (GraphPad Prism, San Diego, CA). Statistical significance was assumed at $P < 0.05$.

Results

Caffeine and 8Br-cAMP inhibited EGF-stimulated SGHPL-4 cell invasion

EGF stimulated SGHPL-4 invasion of fibrin gels (Fig. 1; $P < 0.05$; n = 3). Caffeine reduced basal trophoblast invasion, but this did not reach statistical significance. Inhibition of EGF-stimulated invasion was observed at 2 mM

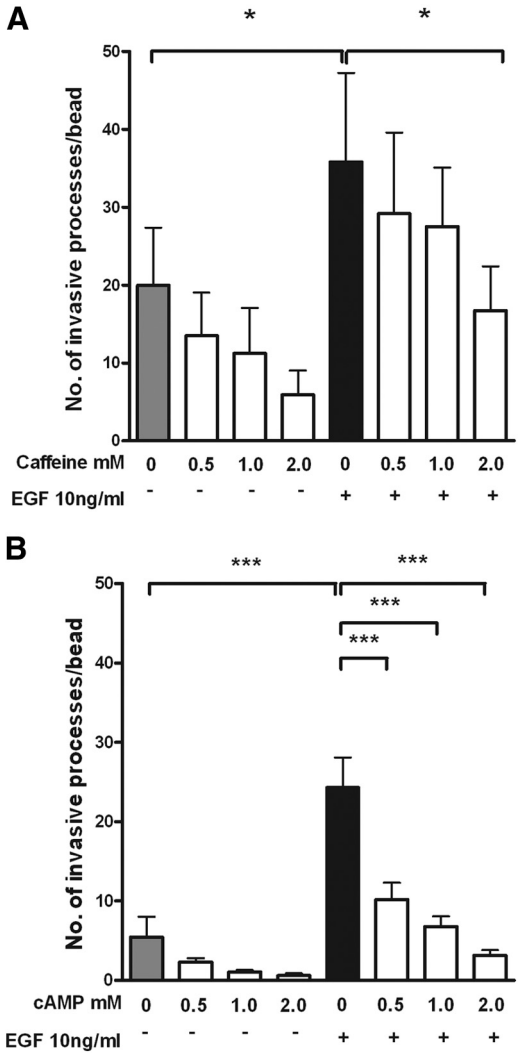

FIG. 1. The effect of caffeine and 8Br-cAMP on SGHPL-4 cell invasion. SGHPL-4 cells were grown on gelatin-coated Cytodex beads embedded in fibrin gels and cultured for 16 h in medium with 0.5% (vol/vol) FBS. Before stimulation with EGF (10 ng/ml), the cells were incubated with either caffeine (A) or 8Br-cAMP (B) for 30 min. After 48 h, images of 20 beads per treatment were taken at random. Both the number and length of invasive processes were determined. The results are expressed as means + SEM. *, $P < 0.05$; ***, $P < 0.001$.

caffeine (Fig. 1A; $P < 0.05$; n = 3). Because one of the most studied effects of caffeine is its inhibitory effect on cyclic nucleotide phosphodiesterases, which results in increased intracellular cAMP, we examined the effect of the membrane-permeable phosphodiesterase-resistant analog of cAMP, 8Br-cAMP. This compound significantly inhibited EGF-stimulated trophoblast invasion of fibrin gels at 0.5, 1, and 2 mM (Fig. 1B; $P < 0.001$; n = 3).

Caffeine and 8Br-cAMP inhibited EGF-stimulated EVT and SGHPL-4 cell motility

Cellular motility is an important component of invasion. To assess the effects of caffeine on primary EVT motility, cells were followed over 24 h using time-lapse microscopy. EGF at 10 ng/ml significantly stimulated primary EVT cell motility (Fig. 2A; $P < 0.01$; n = 5). This was significantly inhibited by pretreatment of cells with 2 mM caffeine for 30 min before the addition of EGF (Fig. 2A; $P < 0.05$; n = 5). As previously reported (20), 10 ng/ml EGF significantly stimulated SGHPL-4 cell motility (Fig. 2B; $P < 0.001$; n = 6), and this was inhibited in a dose-dependent manner by caffeine. This reached statistical significance at 1 mM ($P < 0.05$; n = 6). There was also a small but significant inhibition of basal trophoblast cell motility at the highest concentration of caffeine used (2 mM; $P < 0.05$; n = 6). 8Br-cAMP significantly inhibited both basal and EGF-stimulated motility at both concentrations used (Fig. 2C). To determine whether caffeine exerts its inhibitory effect via the elevation of cAMP and the activation of protein kinase A, we repeated the motility experiments after preincubation of SGHPL-4 cells with the protein kinase A inhibitor Rp-cAMP, but we were unable to overcome the inhibitory effect of caffeine on SGHPL-4 motility (Fig. 2D). Caffeine had no effect on either the viability or the proliferation of trophoblast during the course of the experiments presented (data not shown).

Caffeine but not 8Br-cAMP inhibited EGF-stimulated phosphorylation of Akt

To establish a mechanism for the inhibition of EGF-stimulated motility seen with both caffeine and 8Br-cAMP, we examined signaling pathways known to be activated after stimulation with EGF (20). We have previously shown that activation of Akt is fundamental to the stimulation of trophoblast motility by EGF, and inhibiting phosphorylation at Ser^{473} inhibits motility (20). Stimulation of SGHPL-4 cells with EGF for 5 min led to phosphorylation of Akt at Ser^{473} and Thr^{308}. Caffeine inhibited Akt phosphorylation at both sites in a dose-dependent manner (Fig. 3, A and B). Densitometric analysis of the phosphorylation of Akt at Ser^{473} indicated significant inhibition was reached with caffeine concentrations greater than 1 mM ($P < 0.05$; n = 5) and 2 mM, respectively ($P < 0.01$; n = 3), whereas caffeine at 2 mM significantly inhibited phosphorylation at Thr^{308} ($P < 0.05$; n = 5). 8Br-cAMP had no effect on the phosphorylation at Ser^{473}, suggesting a difference in the mechanisms employed by the two compounds (Fig. 3C; n = 3) and supporting the data obtained with Rp-cAMP (Fig. 2D). In subsequent experiments, only caffeine was used.

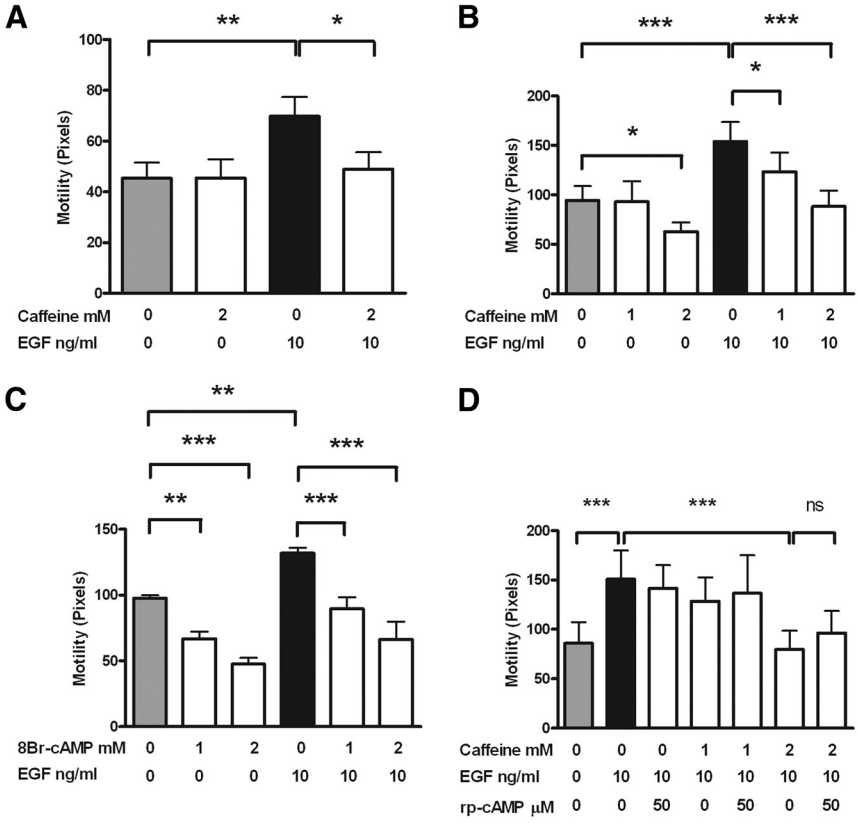

FIG. 2. Effect of caffeine and 8Br-cAMP on EGF-stimulated primary first-trimester EVT and SGHPL-4 cell motility. Primary EVT (A) and SGHPL-4 (B–D) were cultured as stated. Before stimulation with EGF (10 ng/ml), cells were incubated for 30 min with caffeine, 8Br-cAMP, or Rp-cAMP. Images were taken every 15 min over 24 (primary EVT) and 6 (SGHPL-4) hours. Cells were selected at random and motility tracked using Image Pro-Plus software. The data presented are the mean distance moved in pixels + SEM from at least n = 5 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

mTOR, Akt phosphorylation, and trophoblast motility

mTOR is associated with two complexes, mTOR complex (mTORC) 1 and 2, which act both downstream and

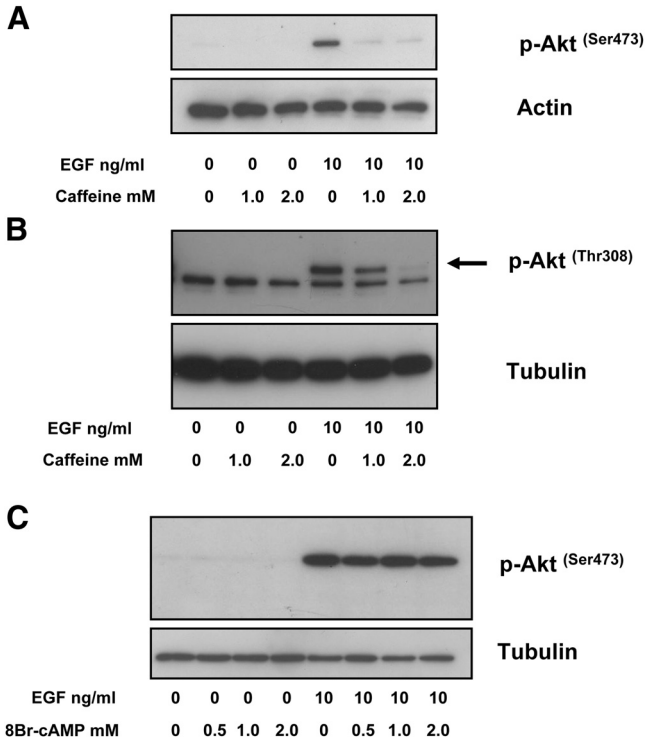

FIG. 3. Caffeine but not 8Br-cAMP inhibited EGF-stimulated AKT phosphorylation at Ser^{473}. SGHPL-4 cells were grown in 9-cm dishes until they reached approximately 80% confluence. Medium was removed and replaced with 0.5% serum for 16 h. Cells were incubated in serum-free medium with or without caffeine (A and B) or 8Br-cAMP (C) at the stated concentrations. After 30 min, EGF (10 ng/ml) was added where appropriate for 5 min. Cell lysates were collected, and equal amounts of total protein were subjected to Western blot analysis for the phosphorylation of Akt. The results presented are representative of at least three independent experiments.

upstream of Akt, respectively. Inhibition of mTORC2 phosphorylation by endostatin has recently been shown to inhibit IGF-II-stimulated trophoblast invasion (23). To assess the role of mTOR in the action of caffeine, we first examined the role of mTOR in EGF-stimulated trophoblast motility using two inhibitors: rapamycin, which targets mTORC1, and KU-0063794, which inhibits both mTORC1 and mTORC2. KU-0063794, but not rapamycin, significantly inhibited trophoblast cell motility (Fig. 4, A and B). Phosphorylation of Akt-Ser^{473} was inhibited by KU-0063794, reaching significance at 1 μM ($P < 0.01$; n = 5) but was not significantly affected by rapamycin (Fig. 4C; n = 6). Similarly, EGF-stimulated phosphorylation of Akt-Ser^{473} in primary EVT was significantly inhibited by KU-0063794 at 1 μM ($P < 0.05$; n = 4) but was not significantly inhibited by rapamycin (Fig. 4D). Stimulation of trophoblasts with EGF led to a rapid increase in the phosphorylation of mTOR at Ser^{2481}, which persisted for at least 30 min (Fig. 5A). To further probe the involvement of mTORC2 in EGF-stimulated trophoblast motility, we examined the phosphorylation of rapamycin-insensitive companion of mTOR (RICTOR), a key regulatory protein in the mTORC2 that is absent from mTORC1. EGF stimulation of trophoblasts resulted in a rapid increase in the phosphorylation of RICTOR at Thr^{1135}. Preincubation of trophoblasts with caffeine significantly inhibited phosphorylation of RICTOR-Thr^{1135} at both 1 and 2 mM (Fig. 5B; $P < 0.001$; n = 3).

Effect of inhibiting phosphatidylinositol 3 (PI_3)-kinase on mTORC2 activation

To determine whether activation of PI_3-kinase could lead to mTORC2 activation, we examined the effect of EGF on RICTOR phosphorylation in the presence and absence of the PI_3-kinase inhibitor Ly294002. As determined previously, EGF stimulated the phosphorylation of RICTOR at Thr^{1135} within 5 min, and phosphorylation of Akt-Ser^{473} was also observed. Incubation of SGHPL-4 cells with 50 μM Ly294002 for 30 min before the addition of EGF significantly inhibited both the phosphorylation of RICTOR and Akt (Fig. 5C; $P < 0.01$; n = 5).

Effect of caffeine on the activation of PI_3-kinase

We have previously shown that activation of the p85 subunit of PI_3-kinase by the membrane-permeable peptide 740y-p stimulates the motility of SGHPL-4 cells (22). To determine whether caffeine inhibits this pathway before or after the activation of PI_3-kinase, we used 740y-p, which directly activates the p85 subunit. At 25 and 50 μg/ml, 740y-p produced a 1.37-fold ($P < 0.05$, n = 6) and 1.49-fold ($P < 0.01$, n = 6) increase in trophoblast motility, respectively, which was significantly inhibited by caffeine (Fig. 6).

Discussion

Poor trophoblast function is associated with a number of pregnancy disorders including preeclampsia, IUGR, and early pregnancy loss. Several studies have sought to establish a link between the incidence of these pregnancy disorders and external factors such as smoking, alcohol, and caffeine consumption. Caffeine is the most common xenobiotic consumed during pregnancy and has been the subject of a number of conflicting epidemiological studies (5, 6, 8).

In mouse studies, caffeine reduced trophoblast outgrowth (24); however, to date, there are no reports of the effect it has on human trophoblast biology. In the present study, both basal and EGF-stimulated trophoblast invasion was inhibited by caffeine. Caffeine modulates a number of intracellular signaling pathways in a dose-dependent manner. At concentrations greater than 0.2 mM,

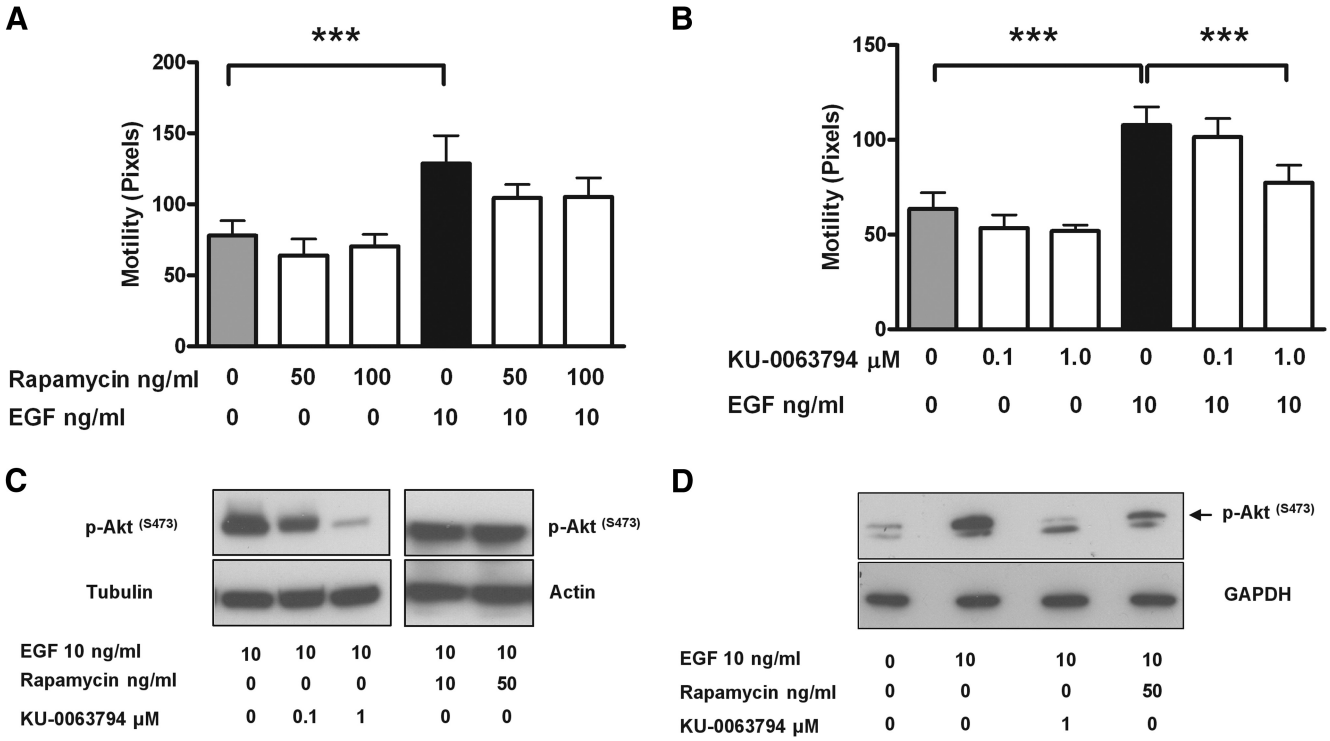

FIG. 4. The regulation of trophoblast motility and phosphorylation of AKT^{Ser473} by mTOR. A and B, Time-lapse digital microscopy was performed on cells that were pretreated for 30 min with the indicated doses of either vehicle (DMSO) or rapamycin (A) or KU-0063794 (B). Cells were then stimulated with EGF (10 ng/ml), and motility was monitored over a 6-h period. The data presented are the mean distance moved in pixels + SEM from at least n = 5 independent experiments. ***, $P < 0.001$. C and D, SGHPL-4 cells (C) or primary EVT (D) were incubated in serum-free medium with KU-0063794 or rapamycin for 30 min and then stimulated with EGF for 5 min. Cell lysates were collected, and equal amounts of total protein were subjected to Western blot analysis and probed for phospho-Akt^{Ser473}. The results presented are representative of at least three independent experiments.

caffeine acts as a phosphodiesterase inhibitor, leading to elevated intracellular cAMP (25). The membrane-permeable analog of cAMP, 8Br-cAMP, mimicked the inhibitory effect of caffeine on EGF-stimulated trophoblast invasion of fibrin gels. Although the mechanism responsible for this is not known, evidence from other cell systems suggests that both caffeine and cAMP can inhibit the expression and or activation of matrix metalloproteinase-2 and -9 (26, 27). Because both enzymes are expressed by trophoblasts and are active in regulating trophoblast invasion, their role warrants additional study (28, 29).

Cellular motility is a key component of invasion. We have shown for the first time that caffeine can inhibit EGF-stimulated motility of both primary EVT cells and the EVT-derived cell line SGHPL-4. The binding of EGF to its receptor induces receptor homo- and heterodimerization that is essential for the phosphorylation of multiple tyrosine (Tyr) residues and the activation of Tyr kinases. These Tyr residues provide docking sites for specific Src homology 2-containing proteins (30–33). Stimulation of trophoblasts with EGF results in the parallel activation of intracellular pathways involving both PI_3-kinase/Akt and p42/44 MAPK (17). In this study, we investigated how caffeine might influence pathways leading to the activation of Akt.

From previous studies, it is known that activation of Akt after phosphorylation at Ser^{473} is key to the regulation of EGF-stimulated motility in these cells (17, 20). We therefore investigated whether caffeine might act by modulating the activity of this protein. The current model of Akt activation proposes that, after EGF receptor occupancy, PI_3-kinase is activated and phosphatidylinositol 3,4,5-trisphosphate is generated, which, in turn, mediates the recruitment of Akt to the plasma membrane. Maximal activation of Akt is dependent on the phosphorylation of two residues, Thr^{308}, which is dependent on the activity of the enzyme PI_3-kinase-dependent kinase 1, and Ser^{473}. We have found previously that phosphorylation of Akt on Ser^{473} is important in regulating EGF-stimulated trophoblast motility (20). In this study, we demonstrate that caffeine reduces the phosphorylation of Akt at both Thr^{308} and Ser^{473}. Although we also found a similar inhibitory effect on motility after stimulation in the presence of 8Br-cAMP, there was no change in the phosphorylation of Akt Ser^{473}, indicating that in trophoblasts the target of inhibition of these two compounds was different. The differ-

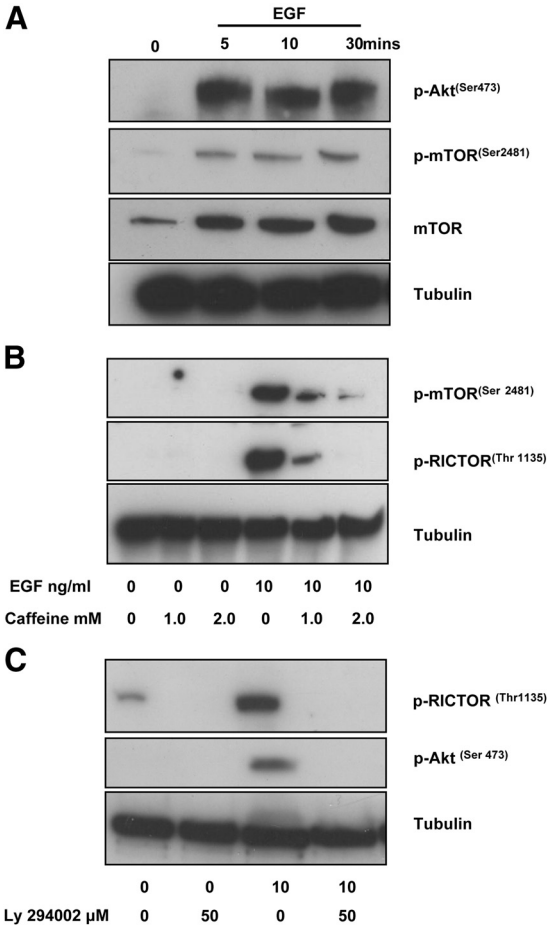

FIG. 5. Effect of caffeine and Ly294002 on the phosphorylation of Akt and mTORC2. A, SGHPL-4 cells were grown in media with 0.5% (vol/vol) serum for 16 h before incubation with EGF (10 ng/ml) for between 0 and 30 min. Cell lysates were collected, and equal amounts of total protein were subjected to Western blot analyses for phospho-Akt^{Ser473}, phospho-mTOR^{ser2481}, and mTOR. B and C, Cells were incubated in serum-free media with either caffeine (B) or Ly294002 (C) for 30 min before the addition of EGF for 5 min. Cell lysates were collected, and equal amounts of total protein were subjected to Western blot analysis and probed for phospho-Akt-^{Ser473}, phospho-mTOR^{Ser2481}, or phospho-RICTOR^{Thr1135}. The results presented are representative of three independent experiments.

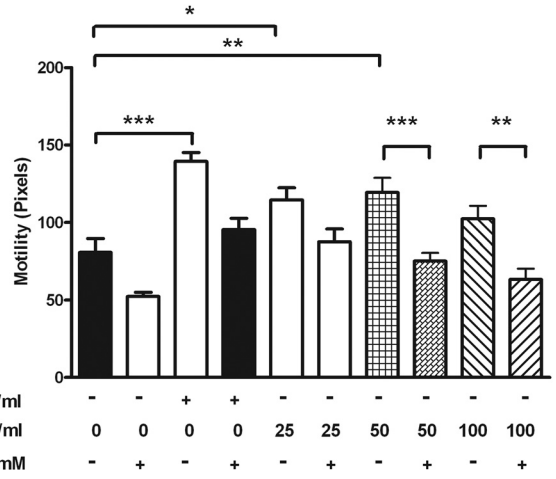

FIG. 6. The effect of caffeine on PI_3-kinase-mediated trophoblast motility. Time-lapse microscopy was performed on cells that were pretreated for 30 min with caffeine. Cells were then stimulated with 740y-p, the membrane-permeable activator of the p85 subunit of PI_3-kinase, and motility was monitored over a 6-h period. The data presented are the mean distance moved in pixels + SEM from at least n = 5 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

ence in the effect of caffeine and cAMP on Akt phosphorylation seen in this study has also been shown in other cell types including human endometrial stromal cells (34). In contrast, however, both caffeine and raised intracellular cAMP lead to an inhibition in the phosphorylation of Akt in both thyrocytes and HEK293 cells (35, 36).

Activation of Akt in response to EGF results from the activation of mTORC2 (37), a complex containing mTOR and RICTOR (38). After stimulation of trophoblasts with EGF, we were able to demonstrate a rapid and sustained increase in the phosphorylation of mTOR at Ser^{2481}. Although phosphorylation of mTOR at Ser^{2481} in response to stimulation has been associated with activation of both mTORC1 and mTORC2, we were able to exclude the involvement of complex 1 as EGF stimulated motility, and phosphorylation of Akt^{ser473} was unaffected by the mTORC1-specific inhibitor rapamycin. Phosphorylation of mTOR at Ser^{2481} in response to EGF was sensitive to inhibition with caffeine. The role of RICTOR in the regulation of mTORC2 is unclear, because phosphorylation of Thr^{1135} has been implicated in both the inhibition and activation of mTORC2 (39, 40). From our study, we were able to demonstrate that EGF stimulated the phosphorylation RICTOR Thr^{1135} in a caffeine-sensitive manner, suggesting that the target of caffeine is either one of the other, as yet unidentified, components of mTORC2 or a component of the pathway that lies upstream of mTORC2 (41).

Ly294002 regulates the PI_3-kinase/Akt pathway by targeting the binding of ATP to the p110 catalytic subunit of PI_3-kinase (42). To date, evidence would indicate that EGF receptor activation leads to activation of PI_3-kinase-dependent kinase 1 and phosphorylation of Akt on Thr^{308} in a PI_3-kinase-dependent fashion, whereas activation of mTORC2 results in the phosphorylation of Akt Ser^{473}. However, we have previously shown that in trophoblasts, inhibition of PI_3-kinase with Ly294002 inhibits motility and also phosphorylation of Akt at Ser^{473} (20). Consistent with this finding, we demonstrate here that inhibition of PI_3-kinase with Ly294002 significantly inhibited the phosphorylation of both Akt Ser^{473} and RICTOR Thr^{1135}. Furthermore, we were able to demonstrate stimulation of trophoblast motility after activation of the p85 regulatory subunit of PI_3-kinase with the cell-permeable peptide 740y-p could be inhibited by caffeine, suggesting

the site of action of caffeine was either at the site of or downstream from PI_3-kinase. This supports a previous study of purified recombinant PI_3-kinase, indicating a direct inhibitory effect of caffeine on the p110 subunit (41).

In conclusion, we have investigated whether caffeine had any detrimental effect on trophoblast biology, a crucial determinant of pregnancy success. Using first-trimester EVT and the EVT-derived cell line SGHPL-4, we examined the effect of caffeine on trophoblast cell motility and invasion. The most significant findings of this study were that caffeine significantly inhibited basal and EGF-stimulated trophoblast invasion and motility in a dose-dependent manner. Furthermore, this mechanism was largely independent of the generation of cAMP but was mediated via inactivation of PI_3-kinase and mTORC2, resulting in a failure to activate Akt, a key intermediate in the intracellular pathway leading to trophoblast invasion and motility. This suggests there may be a cellular basis for the concerns over the consumption of caffeine during early pregnancy.

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