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Title:
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Date:
2014-01-01

Citation:
Docanto, M. M., Yang, F., Callaghan, B., Au, C. M. C., Ragavan, R., Wang, X., Furness, J. B., Andrews, Z. B. & Brown, K. A. (2014). Ghrelin and des-acyl ghrelin inhibit aromatase expression and activity in human adipose stromal cells: Suppression of cAMP as a possible mechanism. Breast Cancer Research and Treatment, 147 (1), pp.193-201. https://doi.org/10.1007/s10549-014-3060-1.

Persistent Link:
https://hdl.handle.net/11343/59316

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Ghrelin and des-acyl ghrelin inhibit aromatase expression and activity in human adipose stromal cells: suppression of cAMP as a possible mechanism.

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Abstract

Purpose: Aromatase converts androgens into estrogens and its expression within adipose stromal cells (ASCs) is believed to be the major driver of estrogen-dependent cancers in older women. Ghrelin is a gut-hormone that is involved in the regulation of appetite and known to bind to and activate the cognate ghrelin receptor, GHSR1a. The unacylated form of ghrelin, des-acyl ghrelin, binds weakly to GHSR1a but has been shown to play an important role in regulating a number of physiological processes. The aim of this study was to determine the effect of ghrelin and des-acyl ghrelin on aromatase in primary human ASCs. **Methods:** Primary human ASCs were isolated from adipose tissue of women undergoing cosmetic surgery. Real-time PCR and tritiated water-release assays were performed to examine the effect of treatment on aromatase transcript expression and aromatase activity, respectively. Treatments included ghrelin, des-acyl ghrelin, obestatin and capromorelin (GHSR1a agonist). GHSR1a protein expression was assessed by Western blot and effects of treatment on Ca^{2+} and cAMP second messenger systems was examined using the Flexstation assay and the Lance Ultra cAMP kit, respectively. **Results:** Results demonstrate that pM concentrations of ghrelin and des-acyl ghrelin inhibit aromatase transcript expression and activity in ASCs under basal conditions and in PGE_2-stimulated cells. Moreover, the effects of ghrelin and des-acyl ghrelin are mediated via effects on aromatase promoter PII-specific transcripts. The GHSR1a-specific agonist capromorelin and the ghrelin gene by-product obestatin had no effect on aromatase transcript expression or activity. Moreover, GHSR1a protein was undetectable by Western blot and neither ghrelin nor capromorelin elicited a calcium response in ASCs. Finally, ghrelin caused a significant decrease in basal and forskolin-stimulated cAMP in ASC. **Conclusions:** These findings suggest that ghrelin acts at alternate receptors in ASCs by decreasing intracellular cAMP levels. Ghrelin mimetics may be useful in the treatment of estrogen-dependent breast cancer.

Keywords: ghrelin, des-acyl ghrelin, aromatase, estrogens, adipose, breast cancer

Introduction

In postmenopausal women, where ovarian steroid biosynthesis has ceased, the adipose tissue is the major site of estrogen biosynthesis. This occurs predominantly in the adipose stromal cells (ASCs) and is heavily dependent on an increase in expression of the enzyme responsible for converting androgens into estrogens, aromatase [1]. The aromatase gene, *CYP19A1*, encompasses 123 kb and is composed of nine coding exons, II-X. The regulation of aromatase expression is mediated by the presence of tissue-specific promoters, which give rise to transcripts with unique 5'-untranslated regions [reviewed in 2]. We have previously demonstrated that a number of factors, known to be altered in obesity, are involved in the regulation of aromatase expression, including inflammatory factors such as prostaglandin E_2 (PGE_2) and adipokines such as leptin [3-5]. These factors stimulate the activity of aromatase proximal promoter II (PII), the same promoter demonstrated to drive aromatase expression in adipose tissue in relation to breast and endometrial cancer [reviewed in 2,6].

Ghrelin is a 28 amino-acid octanoylated peptide whose physiological roles in the stimulation of appetite and growth hormone (GH) release are well established [7,8]. However, the mechanisms of ghrelin signaling in the periphery are still largely uncharacterized. Ghrelin is derived from a pro-hormone that can be processed to a number of peptide products, amongst which is the non-acylated precursor of ghrelin, usually referred to as des-acyl ghrelin, as well as obestatin for which biological roles, if any, are not well characterized. Des-acyl ghrelin is converted to ghrelin by addition of a fatty acid (octanoyl) side chain at serine 3. This reaction is catalysed by the intracellular enzyme GOAT (ghrelin O-acyl transferase), for which des-acyl ghrelin is the only known substrate [9]. The cognate ghrelin receptor, GHSR1a, is a typical 7 transmembrane spanning G-protein coupled receptor (GPCR) known to lead to the intracellular release of Ca^{2+}, and is the only receptor for ghrelin that has been molecularly defined [10-12]. Nevertheless, there is strong evidence to suggest that there are agonist effects of ghrelin that cannot be explained by the actions of ghrelin at GHSR1a, and des-acyl ghrelin is about 1000 times less potent than ghrelin at GHSR1a [13-15]. Similar to adiponectin, plasma ghrelin levels are inversely correlated with BMI, percentage body fat, insulin and leptin levels [16]. However, there have been no reports examining the effect of ghrelin on aromatase, as a possible contributing factor to obesity-associated breast cancer.

The present work aimed to examine the effect of ghrelin on aromatase expression and activity in ASCs, and to determine whether effects are mediated via traditional GHSR1a signaling.

Methods

Human primary ASC isolation, culture and treatments

Primary human ASCs were isolated by collagenase digestion of subcutaneous adipose tissue from women undergoing abdominoplasty and cultured in Waymouth's medium (Invitrogen, USA), as previously described [17]. The studies have been approved by Southern Health Human Research Ethics Committee B. Patients have provided upfront consent for the use of their tissue and the publication of this study. Prior to treatment, cells were serum starved for 24 hours in medium containing 0.1% bovine serum albumin. Cells were treated for 6 or 24 hours, in which case media was replaced twice, prior to collection of cells for RNA extraction or aromatase activity assays. Treatments included prostaglandin E_2 (PGE_2, 1μM; Cayman Chemicals), ghrelin (Ghr; NeoMPS, Strasbourg, France), des-acyl ghrelin (DAG; NeoMPS, Strasbourg, France), obestatin (Ob; Sigma-Aldrich Pty, Sydney, Australia) and the GHSR1a-specific agonist capromorelin (Cap; Pfizer Pharmaceuticals, Sandwich, UK).

Reverse transcription and quantitative real-time PCR (QPCR)

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Germany) and reverse transcribed using the AMV RT Kit and oligo-dT (Promega, USA) as directed by the manufacturer. DNA was digested using the DNA-free DNase Treatment and Removal Kit (Ambion, USA). QPCR was performed on the LightCycler using LightCycler FastStart DNA Master SYBR Green l kit (Roche, Germany). Quantification of human aromatase was carried out using primers hArom F: 5'-TTGGAAATGCTGACCCGAT-3', hArom R: 5'-CAGGAATCTGCCGTGGGAGA-3', and primers specific for PII-specific transcripts PII F: 5'- GCAACAGCAGCTATAGATGAAC -3' and PII R: 5'-CACCCGGTTGTAGTAGTTGCAGGCACTGCC -3', and normalized to housekeeping gene β-actin using primers: β-actin F: 5'- TGCGTGACATTAAGGAGAAG-3', β-actin R: 5'- GCTCGTAGCTCTTCTCCA -3'. Cycling conditions for aromatase were one cycle at 95 C for 10 min followed by a variable number of cycles at 95 C for 10 sec, 60 C for 5 sec and 72 C for 10 sec, 95 C for 10 sec, 65 C for 10 sec and 72 C for 10 sec for PII and 95 C for 10

sec, 59 C for 5 sec and 72 C for 10 sec for β-actin. All the samples were quantified using standards of known concentrations and corrected for abundance using the housekeeping gene β-actin.

Aromatase activity assays

Primary human ASCs, cultured in 6-well plates, were serum-starved for 24 hours and treated with specified reagents for 24 hours. Aromatase activity was measured by tritiated water-release assay using radiolabeled androst-4-ene-3, 17-dione (NET926001MC, PerkinElmer, USA) as a substrate, as previously described [18]. Specific activity was normalized to total protein amount.

Western blotting

Whole ASC protein extracts were obtained as previously described [3]. Twenty micrograms of protein were diluted in sample buffer containing β-mercaptoethanol, run on a 10% denaturing polyacrylamide gel and transferred to nitrocellulose for Western blotting. GHSR1a protein expression was assessed by Western blot using a GHSR1a-specific antibody (sc-10359; 1/500 dilution, Santa Cruz) and visualized using anti-goat Alexa Fluor 680 (1/10,000 dilution, Invitrogen, USA). Human pituitary gland whole cell lysate (AB29749; 3.75mg/ml; Abcam) was used as positive control. Total protein amount was normalized to mouse β-tubulin (MAB3408; 1/10,000 dilution, Millipore). Membranes were scanned using the Odyssey infrared imaging system (LI-COR Biosciences, USA).

Intracellular Ca^{2+} and cAMP measurements

Intracellular Ca2+ levels were measured in ASCs and GHSR1a-expressing HEK293 cells by fluorescence using the Flexstation (Molecular Devices, Sunnyvale, CA) as previously described [19]. Briefly, cells were plated, allowed to reach 50-70% confluency, and loaded with 2 μM fura 2-AM for 1 h in the presence of 2.5 mM probenecid and 0.01% pluronic F-127 (Invitrogen) at 37°C. Cells were then washed twice with assay buffer, and fluorescence was measured over 100 s using excitation wavelengths of 340 and 380 nm and emission of 520 nm. The Lance Ultra cAMP kit was used (Perkin Elmer). All kit components were prepared according to manufacturer's specifications. Briefly, ASCs (475 cells/well) were incubated at room temperature for 30 minutes with ghrelin (1-1000nM), alone or in the presence of forskolin (1mM; Sigma). The Eu-cAMP tracer and Ulight-anti cAMP reagents were then added for 1 h at room temperature. The plate was then read using the Envision TRF capable reader.

Statistical analyses

All data were expressed as mean ± standard error (SE). Statistical analysis was done using one-way ANOVA followed by Dunnett's test whereby columns were compared to control. For experiments where treatment was examined under basal condition, results were compared to vehicle control (VC). For experiments where cells were stimulated with PGE_2, data were compared to PGE_2. Statistical significance was defined as *, $P < 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$, ****, $P \leq 0.0001$. Data analysis was performed using GraphPad Prism version 5.02 (GraphPad Software, San Diego, CA, USA).

Results

Ghrelin and des-acyl ghrelin, but not obestatin or capromorelin, inhibit aromatase under basal conditions.

Total aromatase transcript expression was examined in ASCs treated with ghrelin, des-acyl ghrelin, obestatin and capromorelin. Treatment of ASCs with ghrelin (Ghr; 10, 100, 1000 pM) led to a significant dose-dependent reduction in aromatase transcript expression (Fig. 1A). Similarly, des-acyl ghrelin (DAG; 10, 100, 1000 pM) inhibited aromatase transcript expression in unstimulated cells (Fig. 1B). Of interest, neither obestatin (Ob) nor the GHSR1a-specific agonist capromorelin (Cap) affected aromatase mRNA expression in ASCs at the concentrations examined (Fig. 1C & 1D, respectively). Aromatase activity was also examined in ASCs treated with ghrelin (Fig. 1E) and capromorelin (Fig. 1F). Results demonstrate that ghrelin (1000 pM), but not capromorelin, significantly inhibits aromatase activity, similar to what was observed at the transcript level.

Ghrelin and des-acyl ghrelin inhibit the PII-specific transcript expression of aromatase

Aromatase transcripts derived from promoter PII are increased in obesity and cancers of the breast and endometrium. Using primer pairs specific for the 5'-untranslated region of PII-derived transcripts, the effect of ghrelin and des-acyl ghrelin on the PII-driven expression of aromatase was examined. Similar to results obtained for total aromatase (Fig. 1 & 2), ghrelin and des-acyl ghrelin inhibited PII-specific transcript expression under basal conditions (Fig. 2A & B, respectively).

Ghrelin inhibits the PGE2-mediated aromatase mRNA expression and activity

The effect of ghrelin on total and PII-specific aromatase mRNA and activity in PGE_2-stimulated cells was also examined. In the presence of 1μM PGE_2, 10 pM ghrelin (Ghr) significantly inhibited total and PII-specific aromatase transcripts (Fig. 3A and 3B, respectively). Consistently, ghrelin also inhibited the PGE_2-stimulated aromatase enzymatic activity (Fig. 3C).

GHSR1a is undetectable in ASCs and ghrelin does not elicit a Ca2+ response under basal conditions, but inhibits cAMP

When added to human ASCs, ghrelin, des-acyl ghrelin or capromorelin did not induce any change in intracellular calcium concentration that could be detected by the fluorescence based Flex station II assay (Fig. 4A). Angiotensin II (ATII) was used as a positive control to confirm that activation of G protein coupled receptor induced mobilisation of intracellular calcium could be detected in the ASCs (Figure 4A). To confirm activity of ghrelin and capromorelin, but not des-acyl ghrelin at the GHSR1a, experiments were also performed in HEK293 cells transfected with the human GHSR1a. As expected, ghrelin and capromorelin were potent agonists at the human GHSR1a expressed in HEK 293 cells (Figure 4B), des-acyl ghrelin was considerably less potent, the EC50 being 2.4 μM, the same potency as reported previously on rat GHSR1a expressing HEK293 cells [20]. The pEC50 values were -8.2 ± .21 (7 nM) for ghrelin, −9.4± 0.20 (426 pM) for capromorelin and −4.6 ± 0.8 (2.4 μM) for des-acyl ghrelin. Western blot analysis was performed to determine relative abundance of the cognate ghrelin receptor GHSR1a in ASCs. Results demonstrate that GHSR1a is undetectable in ASCs, with and without PGE_2, compared to pituitary cell extracts (Fig. 4C). Ghrelin significantly inhibited intracellular cAMP in ASCs under basal conditions and in forskolin-stimulated cells (Fig. 4D).

Discussion

In postmenopausal women, the relationship between obesity and breast cancer relies on adipose-derived estrogens. We have previously demonstrated that inflammatory factors and adipokines, known to be altered in obesity, are key regulators of aromatase, and hence estrogen biosynthesis, in adipose stromal cells. The present manuscript describes, for the first time, the regulation of aromatase expression and activity by ghrelin in these cells.

Circulating levels of ghrelin and des-acyl ghrelin are inversely associated with BMI [21]. According to Shiiya *et al.*, ghrelin levels in normal weight individuals are on average 132.4 pM whereas obese individuals have been reported to have significantly lower levels, reaching 68% that of their normal weight counterparts [22]. Consistent with the BMI correlation, ghrelin levels are inversely related to breast cancer histological grade and tumor size [23], suggesting that ghrelin is protective. We discovered that ghrelin inhibits aromatase transcript expression and activity in ASCs. Suppression of aromatase activity under basal conditions is significant but modest compared to effects of ghrelin on aromatase transcript. This discrepancy may in part be due to the degree of stability of the aromatase protein compared to transcript, and longer treatment times may be necessary to see comparable effects. Our findings suggest that the higher levels of circulating ghrelin found in lean individuals would contribute to maintaining adipose-derived aromatase levels low, which would contribute to a reduction in the severity of estrogen-dependent breast cancers. Our work also predicts that decreased levels of ghrelin found in obesity would prevent aromatase suppression and lead to increased levels of estrogens and increase the risk of estrogen-dependent cancers, including that of the breast.

The effect of other products of the ghrelin gene on aromatase expression was also examined. Des-acyl ghrelin, but not obestatin, inhibited aromatase expression. We also demonstrate that ghrelin and des-acyl ghrelin are equipotent in reducing aromatase transcript levels in adipose stromal cells. Des-acyl ghrelin is found to circulate at approximately 4-times that of ghrelin and is known not to act via GHSR1a [24]. These findings therefore suggest that the mechanism of action of ghrelin in adipose stromal cells differs from well characterized actions mediated via GHSR1a in the central nervous system. The suggestion that ghrelin and des-acyl ghrelin may be acting at alternate receptors is also strengthened by the observation that capromorelin, known to act specifically at GHSR1a [19], has no effect on aromatase expression and activity under basal conditions in stromal cells. Moreover, GHSR1a protein was not detected in stromal cells and treatment of stromal cells with ghrelin and capromorelin did not elicit a Ca2+ response, whereas both compounds elicited the expected Ca^{2+} response when added to HEK293 cells expressing human GHSR1a. These findings are consistent with previous reports demonstrating the presence of alternate ghrelin receptors and effects of des-acyl ghrelin. Specifically, des-acyl ghrelin has been shown to promote skeletal muscle regeneration after ischemia[25], improve glucose metabolism and inhibit lipolysis in humans [26], as well as have effects on islet [27] and endothelial cell function [28]. Ghrelin is potent at the alternate ghrelin receptor of ASCs,

causing significant reduction of aromatase expression at 10 pM, and reducing aromatase expression by 50% at 100 pM. This indicates that normal ghrelin plasma levels, which are 60-150 pM [21], probably regulate ASC aromatase.

We also demonstrate that ghrelin inhibits basal and forskolin-stimulated cAMP levels in adipose stromal cells. This would suggest that alternate ghrelin receptors of adipose stromal cells signal via the Gαi subtype of G protein-coupled receptors. These results are consistent with effects of ghrelin to decrease intracellular cAMP levels in islet β-cells [29,30] and also provide a mechanism for the observed decrease in aromatase expression. Specifically, the promoter II-driven expression of aromatase in adipose stromal cells has been shown to be dependent on signaling involving cAMP. Activation of PKA in response to increased cAMP levels has been shown to result in the increased phosphorylation of CREB, the increased nuclear localization of the CREB co-activator CRTC (CREB-regulated transcription coactivator) proteins, and their increased binding to the promoter II region of the aromatase gene [3,31-33]. One factor that has extensively been studied for its role in stimulating aromatase in adipose stromal cells and known to act by stimulating intracellular cAMP levels is PGE_2.

Low grade chronic inflammation, as seen in obesity and cancer, has been shown to be accompanied by an increase in PGE_2 within the adipose which in turn leads to the 4-5-fold increased expression of aromatase driven by promoter II (PII) [34,35]. It is believed that this increase in aromatase expression is responsible for sustaining the growth of estrogen-dependent cancers, including that of the breast [6]. Our findings demonstrate that ghrelin and des-acyl ghrelin also inhibit the PGE_2-mediated expression and activity of aromatase and the effects of ghrelin and des-acyl ghrelin on aromatase occur via effects on promoter PII.

Aromatase inhibitors are considered first-line therapy for hormone receptor-positive breast cancer. [36]. However, their efficacy in suppressing estrogen biosynthesis throughout the body often leads to the development of side-effects related to estrogen deficiency, namely arthralgia, profound osteoporosis, severe hot flushes and possible cardiovascular and cognitive defects. As a result, many women cease use of aromatase inhibitors, which adds risk because cessation is associated with a higher risk of recurrence [37]. There is thus a need to explore alternatives to currently used aromatase inhibitors. Our findings demonstrate that ghrelin and des-acyl ghrelin inhibit the breast cancer-specific promoter of aromatase and hence, may inhibit estrogen production at sites where estrogens drive

tumor growth and leave unaffected sites such as the bone, brain and cardiovascular system where estrogens are beneficial.

Ghrelin has been shown to influence the proliferation of a number of cancer cell types, including that of the breast and endometrium. In the ER-positive MCF7 breast cancer cell line, ghrelin and des-acyl ghrelin were shown to inhibit 17β-estradiol-stimulated cell proliferation in the absence of detectable GHSR1a [38]. Conversely, ghrelin has also been shown to stimulate the proliferation of MDA-MB-231 cells [39] and endometrial cancer cells *in vitro* [40]. These results and ours suggest that des-acyl ghrelin analogs may prove superior over ghrelin analogs for use clinically. Notably, des-acyl ghrelin analogs have been developed for the treatment of metabolic disorders that have many beneficial effects of des-acyl ghrelin [41] but with improved stability and bioavailability [42] and it will be of interest to determine whether these and other ghrelin receptor agonists may prove useful for the treatment or possibly even the prevention of postmenopausal ER-positive breast and endometrial cancers.

Conclusions

Our findings provide a novel link between obesity and breast cancer via the role of ghrelin to regulate aromatase expression and activity. Our results also suggest that ghrelin analogues may be useful for the treatment and possibly the prevention of hormone-dependent breast cancer. This is of significance considering the high prevalence of obesity and the prospect of increased breast cancer cases as this population ages.

Acknowledgements

This research was supported by NHMRC (Australia) Project Grant # GNT1005735 and by a grant from the National Breast Cancer Foundation (NC-14-011) to KAB, and by the Victorian Government Operational Infrastructure Support Program. KAB is supported by an NHMRC (Australia) Career Development Award GNT1007714. We thank Mr Seungmin Ham for his technical support in cell isolation.

Ethical standards

The experiments detailed in the present manuscript were performed in accordance with National Health and Medical Research Council of Australia (NHMR) guidelines.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Legend

Figure 1: Ghrelin and des-acyl ghrelin, but not obestatin or capromorelin, inhibit aromatase under basal conditions. The effect of ghrelin (A), des-acyl ghrelin (B), obestatin (C) and capromorelin (D) on aromatase transcript expression was examined by QPCR in primary human ASCs. The effect of ghrelin (E) and capromorelin (F) on aromatase activity was examined in ASCs using the tritiated water release assay. The effect of treatment was compared to that of vehicle control. Ghr: ghrelin, DAG: des-acyl ghrelin, Ob: obestatin; Cap: capromorelin; vc: vehicle control. N=3, experiment repeated twice.

Figure 2: Ghrelin and des-acyl ghrelin inhibit the PII-specific transcript expression of aromatase. The effect of ghrelin (A) and des-acyl ghrelin (B) on PII-specific aromatase transcript expression was examined by QPCR in ASCs under basal conditions. The effect of treatment was compared to vehicle control. Ghr: ghrelin, DAG: des-acyl ghrelin, vc: vehicle control. N=3, experiment repeated twice.

Figure 3: Ghrelin inhibits the PGE_2-mediated aromatase mRNA expression and activity. The effect of ghrelin on the PGE_2-stimulated expression of (A) total and (B) PII-specific aromatase transcript was examined by QPCR in primary human ASCs. (C) The effect of ghrelin on PGE_2-mediated aromatase activity was also examined in ASCs using the tritiated water release assay. The effect of treatment was compared to that of PGE_2 treatment alone. Ghr: ghrelin. N=3, experiment repeated twice.

Figure 4: GHSR1a is undetectable in ASCs and ghrelin does not elicit a Ca2+ response under basal conditions, but inhibits cAMP. (A) Changes in intracellular Ca^{2+} in primary ASCs in responses to ghrelin, des-acyl ghrelin, capromorelin and angiotensin II. Only angiotensin II caused an increase in intracellular Ca^{2+}. (B) Changes in intracellular Ca^{2+} in HEK293 cells transfected with human GHSR1a in response to ghrelin, des-acyl ghrelin and capromorelin. (C) GHSR1a Western blot on primary ASCs from four different women, with and without PGE_2 treatment, compared to pituitary extract (positive control). (D) Changes in intracellular cAMP in primary ASCs in response to ghrelin with and without forskolin treatment. vc: vehicle control; FSK: forskolin. *Asterisks* denote effects that are significantly different from vc. *Hashtag* denotes effects that are significantly different from FSK. For Ca^{2+} and cAMP assays, N=3, experiments repeated at least twice.

FIGURE 1

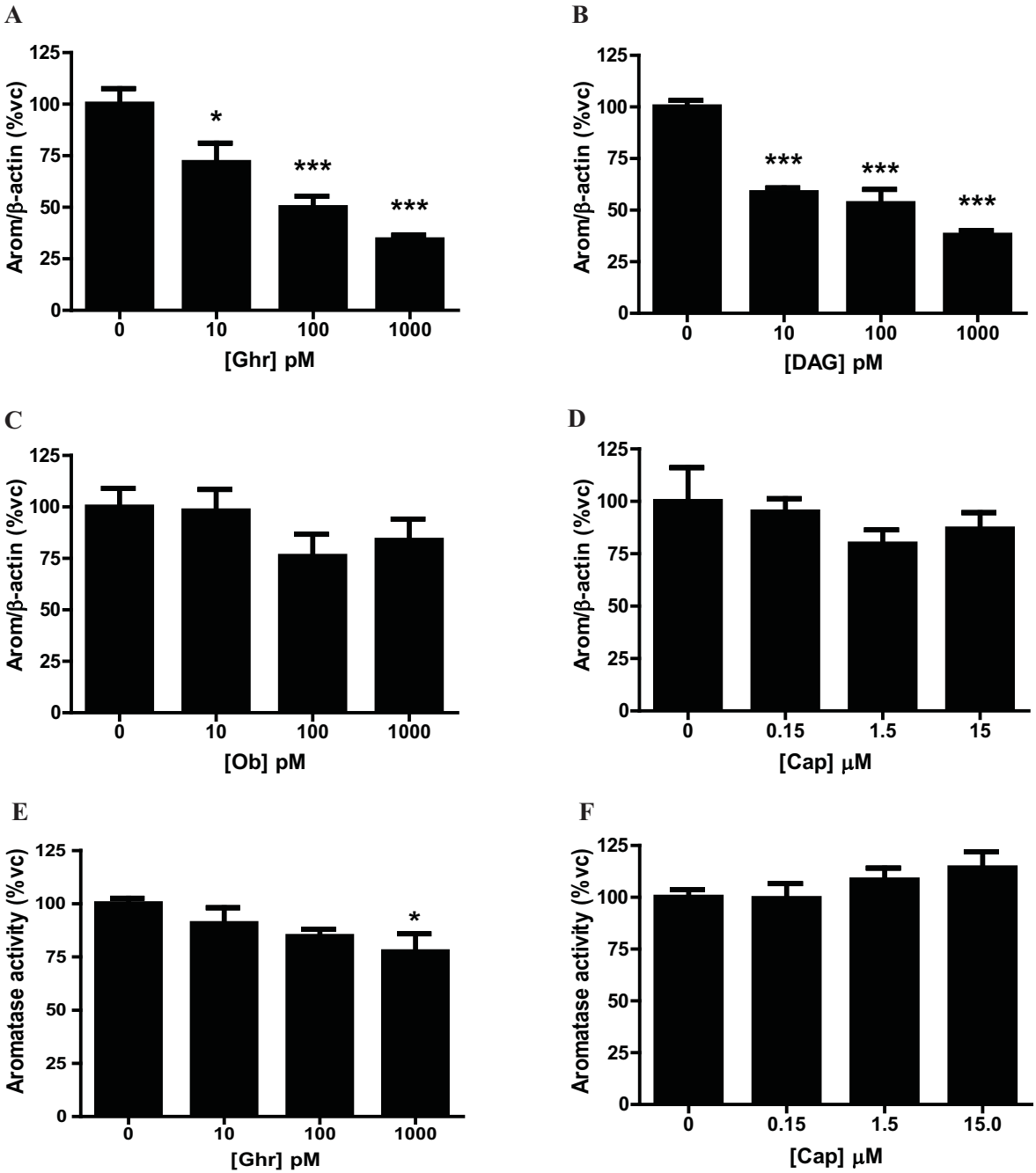
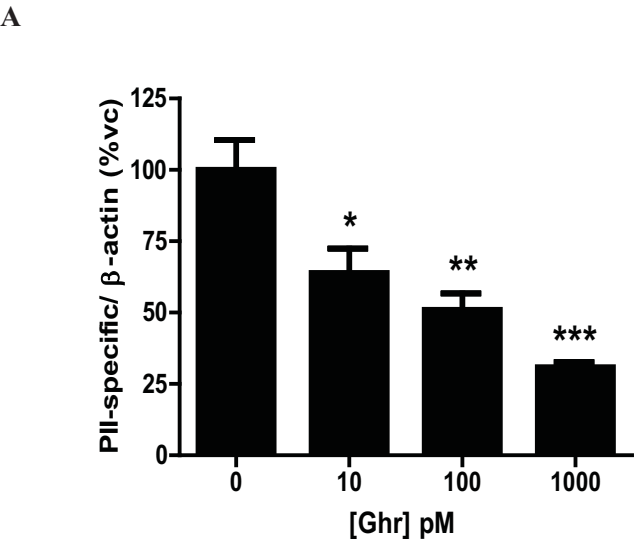

A
Arom/β-actin (%vc)
125
100
75
50
25
0
*
***
***
0
10
100
1000
[Ghr] pM
B
Arom/β-actin (%vc)
***
***
***
[DAG] pM
C
Arom/β-actin (%vc)
[Ob] pM
D
Arom/β-actin (%vc)
0.15
1.5
15
[Cap] μM
E
Aromatase activity (%vc)
*
F
Aromatase activity (%vc)
15.0

FIGURE 2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PII-specific/ β-actin (%vc)
125
100
75
50
25
0
*
**
***
0
10
100
1000
[Ghr] pM

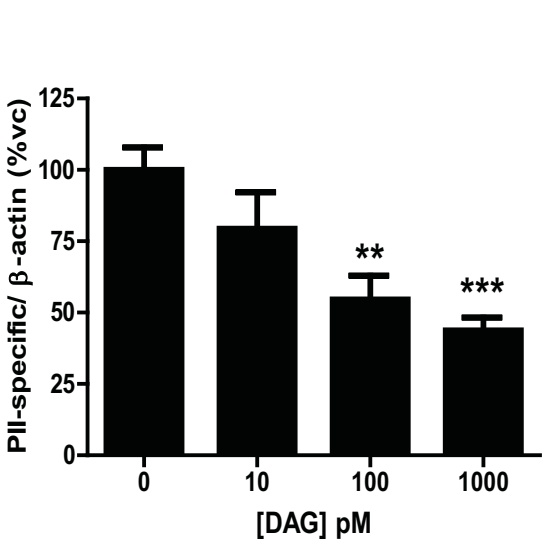

B
PII-specific/ β-actin (%vc)
125
100
75
50
25
0
**
***
0
10
100
1000
[DAG] pM

FIGURE 3

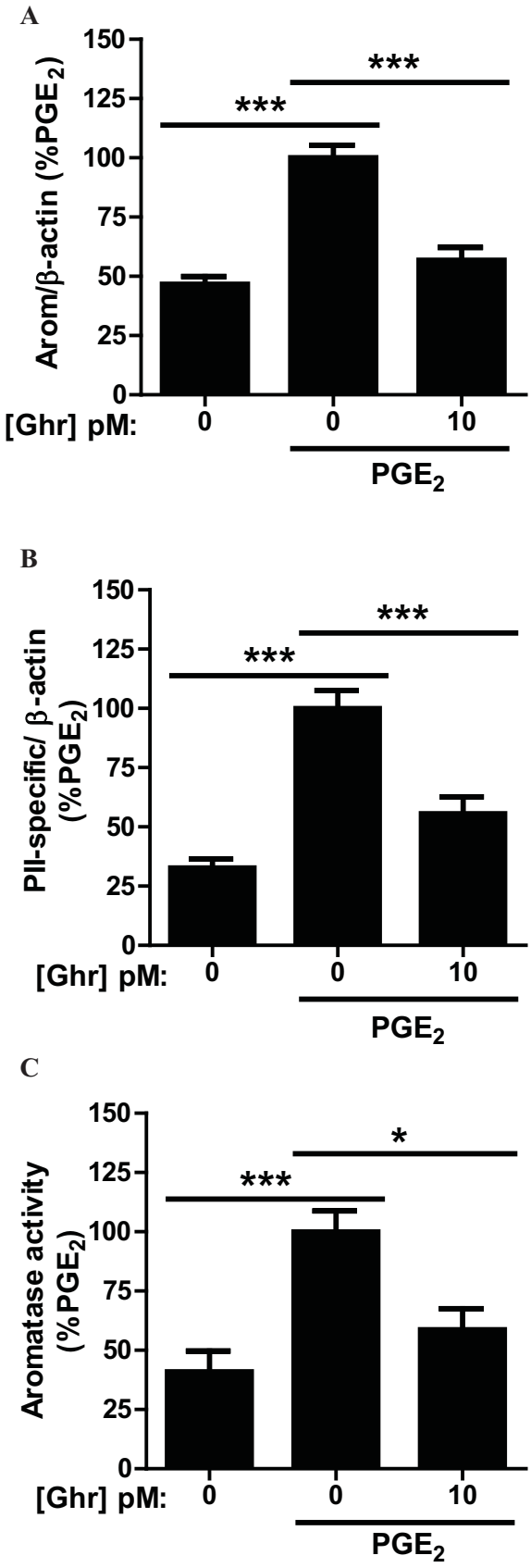
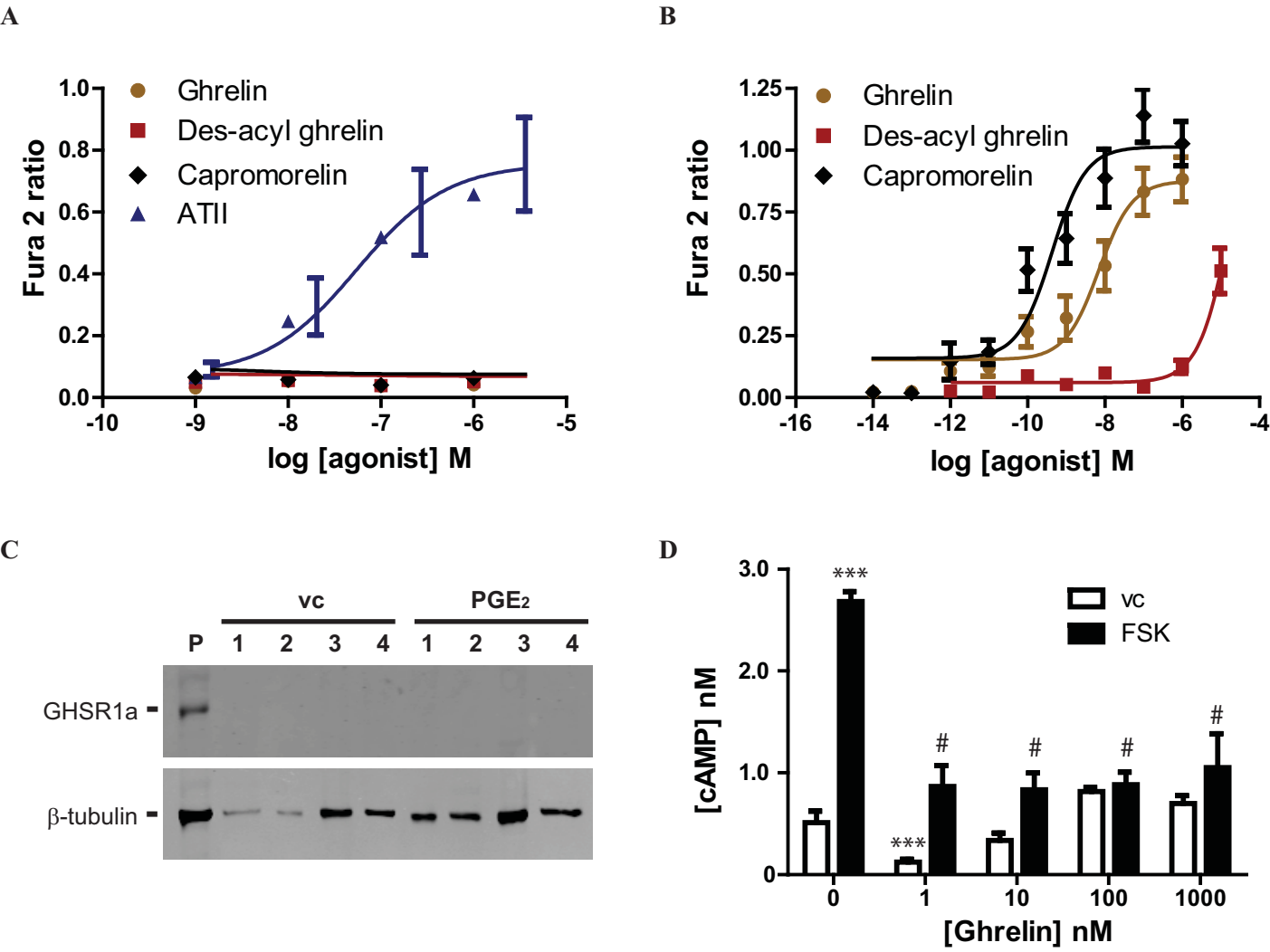

A
150
125
100
75
50
25
0
Arom/β-actin (%PGE2)
***
***
[Ghr] pM:
0
0
10
PGE2
B
150
125
100
75
50
25
0
PII-specific/ β-actin (%PGE2)
***
***
[Ghr] pM:
0
0
10
PGE2
C
150
125
100
75
50
25
0
Aromatase activity (%PGE2)
***
*
[Ghr] pM:
0
0
10
PGE2

FIGURE 4


A
Ghrelin
Des-acyl ghrelin
Capromorelin
ATII
Fura 2 ratio
log [agonist] M
B
Ghrelin
Des-acyl ghrelin
Capromorelin
Fura 2 ratio
log [agonist] M
C
vc
PGE2
P 1 2 3 4 1 2 3 4
GHSR1a
β-tubulin
D
vc
FSK
[cAMP] nM
[Ghrelin] nM