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Prolactin and epidermal growth factor stimulate adipophilin synthesis in HC11 mouse mammary epithelial cells via the PI3-kinase/Akt/mTOR pathway

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ABSTRACT

The aim of the present study is to estimate the role played by cortisol, prolactin (PRL) and epidermal growth factor (EGF) in the synthesis of adipocyte differentiation-related protein (ADRP) as compared to the well-studied regulation of β-casein synthesis by these hormones in the mammary epithelial cell line HC11. This comparison between a cytoplasmic lipid droplet-associated protein, which is strictly specific to both lipid accumulation and secretion by lactating mammary epithelial cells, and an archetypal milk protein is useful for evaluating the extent to which a mechanistic relationship exists between biosynthesis, transport and secretion of these two major milk components. We found that cortisol inhibits PRL-stimulated ADRP synthesis, as opposed to its known stimulating effect on β-casein synthesis. The involvement of PRL and EGF in ADRP synthesis was explored by means of a battery of inhibitors. The Jak2 inhibitor AG490 provoked a stimulation of ADRP synthesis whereas it totally suppressed that of β-casein. The use of AG1478, a specific inhibitor of EGF receptor phosphorylation, or of PD98059, a specific MEK inhibitor, revealed that the Ras/Raf/MEK/ERK1/2 pathway has no significant influence on ADRP levels. Inhibition of JNK was also ineffective. In contrast, incubation of the cells with SB 203580, a specific inhibitor of p38, slightly stimulated ADRP synthesis and induced a proportional dose–response inhibition of PRL-induced β-casein synthesis. Finally, cell treatment with wortmannin or LY294002 revealed that both PRL and EGF positively regulate ADRP and β-casein synthesis through PI3-kinase signaling. Because both the Akt inhibitor MK-2206 and the mTOR inhibitor rapamycin provoked a strong diminution of PRL-induced synthesis of the two proteins, and because oleate induced phosphorylation of Akt, we concluded that, in the mammary epithelial cell line HC11, the PI3-kinase/Akt/mTOR signaling pathway strongly participates in β-casein synthesis and is a main regulator of ADRP expression.

1. Introduction

During lactation, mammary epithelial cells synthesize and secrete huge quantities of proteins and lipids. These two types of constituents are both synthesized in or close to the same cellular compartment, the endoplasmic reticulum. Milk lipids are packaged in cytoplasmic lipid droplets before being released as milk fat globules by a budding process at the apical plasma membrane [1]. On the other hand, upon their export from the Golgi apparatus, milk proteins (mostly casein micelles), are carried through the cytoplasm in secretory vesicles and are then released in the lumen of mammary acini by exocytosis. Hormones such as epidermal growth factor (EGF) and prolactin (PRL) affect the synthesis of these milk constituents. In brief, EGF plays a crucial role in mammogenesis during gestation and, during lactation, PRL in synergy with glucocorticoids, induces the synthesis of milk proteins including caseins, whey acidic protein and α-lactalbumin. The most extensively studied signaling pathway induced by PRL is the Janus kinase (Jak)-signal transducer and activator of transcription (Stat) pathway [2]. PRL binding to the PRL receptor (PRLr) triggers its dimerization and activation of the receptor-associated Jak2. The latter mediates phosphorylation of Stat5 which dimerizes and translocates to the nucleus to exert transcriptional effects. Glucocorticoids (cortisol, dexamethasone) have been shown to enhance Stat5-mediated gene expression either by direct synergistic interactions of the glucocorticoid receptor with Stat5 [3,4] or by increasing Stat5 tyrosine phosphorylation and nuclear retention [5]. PRL is also able to stimulate other signaling pathways such as the

Abbreviations: A.U., arbitrary unit; ADRP, adipocyte differentiation-related protein; BSA, bovine serum albumin; CIS, cytokine-inducible SH2-containing proteins; ECL, electrochemiluminescence; EGF, epidermal growth factor; EGFr, EGF receptor; ER, endoplasmic reticulum; ERK1/2, extracellular regulated kinases 1/2; Jak, Janus kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; mTOR, mammalian target of rapamycin; PBS, phosphate-buffered saline; PI3-K, phosphatidylinositol 3-kinase; PPARγ, peroxysome proliferator activated receptor gamma; PPREs, peroxisome proliferator response elements; PRL, prolactin; PRLr, PRL receptor; s.d., standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOCS, suppressors of cytokine signaling; Stat, signal transducer and activator of transcription; TRITC, Tetra methyl Rhodamine Iso Thio Cyanate

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Src, PI3-kinase and RAS/RAF/MEK/ERK1/2 pathways [6,7]. The hierarchical relationships between Jak2 and these other kinases are not still well understood [8,9] although Src seems to be activated by PRLr signaling in a Jak2-independent manner [10].

EGF binding onto the EGF receptor (EGFr) activates three major signaling pathways. One includes the Ras/Raf/MEK/ERK pathway [11,12]. A second pathway involves PI3-kinase and Akt [13,14]. The third pathway triggers the stress-activated protein kinase pathway, involving Jak/Stat and protein kinase C [15]. On the other hand, EGF inhibits PRL-induced β-casein synthesis [16]. Activation of Stat5 and its subsequent interaction with the β-casein promoter are blocked in the presence of EGF [17,18]. EGF stimulation has also been shown to promote nuclear translocation and activation of Stat5 binding to other DNA regulatory elements. Thus, both positive and negative regulation of the Stat5 pathway by EGF appear to be possible [19,20].

Lipid biosynthesis is also stimulated by the combined actions of PRL, insulin, and glucocorticoids in mammary gland explants [21]. PRL enhances mammary production of lipids by coordinating the activities of key enzymes such as lipoprotein lipase, pyruvate dehydrogenase, acetyl-CoA carboxylase and fatty acid synthase [22]. The stimulatory effects of PRL on both milk lipid and protein production by the cell suggest that they are coupled and co-regulated during lactation. However, the formation of the two main milk constituents seem to be dissociated in various experimental conditions and during differentiation. This suggests that coupling of their production within the cell is not as strict as we may think [23,24].

The aim of the present study was to estimate the role played by hormones on the synthetic pathway of milk lipids and its connections with the protein synthetic pathway. To address this issue we used the mouse mammary epithelial cell line HC11. This clonal derivative of the COMMA-1D cell line [25] has proven to be an important model for understanding hormonal regulation of mammary epithelial cell differentiation and milk protein gene expression such as β-casein. To monitor the lipid synthetic pathway as a whole, we have taken advantage of the fact that the cytoplasmic lipid droplets present a specific protein marker, the adipose differentiation-related protein (ADRP) also called ADPH, ADFP and now perilipin 2 (PLIN-2) [26], at their surface. This marker protein has been used as a surrogate measure of the abundance of cellular cytoplasmic lipid droplets [27]. Instead of evaluating the mRNA levels of both β-casein and ADRP or of using reporter genes by means of engineered cells, we have chosen to directly study the final product of the representative marker protein of each pathway.

2. Materials and methods

2.1. Reagents

Ovine PRL, EGF, fatty acid-free bovine serum albumin (BSA), Tween 20, AG490, poly-L-lysine, wortmannin, phosphatase inhibitor cocktail and all standard chemicals were obtained from Sigma (La Verpillère, France). Both PD98059 and AG1478 were from Calbiochem (Merck, Lyon, France). MK-2206, SB203580, LY294002 and SP600125 were from Selleck (Euromedex, Souffelweyersheim, France). Nitrocellulose Hybond-C Extra and ECL were purchased from GE-Healthcare (Buc, France). Oleic acid conjugated with fatty acid-free BSA at a ratio of 6 mol of oleic acid/mol of albumin was prepared as follows. Oleic acid was directly saponified by stoichiometric amounts of NaOH, solubilized in warm PBS and supplemented with 1 vol of BSA in PBS. The mixture was filtered through a 0.22 μm filter and stored at 4 °C.

2.2. Antibodies

Guinea-pig anti-ADRP (GP40) antibodies were purchased from Progen Biotechnique (Maurepas, France). Rabbit antibodies against mouse milk proteins (RAM/MSP) were from Nordik Immunological Laboratories (Tilburg, The Netherlands). Mouse monoclonal anti-β-actin antibodies and horseradish peroxidase-conjugated sheep anti-mouse IgG were obtained from Sigma (La Verpillère, France). Rabbit polyclonal Akt and phospho-Akt (Ser473) antibodies (#9272 and #9271, respectively) were from Cell Signaling (Ozyme, France). Horseradish peroxidase-conjugated goat anti-rabbit and anti-Guinea pig IgG were obtained from Jackson Immunoresearch (Tebu, France) and Santa Cruz (Tebu, France), respectively. TRITC-conjugated goat anti-Guinea pig IgG were obtained from Santa Cruz (Tebu, France).

2.3. Cell culture

The mouse mammary epithelial cell line HC11 [25] was obtained from Dr. B. Groner (Frankfurt am Main, Germany). Cells were grown until confluent in RPMI 1640 medium containing 2 mM L-glutamine, supplemented with 5% fetal calf serum (HyClone, Perbio) and 4 μg/ml insulin in 5% CO_2 at 37 °C. To promote differentiation, i.e. β-casein synthesis, confluent cells were first grown for 2 days in RPMI 1640 medium supplemented with 10% horse serum, 4 μg/ml insulin, 1 μM cortisol and then incubated for 4 days in the same medium in the absence or the presence of 200 ng/ml PRL with medium renewal every other day. During this 4 day-phase treatment, medium was supplemented with 300 μM oleate to promote formation of cytoplasmic lipid droplets (standard procedure). Inhibitors and EGF, if any, were also added at this step. All inhibitors were dissolved in DMSO and a control assay containing an equivalent amount of DMSO was performed each time. Toxicity of all inhibitors and percent viability of HC11 cells was assayed by trypan blue exclusion [28].

2.4. Immunofluorescence

HC11 cells were cultured as described above on poly-L-lysine-coated glass coverslips. All steps were performed at room temperature. Following respective treatments, cells were rinsed with PBS, fixed for 45 min with freshly prepared 3% PFA in PBS (32% formaldehyde stock solution EMS, Hatfield, USA), quenched for 20 min with 50 mM NH_4Cl, rinsed and permeabilized for 30 min with 0.1% digitonin in PBS [29]. Cells were washed 3 times in PBS and incubated for 45 min with anti-ADRP diluted 1:100 in PBS-0.2% gelatine. After 3 washes in PBS-0.2% gelatine, cells were incubated for 20 min with TRITC coupled-fluorescent secondary antibodies diluted at 1:200 in PBS-0.2% gelatine. Bodipy 493/503 (Molecular Probes, Inc.), which specifically stains neutral lipids, was dissolved in ethanol at 1 mg/ml and added to secondary antibody solution to a final concentration of 0.5 μg/ml. Nuclei were stained with 1 μg/ml DAPI in PBS. Coverslips were mounted on glass slides using VectaShield (Vector, USA). Images were acquired using an ApoTome (Zeiss) microscope (MIMA2 Platform, INRA, Jouy-en-Josas, France).

2.5. Western blot analysis

Cells were rinsed 3 times with PBS, lysed for 1 h at room temperature with 200 μl Laemmli sample buffer [30] per 3.5 cm diameter Petri dish, sonicated and then boiled for 5 min. For Akt phosphorylation experiments, lysis buffer was supplemented by 2 μl phosphatase inhibitor cocktail per plate. Cellular extracts (10 μl) were analyzed using 12.5% SDS-PAGE and electrotransferred to a nitrocellulose membrane [31]. The membrane was blocked for 1 h in PBS, 10% skimmed milk, and then incubated for 2 h with primary antibodies (Guinea pig anti-ADRP antibody: 1:5000; rabbit anti-mouse milk proteins: 1:10,000) in PBS, 10% skimmed milk, 0.3% Tween 20. After a final wash, the blots were incubated with their respective secondary horseradish peroxidase-conjugated antibodies. Visualization of peroxidase positive bands was performed using enhanced chemiluminescence substrate ECL (GE Healthcare, Buc, France) and X-ray film

(XBM Retina, Onde & Rayons, France). Following film exposure, development and scan (300 dpi), densitometry quantification of ECL signals was performed using the ImageJ 1.33u software (Wayne Rasband, NIH, USA http://rsb.info.nih.gov/ij/). Integrated density of the band of interest was measured. Band intensities were normalized to that of β-actin (mouse monoclonal anti-β-actin 1:10,000) obtained from reprobed blots. Each experimental condition was performed at least in triplicate and data are presented as means ± s.d. arbitrary unit (A.U.) densitometry. Student's *t*-test was used to compare treated samples to control. Statistical significance for all comparisons was indicated. $p<0.05$ was considered statistically significant.

3. Results

3.1. PRL stimulates and cortisol inhibits ADRP synthesis independently of that of β-casein

The mouse mammary epithelial cell line HC11 expresses β-casein when treated with PRL plus cortisol (or dexamethasone) [25,32]. To obtain further evidence for potential hormonal links between milk protein and lipid synthesis, cells were treated or not with PRL and/or cortisol in the presence of 300 μM oleate to enhance the basal level of cytoplasmic lipid droplets, and therefore ADRP. Cellular proteins were then analyzed by SDS-PAGE and Western blot to estimate the levels of ADRP and β-casein as landmark protein markers of milk lipid and protein synthetic pathways, respectively. As shown in the upper part of Fig. 1, in the presence of cortisol, PRL induced the expected β-casein response evidenced by the typical protein doublet which corresponds to immature forms of mouse β-casein [33]. Interestingly, ADRP synthesis also appeared stimulated by PRL in these conditions. In the absence of cortisol, the β-casein response was no longer observed, as anticipated, and a smaller enhancement of the ADRP signal was detected in PRL-treated cells as compared to that obtained in the absence of PRL (lower part of Fig. 1). In fact, cortisol clearly inhibited the basal level of ADRP synthesis in the absence of PRL. ADRP synthesis, however, was always stimulated by PRL, with or without cortisol, when compared with the respective control. We concluded from these results that ADRP synthesis is stimulated by PRL independently of the presence of cortisol, a regulation which was apparently not linked to the induction of β-casein gene expression by the hormone, and that cortisol has an inhibitory action on basal ADRP synthesis.

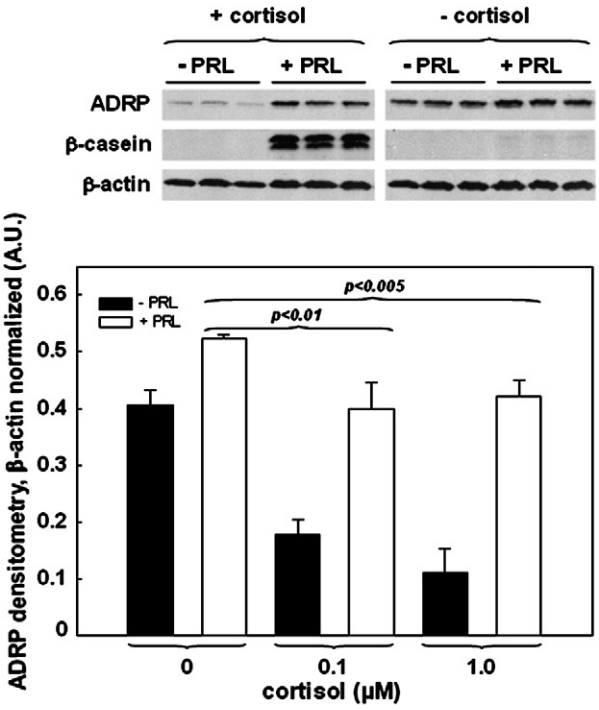

Fig. 1. PRL stimulates, but cortisol inhibits ADRP synthesis independently of that of β-casein. HC11 cells were grown to confluence, then differentiated for 2 days in RPMI-10% horse serum containing 4 μg/ml insulin in the absence or the presence of 1 μM cortisol and then grown for 4 days in the same media containing 300 μM oleate, with or without 1 μg/ml PRL. Cellular proteins were analyzed by SDS-PAGE and immunoblot. The upper part shows immunoblots successively probed with antibodies against ADRP, β-casein, β-actin and their respective HRP-conjugated secondary antibodies. The lower part displays the relative amount of ADRP in the absence (■) or the presence (□) of PRL, in the absence or presence of 0.1 or 1 μM cortisol. The amount of ADRP was quantified by densitometry and all values were normalized to the amount of β-actin. Each experimental condition was performed in triplicate and data are presented as means ± s.d. arbitrary units (A.U.).

3.2. Comparative analysis of the action of PRL on both ADRP and β-casein synthesis

To get more insight into the effect of PRL treatment on both ADRP and β-casein synthesis, we first established the dose–response relationship of PRL on the synthesis of the two protein markers, both in the presence of 1 μM cortisol. As shown in Fig. 2 (upper part), β-casein synthesis appeared enhanced for PRL concentrations above

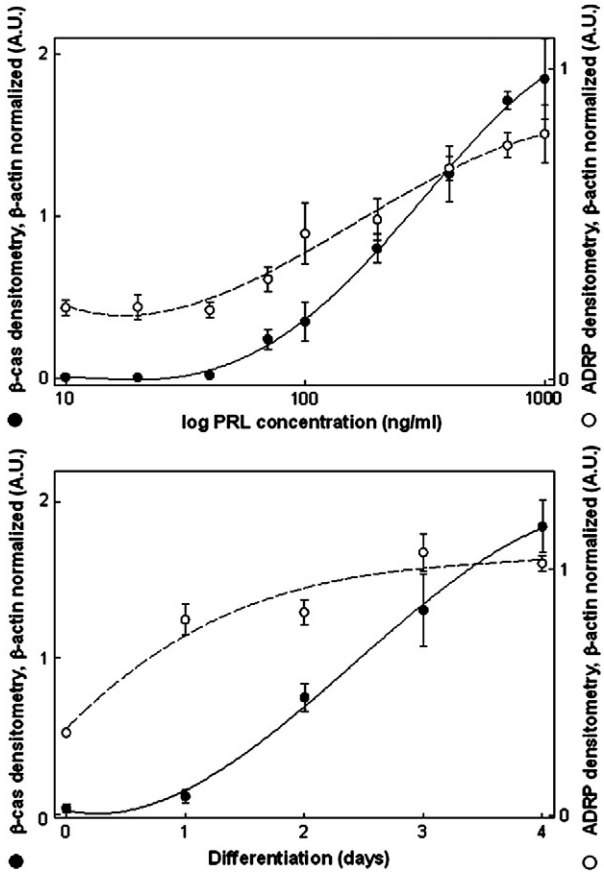

Fig. 2. PRL stimulates both ADRP and β-casein synthesis. Confluent HC11 cells were treated for 2 days in RPMI-10% horse serum containing 4 μg/ml insulin, 1 μM cortisol and then for either 4 days (upper panel) or for 1 to 4 days (lower panel) in the same medium containing 300 μM oleate with either 0 to 1000 ng/ml PRL (upper panel) or with 1 μg/ml of PRL (lower panel). Cellular proteins were analyzed by SDS-PAGE, followed by immunoblotting for ADRP, β-casein and β-actin. The amount of each protein was quantified by densitometry and ADRP and β-casein values were normalized to the amount of β-actin. Each experimental condition was performed in triplicate and data is presented as means ± s.d. arbitrary units (A.U.). Linear regression for each curve is indicated (n = 3).

40 ng/ml. This result is in total agreement with previous work (see Fig. 2 in reference [25]). The basal level of ADRP was not null, as already shown by others and us. Upon PRL treatment, ADRP and β-casein synthesis increased together according to almost the same dose–response curve. In a second set of experiments, we established the time-course of the effect of PRL treatment on the synthesis of the two protein markers. Particularly, we evaluated the lag time needed before any enhancement of both ADRP and β-casein synthesis could be observed following the addition of PRL to the cells. The signal for ADRP appeared clearly amplified after one day of PRL treatment whereas β-casein synthesis needed one extra day to be detected (Fig. 2, lower part) as previously observed [25]. These results were in favor of independent molecular machineries in charge of the regulation of ADRP and β-casein synthesis.

3.3. Blocking the Jak2/Stat5A signal pathway inhibits β-casein synthesis but enhances ADRP production

To determine which signal transduction pathways regulate ADRP synthesis independently of that of β-casein, a battery of specific chemical inhibitors was used to analyze the contribution of individual signaling pathways to the expression of ADRP when HC11 cells were treated with PRL. It is noteworthy that none of these inhibitors had adverse effects on cell viability even after a four-day treatment on plastic plates, as observed from trypan blue exclusion (data not shown). Since the Jak2/Stat5A signal pathway is the major signaling cascade involved in PRL-induced mammary cell differentiation, we used AG490, a Jak2-selective inhibitor, to block it [34]. As shown in Fig. 3 (upper part), following PRL treatment according to the standard procedure, the cellular content of β-casein was maximum and reduced in the presence of oleate as previously described [24]. When 50 μM of AG490 were added throughout the differentiation phase, β-casein synthesis was totally suppressed independently of the presence of oleate. This experiment clearly confirmed that blockage of the Jak2/Stat5A signal pathway with AG490 led to the failure of HC11 cell differentiation as previously observed [35].

Quantification of the ADRP signal (Fig. 3, lower part) indicated that oleate is the key stimulator of ADRP synthesis. In the absence of oleate, neither PRL nor AG490 had any effect. In the presence of oleate, the highest ADRP response was measured by treatment with PRL plus AG490. An intermediate response was observed with PRL or AG490 alone. We concluded from these data that: i) the Jak2/Stat5A pathway is not involved in the stimulation of ADRP synthesis but rather seems to inhibit it; ii) the stimulating action of PRL on ADRP synthesis must use another signaling pathway.

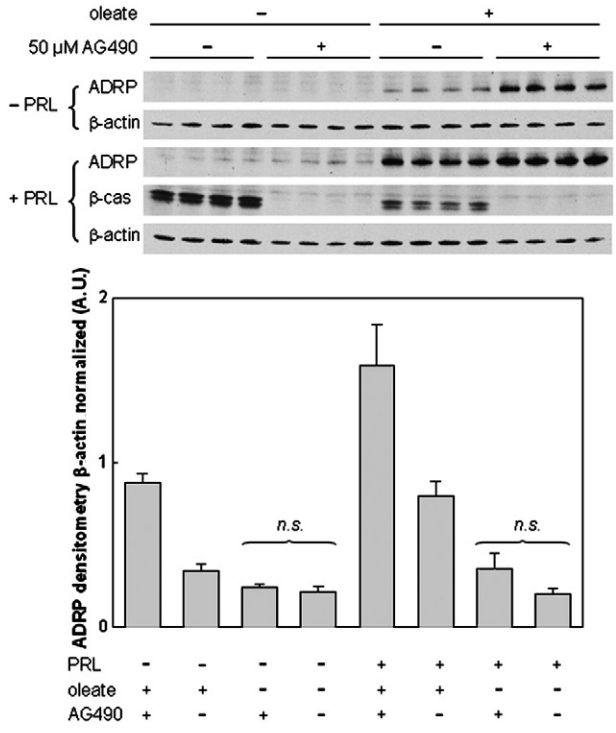

Fig. 3. The Jak2 inhibitor AG490 inhibits β-casein synthesis but enhances ADRP production. Confluent HC11 cells were grown according to the standard procedure (see Materials and methods) in the absence or presence of oleate and/or 50 μM AG490. Cellular proteins were analyzed by SDS-PAGE followed by immunoblotting for ADRP, β-casein and β-actin. The ADRP signal was quantified by densitometry and normalized to that of β-actin. Lower panel: each experimental condition was performed in quadruplicate and data are presented as means ± s.d. arbitrary units (A.U.). *n.s.* non significant.

3.4. The Ras/Raf/MEK/ERK1/2 signal pathway has no impact on ADRP synthesis

Activation of the EGFr in HC11 cells promotes proliferation and confers competence to respond to the lactogenic hormones while inhibiting differentiation [36]. Indeed, differentiation of HC11 cells is induced after removal of EGF by the synergistic actions of insulin, glucocorticoids, and PRL following the standard procedure used in the present study [37]. The Ras/Raf/MEK/ERK1/2 cascade is the main signaling pathway activated by EGF, mostly responsible for mammary epithelial cell proliferation in vivo [38]. The inhibitor PD98059 selectively blocks MEK activation in response to different growth factors, including EGF [39]. When HC11 cells were incubated in the presence of EGF (1 ng/ml), treatment with 50 μM PD98059 rescued part of the β-casein signal, the synthesis of which was clearly blocked by EGF, but had no significant effect on the levels of ADRP (Fig. 4). We obtained more significant results with AG1478, a selective inhibitor of EGFr tyrosine kinase activity. With 1 μM AG1478 the β-casein signal acquired in the presence of PRL + EGF appeared analogous to that with PRL alone. On the other hand AG1478 suppressed the stimulation of ADRP synthesis by EGF but did not affect that induced by PRL. These results confirmed that the Ras/Raf/MEK/ERK1/2 cascade is one of the major pathways used for EGF signal transduction but this pathway has no real influence on PRL-induced ADRP levels.

3.5. Differential role played by c-jun and p38 on ADRP and β-casein synthesis

It was important to determine whether distal transduction events possibly activated by both hormone receptors encompass other mitogen-activated protein kinases. Some other ERKs, including c-Jun

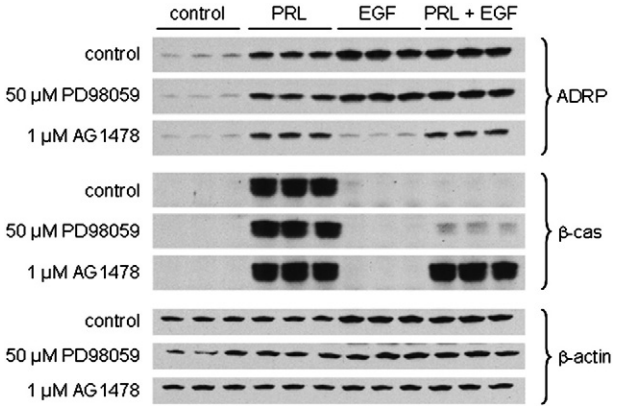

Fig. 4. Effects of PD98059 or AG1478 on ADRP and β-casein synthesis. Confluent HC11 cells were grown according to the standard procedure in the absence or presence of 4 ng/ml EGF, with or without 1 μM AG1478 or 50 μM PD98059. Cellular proteins were analyzed by SDS-PAGE followed by immunoblotting for ADRP, β-casein and β-actin. As shown, each experimental condition was performed in triplicate.

N-terminal kinases (JNKs) and p38, have been implicated in transcriptional regulation by PRL [40]. To address this point we used SP600125 and SB203580, inhibitors of JNK and p38, respectively. SP600125 is a reversible ATP-competitive inhibitor against JNK1-3 which inhibits the phosphorylation of c-Jun [41]. Addition of 5 μM SP600125 (IC50 = 40–90 nM) together with PRL to the culture medium neither affected the PRL-induced β-casein synthesis nor the cellular content of ADRP. Even with 50 μM SP600125 (at least 5000 fold the IC50), we only found 18% and 38% decrease of β-casein and ADRP signal, respectively. These results indicated that JNK was not significantly involved (Fig. 5, left panel). To test p38, we used SB 203580, a specific inhibitor of the MAP kinase homologues p38-α, -β and -β2, which has no significant effect on the activities of ERKs and JNKs [42]. As shown in Fig. 5, right panel, this inhibitor induced a linear dose–response inhibition of the PRL-induced β-casein synthesis (90% inhibition for 25 μM (IC50 = 0.6 μM)). It is interesting to note that at this concentration the inhibitor stimulated ADRP synthesis by 20%.

3.6. The PI3-kinase/Akt/mTOR signal pathway is critical for the control of both ADRP and β-casein synthesis

The PI3-kinase/Akt/mTOR cascade is another important signaling pathway activated by PRL and EGF [43,44]. To determine whether one or both hormones regulate ADRP and β-casein synthesis through this signaling cascade, we examined the effect of inhibition of PI3-kinase and its downstream mediators Akt and the mammalian target of rapamycin (mTOR). With this aim, we used wortmannin and LY294002, two inhibitors of PI3-kinase. As shown in Fig. 6, addition of 100 nM wortmannin together with PRL to the culture medium resulted in a strong diminution of the PRL-induced β-casein synthesis (20% of control). Concerning ADRP, wortmannin induced a smaller decrease of the signal with 67% of control for PRL, 89% of control for EGF, or 67% of control for PRL + EGF treated cells. In line with this, addition of 50 μM LY294002, a PI3-kinase inhibitor with higher stability and specificity provoked the complete arrest of PRL-induced β-casein synthesis and a drastic inhibition of that of ADRP (22% of control for PRL, 1% of control for EGF, or 28% of control for PRL + EGF). To strengthen the role of the PI3-kinase/Akt transduction cascade in the control of both the β-casein and ADRP synthesis, we assayed the highly potent and selective Akt inhibitor, MK-2206 [45]. As shown in Fig. 7A, 1 and 10 μM MK-2206 inhibited both synthetic pathways

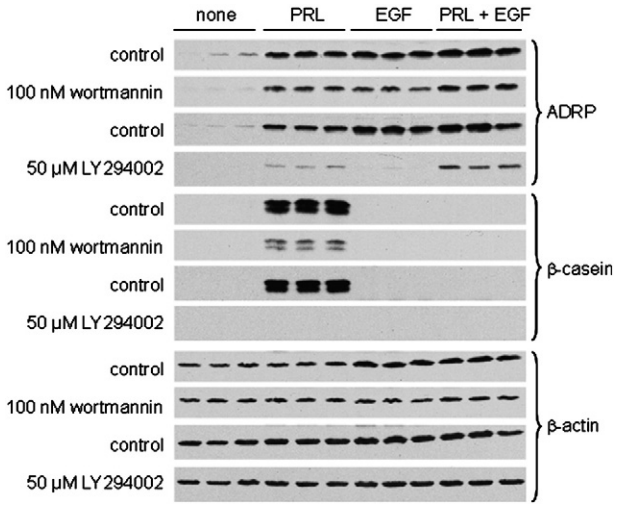

Fig. 6. Comparative effects of wortmannin and LY294002 on ADRP and β-casein synthesis. Confluent HC11 cells were treated according to the standard procedure in the absence or presence of 4 ng/ml EGF either with or without 100 nM wortmannin or 50 μM LY294002. Cellular proteins were analyzed by SDS-PAGE and immunoblot successively probed with anti-ADRP, anti-β-casein, anti-β-actin antibodies and their respective HRP-conjugated secondary antibodies. ECL signals were quantified by densitometry and normalized to β-actin. Each experimental condition was performed in triplicate. Percentages of inhibition for both inhibitor and hormonal treatments are indicated in the text.

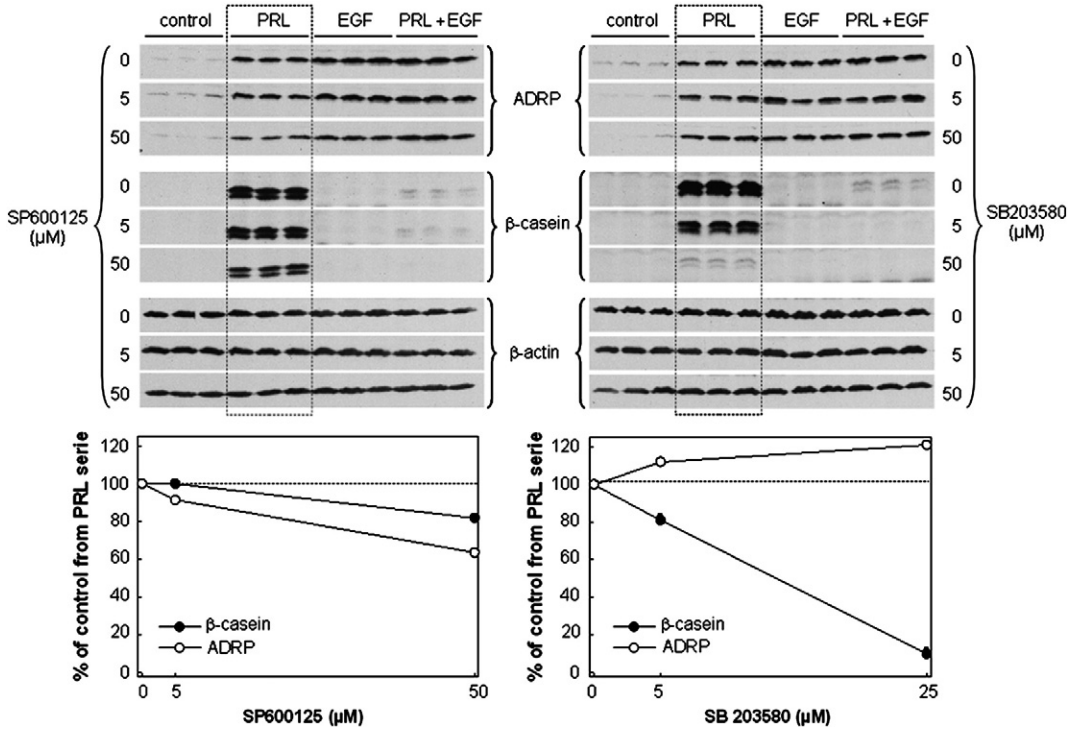

Fig. 5. Comparative effects of SP600125 and SB203580 on ADRP and β-casein synthesis. Confluent HC11 cells were treated according to the standard procedure in the absence or presence of 4 ng/ml EGF, either minus or plus 5 or 50 μM SP600125 (left panel), or 5 or 25 μM SB203580 (right panel). Cellular proteins were analyzed by SDS-PAGE and the immunoblots were successively probed with anti-ADRP, anti-β-casein, anti-β-actin antibodies and their respective HRP-conjugated secondary antibodies. Each experimental condition was performed in triplicate. ADRP and β-casein ECL signals from the PRL series (dashed rectangles) were quantified by densitometry, normalized to β-actin and expressed as percentage of untreated cells.

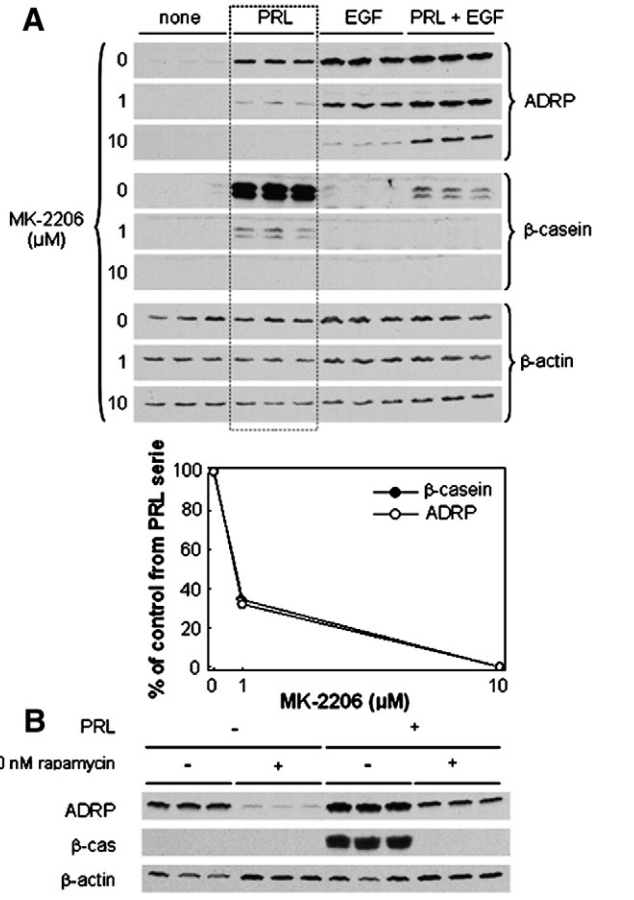

Fig. 7. Effects of MK-2206 and rapamycin on ADRP and β-casein synthesis. Confluent HC11 cells were grown according to the standard procedure in the absence or presence of 4 ng/ml EGF with or without 1 or 10 μM MK-2206 (panel A) or 50 nM rapamycin (panel B). Cellular proteins were analyzed by SDS-PAGE and the immunoblots were successively probed with anti-ADRP, anti-β-casein, anti-β-actin antibodies and their respective HRP-conjugated secondary antibodies. Each experimental condition was performed in triplicate. Part A: ECL signals from the PRL series (dashed rectangle) were quantified by densitometry, normalized to β-actin and expressed as percentage of control without MK-2206 treatment.

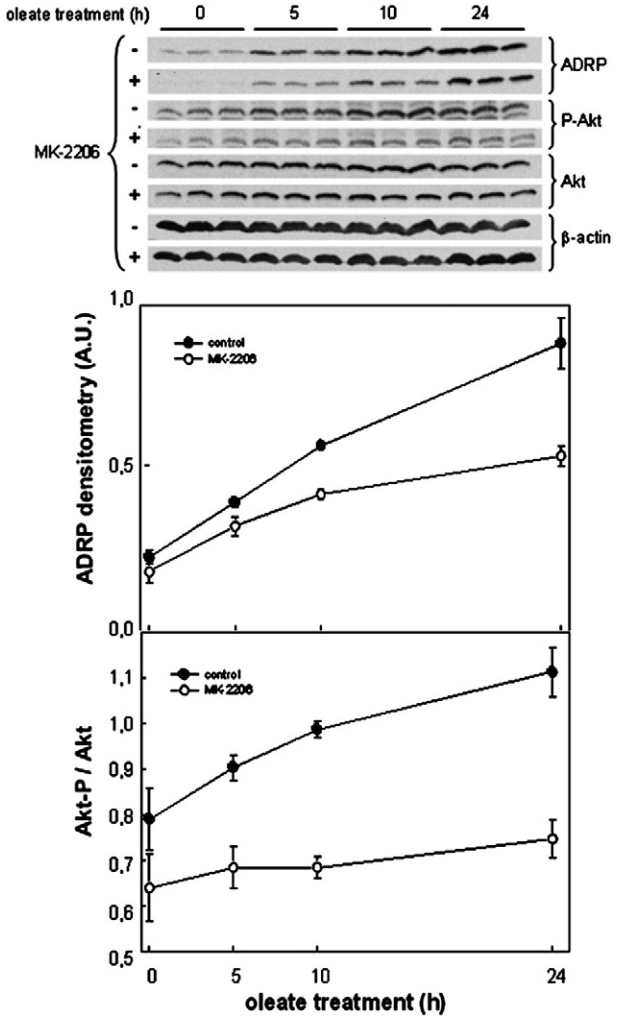

Fig. 8. Effects of oleate on the phosphorylation of Akt. HC11 cells were grown to confluence in RPMI-5% fetal calf serum containing 4 μg/ml insulin in the absence of cortisol and PRL. Cells were then preincubated for 12 h in the absence or presence of 2 μM MK-2206 and further treated for 0, 5, 10 or 24 h in the same medium containing 300 μM oleate. Cellular proteins were analyzed by SDS-PAGE and the immunoblots were successively probed with anti-phospho Akt, anti Akt, anti ADRP, anti-β-actin antibodies and their respective HRP-conjugated secondary antibodies. Each experimental condition was performed in triplicate (upper panel). For each protein, the ECL signal was quantified by densitometry, normalized to that of β-actin and data are presented as means ± s.d. arbitrary units (A.U. for ADRP) or as the ratio of phospho-Akt/Akt.

with a complete lack of β-casein signal, whereas that of ADRP remains detectable notably in the presence of EGF and PRL. Finally, we tested mTOR, the downstream mediator of this pathway which plays an essential role for both proliferation and differentiation of mammary epithelial cells [46]. In agreement with the above results, 50 nM rapamycin totally inhibited β-casein synthesis and decreased that of ADRP to the level observed in the absence of both rapamycin and PRL (Fig. 7B).

3.7. Oleate stimulates phosphorylation Akt

Given the above observations, we wished to investigate whether activation of the PI3-kinase/Akt/mTOR signal pathway by oleate could be directly demonstrated. We evaluated the phosphorylation status of Akt during the time-course of oleate treatment of HC11 cells. We also tested the action of MK-2206 which inhibits Akt phosphorylation [47]. Cells were pretreated in the presence or absence of 2 μM MK-2206 for 12 h and then further incubated with 300 μM oleate for 0, 5, 10 or 24 h. As shown in Fig. 8, phosphorylation of Akt in control cells was observed at detectable levels under basal culture conditions and gradually increased over oleate incubation time. ADRP levels regularly grew according to a similar time-course. In the presence of MK-2206, the phosphorylated Akt/whole Akt ratio remained steady. Altogether, these data pinpoint the role of the PI3-kinase/Akt/mTOR signal pathway on the oleate-stimulated formation of lipid droplets.

3.8. Visualization of the action of inhibitors by immunofluorescence microscopy

Given the above results, it was important to determine the effect of inhibitors on the formation of cytosolic lipid droplets. With this aim, we conducted immunofluorescence microscopy observations of lipid-laden HC11 cells treated according to the standard procedure in the absence or presence of inhibitors. It must be noted that HC11 cell cultures displayed variations in their morphology when cultivated on poly-L-Lysine-coated glass coverslips versus plastic plates. Thus, with some inhibitors (MK-2206, LY294002 and AG490), cell layers exhibited large empty areas. In agreement with this, it has been shown that MK-2206 causes growth inhibition of eight cell lines with an IC50 that ranges between 3.4 and 28.6 μM [45]. MK-2206 also provoked a perturbation of cell organization; all other inhibitors maintained it. These phenomena were not observed on plastic plates and biochemical analysis

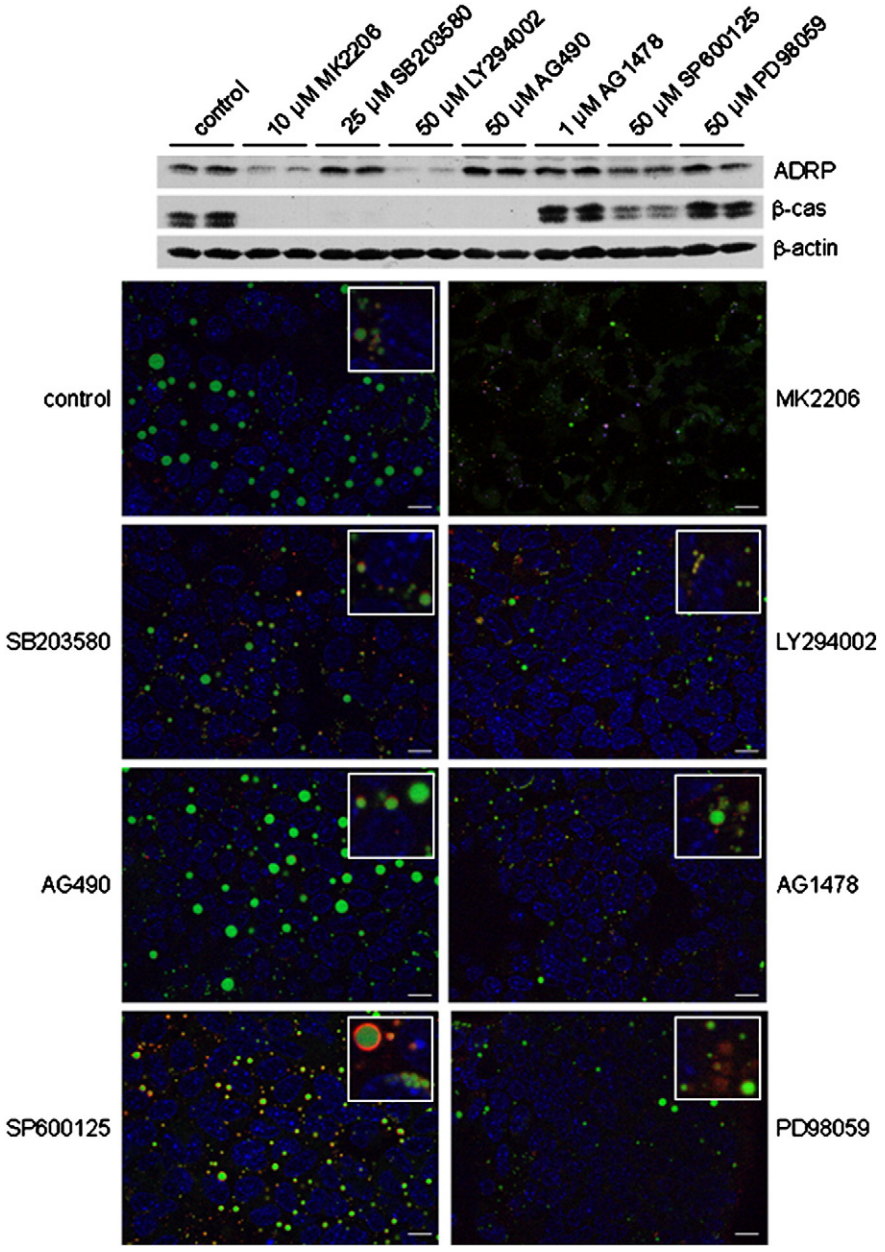

Fig. 9. Comparative effects of inhibitors by immunofluorescence microscopy study. Confluent HC11 cells were grown on poly-L-lysine-coated glass coverslips (immunofluorescence) and on plastic plates (biochemical control) and then treated with inhibitors according to the standard procedure. Upper part: the biochemical action of the inhibitors was tested to validate the immunofluorescence results. Cellular proteins were analyzed by SDS-PAGE and the immunoblots were successively probed with anti-ADRP, anti-β-casein, and anti-β-actin antibodies and their respective HRP-conjugated secondary antibodies. Each experimental condition was performed in duplicate. Lower part: cells were fixed, permeabilized and subjected to immunofluorescence microscopy using antiserum against ADRP and TRITC-conjugated secondary antibody (red). Neutral lipids and nuclei were labeled with Bodipy 493/503 (green) and Dapi (blue), respectively. Observations were made using ApoTome microscopy. Bar = 1 μm. Inset shows lipid droplet details (3×).

conducted at the same time indicated that inhibitors worked as previously observed (Fig. 9, upper part). Although immunofluorescence microscopy is not quantitative, the Bodipy 493/503-stained cytoplasmic lipid droplet content clearly decreased after the action of certain inhibitors (Fig. 9 lower part, right column). As previously observed, whole ADRP levels, estimated by Western blot, decreased only in the presence of MK-2206 and LY294002. In most cases, ADRP decorated the surface of small lipid droplets and appeared as little patches on large cytoplasmic lipid droplets (Fig. 9 lower part). With the exception of SP600125, which induced a strong ADRP coating of almost all cytoplasmic lipid droplets (although this inhibitor did not increase ADRP synthesis), variation in the distribution of ADRP at the surface of lipid droplet was difficult to estimate, notably because the signal was faint and uneven.

4. Discussion

Mammary epithelial cell secretion is regulated through complex integration of transduction signals by multiple hormones including PRL and EGF. Herein we report that a finely tuned hormonal balance leads to differential regulation of the synthesis of ADRP and β-casein, two landmark components of lipid and protein synthetic pathways, in the mouse mammary epithelial cell line HC11. Although a vast literature is available on the role played by cortisol, PRL and EGF in the functioning of the mammary gland, to our knowledge, this is the first comparative study of their action on the synthetic pathways of the two main milk constituents in the HC11 cell system. Most importantly, our results clearly demonstrate that the PRL-induced

stimulation of ADRP synthesis is independent of that of β-casein synthesis. As a first example, cortisol is required for β-casein expression, whereas it reduces ADRP synthesis. This inhibitory effect of cortisol on ADRP synthesis was unexpected. In addition to its role in activating glucocorticoid receptor to initiate β-casein transcription synergistically with Stat5, it has been shown that dexamethasone inhibits the phosphorylation of p70S6 kinase in HC11 cells, thereby decreasing protein synthesis [48]. It has also been reported that treatment of bovine mammary epithelial cells with dexamethasone (50–250 nM) induces downregulation of both the hormone-sensitive lipase and the uncoupling protein (UCP 2) [49]. However, no common mechanism seems to emerge from these reports. The molecular mechanisms involved in the inhibition of ADRP synthesis by cortisol need further investigation.

The use of specific pharmacological inhibitors allowed us to gain new insights into the mechanism by which PRL and EGF regulate β-casein and ADRP synthesis. AG490, known to attenuate Jak2 phosphorylation activity, induced the inhibition of PRL-induced β-casein synthesis, as previously observed in the HC11-Lux model system [35]. Intriguingly, AG490 enhanced ADRP production. This indicates that the Jak2/Stat5A pathway, in contrast to its effect on ß-casein synthesis, is not involved in the induction of ADRP synthesis upon stimulation of the cells with PRL. Moreover, the Jak2/Stat5A pathway appears to negatively control ADRP expression. This result raises the question of the role played by Stat5 and, more precisely, by its phosphorylation status, in the regulation of milk lipid synthesis and trafficking. The involvement of peroxisome proliferator activated receptor gamma (PPARγ) a transcription factor expressed in many cell systems including mammary epithelial cells, in these processes is likely. PPARγ has a binding domain specific for natural lipid ligands such as eicosanoids, poly-unsaturated fatty acids and oleate [50]. Following activation by these ligands, PPARγ binds to peroxisome proliferator response elements (PPREs) in upstream promotor domains of target genes to stimulate their transcription. PPRE sequences have been found upstream of the *ADRP* and *Stat5A* genes [51,52]. Moreover, it has been suggested that the EGF-mediated suppression of Stat5A expression is totally or partially mediated through inhibition of PPARγ activity [52]. On the other hand, binding of PRL to the PRLr also activates negative regulators such as tyrosine phosphatases and a family of proteins known as suppressors of cytokine signaling (SOCS) or cytokine-inducible SH2-containing proteins (CIS) [53–55]. It is an open question whether SOCS and/or PPARγ are involved in the regulation of ADRP synthesis.

The cytokine receptors that turn on the Jak2/Stat5 pathway, including the PRLr, also activate the Ras/Raf-1/MEK/ERK1/2 pathway. These mitogen-activated protein kinases (MAPK) are also triggered upon stimulation of the cells by insulin and growth factors. In agreement with this, we found that the Ras/Raf-1/MEK/ERK1/2 pathway contributes to the inhibition of the lactogenic hormone-induced differentiation of HC11 by EGF as previously described [38], but this is not the case for ADRP synthesis. JNK and p38 have been implicated in transcriptional regulation by both PRL and EGF [40,56]. PRL activates JNK whereas glucocorticoid prevents this activation [57,58]. While JNK inhibition had a limited effect, p38 inhibition slightly increased the cellular content of ADRP and inhibited PRL stimulated β-casein synthesis in a dose-dependent manner. Such a dual effect has been previously reported, as SB 203580 induces an activation of c-Raf independently of p38 inhibition [59]. It has also been reported that ERK2 and phospholipase D1 are both required in the formation of cytosolic lipid droplets in a cell-free system. ERK2 increases the phosphorylation of dynein which, in turn, increases the amount of ADRP on cytoplasmic lipid droplets [60]. Determination of the role played by ERK1/2 in cytoplasmic lipid droplet formation in HC11 cells will need further investigation.

Upon activation, PI3-kinase translocates from the cytosol to the membrane where it phosphorylates various substrates including Akt, a central signaling molecule in mammary epithelial cell development and function. Akt is activated in response to a number of hormones and growth factors, including EGF and PRL [61,62]. Overexpression of an activated form of Akt in the mammary glands of transgenic mice induces an increase in lipid synthesis during both pregnancy and lactation, and is associated with a lactation defect that retards pup growth. This suggests that Akt may contribute to the regulation of milk secretion by modifying the formation and/or accumulation of cytoplasmic lipid droplets in mammary epithelial cells [63]. Thus, PRL acts both through the Akt pathway and independently, since it is also known to stimulate milk protein synthesis via Stat5 activation [64]. Akt is specifically required for lactating mice to synthesize sufficient quantities of milk to support the growth of their offspring. Loss of Akt provokes a failure to upregulate glucose uptake, a defect in lipid synthesis and expression of multiple lipogenic enzymes, as well as a failure to downregulate lipid catabolic enzymes [65]. Activation of Akt can link mitogenic signaling with nutrient sensing pathways that regulate protein synthesis and cell size via a signaling pathway that includes mTOR [66]. These observations suggest that the PI3-kinase/Akt/mTOR pathway is critical as a central control system for the integrated coordination of both β-casein and ADRP synthesis. In agreement with this, the PI3-kinase inhibitor wortmannin appears to impede the PRL-induced stimulation of casein, lipid, and lactose synthesis in mouse mammary gland explants in a dose-dependent manner [67]. Moreover, previous experiments in our laboratory indicate that wortmannin diminishes casein secretion by 50% in rabbit mammary gland explants (Boisgard, unpublished results). Here, we showed that wortmannin and, most potently, LY294002 inhibit both ADRP and β-casein synthesis. On the other hand, rapamycin has been shown to impede the PRL-induced stimulation of casein, lipid and lactose synthesis in mouse mammary explants in a dose-dependent manner. This effect is also inhibited by wortmannin, indicating that it is mediated by the PI3-kinase/Akt pathway [48,68,69]. Furthermore, rapamycin prevents the lactogenic hormone-induced expression of milk proteins in HC11 cells [46]. In agreement with these data we found that the inhibition of either Akt by MK-2206 or mTOR by rapamycin equally hinders β-casein and ADRP synthesis.

We attempted to evaluate the effects of these inhibitors on the subcellular localisation of ADRP using immunofluorescence microscopy. However, these experiments were hampered by the fact that the ADRP signal at the lipid droplet surface was often faint and uneven. This, coupled with the inherent difficulty in estimating protein amounts by immunofluorescence, did not allow reliable quantitation of ADRP distribution relative to lipid droplet numbers. Moreover, it must be considered that the permeabilization step required for the immunofluorescence procedure could induce a leak of both cytoplasm and small lipid droplets together with an unknown pool of ADRP. These experiments, however, revealed that several inhibitors induced a clear reduction in the number and/or size of cytoplasmic lipid droplets. In agreement with the results obtained with PD98059 treatment, MAP kinases are known to play a role in the regulation of lipid droplet formation. Similarly, the observation that AG1478 treatment resulted in a decrease in lipid droplet formation could be due to inhibition of the Ras/Raf/MEK/ERK1/2 cascade, the main signaling pathway activated by EGF. An alternative explanation is that inhibition of EGFr phosphorylation impairs activation of the PI3-kinase/Akt/mTOR signaling cascade. Finally, the latter signaling pathway was directly affected by the two other inhibitors that induced reduction in the production of lipid droplets by HC11 cells, namely LY294002 and MK-2206. As discussed above, this effect was expected. In this context, it is also worth noting that we observed a high decrease in total ADRP content in these last conditions. The second clear effect that we observed by immunofluorescence was a drastic (re)localization of ADRP at the surface of lipid droplets following HC11 treatment with SP6000125. One possible explanation for this finding is that the degradation of the lipid droplet-unbound ADRP pool by the proteasome is inhibited in these conditions, as it has been reported that JNK is a general regulator of cotranslational

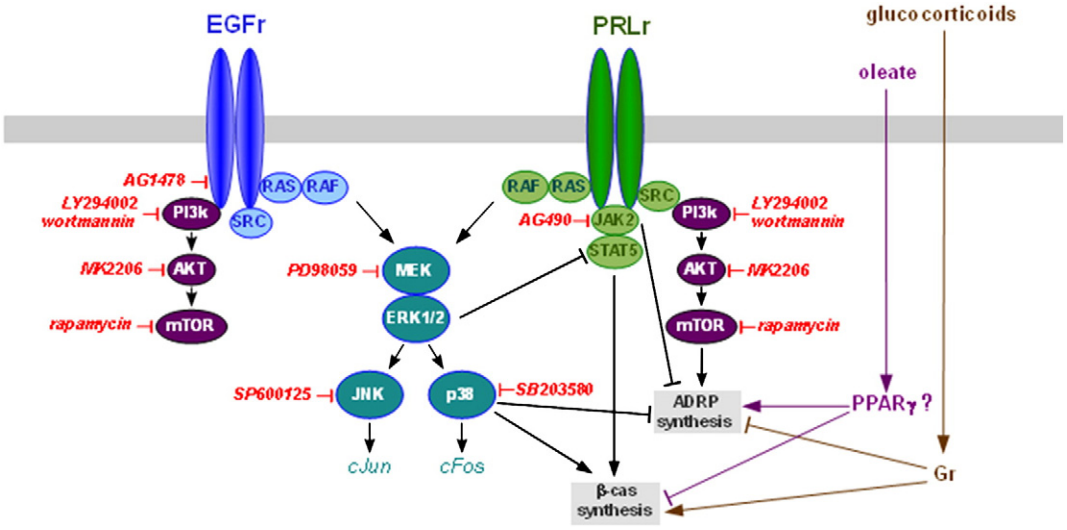

Fig. 10. Integrated model. This model of the signaling cascades regulating β-casein and ADRP synthesis summarizes current knowledge and the new results described in the present paper. Events that stimulate activity are noted with lines terminating in arrowheads, whereas inhibitory events are marked with lines ending with bars. For the sake of clarity, the signaling connections between the PI3-kinase/Akt/mTOR pathway and β-casein synthesis, as well as between the EGFr/PI3-kinase pathway and ADRP synthesis are not illustrated.

degradation [70]. As estimated by immunoblotting, however, the total cellular levels of ADRP appeared not to increase in these conditions. Further study will be needed to appraise the functional significance of this increase in ADRP localization at the lipid droplet surface and the molecular mechanisms underlying this process. In Fig. 10, we depict the pathways involved in the regulation of β-casein and ADRP synthesis, with their respective inhibitors.

Though the precise role played by oleate as a signaling molecule for the regulation of ADRP cell level remains poorly understood, it appears to control many enzymes involved in transduction mechanisms. It has been reported that oleate triggers a time- and dose-dependent phosphorylation of EGFr and subsequent ERK1/2 activation in the ECV-304 endothelial cell line. This process was found to be independent of fatty acid metabolism and inhibited by AG1478 [71,72]. It has also been shown that oleate enhances phosphorylation of ERK 1/2 and Akt kinase in bovine mammary epithelial cells [73]. However, the inhibition of β-casein synthesis in PRL-treated HC11 cells in the presence of oleate [24] is not the consequence of an activation of EGFr by oleate, since AG1478 does not enhance the amount of ADRP in control or PRL-treated cells. It has also been claimed that oleate stimulates cell proliferation in human mammary cancer cells by increasing PI3-kinase activity [74]. Finally, our finding that oleate alone stimulated the phosphorylation of Akt concomitantly with an increase in ADRP levels is also in favor of the activation of the PI3-kinase/Akt/mTOR pathway by this lipid. How ADRP synthesis is increased upon PRL stimulation in the presence of oleate when Jak2 is inhibited by AG490 remains to be explained [52]. Our next goal will be to determine the precise mechanisms by which oleate activates the PRL and EGF signal transduction cascades in the context of the regulation of ADRP expression.

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