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Integrating leptin and cAMP pathways in triple-negative breast cancer cells

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1. ABSTRACT

Triple-negative breast cancers are characterised by an aggressive phenotype, are often found in younger women and have been associated with poor prognosis. Because triple-negative breast cancer patients are unresponsive to current targeted therapies and other treatment options are only partially effective, new pharmacological approaches are warranted. The obesity-linked adipokine, leptin, is a well known mitogen/survival factor in breast cancer cells and several studies have addressed its role in breast cancer. Surprisingly, recent in vitro studies have shown that leptin enhances the anti-proliferative effects of cAMP elevation in triple-negative breast cancer cells by apoptosis induction. In the current review, we discuss on the role of cAMP as a growth suppressor and of leptin as a growth promoting factor in breast cancer cells and we will focus on the molecular pathways involved in the antiproliferative interaction between leptin and cAMP elevation. The rationale for the possible development of a simple, cheap and innovative approach for therapeutic intervention in triple-negative breast cancer, based on the use of cAMP elevating drugs at tolerable doses, will be discussed.

2. INTRODUCTION

Breast cancer is one of the most common malignancies and a major cause of cancer death in women throughout the world, with an estimated 1 million women diagnosed annually. According to the American Cancer Society, nearly 230,000 new cases and 40,000 deaths are estimated to occur in the United States in 2011 (1).

Localized breast cancer can be cured by surgery. However, the high mortality rate associated with breast cancer is due to a propensity of the tumor to metastasize when the primary tumor is small or undetectable (2). Although hormone therapy is effective for the treatment of most patients with estrogen receptor (ER)-positive breast cancer, resistance to hormones is frequent (3). In addition, a number of tumors do not express these receptors and do not respond to anti-hormone therapy (4). Drugs targeting other pathways involved in breast carcinogenesis, such as trastuzumab, an antibody against ErbB2, or oral tyrosine kinase inhibitors are actually used in therapy (5). Nevertheless, these drugs carry significant adverse effects along with their known benefits. Tamoxifen shows a positive effect on bone decreasing the osteoporosis, but on

the other hand increases the risk of endometrial cancer and venous thromboembolism. Trastuzumab has potential concern of severe cardiac dysfunction.

Overall, chemotherapy is still a major treatment modality for both hormone-refractory and ER-negative breast cancer. However, women with advanced metastatic breast cancer, that is resistant to hormone therapy, usually respond poorly to conventional chemotherapy and to other current targeted therapies (6). Therefore, new effective therapies are warranted for the treatment of metastatic breast cancer (7).

Evidence suggests that different hormones and peptide growth factors might cooperate in promoting mammary carcinogenesis. Since the circulating levels of leptin are elevated in obese individuals, and excessive body weight has been shown to increase breast cancer risk in women, several studies have addressed the role of leptin in breast cancer.

Leptin is the peptide hormone product of the obesity (ob) gene (8). Leptin is a well-known factor involved in the regulation of body weight and body composition and is an important mediator of obesity. The circulating leptin acts as a regulator of food intake via hypothalamic-mediated effects (9). Leptin is secreted mainly from adipose tissue as well as from normal or malignant breast tissue and from secondary sources like placenta, stomach, and skeletal muscle (10). The plasma leptin levels are strongly correlated with both BMI and insulin (11).

Remarkably, both leptin and its receptors are overexpressed in breast cancer, especially in high-grade tumors including triple-negative cancers and leptin acts as a mitogen/survival factor in breast cancer cells (12-17). Leptin may influence breast cancer development in relation to estrogen receptor status as well as to the presence or absence of HER2 (18). In obese humans, high plasma levels of leptin are correlated with increased fat mass and the development of resistance to insulin and hyperinsulinemia (11, 19, 20). Leptin expression is induced by obesity, insulin, TNF-a and glucocorticoids; on the other hand, it is negatively regulated by b-adrenergic agonists and thiazolidinediones (21).

Leptin is associated with increased aromatase activity leading to a functional cross-talk relationship with estrogen (22). Total body aromatization in postmenopausal breast cancer patients is strongly correlated with plasma leptin levels (23). There are several other factors such as adiponectin may be involved in the relationship between leptin and breast cancer (24-26). In addition, leptin can regulate endothelial cell proliferation and promote angiogenesis (27, 28). Leptin also has a significant association with the progression and poor survival of breast cancer patients (29-32).

Overall, leptin is strongly proposed as cytokine link between obesity and breast cancer (33, 34). Importantly, leptin system has emerged as a new and promising therapeutic target for breast cancer (16, 35).

Surprisingly, our recent *in vitro* studies have shown that leptin enhances the anti-proliferative effects of cAMP elevation in triple-negative breast cancer cells (that lack oestrogen and progesterone receptors, ER, PgR, and do not express or express low levels of the oncogenic receptor HER2) by apoptosis induction (36, 37).

Despite their potent antiproliferative and antiangiogenetic effects in many cancer cells, agents that increase intracellular cAMP levels are not recommended to be used as anti-cancer drugs because of their high cytotoxicity (38). Therefore, finding new strategies able to potentiate the antitumor activity of these agents in order to reduce their dosage required to obtain antiproliferative effects while limiting the potential toxicity would be interesting (39). On the other hand, the activation of apoptosis of cancer cells is considered a key mechanism of anti-cancer therapy.

Very interestingly, we demonstrated that leptin causes a large pro-apoptotic action when used in combined treatments with cAMP elevating agents in triple-negative breast cancer cells (36, 37). This positive pharmacological interaction between leptin and cAMP elevating compounds allows a reduction in the effective doses of cAMP elevating drugs *in vitro*, thus potentially decreasing their undesirable side-effects *in vivo*.

Importantly, the costs of therapies and their economic impact are increasingly becoming more relevant for National Health Services.

In this review, we discuss on the role of cAMP as a growth suppressor and of leptin as a growth promoting factor in breast cancer cells and we will focus on the molecular pathways involved in the antiproliferative interaction between leptin and cAMP elevation.

The rationale for the possible development of a simple, cheap and innovative approach for therapeutic intervention in triple-negative breast cancer, based on the use of cAMP elevating drugs at lower and tolerable doses, will be also discussed.

3. BREAST CANCER: A GENERAL OVERVIEW

Breast cancer remains a widespread disease and a major cause of death in the United States as well as the rest of the world. Nevertheless, a decline in mortality rate has been observed during the last few years. This decline is due to mammographic screening, more precise diagnosis, and an increase in the number of women receiving the best treatment for their condition, like the extensive use of tamoxifen (40-42).

The causes leading to breast cancer and the identification of prevention strategies are still elusive. Association of the risk of breast cancer with age at first birth and parity was proposed several years ago and

confirmed by subsequent studies (2). Additional risk factors have been added in recent years. These include genetic factors, geographical location, exposure to ionising radiation, particularly during puberty, absence or short lifetime duration of breastfeeding (typical of women in developed countries), use of oral contraceptives, hormone-replacement therapy, high body-mass index and dietary factors, such as alcohol abuse (43). Progression from healthy mammary tissue to invasive carcinoma is still a debated process. The pre-neoplastic potential of benign, proliferative lesions of breast and dysplastic changes present in different non-malignant breast diseases is not defined. To date, *in situ* carcinomas (either ductal or lobular) are morphologically identifiable as neoplastic transformation, whereas stromal invasion and metastasis to regional lymph nodes or distant organs are the hallmarks of developed breast cancer (2).

Breast cancer is a heterogeneous disease with varied morphological appearance, molecular features, behaviour and response to therapy (4, 44). However, it has become clear that patients with apparently similar features may vary in their outcomes (44-46). In an attempt to subdivide patients into clearly defined categories that can be used to support management decisions, various well established prognostic factors have been combined to constitute prognostic indices. Subgrouping based on molecular biomarkers such as hormone receptor (HR) has the advantage of avoiding the subjectivity inherent in histopathology. Currently, expression of HR, i.e. oestrogen receptors (ER) and progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2) play an important part in breast cancer classification, at least partly because they predict the response to treatment (47, 48). ER-positive (ERþ) tumours account for up to 65% of breast tumours in women aged <50 years, and 80% in older women. PR-positive (PRþ) tumours account for about 60% of breast cancers (49). Tumours that are HRþ and HER2-negative comprise the largest proportion of breast cancer and have a better prognosis and response to hormone therapy than the other HR/HER2 subgroups (50). HRþ/HER2þ tumours account for about 6% of breast cancers (51). Preclinical and clinical data suggest that HER2 overexpression in HRþ tumours confers resistance to hormonal treatment and specific chemotherapeutic agents (52, 53). Tumours without either HR or HER2 expression are referred to as triple negative breast cancer (TNBC).

TNBC has several characteristic aggressive clinicopathological features, including young age at onset and large tumour size (6, 54). Its histological hallmarks include high grade, high proliferative activity, focal areas of necrosis, absence of infiltrative margin, absence of gland formation, presence of central scar/fibrotic foci and prominent lymphoplasmacytic inflammatory infiltrate (6, 54)

They have a poor prognosis in terms of disease-free and overall survival and currently there is a lack of targeted therapies for this group of patients (7, 55) .

Because of its expression profile, TNBC is not amenable to treatment with hormone therapy or the anti-HER2 monoclonal antibody trastuzumab, and systemic treatment options are currently limited to cytotoxic chemotherapy. Overall survival, whether in early-stage or advanced disease, is poor compared with that in patients who have other phenotypes. A number of targeted approaches to TNBC are undergoing clinical evaluation, including the use of agents with poly-(ADP-ribose) polymerase inhibitory properties such as iniparib (the United States Adopted Name for the investigational agent BSI-201), olaparib (AZD2281), and veliparib (ABT-888), antiangiogenic agents such as bevacizumab and sunitinib, and epidermal growth factor receptor blockers such as cetuximab and erlotinib. Encouraging results with some of these agents have been reported, thereby offering the promise for improved outcomes in patients with TNBC (6, 7, 55, 56).

However, optimal conditions for the therapeutic assessment of women with triple-negative breast tumours and for the management of their disease have yet to be validated in prospective investigations and new pharmacological approaches for TNBC patients are warranted.

Importantly, the costs of therapies and their economic impact are increasingly becoming more relevant for National Health Services.

Combination chemotherapy has received more attention in order to find compounds that could increase the therapeutic index of clinical anticancer drugs while limiting their potential toxicity.

At this regard, naturally occurring molecules with no or the least toxicity to normal tissues and able to reduce the dosage of “cheap” drugs required to obtain antitumor effects are suggested as very attractive candidates to be investigated.

4. LEPTIN, cAMP AND BREAST CANCER

4.1. cAMP and the cAMP-dependent signaling pathways: an overview

A large number of hormones, neurotransmitters and other signal substances utilize cyclic adenosine 3’5’ cyclic monophosphate (cAMP) as an intracellular second messenger. cAMP was first identified as a small intracellular heat-stable factor mediating the effect of glucagon on the phosphorylation status of glycogen phosphorylase in the 1950s, and the concept of cAMP as an important mediator for many extracellular signaling molecules was rapidly developed (57).

cAMP is intracellularly generated from ATP by adenylate cyclases, and can be induced more than twenty-fold upon activation of ACs by extracellular signals (58).

Degradation of cAMP is mediated by cAMP phosphodiesterases, that hydrolyze cAMP into adenosine 5’- monophosphate and this event is important for controlling cAMP resting state levels (59).

The main intracellular target for cAMP in mammalian cells is the cAMP-dependent protein kinase, PKA, EC 2.7.1.37, we have recently reviewed on (60).

An important additional effector system for cAMP signaling is achieved by the exchange proteins directly activated by cAMP 1 and 2 (Epac1 and -2; also named cAMP-GEFI and -II). These guanine nucleotide exchange factors (GEFs) are specific activators of the small GTPase Rap1. The cAMP-binding domain of Epac can bind one molecule of cAMP, resulting in a conformational change of the protein, which will expose the active site of the catalytic domain, enabling the protein to bind to and activate Rap1 (61).

cAMP, either via a PKA-dependent or PKA-independent manner, affects numerous cellular functions and can exert different biological effects such as cell differentiation, proliferation and apoptosis (62-65). Therefore, a major question for scientists working in the field of the cAMP signaling has been to understand how specificity is maintained in this second messenger system.

The existence of different cAMP downstream effectors and some features of PKA signaling pathway described in previous reviews may contribute to explain how differential discrete effects of cAMP may be obtained (60, 66, 67).

Intracellular concentration of cAMP results from the fine balance between the activities of synthesis and degradation by adenylate cyclases and PDEs, respectively (58, 59). However, the main control of cellular cAMP content lies at the level of its synthesis. The molecular mechanisms regulating cAMP biosynthetic adenylate cyclase activity are highly controlled and play a key role in cAMP dependent functions (58). In this context we have previously described a novel proteasome-mediated regulatory mechanism controlling adenylate cyclase activity/cAMP levels by modulating Gαs protein levels, and provided evidence that proteasome has a physiological role in regulating Gαs-mediated cAMP signaling triggered by β-adrenergic agonist (68). Moreover, some naturally occurring molecules, such as resveratrol and inorganic phosphate, have been shown to regulate adenylate cyclase activity for controlling the proliferation of cancer cells, including breast cancer cells (69, 70).

An important concept is that cAMP concentration and cAMP signaling can change and occur very locally, respectively (71). Localized cAMP-mediated activity is explained by localized induction and degradation of cAMP in specialized cellular compartments such as caveolae and lipid rafts (72, 73). PDEs are important for regulating cAMP concentration in these microdomains as their unique intracellular targeting regulates the availability of cAMP to its effectors (59, 74). Also ACs and GPCRs are not evenly distributed along the membrane. Furthermore, PKA can be targeted by several proteins, including Src family kinases, arrestins, and A-kinase anchoring proteins (AKAPs). AKAPs especially play a role in compartmentalization of PKA isoforms by anchoring to specific sites for AKAP isoform (75). This all contributes to a localized activation of cAMP effectors.

4.2. cAMP and cAMP elevating agents in breast cancer

Elevation of cAMP inhibits the proliferation and expression of transformed phenotype in a wide variety of cancer models, including breast cancer, through the induction of apoptosis and/or cell cycle arrest (76-79).

cAMP elevating compounds include selective agonistic cAMP analogs (dibutyryl-cAMP, 8-bromo-cAMP, 8-chloro-cAMP, 4-chlorophenyltio-cAMP), adenylate cyclase activators (forskolin and derivates) and specific phosphodiesterase inhibitors (3-isobutyl-1-methyl-xanthene, theophylline) (38, 80-82).

Initial reports indicated that dibutryl-cAMP together with arginine suppresses the proliferation of MCF-7 cells (76). Subsequently, it was confirmed that the elevation of cAMP levels produces substantial effects in MCF-7 cells. Addition of 8Br-cAMP or expression of mutant (Q227L)-activated G alphas in MCF-7 cells block the ability of these cells to grow in an anchorage-independent manner, and stable transfection of activated-G alphas in MCF-7 cells reduced both EGF stimulation of MAPK in MCF-7 cells and the ability of the same cells to form tumours in nude mice (77). Subsequent studies have demonstrated that G protein alpha expression inhibits the growth of established human tumors of breast cancer cells in athymic mice by inhibiting the MAPK pathway (79). In addition, these data also imply that targeting of the cAMP/MAPK axis (i.e. by continuous elevation of cAMP) could be used to block tumor formation and offer a clear example that in many instances, cAMP can inhibit cellular proliferation by blocking extracellular signal-regulated kinase (ERK) signalling pathway, which is frequently activated in breast cancer cells (62, 83, 84). The elevation of cAMP also induces the cell cycle inhibitor p27kip1 (85). A decrease in p27kip1 levels is thought to be an important factor in breast tumor progression (86).

However, the signalling mechanisms involved in the antitumor activity of cAMP elevating agents in some cases are still not completely understood.

It has been suggested that differential regulation of PKA isozymes is the major cause of the 8-chloro-cAMP-induced anticancer activity. 8-chloro-cAMP is able to inhibit PKA-I expression and function and to promote PKA-II formation, causing cancer cell growth arrest *in vitro* and *in vivo* (60). In fact, differential expression of PKA-I and PKA-II has been correlated with cell differentiation and neoplastic transformation (87). Preferential expression of PKA-II is found in normal nonproliferating tissues and in growth-arrested cells, while PKA-I is generally overexpressed in cancer cells (88, 89). On the other hand, cAMP could attenuate the antiapoptotic protein Bcl2, upregulate wild p53 expression and suppress different oncogenes, such as myc, ras and erbB-2 (90-93). In addition, cAMP elevating drugs have been reported to induce differentiation in neoplastic cells (94) and inhibition of angiogenesis in breast cancer through down-regulation

of several cytokines (vascular endothelial growth factor: VEGF, fibroblast growth factor: FGF, epithelial growth factor: EGF, transforming growth factor beta: TGF-β) and inhibition of the ability to invade the basement membrane matrix (95, 96). Moreover, cAMP elevation has been shown to be involved in migration of breast cancer cells (97-101).

Unfortunately, despite their potent antitumor effects in many cancer cells, these substances that increase intracellular cAMP levels are not recommended to be used as anti-cancer drugs because of their high cytotoxicity. Therefore, it would be interesting to study new strategies able to potentiate the antitumor activity of these agents in order to reduce the dosage of cAMP elevating compounds required to obtain antiproliferative effects while limiting the potential toxicity.

4.3. Leptin signaling pathways in breast cancer

Leptin, the product of the obesity (ob) gene, is a a 167-aminoacid hormone principally synthesized and secreted by adipose tissue, involved in the regulation of food intake and energy balance (8, 9). This cytokine acts through binding to specific transmembrane receptors. The leptin receptor belongs to the class-I cytokine receptor family. Six leptin receptor isoforms have been identified up to now (102).

Leptin receptor (Ob-R) mRNA and protein expression have been characterised in different breast cancer cell lines irrespective of estrogen receptors status, including MCF-7 and MDA-MB-231 cells (16). The effect of leptin, acting via its receptors, on cell growth is well known, with leptin stimulating proliferation (17,18).

In vitro experiments show that the proliferative activity of leptin is mediated via different signalling pathways in breast cancer cells (24, 33, 34, 103). First, leptin induces the PI3K/Akt survival pathway by activating the phosphorylation of Akt Thr308 or Akt Ser473 and by stimulating the protein expression of PKC-alpha, which is controlled by PI3K. Second, leptin activates the MAPK pathway by inducing ERK1 and ERK2 phosphorylation.

Third, leptin receptor contains docking sites for Janus-family tyrosine kinase 2 (JAK2) that, after leptin activation of the receptor, rapidly phosphorylates specific members of the signal transducers and activators of transcription (STAT) family, particularly STAT3. Once activated, STAT proteins combining their Src homology 2 domains form homodimers and translocate to the nucleus, where they induce the expression of suppressor of cytokine signalling 3 (SOCS3) and other genes, including c-myc.

Leptin also up-regulates cdk2 and cyclin D1 (genes promoting cell cycle transition G1/S), indicating that cell proliferation may be activated through the alteration of cell checkpoints that accelerate cell cycle progression. Recently, Perera *et al.* have identified more than 64 leptin-regulated genes, including those for growth factors, cell cycle regulators, extracellular matrix (ECM) proteins and genes associated with metastasis (104). In breast cancer, the pro-carcinogenic effect of leptin results not only from an enhanced activity of signalling pathways involved in proliferation process but also from a probable down-regulation of the apoptotic response (24). Readers can refer to more exhaustive reviews available for clarifying a number of leptin signalling features only partially discussed here (24, 33, 34, 103).

4.4. Integration between cAMP and leptin signaling pathways in triple negative breast cancer cells

Recently, we have found that exposure of triple negative human breast cancer cell line MDA-MB-231, a well-established model system of highly invasive breast cancer, to leptin resulted in a stimulation of growth and in a strong phosphorylation of both ERK1/2 and STAT3 proteins (36, 37), in agreement with previous studies in various breast cancer models (17, 18).

Surprisingly, we demonstrated that in MDA-MB-231 cells, intracellular cAMP elevation completely abrogated both ERK1/2 and STAT3 phosphorylation in response to leptin and provided evidence that leptin, when cAMP levels are increased, drives cells towards apoptosis accompanied by a marked down-regulation of Bcl2 protein levels (36).

The extracellular-signal-regulated kinase (ERK)-dependent signaling pathway is relevant to breast cancer, and several studies demonstrate it is frequently activated (105). A variety of extracellular stimuli other than growth factors of the EGF gene family, including ligands of G protein coupled receptors (GPCRs) and of the cytokine receptor family, as well as estradiol, progesterone and androgens affect Ras/Raf/ERK signaling cascade (83, 84, 106-108). Indeed the occurrence of network between multiple signal transduction pathways and its importance in the control of proliferation, including that of breast cancer cells is largely known. These signaling connections play an important role in cancer biology and a combined blockade of such signaling pathways is considered a relevant strategy for therapeutic intervention.(109).

Proliferative signal transmission through the Ras/Raf/ERK pathway is blocked by the elevation of cellular cAMP levels mainly via PKA-mediated Raf inhibition (62). Accordingly, we found that exposure of MDA-MB-231 breast cancer cells to cAMP elevating compounds resulted in a rapid time-dependent decrease of basal ERK1/2 activity that was almost completely abrogated after 6 hours, whereas CREB protein, a major substrate of PKA, was strongly phosphorylated in response to cAMP elevation. Importantly, we also found that exposure of cells to leptin plus cAMP elevating agents resulted in a dramatic decrease of ERK1/2 phosphorylation compared to leptin alone. In other terms in the presence of cAMP elevation, irrespective of cAMP elevating agent used, leptin lacks to trigger ERK1/2 activation (36, 37).

Multiple lines of evidence place Signal Transducer and Activator of Transcription 3 (STAT3) at a central node in the development, progression, and maintenance of many human tumors, including breast

cancer and STAT3 has been validated as an anticancer target in several contexts (110). STAT3 modulates the transcription of responsive genes involved in the regulation of a variety of critical functions, including cell differentiation, proliferation, apoptosis, angiogenesis, metastasis, and immune responses (111).

Cellular transformation by the viral oncogene v-src requires activated STAT3, and the transfection and expression of a constitutively activated form of STAT3 is sufficient to transform immortalized fibroblasts and normal epithelial cells derived from breast and prostate tissue. Most of the major human malignancies, including breast cancer, manifest elevated levels of constitutively activated STAT3 as well as transcriptional profiles that are consistent with STAT3-regulated gene expression. For many cancers, elevated levels of activated STAT3 have been associated with a poor prognosis. STAT3-activated genes block apoptosis, favor cell proliferation and survival, promote angiogenesis and metastasis, and inhibit antitumor immune responses. Importantly, approaches that disrupt STAT3 signaling lead to growth inhibition and apoptosis in tumor cell lines and can impair tumor growth in mouse xenograft breast cancer model, as well as in other cancer models. Although knockout of STAT3 leads to embryonic lethality in mice, the cumulative data in postnatal mice (conditional knockouts) indicates that STAT3 may be dispensable for the function of normal cells and tissues. The ability of nontransformed cells to withstand inhibition of STAT3 signaling suggests that a potential anticancer drug targeting STAT3 might be well tolerated. Recently, approaches that have been pursued to target STAT3 for developing anticancer drugs that might therapeutically inhibit the STAT3 signaling pathway have been straighforwardly reviewed (112).

Leptin-induced STAT3 activation is primarily involved in induction of proliferation of triple negative breast cancer cells, as well as in their migration and invasion (113-115).

Importantly, we found that exposure of MDA-MB-231 cells to leptin plus cAMP elevating agents resulted in a dramatic decrease of STAT3 phosphorylation compared to leptin alone (36, 37). In other terms in the presence of cAMP elevation, irrespective of cAMP elevating agent used, leptin lacks to trigger STAT3 activation. Moreover, we also reported that the large inhibition triggered by cAMP elevation of ERK1/2 and STAT3 phosphorylation in response to leptin is mediated by PKA (37). Interestingly, our findings are in agreement with the evidence that the expression of SOCS3 can also be upregulated in a STAT3-independent mechanism that involves elevated intracellular cAMP levels (116-118). For example, G protein–coupled receptors (GPCRs) that signal through Gs proteins (e.g., PGE2, β adrenergic, and prostacyclin receptors) produce increased levels of cAMP, which activate PKA and trigger the accumulation of SOCS3, thereby inhibiting interleukin-(IL)-6–induced STAT3 activation (116-117).

SOCS proteins behave as classical feedback inhibitors, induced by the cytokine-mediated activation of the JAK/STAT pathway that they function to inhibit (119, 120). Furthermore, the transcription of SOCS3 is activated mainly by STAT3. SOCS3 upregulation, either by STAT3 or STAT3-independent ways, can block STAT3 signaling by any of three mechanisms: by direct binding and inhibition of JAKs, by competing with STAT3 for pY-binding sites on activated receptor chains, or by binding signaling proteins and targeting them for proteasomal degradation (112).

Importantly, whatever the exact mechanism, we found that cAMP elevation prevents leptin-induced STAT3 activation and that PKA inhibition completely counteracts the effects of cAMP elevation (36, 37). The role of SOCS3 in inhibiting STAT3 function in response to cAMP/PKA activation is actually under our investigation (Figure 1).

By the way, the lack of leptin-triggered ERK1/2 and STAT3 activation upon cAMP elevation was followed by a strong lowering of protein levels of both regulatory RIα and catalytic subunits of protein kinase A, and by a consistent CREB phosphorylation reduction. Remarkably, all above effects, including enhancement of the antiproliferative action of cAMP elevating agents by leptin, were prevented by KT5720 and/or H89 PKA inhibitors (37).

In the aggregate, our studies indicate that upon cAMP elevation leptin lacks to trigger ERK1/2 and STAT3 activation, signals differently downstream and triggers a proteasome-mediated RIα protein levels down-regulation. These early events required PKA activity. Then, RIα down-regulation leads to PKA calalytic subunits down-regulation, too. The resulting down-regulation of PKA function causes a CREB phosphorylation decrease, followed by change in expression of CREB-regulated genes, including Bcl2, and apoptosis induction (36, 37).

Currently, we are accumulating evidence that cAMP elevation modify behaviours relative to invasiveness attitudes of MDA-MB-231 triple-negative breast cancer cells in response to leptin, too (manuscript in preparation).

5. SUMMARY AND PERSPECTIVES

Triple-negative breast cancers (TNBC) are characterised by an aggressive phenotype and have been associated with poor prognosis. Triple-negative breast cancer patients are unresponsive to current targeted therapies and other treatment options are only partially effective. Therefore, new pharmacological approaches are needed. Importantly, the costs of therapies and their economic impact are increasingly becoming more relevant for National Health Services.

Due to the heterogeneity of triple-negative tumours and the complexity of the molecular pathways involved in their development and progression, it is postulated that the optimal therapeutic concept for TNBC will eventually comprise a combination approach of cytotoxic and targeted agents in an individual tailored pattern.

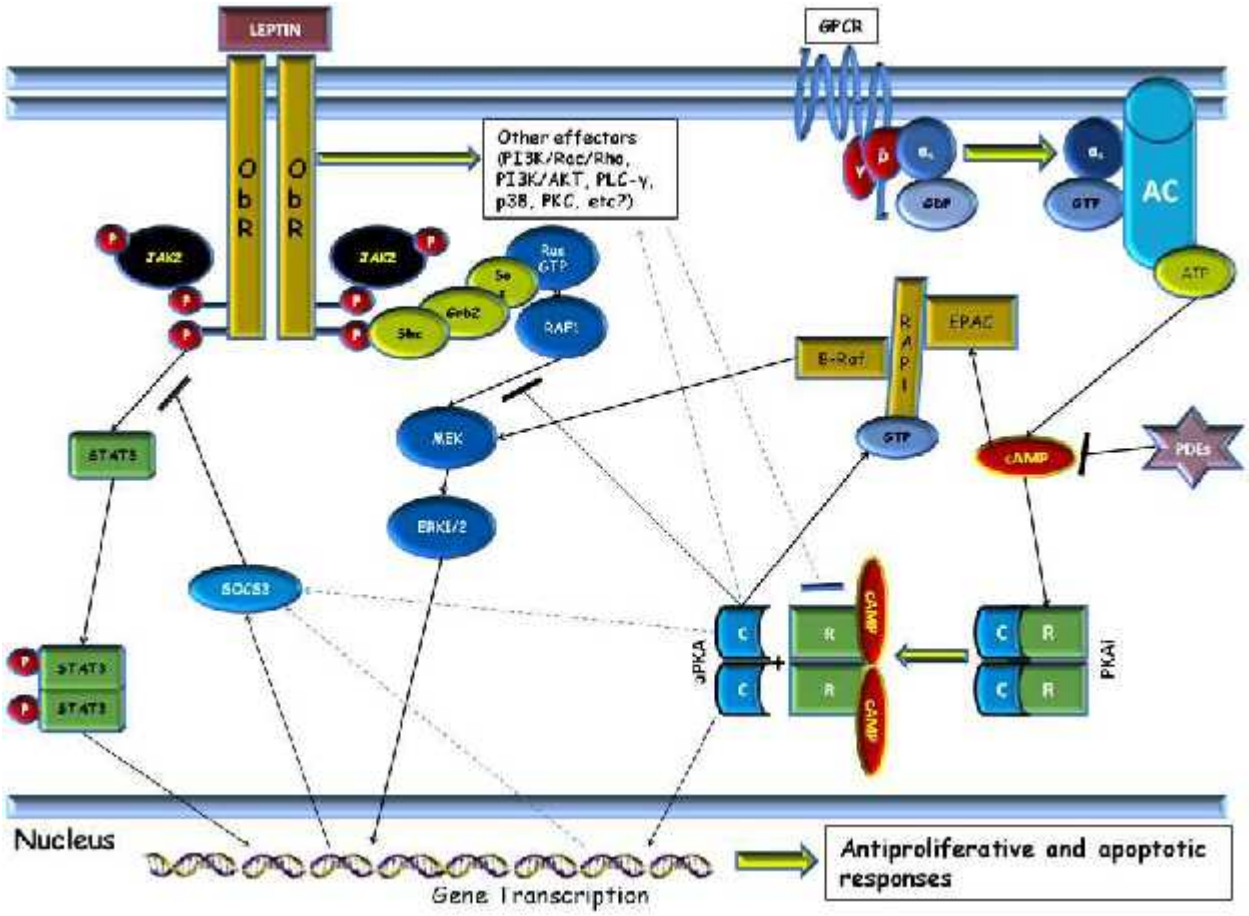

Figure 1. Cross-talk between leptin and cAMP signalling pathways in triple-negative MDA-MB-231 breast cancer cells. Leptin binding to ObR results in conformational changes and receptor oligomerization. These events stimulate tyrosine phosphorylation and activation of JAK2 that is constitutively associated with the receptor. JAK2 phosphorylates the intracellular domain of ObR, especially tyrosines within the SHP2 and STAT3 binding sites. Recruitment of SHP2 leads to its tyrosine phosphorylation, binding to GRB2 and activation of the guanine nucleotide exchange factor Sos and in turn the small GTP-binding protein, Ras; recruitment of Raf-1 to the plasma membrane by Ras leads to its activation triggering ERK1/2 cascade and proliferation. In parallel, binding of STAT3 to ObR induces STAT3 tyrosine phosphorylation, dimerization, nuclear translocation, and induction of target genes. These include suppressor of cytokine signalling 3, socs3. Induction of JAK2 can also stimulate PI-3K. Activation of PI-3K can stimulate the major growth/survival pathway Akt. ObR could activate other effectors, such as Rac/Rho, PLC-gamma, PKC and p38 kinases. Binding of an extracellular ligand to a transmembrane receptor (G protein-coupled receptor, GPCR) alters the conformation of the associated heterotrimeric G protein, causing dissociation of the Gα and Gβγ subunits and initiating a cascade of cellular events. The subunit Gαs activates the adenylate cyclase enzyme which converts ATP into cAMP. cAMP activates PKA binding to the regulatory (R) subunits, which causes a conformational change that releases the active catalytic (C) subunits. The catalytic subunits of PKA phosphorylate proteins at specific Ser or Thr side chains such as the transcription factors of cAMP response element binding (CREB) family. PKA represents the main intracellular effector of cAMP signaling. However, cAMP can also activate guanine nucleotide-exchange factors (GEFs) that are known as exchange proteins activated by cAMP (EPACs). When bound to cAMP, EPAC catalyses the exchange of GDP for GTP on Rap1, a small molecular weight GTPase of the Ras family. The activated form of Rap-GTP is then capable of activates the kinase B-Raf promoting MAPK cascades. Alternatively, PKA may directly activate Rap-1, sequestering Raf1 and preventing its activation by Ras. On the other hand, phosphorylation on distinct sites within Raf1 by PKA can down-regulate Raf1 activity either by direct inhibition or impairing its interaction with Ras. Finally, signal-termination enzymes such as phosphodiesterases (PDEs) degrade cAMP and limit PKA activation.The role of SOCS3 in inhibiting STAT3 function in response to cAMP/PKA activation is possible and is actually under our investigation. Upon cAMP elevation leptin lacks to trigger ERK1/2 and STAT3 activation, signals differently downstream and triggers a proteasome-mediated RIα protein levels down-regulation. These early events required PKA activity and occur through yet unclear mechanisms. Then, RIα down-regulation leads to PKA calalytic subunits down-regulation, too. The resulting down-regulation of PKA function causes a CREB phosphorylation decrease, followed by change in expression of CREB-regulated genes, including Bcl2, and apoptosis induction.

Combination chemotherapy has received more attention in order to find compounds that could increase the therapeutic index of clinical anticancer drugs while limiting their potential toxicity.

At this regard, naturally occurring molecules with no or the least toxicity to normal tissues and able to reduce the dosage of "cheap" drugs required to obtain antitumor effects are suggested as very attractive candidates to be investigated.

In this context, further positive results from *in vivo* studies could indicate that the integration between leptin and cAMP signalling pathways might provide the rationale for the development of a simple, innovative and cheap way for therapeutic intervention in triple negative breast cancer, and potentially in other tumors, implying the use of cAMP elevating drugs at lower and more tolerable doses.

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Abbreviations: cAMP, 3'-5'-cyclic adenosine monophosphate, PKA, Protein Kinase A, ERK, extracellular signal-regulated kinases, MAPK, mitogen activated protein kinases; MEK-1, mitogen-activated kinase kinase, Rap1, Ras-associated protein-1, Epac, exchange proteins activated by cAMP, SR, steroid receptor; ER, estradiol receptor, PgR, progesterone receptor; AR, androgen receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PI3-K, phosphatidylinositol-3-kinase; GPCRs, G protein coupled receptors; PKC, protein kinase C; suppressor of cytokine signalling 3, SOCS3.

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Key Words: Breast Cancer, STAT3, Novel Anticancer Therapy, cAMP elevating agents, SOCS3

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