β-arrestin-1 mediates the endothelin-1-induced activation of Akt and integrin-linked kinase[1]

Roberta Cianfrocca, Laura Rosanò, Francesca Spinella, Valeriana Di Castro, Pier Giorgio Natali, and Anna Bagnato

Abstract: The contribution of the endothelin-1 (ET-1)/ET A receptor (ET_AR) axis in tumor growth and progression is investigated in many tumor types, including ovarian carcinoma. In ovarian cancer cells, ET-1 acts as an autocrine growth factor selectively through the ET_AR triggering the concomitant activation of multiple pathways. In these cells, the involvement of β-arrestin-1 as signal transducer in ET-1-dependent signalling pathways has been recently highlighted. Because several G protein-coupled receptors have been shown to activate signalling pathways in a β-arrestin-dependent manner, in this study we explored whether β-arrestin-1 is involved in a distinct signalling mechanism linking the ET_AR to phosphoinositide 3-kinase (PI3K)/integrin-linked kinase (ILK)/Akt in HEY ovarian cancer cells. The inhibitory effects of ZD4054 (zibotentan), a specific ET_AR antagonist, in ET-1-dependent phosphorylation of ILK, Akt, and glycogen synthase kinase (GSK-3β) demonstrated the involvement of the ET_AR in these effects. By using a kinase assay, we demonstrate that β-arrestin-1 silencing inhibits the ET-1-induced ILK activity in a time-dependent manner and downstream Akt and GSK-3β phosphorylation. These results reveal that β-arrestin-1 is implicated as an ET_AR-transducer in the activation of ILK and Akt and in the inactivation of GSK-3β in response to ET-1 and further support the role of β-arrestin-1 as a multifunctional adaptor facilitating interprotein interactions critically involved in ET_AR-mediated signalling that regulate invasive and metastatic behaviour of ovarian cancer.

Key words: ovarian cancer, ET-1, ET_A receptor, β-arrestin-1, ILK, Akt.

Résumé : Le rôle de l'axe endothéline-1/récepteur A de l'ET (RET_A) dans la croissance et la progression des tumeurs a été examiné dans de nombreux types de tumeurs, y inclus le carcinome ovarien. Dans les cellules de cancer ovarien, l'ET-1 agit comme un facteur de croissance autocrine, par l'intermédiaire du RET_A, pour déclencher l'activation concomitante de multiples voies. De récents travaux ont mis en évidence l'implication de la β-arrestine-1 comme transducteur de signal dans les voies de signalisation dépendantes de l'ET-1. Comme il a été démontré que plusieurs récepteurs couplés aux protéines G activent les voies de signalisation d'une manière dépendante des β-arrestines, notre étude a eu pour but d'examiner si la β-arrestine-1 est impliquée dans un mécanisme de signalisation distinct liant le RET_A à la voie phosphoinositide 3-kinase (PI3K)/kinase liée aux intégrines (ILK)/Akt dans les cellules ovariennes HEY. Les effets inhibiteurs du ZD4054 (zibotan), un antagoniste spécifique du RET_A, dans la phosphorylation ET-1 dépendante de l'ILK, de l'Akt et de la glycogène synthase kinase (GSK-3β) ont mis en lumière l'implication du RET_A. Nous démontrons, à l'aide d'un essai kinase, que l'inactivation de la β-arrestine-1 inhibe l'activité de l'ILK induite par l'ET-1 d'une manière dépendante du temps et en aval de la phosphorylation de GSK-3β et de l'Akt. Ces résultats indiquent que la β-arrestine-1 intervient en tant que transducteur du RET_A dans l'activation de l'ILK, de l'Akt et dans l'inactivation de la GSK-3β, en réponse à l'ET-1. De plus, ils confortent le rôle de la β-arrestine-1 comme adaptateur multifonctionnel facilitant les interactions interprotéines impliquées dans la signalisation véhiculée par le RET_A qui régulent le comportement invasif et métastatique du cancer ovarien.

Mots-clés : cancer ovarien, ET-1, récepteur ET_A, β-arrestine-1, ILK, Akt.

[Traduit par la Rédaction]

Introduction

Elevated endothelin-1 (ET-1) levels are commonly observed in human ovarian tumors (Bagnato and Rosanò 2008). In this tumor histotype, ET-1 manifests its biological activities by binding to the ET A receptor (ET_AR), a G protein-coupled receptor (GPCR) (Levin 1995; Masaki 2000). Studies in ovarian cancer models by using gain/loss of function approaches and (or) receptor antagonists have indicated the role of the ET-1/ET_AR axis in tumor development and progression by the activation of multiple signalling path-

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R. Cianfrocca, L. Rosanò,[2] F. Spinella, V. Di Castro, P.G. Natali, and A. Bagnato. Laboratory of Molecular Pathology, Regina Elena Cancer Institute, via Delle Messi D'Oro 156, Rome, Italy.

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[2]Corresponding author (e-mail: rosano@ifo.it).

ways (Bagnato et al. 2005, 2008). To dissect the molecular mechanisms occurring after ET_AR activation, we focused our attention on the cytosolic adaptor protein β-arrestins (Lefkowitz and Shenoy 2005). β-arrestin-1 and -2 were originally identified as signal terminators for GPCR signalling. Their binding to the receptor sterically inhibits receptor coupling to G proteins leading to inactivation of effectors such as second messenger generating enzymes (Lefkowitz and Shenoy 2005). Besides this classical function, evidence recently accumulated has revealed novel functions of β-arrestins as signal transducers in various signalling pathways (Lefkowitz and Shenoy 2005; Barki-Harrington and Rockman 2008). Among different GPCRs, it has been previously shown that β-arrestins are involved in the regulation of the ET-1 receptors, in terms of internalization and intracellular trafficking pathways, demonstrating that agonist-activated ET_AR is able to recruit, with different affinities, both β-arrestin-1 and -2 to the plasma membrane (Bremnes et al. 2000; Oakley et al. 2000). Moreover, ET-1 via the ET_AR forms a molecular complex with the Src family tyrosine kinase Yes and β-arrestin-1 in adipocytes (Imamura et al. 2001). In this context, our recent work demonstrates that the ET_AR–β-arrestin complexes can also contribute to receptor-mediated signalling, including the activation of Src, epidermal growth factor receptor (EGFR), and mitogen activated protein kinase (MAPK) (Rosanò et al. 2009; Rosanò and Bagnato 2009). Indeed, we show that upon ET-1 stimulation of ET_AR, β-arrestin is recruited to the membrane, where it can functionally interact with the ET_AR and Src, resulting in the activation of Src and downstream EGFR transactivation. This promotes tyrosine phosphorylation of β-catenin, thereby promoting its stabilization. In a parallel way, the ET_AR–β-arrestin complex binds directly to Axin contributing to destabilization of the degradation complex resulting in the stabilization of β-catenin. Ultimately, stabilized β-catenin translocates into the nucleus to form a nuclear complex β-catenin–TCF-4 that, in turn, increases its transcriptional activity, cell invasion, and metastatic activities (Rosanò et al. 2009). In this regard, we previously demonstrated that, in ovarian cancer cells, the ET-1/ET_AR autocrine pathway drives epithelial-to-mesenchymal transition (EMT), by inducing a fibroblastoid and invasive phenotype. In particular, activation of ET_AR, by mimicking the canonical Wnt signalling, triggers a signalling pathway mediated by integrin-linked kinase (ILK), an intracellular protein serine/threonine kinase that coordinates signalling elicited by integrins and growth factors. ILK activity induced by ET-1 mediates the signalling of 2 downstream kinases, Akt and glycogen synthase kinase (GSS-3β), leading to Snail and β-catenin stabilization and transcriptional programs related to EMT (Rosanò et al. 2005). Thus, ET_AR-mediated activation of PI3K/ILK/Akt contributes to promote β-catenin stabilization through inactivation of GSK-3β (Rosanò et al. 2005, 2006).

Because β-arrestin-1 is reported to exert a functional role in ET-1-dependent multiple signalling pathways including p42/44 MAPK and Akt activation, in this study we examined the possible involvement of β-arrestin in ET-1-induced ILK activity and downstream substrates Akt and GSK-3β through the ET_AR.

Materials and methods

Cell culture and siRNA transfection experiments

Human ovarian carcinoma cell line HEY, generously gifted by Giovanni Scambia (Catholic University School of Medicine, Rome, Italy), previously characterized for ET-1 receptor expression and for ET-1 production (Bagnato et al. 1995, 1999; Rosanò et al. 2005), was cultured in RPMI containing 10% fetal calf serum (FCS) and 1% penicillin–streptomycin at 37 °C under 5% CO_2 – 95% air. All culture reagents were from Invitrogen (Paisley, Scotland, United Kingdom). The cells were serum starved by incubation for 24 h in serum-free RPMI and then treated with 100 nmol/L ET-1 (Peninsula Laboratories, Belmont, Calif.) for different times (i.e., 5, 15, 30 min). Clinical grade zibotentan, ZD4054, kindly provided by AstraZeneca (Macclesfield, UK), was added 15 min before the agonist.

For the silencing of β-arrestin-1, cells were transiently transfected with duplex siRNAs (30 nmol/L) targeting human β-arrestin-1 (Hs_ARRB1_11 HP validated siRNA; Qiagen, Milan, Italy) or the negative control (scrambled sequence), using RNAiFect transfection reagent (Qiagen). The specificity of the siRNA sequences for β-arrestin-1 has been previously validated (Ahn et al. 2003). After 48 h of incubation, cells were divided into 6-well plates for further experiments and for β-arrestin immunoblotting. Each knock-down experiment described herein was monitored for reduced expression of β-arrestin-1 (75%–90%) with anti-β-arrestin-1 (Santa Cruz Biotechology Inc., Heidelberg, Germany).

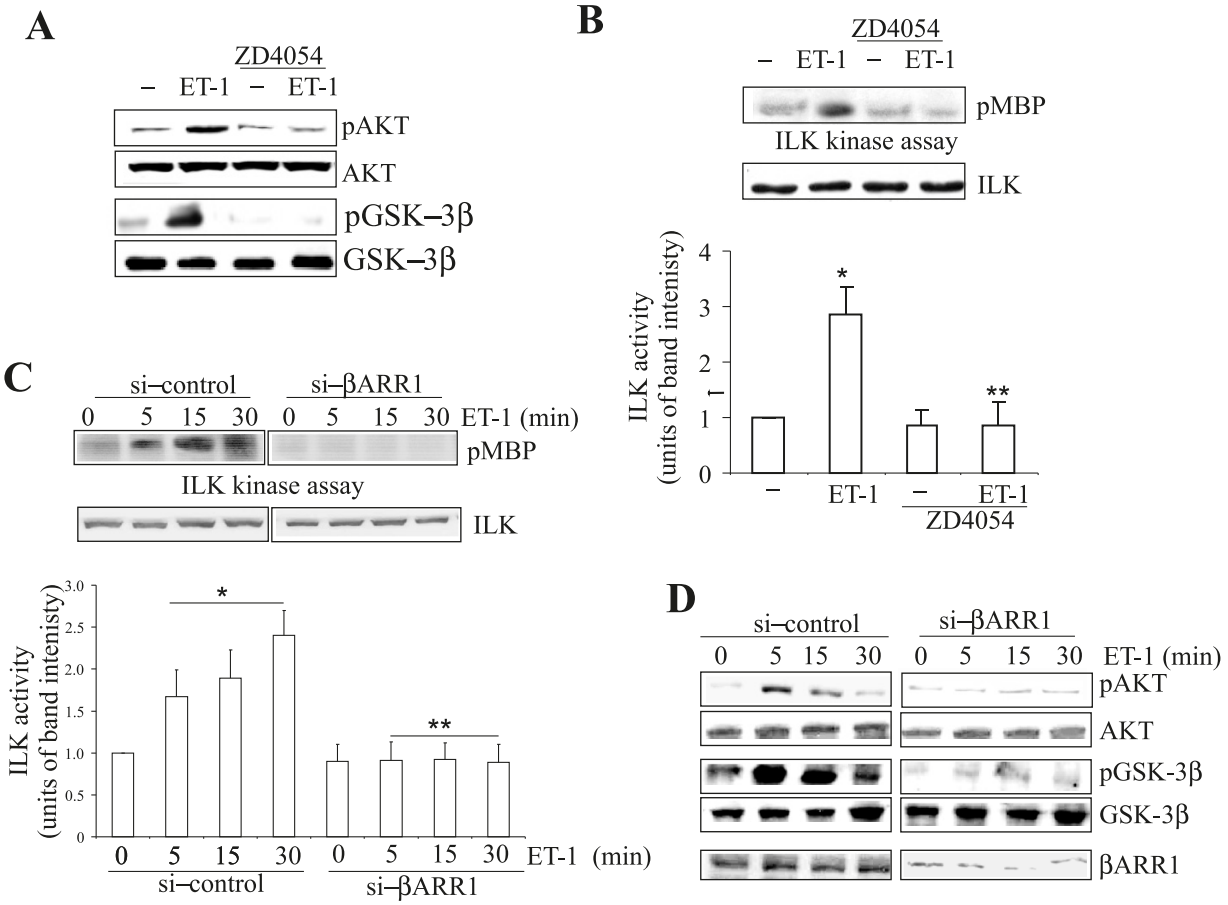
ILK immune complex kinase assay

Cell lysates (0.25 to 1.0 mg of protein) were immunoprecipitated with 1 μg of affinity purified rabbit anti-ILK (Millipore, Billerica, Mass.) overnight at 4 °C with rotation. Protein A – Sepharose (Sigma–Aldrich, St. Louis, Mo.), preswollen in NP-40 lysis buffer (150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 50 mmol/L HEPES (pH 7.4), 1 μg leupeptin/mL, 1 μg aprotinin/mL, and 3 mmol/L phenylmethylsulfonyl fluoride) was added for 2 h at 4 °C to capture the antibodies. After 2 washes with NP-40 lysis buffer and 2 washes with kinase wash buffer (10 mmol/L $MgCl_2$, 10 mmol/L $MnCl_2$, 50 mmol/L HEPES (pH 7.5), 0.1 mmol/L sodium orthovanadate, and 1 mmol/L dithiothreitol), assays were performed directly on the protein A beads in a 25 μL reaction volume containing 10 mmol/L $MgCl_2$, 10 mmol/L $MnCl_2$, 50 mmol/L HEPES (pH 7.5), 0.1 mmol/L sodium orthovanadate, 2 mmol/L sodium fluoride, 5 μCi of γ-[^{32}P] (Amersham Pharmacia Biotech, Buckinghamshire, UK), and 2.5 μg of myelin basic protein (MBP) as substrate (Upstate). Incubation was for 30 min at 30 °C. The reaction was stopped with 10 μL of sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) nonreducing stop buffer and heated for 5 min at 95 °C. Phosphorylated MBP bands were visualized by autoradiography of dried SDS – 10% PAGE gels. Autoradiograms were scanned and quantified using NIH image program (Scion, Frederick, Md.). The results show the mean ± SD of 3 experiments.

Immunoblotting

Total cell lysates were subjected to SDS–PAGE and processed by immunoblotting using antibodies specific to

Fig. 1. (A) ET A receptor (ET_AR) blockade by ZD4054 inhibits the endothelin-1 (ET-1)-induced glycogen synthase kinase 3β (GSK-3β) and Akt activation. Serum-starved HEY cells were treated with ET-1 (100 nmol/L) for 30 min and (or) ZD4054 (1 μmol/L). Lysates were immunoblotted for phosphorylated GSK-3β (Ser-9) (pGSK-3β) and total GSK-3β and for phosphorylated Akt (Ser-473) (pAkt) and total Akt. (B) ZD4054 inhibits ET-1-induced integrin-linked kinase (ILK) activity. ILK activity was indicated by the amount of ^{32}[P] labelling of myelin basic protein (pMBP) of the immunoprecipitates with anti-ILK of HEY cell lysates, treated as in (A). Cell lysates were also immunoblotted for ILK expression. Histograms represent the average band intensity of ILK activity from 3 independent kinase assays. *, $p < 0.01$ compared with untreated cells and **, $p < 0.05$ compared with ET-1. (C) ET-1 promotes ILK activity through β-arrestin-1. Serum-starved HEY cells, transfected with scrambled (si-control) or β-arrestin-1 siRNA (si-β-ARR1), were treated with ET-1 (100 nmol/L) for the indicated times. ILK activity was indicated by the amount of ^{32}[P] labeling of pMBP of the immunoprecipitates with anti-ILK. Cell lysates were also immunoblotted for ILK expression. Histograms represent the average band intensity of ILK activity from 3 independent kinase assays. *, $p < 0.01$ compared with untreated cells and **, $p < 0.05$ compared with ET-1. (D) ET-1 induces time-dependent phosphorylation of GSK-3β and Akt through β-arrestin-1. Lysates of HEY cells, treated as in (C), were immunoblotted for pGSK-3β, GSK-3β, pAkt, and Akt. Lysates were also analyzed by immunoblotting to evaluate β-arrestin-1 expression in cells transfected with scrambled siRNA (control) or with specific siRNA.



phospho-GSK-3β (pSer-9), GSK-3β, phospho-Akt (Ser-473), Akt (Cell Signaling, Beverly, Mass.), ILK (Upstate), and β-arrestin-1 (Santa Cruz Biotechology Inc.). The proteins were visualized by ECL (Amersham Pharmacia Biotech).

Statistical analysis

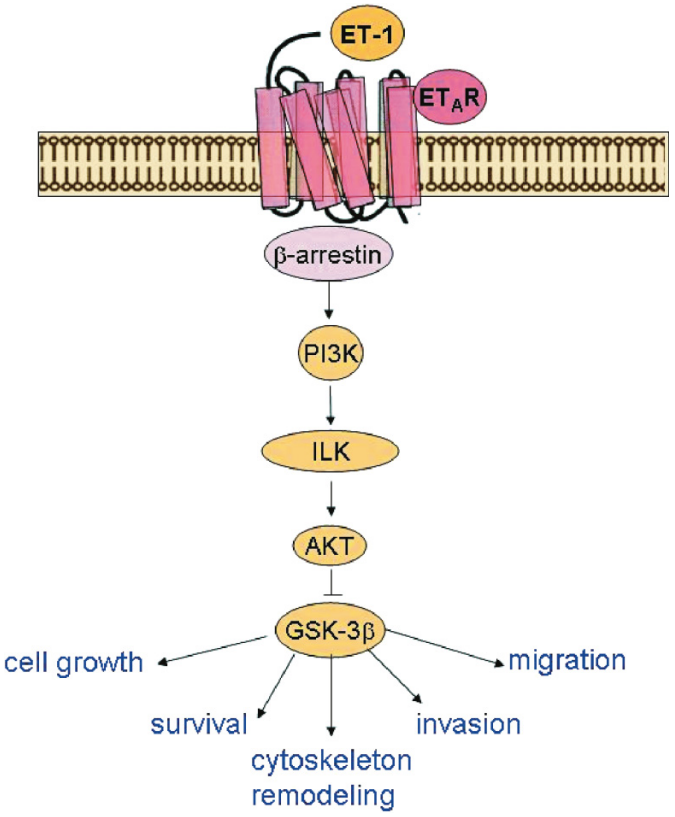
Results are representative of at least 3 independent experiments with each performed in triplicate. The statistical analysis was assessed using a 2-tailed Student's *t* test. A probability value of <0.05 was considered statistically significant. All statistical analyses were performed using commercial software (SPSS 11, Chicago, Ill.).

Results

ET-1/ET_AR induces β-arrestin-1-dependent ILK activity leading to Akt and GSK-3β phosphorylation

To explore whether β-arrestin-1 is involved in a distinct signalling mechanism linking the ET_AR to the PI3K/ILK/Akt pathway in ovarian cancer cells, we first analyzed the activation status of these signalling proteins upon ET-1 stimulus and the ability of a specific ET_AR antagonist, ZD4054, to control these signalling pathways. Immunoblotting with phosphospecific antibodies demonstrated that the ET-1-dependent phosphorylation of Akt at Ser-473 and GSK-3β at Ser-9 were inhibited upon treatment with ZD4054

Fig. 2. A diagram of the signalling pathway activated by the ET-1/ET_AR axis through β-arrestin-1. Binding of ET-1 to ET_AR in ovarian cancer leads to the recruitment of β-arrestin to the activated receptor, which mediates the ET_AR-activated PI3K–ILK–Akt signalling route, causing the phosphorylation and inactivation of GSK-3β, thereby promoting cell growth, survival, cytoskeleton remodelling, invasion, and migration.



(Fig. 1A). In a similar way, by using an immunocomplex kinase assay, we observed that the ET-1-dependent activation of ILK, which reached about a 3-fold increase compared with the control as shown by densitometric analysis of the autoradiograms, was blocked upon treatment with ZD4054, demonstrating that these events are mediated by ET_AR (Fig. 1B).

To evaluate the potential involvement of β-arrestin-1 in ET-1-induced ILK activity, we examined the effects of siRNA-mediated β-arrestin-1 knock-down. The ET-1-dependent ILK activation, starting at 5 min and reaching an approximate 2.5-fold increase at 30 min of treatment, was completely inhibited upon silencing of β-arrestin-1, demonstrating an essential role of β-arrestin-1 in ET-1-induced ILK activity (Fig. 1C). Specificity of siRNA oligos for β-arrestin-1 was previously confirmed (Rosanò et al. 2009) and the Western blot analysis showed an 80% knock-down of β-arrestin-1 (Fig. 1D). On the other hand, the kinetic pattern of ET-1-stimulated phosphorylation of Akt and GSK-3β was inhibited after transfection with β-arrestin-1 siRNA that paralleled the inhibition of ILK activation (Fig. 1D). These results collectively indicate that activation of the ET_AR by ET-1 promotes ILK-dependent phosphorylation of its downstream targets, GSK-3β and Akt through β-arrestin-1, that may lead to the activation of multiple activities, including the stability of β-catenin, in ovarian cancer cells.

Discussion

The present study offers interesting findings related to the involvement of β-arrestin-1 in response to ET-1 in ovarian cancer, supporting the view that β-arrestin-1 acts as both a scaffolding and signalling transducer in ET-1-mediated signalling. We demonstrate that ET-1-induced ILK phosphorylation requires the recruitment of β-arrestin-1 to the activated ET_AR, resulting in the phosphorylation of Akt and GSK-3β.

The ET_AR remains a key signalling node that regulates the control of cell proliferation, survival, invasion, and metastasis in ovarian cancer cells through a variety of downstream targets (Bagnato and Rosanò 2008). Thus, the knowledge of the exact molecular mechanism by which signals from this receptor are regulated in space and time is critical for understanding the interactions between signalling cascades and for developing new pharmacological approaches for controlling ET_AR-mediated responses. Although several proteins have been identified, we recently focused our attention on a new emerging paradigm in which β-arrestin directly initiates signalling through a variety of cellular pathways (Lefkowitz and Shenoy 2005). β-arrestin-mediated signalling usually requires the formation of multiprotein signalling complexes, known as a signalosomes, in which β-arrestin-1 acts as a scaffold, adaptor, and signal transducer. In elucidating the role of β-arrestin-1 in ET-1 signalling, we recently found that once ET_AR is activated, a signalling pathway is mediated by β-arrestin involving the recruitment and activation of Src into a β-arrestin-scaffolded complex containing ET_AR (Rosanò et al. 2009). Thus, Src functions as a switch that activates EGFR to induce the phosphorylation of β-catenin promoting its accumulation. In addition to this complex, ET-1 mediates the formation of the ET_AR/β-arrestin-1/Axin signalling complex that regulates the stability of β-catenin by displacing GSK-3β from the degradation complex. Intracellular β-catenin accumulation results in its nuclear translocation, where it works as a cofactor for transcription factors of the TCF/LEF family, modulating the expression of a broad spectrum of target genes affecting invasive and metastatic behaviour of ovarian cancer cells. The results of this study support the model by which ET_AR activation presumably places β-arrestin-1 in close proximity to PI3K ensuring the activation of ILK that, in turn, promotes phosphorylation of Akt and GSK-3β, as shown by the inhibition of these phosphorylations after depletion of β-arrestin-1 (Fig. 2). Moreover, these findings highlight the importance of β-arrestin-1 in the dynamic changes of intracellular signalling dependent on ET-1/ET_AR activation, adding complexity to the signalling from the ET_AR that leads to the activation of PI3K/β-arrestin-1, src/β-arrestin, and Axin/Akt signalling pathways that concur to activate β-catenin and invasive cell behaviour. Although Src inhibition by PP2 affects Akt and GSK-3β phosphorylation following ET_AR activation (data not shown) indicating that Src activity is upstream of Akt and GSK-3β, the possibility of cross-talk between these pathways is still not completely elucidated.

Thus, a better understanding of how these pathways could interrelate, in terms of synergistic or convergent effects, regulating the intensity and duration of signalling activated in tumor progression should be better defined.

All our findings demonstrating that distinct pathways act in concert to control cell motility in ovarian cancer cells, highlight the previously unappreciated levels of complexity of β-arrestin-1 in cytoskeleton remodelling and in invasive cellular behaviour, in which multiple pathways are probably interlinked, serving slightly different and complementary purposes implicated in the fine tuning of tumor growth and progression.

Moreover, the knowledge of β-arrestin-1 in ET-1-mediated Akt signalling expands our understanding of the roles of β-arrestin in determining the outcomes of signalling initiated from the ET_AR and implicates the putative role of β-arrestin-1 in other ET_AR-dependent processes regulated by Akt signalling, such as apoptosis (Del Bufalo et al. 2002). In agreement with our results, prostaglandin E2, insulin-like growth factor-1 (IGF-1), α-thrombin, and β1-adrenergic receptors activate the Akt pathway through a β-arrestin-1 dependent mechanism (DeWire et al. 2007). Regarding its function in apoptosis, in mouse embryonic fibroblasts, deficiency of β-arrestin-1 and -2 leads to loss of Akt activation and anti-apoptotic effects following IGF-1 stimulation (Povsic et al. 2003). Recently, Ahn et al. (2009) demonstrated that β-arrestin-2 plays a crucial role in angiotensin II-stimulated anti-apoptotic responses and delineated the ERK-p90RSK and PI3K-Akt downstream biochemical pathways responsible for this β-arrestin-2-mediated cytoprotection from apoptotic challenge, providing us with a more comprehensive view of the molecular mechanisms used by cancer cells to grow and spread. The understanding of the interaction of ET_AR, β-arrestin, and other critical signalling molecules offers a better knowledge of how to treat ET_AR-overexpressing tumors that have a highly metastatic phenotype. In this context, these findings demonstrating the inhibitory effects of the selective ET_AR antagonist ZD4054 in the activation of β-arrestin-dependent signalling pathways, suggest that this treatment, by disabling different sets of biological interactions activated by ET_AR in a β-arrestin-dependent manner (Rosanò et al. 2009; Rosanò and Bagnato 2009), may represent an antimetastatic strategy.

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