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Cooperation of amphiregulin and insulin-like growth factor-1 inhibits Bax- and Bad-mediated apoptosis via a protein kinase C-dependent pathway in non-small cell lung cancer cells

Hurbin Amandine [1] , Coll Jean-Luc [1] , Dubrez-Daloz Laurence [2] , Mari Bernard [3] , Auberger Patrick [3] , Brambilla Christian [1] , Favrot Marie-Christine [1 *]

[1] *Groupe de Recherche Sur Le Cancer du Poumon : Bases Moléculaires de la Progression Tumorale, Dépistage et Thérapie Génique INSERM : U578, Institut Albert Bonniot, Rond Point de La Chantourne 38706 LA TRONCHE CEDEX,FR*

[2] *Mort cellulaire et cancer INSERM : U517, IFR100, Université de Bourgogne, Faculte de Medecine 7, Boulevard Jeanne D'Arc 21079 DIJON CEDEX,FR*

[3] *Physiopathologie de la survie et de la mort cellulaire et infection virale INSERM : U526, IFR50, Université de Nice Sophia-Antipolis, Faculte de Médecine 28, Avenue de Valombrose 06107 NICE CEDEX 2,FR*

* Correspondence should be adressed to: Marie-Christine Favrot <MCFavrot@chu-grenoble.fr>

Abstract

Amphiregulin (AR) and insulin-like growth factor-1 (IGF1) are growth factors known to promote non-small cell lung cancer (NSCLC) survival. We have previously published that 1) AR and IGF1, secreted by H358 NSCLC cells, cooperate to protect those cells and H322 NSCLC cells from serum-starved apoptosis; 2) H358 cells resist to Bax-induced apoptosis through an inhibition of Bax conformational change. We show here that the anti-apoptotic activity of AR/IGF1 combination is specifically abolished by the PKC inhibitors calphostin C and staurosporine, but not by the MAPK and PI_3K inhibitors PD98059 and wortmannin, suggesting the involvement of a PKC-dependent, MAPK- and PI_3K-independent survival pathway. The PKC inhibitor rottlerin restores apoptosis induced by serum deprivation. In addition, phosphorylation of PKC and PKC/, but not of $PKC/_{II}$, increases in serum-starved H358 cells and in H322 cells treated with AR/IGF1 combination and is blocked by calphostin C. Combination of AR and IGF1 increases $p90^{Rsk}$ and Bad phosphorylation as well as it inhibits the conformational change of Bax by a PKC-dependent mechanism. Finally, PKC, PKC or $p90^{Rsk}$ siRNAs block the anti-apoptotic activity of AR/IGF1 combination but have no effect on partial apoptosis inhibition observed with each factor used alone. Constitutively active PKC expression inhibits serum deprivation-induced apoptosis, whereas a catalytically inactive form of $p90^{Rsk}$ restores it. Thus, AR and IGF1 cooperate to prevent apoptosis by activating a specific PKC-$p90^{Rsk}$-dependent pathway, which leads to Bad and Bax inactivation. This signalling pathway is different to that used by single factor.

MESH Keywords 1-Phosphatidylinositol 3-Kinase ; antagonists & inhibitors ; Androstadienes ; pharmacology ; Apoptosis ; drug effects ; physiology ; Carcinoma, Non-Small-Cell Lung ; pathology ; physiopathology ; Carrier Proteins ; antagonists & inhibitors ; physiology ; Cell Line, Tumor ; Culture Media, Serum-Free ; Enzyme Inhibitors ; pharmacology ; Flavonoids ; pharmacology ; Glycoproteins ; physiology ; Humans ; Insulin-Like Growth Factor I ; physiology ; Intercellular Signaling Peptides and Proteins ; physiology ; Isoenzymes ; antagonists & inhibitors ; physiology ; Lung Neoplasms ; pathology ; physiopathology ; MAP Kinase Signaling System ; drug effects ; Models, Biological ; Naphthalenes ; pharmacology ; Protein Kinase C ; antagonists & inhibitors ; physiology ; Proto-Oncogene Proteins c-bcl-2 ; antagonists & inhibitors ; physiology ; Signal Transduction ; drug effects ; Staurosporine ; pharmacology ; bcl-2-Associated X Protein ; bcl-Associated Death Protein

In non small cell lung cancer (NSCLC), growth factors that bind to tyrosine kinase receptors, have been characterized for their ability to inhibit apoptosis and promote cell survival through autocrine/paracrine loops (1). They represent promising targets for new therapeutic strategies as illustrated by the clinical development of epidermal growth factor (EGF) receptor signaling pathway inhibitors and they might be an alternative to conventional chemotherapy which fails to improve survival in most patients with NSCLC. A significant percentage of tumorigenic human lung epithelial cells overexpress amphiregulin (AR) and EGF-related cytokines (2) as well as members of the erbB receptor family, in particular the EGF receptor (erbB-1/HER1) that binds AR, and erbB-2/neu (HER2) (3,4). Such overexpression has been associated with shortened survival in patients with NSCLC (3–6). Type 1 insulin like-growth factor (IGF1) receptor is also overexpressed in NSCLC (7); it is a major survival factor that protects cells from apoptosis induced by number of stimulus (8), including serum withdrawal (9,10). Moreover, we have previously shown, on two NSCLC cell lines H322 and H358 that 1) H358 cells secrete AR and IGF1 that cooperate to inhibit apoptosis induced by serum deprivation in an autocrine manner (11). Those two factors also protect H322 cells from apoptosis in a paracrine manner when cells are incubated with conditioned medium from H358 cells (CM) or with the two growth factors at concentrations found in the H358 CM (11). 2) AR inhibits apoptosis through an IGF1-dependent survival pathway, independently of the EGF receptor (11). 3) H358 cells resist to Bax-induced apoptosis through an inactivation of Bax conformational change (12).

The IGF1 receptor is known to exert its anti-apoptotic function mainly through activation of the phosphatidylinositol 3-kinase (PI_3K)/Akt pathway (13) whereas the EGF receptor usually activates the Ras/Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated

kinase (ERK) pathway (14) and the PI_3K pathway (15). These two growth factor receptors signaling pathways promote NSCLC cells survival (16,17) and converge with the cell death machinery to the phosphorylation and inactivation of Bad, a pro-apoptotic member of the Bcl2 family of proteins (18–21). When phosphorylated on Ser^{112} or Ser^{136}, Bad is complexed to the cytosolic 14.3.3 protein and fails to interact with the anti-apoptotic $BclX_L$ protein, thus favoring cell survival (19). Phosphorylation of Bad on Ser^{155} also directly suppresses its interaction with $BclX_L$ (22). Akt, a kinase activated by growth factors though a PI_3K-dependent mechanism, phosphorylates Bad on Ser^{136} (20,21). Furthermore, mitochondrial membrane-based protein kinase A (PKA) and protein kinase C (PKC) could also be implicated in Bad phosphorylation. PKA is able to phosphorylate Bad on Ser^{112} (19,23,24) and Ser^{155} (22). Finally, the MAPK-activated p90 ribosomal S6 kinase ($p90^{Rsk}$) phosphorylates Bad on Ser^{112} (19,23,24) in a PKC-dependent pathway (25,26).

The PKC family contains at least 12 isotypes classified into three groups according to their structure and cofactor requirement. In the first group, conventional PKC (PKCα, $PKC\beta_I$, $PKC\beta_{II}$ and PKCγ) are diacylglycerol, calcium and phosphatidylserine-dependent. In the second group, novel PKC (PKCδ, PKCε, PKCη, PKCθ and PKCμ) are diacylglycerol and phosphatidylserine-dependent but calcium-independent. In the third group, atypical PKC (PKCζ, PKCι and PKCλ) are activated only by phosphatidylserine (27,28). PKC-dependent signaling pathways can promote cell survival. Overexpression of PKCα, PKCε and PKCθ increase the cells resistance to apoptosis and PKC inhibitors are known to sensitize cells to death (26,29,30). PKCε also interacts with the pro-apoptotic protein Bax and promotes survival of human prostate cancer cells (31).

Based on our previous published results, the aim of the present study was first to characterize the molecular cascade activated by both factors AR and IGF1, acting in cooperation, to protect NSCLC cells H322 and H358 from apoptosis induced by serum deprivation; secondly to see whether or not this molecular cascade leads to Bax inactivation. We show here that combination of AR and IGF1 stimulates a PKCζ- and PKCδ-dependent, MAPK- and PI_3K-independent, survival pathway. PKC inhibit serum deprivation-induced apoptosis through activation of $p90^{Rsk}$ and inactivation of the pro-apoptotic molecules Bax and Bad. Finally, the pathways activated by AR or IGF1, used as single agent, are different from that observed, here, with the two factors used in combination.

EXPERIMENTAL PROCEDURES

Cell culture, serum withdrawal and H358 conditioned medium

The human H358 and H322 cells were cultured in RPMI 1640 medium (Gibco, Cergy Pontoise, France) with 10% heat-inactivated fetal calf serum. For serum deprivation, cells were rinsed twice in PBS and cultured in serum-free medium. After 8h, serum-free medium was changed and cells cultured as described in each experimental condition. Conditioned medium (CM) from H358 cells was collected 72 h after serum-free culture, centrifuged (5000g) at 4°C and stored at −80°C until use.

We have previously shown that in H358 CM obtained from serum-free medium cell culture, IGF1 level was about 1 ng/ml, as usually observed in CM of other analyzed cancer cell lines, but the AR level was very high: 5 ng/ml, 50 more than in CM from other analyzed cancer cell lines (11). In all experiments performed in this manuscript, levels of AR and IGF1 in H358 CM were controlled as described (11) and shown to be similar.

Drug treatment (table 1)

N^6, $O^{2'}$-dibutyryladenosine 3′, 5′-cyclic monophosphate sodium salt (dbcAMP), bisindolylmaleimide I (GF109203X), PD98059 (Alexis Biochemical, Laufelfingen, Switzerland), calphostin C, rottlerin, staurosporine and wortmannin (Sigma-Aldrich, Saint Quentin Fallavier, France), Go6976, U0126, SB203580 and SB202190 (Calbiochem, VWR International S.A.S. France), were dissolved in DMSO; BAPTA/AM (Alexis Biochemical) in acetonitrile; H89 (Sigma-Aldrich) in ethanol:H_2O (1:1); phorbol 12-myristate 13-acetate (PMA, Alexis Biochemical) in methanol. AR and IGF1 recombinant proteins (Sigma-Aldrich) were dissolved in fresh medium just before use. Cells were treated as indicated in figures legends.

Transfections

Short interfering RNA (siRNA) targeting human PKCδ (32) and siRNA non specific control were synthesized by Eurogentec (Liege, Belgium). Transfection of duplex siRNA was performed with oligofectamine™ reagent (Invitrogen, Cergy Pontoise, France), following the manufacturer's instructions. Dharmacon siRNA SMARTpool PKCζ, Rsk1 and siRNA negative control were purchase from Upstate Biotechnology (Euromedex, Mundosheim, France). Transfection of siRNA SMARTpool was performed with siIMPORTER™ reagent (Upstate Biotechnology), following the manufacturer's instructions. SiRNA were transfected into 60% confluent cells at the final concentration of 200 nM. After 96 h or 120 h of transfection, the efficiency of knockdown was assessed by Western blotting.

Transient transfections were carried out using Fugène 6 (Roche Diagnostics, Meylan, France) according to the manufacturer's protocol. H322 cells were transfected with 5 µg of glutathione S-transferase-Bad (Cell Signaling Technology, Ozyme, St Quentin Yvelines) or pcDNA empty vector. 24h after transfections, cells were exposed to the different effectors as indicated. H322 cells, cultured on Lab-Tek® two wells, were transfected with 2 µg of PKC (constitutively active PKCζ or PKCδ isoforms (33)) or Rsk (Rsk2-KN) mutants or GFP-vector. Immunofluorescence was performed 96h after transfection.

Quantification of apoptotic cell death

Cells were incubated with the different effectors diluted in the medium for 96 h at the concentration indicated. The morphological changes related to apoptosis were assessed by fluorescence microscopy after Hoechst 33342 (5 µg/ml, Sigma-Aldrich) staining of cells and the percent of apoptotic cells was scored after counting at least 500 cells. The proteolytic activation of pro-caspase-3 was evaluated by Western Blotting.

Analysis of protein expression by Western blotting

Cells were washed, incubated in lysis buffer (Tris-Hcl 62.5 mM pH 6.8, glycerol 10%, β-mercaptoethanol 5%, SDS 2 %, urea 6M, bromophenol blue) and sonicated. Proteins were subjected to electrophoresis and transferred to polyvinylidene difluoride membrane (PVDF, Millipore, Saint Quentin les Yvelines, France). Bad, $P90^{Rsk}$, p44/42 MAPK and PKC isoforms were detected with phospho-specific antibodies (Cell Signaling Technology, 1:1000–1:3000) or with antibodies that recognizes Bad (Cell Signaling Technology, 1:1000), Rsk1/MAPKAP-K1a (Upstate Biotechnology, 1:1000), p44/42 MAPK (New England Biolabs, Ozyme, St Quentin Yvelines, 1:1000) or the different PKC isoforms (Becton Dickinson Transduction Lab, Le Pont de Claix, France, 1:250–1:1000 and Tebu for PKCζ, Le Perray en Yvelines, France, 1:500) regardless of the phosphorylation state. For immunoblotting of active caspase-3, cells were incubated in lysis buffer (Tris-Hcl 50 mM pH 7.4, Nacl 150 mM, EDTA 1 mM, Nonidet P-40 0.5 %, NaF 1 mM, Na_3VO_4 1 mM, phenylmethylsulfonyl fluoride (PMSF) 0.5 mM, leupeptin, aprotinin and pepstatin 10 µg/ml each) for 30 min on ice. Protein content was assessed by the Bio-Rad D_C Protein Assay kit (Bio-Rad Lab., Ivry sur Seine, France) and 60 µg of cell lysates were subjected to electrophoresis. Caspase-3 was detected with anti-human caspase-3 rabbit polyclonal antibody (Pharmingen, Becton Dickinson, 1:1000). To ensure equal loading and transfer, membranes were also probed for actin using anti-actin rabbit polyclonal antibody (Sigma-Aldrich, 1:1000) or for HSP70 using anti-HSP70 mouse monoclonal antibody (Affinity BioReagents Inc, 1:5000). The immunoreactive proteins were visualized using horseradish peroxidase conjugated goat anti-mouse or anti-rabbit antibodies and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Orsay, France).

Immunofluorescence staining

Cells cultured on Lab-Tek® were transfected as indicated. 96h after transfection, cells were fixed in −20°C acetone for 5 min. After washes, non specific binding sites were saturated in PBS/BSA 2%/normal goat serum 10% and incubation with anti-PKCδ (1/250, Becton Dickinson Transduction Lab) or anti-PKCζ (1/500, Tebu) or anti-Rsk1/MAPKAP-K1a (1/250, Upstate Biotechnology) was carried out for 2h at room temperature in PBS/BSA 2%. After three washes, Alexa™ 488 goat anti-rabbit IgG (H+L) conjugate (Interchim, Montluçon, France, 1:500), was added and cells were further incubated for 30 min in dark. Cells were then washed, counterstained with Hoechst 33342 and observed using an Olympus microscope.

Protein immunostaining by flow cytometry

Cells were fixed in paraformaldehyde 0.5% for 5 min at room temperature, washed and nonspecific binding sites were blocked with 2% BSA in PBS/saponin 0.1 % for 30 min at room temperature. Cells were then incubated for 2 h at room temperature in the presence of an anti-Bax rabbit polyclonal antibody (Santa Cruz Biotechnology, Tebu, 1:100) raised against the peptide sequence amino acids of N terminus Bax protein or rabbit irrelevant IgG (Pharmingen, Becton Dickinson), washed, incubated for 30 min with Alexa™ 488 goat anti-rabbit IgG (H+L) conjugate (Interchim, 1:500), washed again and resuspended in PBS. All antibodies were diluted in PBS containing 0.1% saponin and 1% BSA. Analysis was performed on a FACScan flow cytometer (Becton Dickinson) using Cellquest software. Green fluorescence was detected at 488 nm.

RESULTS

We have previously demonstrated that H358 cells resisted to serum-deprivation apoptosis and that serum-free conditioned medium (CM) from H358 cells protected H322 NSCLC cells from apoptosis, although they are normally sensitive to serum deprivation (11). The anti-apoptotic activity of H358 CM was completely abolished by a pre-incubation with the anti-AR neutralizing antibody and to a lower extent (about 50 %) by the anti-IGF1 antibody. In contrast, neutralization of other putative growth factors did not alter anti-apoptotic activity of H358

CM (11). In addition, AR and IGF1 recombinant proteins, used in combination at the concentrations detected in H358 CM, mimicked the anti-apoptotic activity of H358 CM, whereas AR or IGF1, used as a single agent, partially inhibited apoptosis (11). We have taken advantage of H358 and H322 NSCLC cells properties to analyze here the apoptosis inhibition pathway promoted by AR and IGF1.

H358 CM and combination of AR and IGF1 inhibit apoptosis by a PKC-dependent, MAPK- and PI_3K-independent, survival pathway

The PI_3K, MAPK as well as the PKC and PKA are the prevalent intracellular pathways transmitting receptor-mediated anti-apoptotic signals from the cell surface to the nucleus. In order to determine which of these pathways was responsible of the apoptosis inhibition, specific inhibitors (table 1) were added to H322 cells cultured in H358 CM (with characterized levels of AR and IGF1 as described in methods) or in presence of AR and IGF1 recombinant proteins at the same concentrations as in H358 CM (figure 1).

Incubation of serum-starved H322 cells in the presence of the p42/44 MAPK inhibitor PD98059 and the PI_3K inhibitor wortmannin enhanced their apoptosis but did not affect the protective effect of the H358 CM or of the combination of AR and IGF1 (figure 1A, B, F). Similarly, the anti-apoptotic activity of the H358 CM did not change with U0126, another p42/44 MAPK inhibitor, SB202190 or SB203580, two p38 MAPK inhibitors, and the PI_3K inhibitor LY294002 (data not shown). In contrast, low concentrations of calphostin C (200 nM) and staurosporine (10 nM), which both inhibit the PKC pathway, restored apoptosis of H322 cells cultured in H358 CM or in presence of AR and IGF1, but did not modify the percentage of apoptosis induced by serum deprivation in H322 cells (figure 1C, D, F). The PKA inhibitor H89 also enhanced apoptosis of H322 cells cultured in serum-free condition and of H358 CM but without statistical significance (figure 1E). In addition, dibutyryl 3′, 5′-cyclic adenosine monophosphate (dbcAMP), which activates PKA, did not inhibit serum deprivation-induced apoptosis (figure 1E). PD98059 and wortmannin, but not calphostin C, blocked the partial anti-apoptotic activity of AR alone, used at the same concentration as in H358 CM, whereas wortmannin, but not PD98059 or calphostin C, seemed prevent the partial inhibition of apoptosis observed with single agent IGF1 (figure 1F).

Similar experiments performed on H358 cells also demonstrated that PKC inhibitors staurosporine and calphostin C sensitized H358 cells to serum deprivation-induced apoptosis, while wortmannin and PD98059 had no significant effect (figure 2).

Taken together, these data suggested that combination of AR and IGF1 present in the H358 CM inhibited apoptosis through activation of a PKC-dependent survival pathway in NSCLC cells but not p42/p44 and p38 MAPK nor PI_3K pathways. AR or IGF1 single agent did not activated such PKC-dependent survival pathway.

H358 CM and AR/IGF1 combination involve atypical PKC and novel PKC to inhibit apoptosis

Among the PKC family, several PKC isoforms could be involved. The specific classical PKC inhibitor Go6976, the calcium chelator BAPTA-AM (data not shown) or the inhibitor of both classical and novel PKC basindolylmaleimide I (GF109203X) did not influence the capacity of H358 CM to inhibit the apoptosis induced by serum deprivation in H322 cells (figure 3A, C). The phorbol 12-myristate 13-acetate (PMA), which activates PKC, mimicked the anti-apoptotic effect of H358 CM in serum-starved H322 cells and this effect was totally block by GF109203X, demonstrating that GF109203X was active (figure 3D). Surprisingly, 1 μM of rottlerin, which inhibits novel PKCδ, restored apoptosis of H322 cells cultured in H358 CM (figure 3B). Altogether, these data suggested that apoptosis inhibition involved the atypical PKC and the novel PKCδ but was independent of classical PKC and of the known anti-apoptotic effect of PMA.

In order to control that the PKC-dependent survival pathway was independent of the MAPK pathway, p42/p44 MAPK phosphorylation status was examined in H358 and H322 cells using specific antibody recognizing Thr^{202}/Tyr^{204} phosphorylated residues. As expected, in both cell lines, PD98059 incubation dramatically prevented p42/p44 phosphorylation (figure 3E). In serum-starved H358 cells, p42/p44 MAPK phosphorylation level did not change as compared to control culture condition. Similar levels of phosphorylated p42/p44 were also detected in the presence of specific PKC inhibitors. In H322 cells, as compared to serum-starved condition, H358 CM enhanced the level of phosphorylation of p42/p44 MAPK, but this effect was not abolished by specific inhibitors of PKC calphostin C, staurosporine or rottlerin (figure 3E). This suggested that the PKC-dependent anti-apoptotic pathway induced by H358 CM did not involve p42/p44 MAPK, even if this latter was activated by H358 CM.

H358 and H322 NSCLC cells expressed conventional PKCα/β, novel PKCδ, PKCε and PKCη and atypical PKCζ and PKCι/λ, but not PKC γ and PKCθ (figure 4A). To further investigate the putative involvement of atypical PKC and PKCδ, the phosphorylation status of PKC isoforms was analyzed in H358 and H322 cells in the presence of specific PKC inhibitors (figure 4B). The robust basal phosphorylation of $PKC\alpha/\beta_2$ $Thr^{638/641}$ remained unchanged whatever the stimulus applied to H358 or H322 cells. Serum-starvation of H358 cells and treatment of H322 cells with H358 CM or with a combination of AR and IGF1 induced a strong phosphorylation of PKCδ Thr^{505} as compared to control culture condition. This activation was blocked by the use of PKC inhibitors calphostin C, staurosporine and rottlerin. Phosphorylation of PKCδ

Ser^{653} was not detected (data not shown). The phosphorylation level of PKCζ/λ $Thr^{410/403}$ increased in serum-starved H358 and H322 cells treated with H358 CM or with AR and IGF1 together, as compared to control culture condition and was inhibited by calphostin C and to a lower level by staurosporine. The use of rottlerin did not have a significant effect on PKCζ/λ phosphorylation. In addition, AR or IGF1 used as single agent, did not induce the same phosphorylation of PKCδ Thr^{505} and PKCζ/λ $Thr^{410/403}$ as the combination of AR and IGF1 or H358 CM (figure 4B).

This suggested that the cooperation of AR and IGF1 in H358 CM, but not AR or IGF1 single factor, inhibited serum deprivation-mediated apoptosis through PKCδ and PKCζ/λ activation.

PKC-dependent survival pathway, activated by AR and IGF1, leads to $p90^{Rsk}$ activation and Bad inactivation

It has been shown that phorbol esters prevented apoptosis by activating a PKC- and $p90^{Rsk}$-dependent pathway, MAPK-independent pathway, that led to inactivation of Bad (26). We thus analyzed the phosphorylation status of $p90^{Rsk}$ and Bad in H322 cells.

H358 CM, as well as combination of AR and IGF1 recombinant proteins, in the same ratio as in H358 CM, induced a strong phosphorylation of $p90^{Rsk}$ Ser^{381} and Thr^{573} (figure 5A). This was abolished by the specific PKC inhibitor calphostin C. This increase of phosphorylation was not observed with AR or IGF1 alone. The basal phosphorylation of $p90^{Rsk}$ Thr^{359}/Ser^{363} remained unchanged in the presence of H358 CM with or without PKC inhibitor calphostin C and in presence of AR and IGF1 recombinant proteins together or alone. Serum starvation decreased faintly the phosphorylation level of $p90^{Rsk}$ Thr^{359}/Ser^{363} as compared to control culture conditions (figure 5A). This suggested that the basal phosphorylation of these residues was independent of H358 CM PKC-mediated survival pathway, even if it was decreased by serum-deprivation.

Additionally, serum-starvation decreased Bad phosphorylation at Ser^{112} and Ser^{155} in H322 cells, whereas H358 CM or combination of AR and IGF1 recombinant proteins restored its phosphorylation (figure 5B). AR or IGF1 alone did not restore the phosphorylation of Bad Ser^{112} or Ser^{155}. Calphostin C dramatically reduced H358 CM-mediated phosphorylation of Bad Ser^{112}, but not significantly of Ser^{155}. Phosphorylation of Bad Ser^{136} was not detected (data not shown).

Taken together, these results indicated that the combination of AR and IGF1, but not AR or IGF1 used alone, inhibited serum deprivation-mediated apoptosis via a PKC-dependent pathway involving activation of $p90^{Rsk}$ and inactivation of Bad through phosphorylation.

PKC-dependent survival pathway, activated by AR and IGF1, prevents Bax conformational change

Previous studies have shown that the Bax protein changed of conformation and exposed its N terminus domain during apoptosis (12,34,35). Using an epitope-specific antibody that only recognizes the N terminal extremity of Bax when it is exposed, we showed that serum deprivation increased Bax conformational activation in H322 cells but not in H358 cells (figure 6). H358 CM or combination of AR and IGF1 recombinant proteins prevented Bax conformational-activation; the level of fluorescence, reflecting Bax conformational change, was similar in H322 cells treated with H358 CM or with combination of AR and IGF1 and in untreated control cells (figure 6B). AR or IGF1 used alone did not have the same effect as the combination of the both growth factors. The presence of the specific PKC inhibitor calphostin C in H358 CM or in serum-free medium supplemented with AR and IGF1, enhanced Bax activation and restored the level of Bax N terminus staining to the level of serum-starved H322 cells. Similarly, calphostin C enhanced the staining of Bax N terminus in serum-starved H358 cells (figure 6A).

These observations highly suggested that inhibition of apoptosis by combination of AR and IGF1 came from the inhibition of Bax conformational change by a PKC-dependent mechanism.

AR/IGF1 combination inhibits apoptosis through a PKC-, PKC- and $p90^{Rsk}$-dependent pathway

Taken together, our results suggested that H358 CM and combination of AR and IGF1 inhibited apoptosis-induced by serum deprivation through a PKC- and $p90^{Rsk}$-dependent pathway. This pathway led to inactivation of Bad as well as conformational inactivation of Bax.

In order to confirm the involvement of PKC and $p90^{Rsk}$, we analyzed the effect of silencing subtype-specific PKC and $p90^{Rsk}$ by siRNA in H322 cells (figure 7). Transfections of siRNA targeting PKCδ or PKCζ strongly silenced endogenous PKCδ and PKCζ respectively as compared to transfections of non-specific siRNA. SiRNA for each PKC isoform did not inhibit the expression of the other isoform (figure 7A). Transfection of siRNA targeting PKCδ or PKCζ totally restored apoptosis of H322 cells cultured in H358 CM or in presence of combination of AR and IGF1 (figure 7B, C). PKCζ siRNA appeared to be more potent than PKCδ siRNA. We also observed that the inhibition of serum-starved H322 cells apoptosis by H358 CM or AR and IGF1 was blocked by the double transfection of siRNA targeting PKCδ and PKCζ (data not shown). Moreover, the partial anti-apoptotic activity of AR or IGF1 used as single agent, was not prevented when PKCδ or PKCζ

were knocked-down (figure 7B–C). Transfections of siRNA targeting $p90^{Rsk}$ strongly silenced endogenous $p90^{Rsk}$, as compared to transfections of non-specific siRNA (figure 7A), and greatly increased apoptosis of cells cultured in presence of H358 CM or of combination of AR and IGF1, but not in presence of AR or IGF1 alone (figure 7D).

Altogether, these data suggested that the inhibition of apoptosis by H358 CM or by the combination of AR and IGF1 involved both PKCζ and PKCδ as well as $p90^{Rsk}$, whereas single factor AR or IGF1 acted through different pathway.

In order to verify the activity of PKC and $p90^{Rsk}$ on serum deprivation-mediated apoptosis, H322 cells were transiently transfected with mutant constructs and apoptosis of transfected cells, detected by immunofluorescence, was analyzed (figure 8). Overexpression of constitutively active PKCζ or PKCδ isoforms strongly inhibited apoptosis of serum-starved H322 cells, as compared to GFP-transfected cells (figure 8). The double transfection of constitutively active PKCζ and PKCδ did not increase the inhibition of apoptosis. Moreover, introduction of a catalytically inactive form of Rsk, Rsk2-KN, strongly inhibited the protective effect of H358 CM. These results demonstrated that PKCζ and PKCδ isoforms and $p90^{Rsk}$ protected cells from serum-deprivation-induced apoptosis.

DISCUSSION

In this manuscript, we have shown that, in two NSCLC cell lines H358 and H322, AR and IGF1 cooperate to prevent apoptosis induced by serum deprivation by activating a PKC-dependent survival pathway different to that used by single factor AR or IGF1. Atypical PKCζ, novel PKCδ and $p90^{Rsk}$ are the principal mediators in this signal transduction. This survival pathway leads ultimately to Bad as well as Bax inactivation.

We have previously identified that AR and IGF1 are the key anti-apoptotic molecules in the conditioned medium from H358 cells and that the cooperation of those growth factors inhibits apoptosis via both IGF1 and EGF receptors in NSCLC (11). IGF1 receptor exerts its anti-apoptotic effect essentially through activation of the PI_3K pathway (13), which promotes cell survival by phosphorylating Bad (18–21). In agreement, the IGF1-mediated inhibition of apoptosis in our model of NSCLC cells seems prevented by PI_3K inhibitors (figure 9A). However, alternative pathways have been proposed such as the MAPK pathway or the activation of Raf-1 and its translocation to the mitochondria (36,37). PKC are also involved in IGF1-dependent pathway activated during mitogenesis (38,39), cell migration (40–42), proliferation (43) or transformation (41,44). Moreover PKCα antisense oligonucleotide restores apoptosis, blocked by IGF1, in glioblastoma multiform cells (45). EGF receptor activates both MAPK and PI_3K pathways (14,15). In agreement, we show that MAPK and PI_3K inhibitors block the AR-mediated inhibition of apoptosis (figure 9A). To date, PKC pathway has not been described to directly propagate the EGF receptor signaling. However, EGF receptor triggers PKC activity (46) at least in part downstream of phospholipase signaling (47) and EGF has recently been shown to induce fibroblast contractility and motility via a PKCδ-dependent pathway (48). In our model of NSCLC cells, the anti-apoptotic activity of H358 CM, mediated by AR/IGF1 cooperation, is prevented by PKC inhibitors but not by PI_3K or MAPK inhibitors, demonstrating the activation of an original PKC-dependent survival pathway (figure 9B). Of note, although PKC might activate the MAPK pathway (25,26,49,50), the phosphorylation of ERK1/2 proteins, by serum deprivation in H358 cells and by H358 CM in H322 cells, is independent of PKC; this result confirms that MAPK activation is not involved in the apoptosis inhibition mediated by combination of AR and IGF1 in our model. Similarly, H89 restores apoptosis but the PKA activator dbcAMP has no significant protective effect, suggesting that the PKA pathway is also not involved in cells survival.

Specific PKC isoforms may be either anti- or pro-apoptotic, depending on the nature of the apoptotic stimuli and of the cell type (51). Known anti-apoptotic PKC isoforms include PKCα, $PKC\beta_{II}$, PKCε, PKCλ/ι and PKCζ (29,49,52,53). Conversely, PKCδ has been shown to have anti- or pro-apoptotic actions (54). In our model, conventional PKC are not involved in apoptosis inhibition. Specific inhibitors of atypical PKC are not available, but the use of conventional and novel PKC inhibitors strongly suggests that the atypical PKC play a central role in the survival pathway mediated by AR and IGF1. This hypothesis is confirmed by the inhibition of the AR/IGF1 survival pathway when we use siRNA directed against atypical PKCζ. We also demonstrate that rottlerin and siRNA targeting novel PKCδ restore apoptosis. PKCζ and PKCδ are thus involved in the anti-apoptotic pathway activated by AR and IGF1. In addition, overexpression of constitutively active PKCζ and PKCδ strongly inhibits serum deprivation-induced apoptosis, suggesting that these PKC isoforms are sufficient for apoptosis inhibition. Our results comfort previous data showing that PKCζ plays an essential role in IGF1 regulation of cell proliferation, migration, gene expression (41) and mitogenesis (38,39). The major activation pathway of PKCζ depends on PI_3K but it may also act independently of the PI_3K pathway (41), in the MAPK cascade, the transcriptional factor NFκB activation or Rsk signaling (50). Our results are also in agreement with studies showing that PKCδ promotes cellular survival and chemotherapeutic drug resistance in NSCLC cells (55) or is involved in IGF1 receptor-mediated cell migration (40) and transformation (44).

Caspases can cause limited proteolysis of PKCδ, generating a 40 kDa C-terminal fragment. It was proposed that the catalytic fragment of PKCδ is pro-apoptotic and the holoenzyme is anti-apoptotic (54,56). In our model, we do not detect the 40 kDa fragment, except after PMA treatment (data not shown). In addition, the novel PKC inhibitor GF109203X has no effect on the anti-apoptotic activity of H358 CM, suggesting that the PKCδ-dependent pathway triggered by AR and IGF1 is independent of the anti-apoptotic activity of phorbol esters.

PKCζ or PKCδ siRNAs completely inhibit the survival pathway mediated by H358 CM or combination of AR and IGF1. In addition, transfection of siRNA targeting both PKC isoforms does not increase apoptosis restoration (data not shown), and the double transfection of constitutively active PKCζ and PKCδ does not have additive effect on apoptosis inhibition. This confirmed that both PKC are on a unique pathway. Moreover, rottlerin inhibits the phosphorylation of PKCδ Thr^{505} in serum-starved H358 cells and in H322 cells treated with H358 CM, but does not block the phosphorylation of PKCζ/λ $Thr^{410/403}$ in the same conditions, indicating that the PKCζ may act upstream the PKCδ (figure 9B). This is in agreement with previous observations showing that the function of PKCδ may be influenced by the presence of other PKC isozymes (54) and that PKCζ could control PKCδ phosphorylation (57).

We show here that calphostin C and PKCζ or PKCδ siRNA inhibit survival pathway activated by the combination of AR and IGF1 but have no effect when each growth factor are used as a single agent. This PKC-dependent survival pathway, activated by the combination of AR and IGF1, is different of the pathways activated by AR or IGF1 used as single agent. Each factor activates the classical ways described respectively for the EGF and IGF1 receptors (figure 9A). Altogether, our results suggested that the cooperation between AR and IGF1 takes place upstream of the PKC cascade, probably at the level of receptors. In agreement with this hypothesis, we have previously identified that AR inhibits apoptosis through an IGF1-dependent survival pathway in NSCLC (11), but the mechanism responsible of such cooperation remains to be determined.

In our study, the PKC-survival pathway involves the activation of $p90^{Rsk}$ and the phosphorylation of Bad on Ser^{112} (figure 9B). This is confirmed by AR/IGF1 survival pathway inhibition, when we use siRNA directed against $p90^{Rsk}$. We also demonstrate that overexpression of a catalytically inactive form of Rsk prevents the anti-apoptotic activity of H358 CM, demonstrating the major role of this protein in AR/IGF1 survival pathway. Accordingly, the MAPK-activated $p90^{Rsk}$ has been reported to protect cells from Bad-induced apoptosis by phosphorylation of Bad on Ser^{112} both in vitro and in vivo (23,25). Moreover, phorbol esters promote cell survival essentially through a PKC-$p90^{Rsk}$-dependent, MAPK-independent pathway that lead to phosphorylation and inactivation of Bad (26). Of note, in our model, AR and IGF1 also enhance the phosphorylation of Bad on Ser^{155} independently of the PKC, as it is not modify by calphostin C. Phosphorylation of Bad on Ser^{155} might be mediated by PKA, as previously described (22), since the PKA inhibitor H89 has a pro-apoptotic effect in our model. We do not detect any phosphorylation of Bad Ser^{136} by AR and IGF1 (not shown), a result in agreement with an Akt-dependent phosphorylation of this residue (20, 21). These observations confirm the non-involvement of the PKA and PI_3K in the survival pathway induced by AR and IGF1.

Forced expression of PKCε in prostate cancer cells confers a significant resistance to phorbol esters, associated with an inhibition of Bax conformational rearrangements (31). This study suggested that an association of PKCε with Bax might neutralize apoptotic signals propagated through a mitochondrial death-signaling pathway. We previously showed that H358 NSCLC cells resist to Bax-mediated apoptosis, due to an inhibition of Bax conformational change and of its translocation from the cytosol to the mitochondria (12). Here, we show that Bax conformational change inhibition is due to AR/IGF1 cooperation and the activation of a PKC-dependent pathway (figure 9B).

In conclusion, it is well established that growth factors, in particular EGF-related peptides and IGF1 are involved in survival of malignant human lung epithelial cells through activation of intracellular MAPK and/or PI_3K pathways. Our study has demonstrated for the first time that 1) the two growth factors, AR and IGF1, cooperate to induce an original PKCζ-, PKCδ- and $p90^{Rsk}$-dependent survival pathway in NSCLC cells, independently of other pathways classically described for these two growth factors alone. 2) The PKC-dependent pathway induced by AR/IGF1 cooperation can modify Bad phosphorylation and inactivate Bax conformational change. We thus have identified a new survival pathway mediated by AR/IGF1 cooperation that might contribute to lung tumor development and progression. Further studies on the relevance of this pathway in vivo on fresh tumor samples will allow development of new therapeutic strategies.

Ackowledgements:

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Abbreviations

AR: amphiregulin

dbcAMP: dibutyryl 3′, 5′-cyclic adenosine monophosphate

EGF: epidermal growth factor

GFP: green fluorescent protein

H358 CM: conditioned medium from H358 cells

IGF1: type 1 insulin-like growth factor

MAPK: mitogen-activated protein kinase

NSCLC: non small cell lung cancer

Rsk: ribosomal S6 kinase

PI_3K: phosphatidylinositol 3-kinase

PKA: protein kinase A

PKC: protein kinase C

PMA: phorbol 12-myristate 13-acetate

PMSF: phenylmethylsulfonyl fluoride

PVDF: polyvinylidene difluoride membrane

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Figure 1

Effect of transduction pathway inhibitors on apoptosis induced by serum deprivation in H322 cells

H322 cells were cultured in medium with 10 % serum (10%), serum-free medium (0%), serum-free H358 CM (CM) or serum-free medium supplemented with AR 5 ng/ml (AR) or IGF1 1 ng/ml (IGF1) or with the both recombinant proteins (AR+IGF1). The activity of indicated concentrations of PD98059 (**A**), wortmannin (**B**), calphostin C (**C**), staurosporine (**D**), H89 or dbcAMP (**E**) was analyzed. **F:** The activity of PD98059 10 µM (PD), wortmannin 200 nM (Wort) or calphostin C 200 nM (CalC) was analyzed. Apoptosis was analyzed after 96h by detection of the active caspase-3 by immunoblotting or after counting Hoechst stained cells. Percentages of apoptosis are expressed as the mean ± SD of at least three independent experiments (A–E: *** p<0.0001, statistically less significant than 0%; ** p<0.001, statistically more significant than H358 CM; F: ** p<0.005, * p<0,05, statistically less significant than 0%).

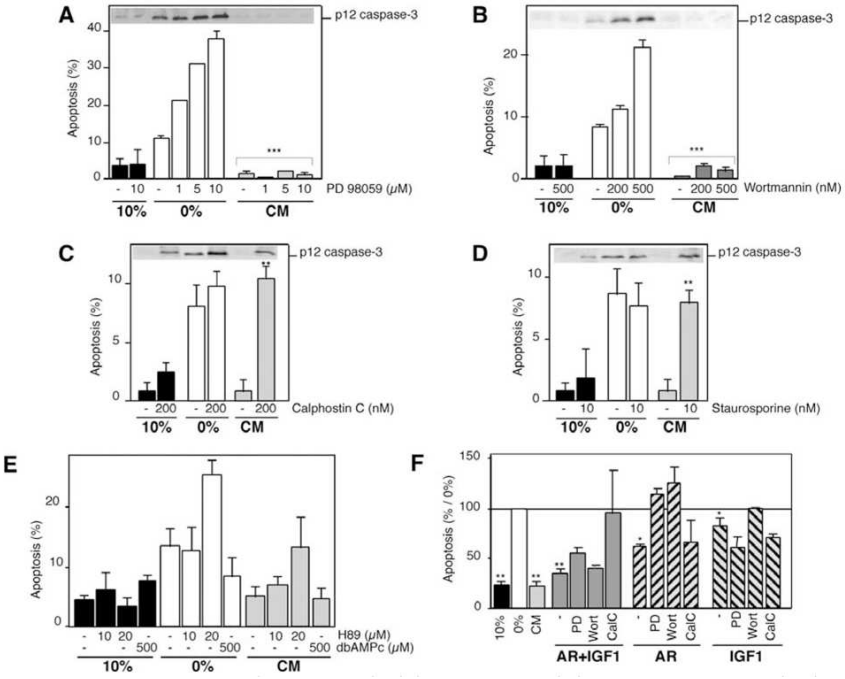

Figure 2

Effect of transduction pathway inhibitors on apoptosis induced by serum deprivation in H358 cells

H358 cells were cultured in presence (10%) or absence (0%) of serum. The activity of indicated concentrations of staurosporine (**A**), calphostin C (**B**), PD98059 or wortmannin (**C**) was analyzed. Apoptosis was analyzed after 96h after counting Hoechst stained cells. Percentages of apoptosis are expressed as the mean ± SD of three independent experiments (*** $p<0.0001$, statistically more significant than 0%).

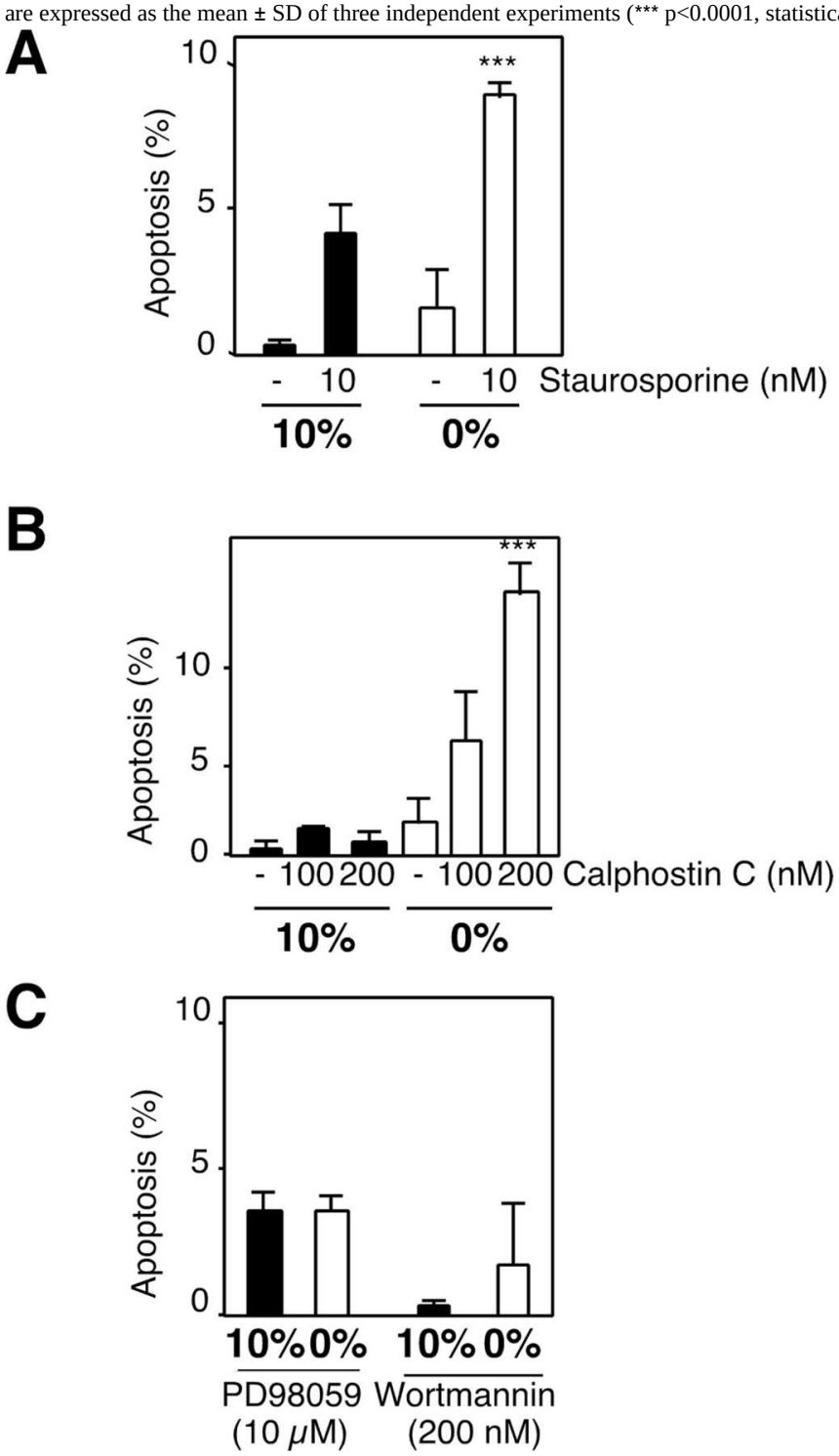

Figure 3

PKC inhibited apoptosis induced by serum deprivation in NSCLC cell lines independently of MAPK pathway

A–D: H322 cells were cultured in medium with 10 % serum (10%), serum-free medium (0%) or H358 CM (CM). The activity of indicated concentrations of Go6976 (**A**), rottlerin (**B**), GF109203X (**C** and **D**) and PMA (**D**) was analyzed. Apoptosis was analyzed after 96h after counting Hoechst stained cells. Percentages of apoptosis are expressed as the mean ± SD of at least three independent experiments. (*** $p<0.001$, statistically less significant than 0%). **E:** Western-blotting analysis of p42/p44 phosphorylated on Thr^{202}/Tyr^{204} in H358 and H322 cells. Cells were incubated in medium with (10%) or without (0%) serum or in H358 CM (CM) supplemented with calphostin C 200 nM (CalC), staurosporine 10 nM (Stsp), rottlerin 1 μM (Rot) or PD98059 10 μM (PD) as indicated. P42/p44 was used as protein level control. Results are representative of three independent experiments.

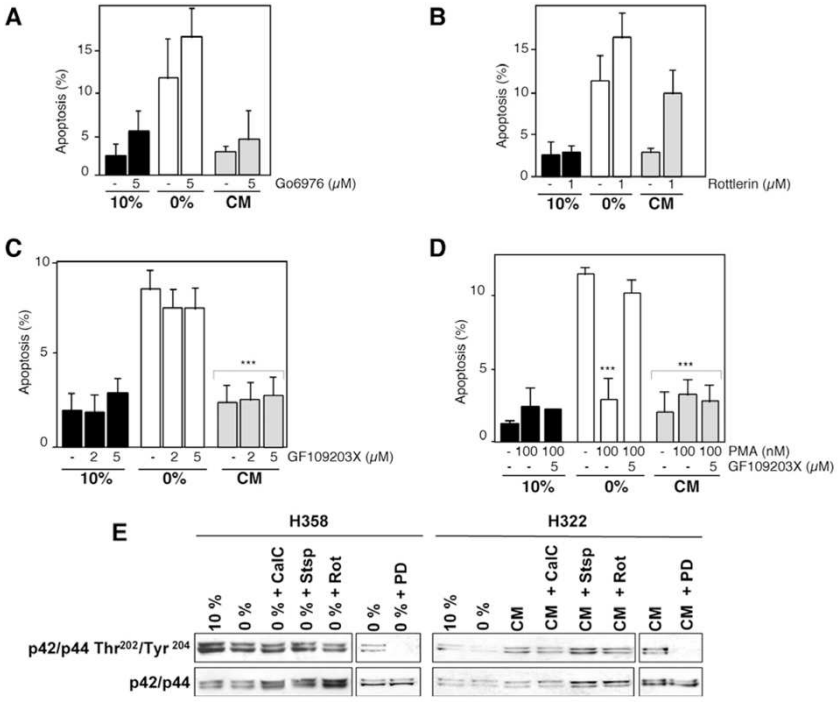

Figure 4

Phosphorylation of PKC isoforms following serum deprivation in NSCLC cells

A: H358 and H322 cells were incubated for 30 min in control culture conditions. **B:** H358 and H322 cells were incubated for 30 min with medium containing 10% serum (10%), serum-free medium (0 %) or H358 CM (CM), supplemented with calphostin C 200 nM (CalC), staurosporine 100 nM (stsp), rottlerin 1 μM (Rot), or serum-free medium supplemented with AR 5 ng/ml (AR), IGF1 1 ng/ml (IGF1) or a combination of the both recombinant proteins (AR+IGF1) as indicated. Cells lysates were separated by electrophoresis. Proteins were then blotted on to PVDF membranes, which were incubated with anti-isoform-specific PKC (**A**) or anti-phospho-specific PKC (**B**) antibodies as indicated. Actin or HSP70 were used as protein level loading control. Results are representative of three independent experiments.

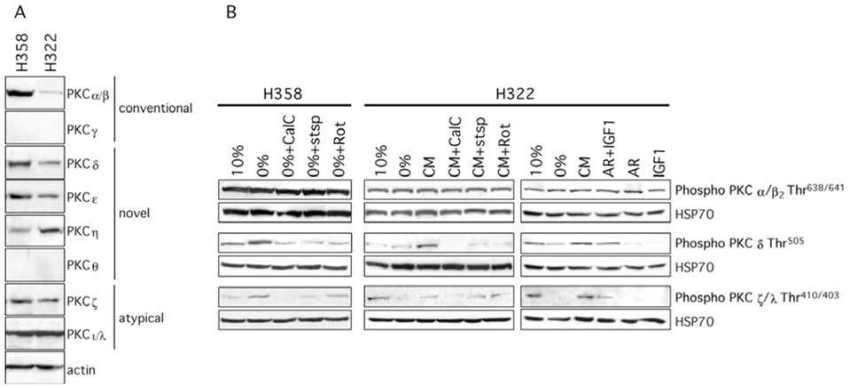

Figure 5

PKC promoted inhibition of apoptosis induced by serum deprivation by phosphorylating $p90^{Rsk}$ and Bad

A: H322 cells were incubated for 30 min with medium containing 10% serum (10 %), serum-free medium (0 %) in presence or absence of AR 5 ng/ml (AR), IGF1 1 ng/ml (IGF1) or the both recombinant proteins (AR+IGF1), H358 CM (CM) in presence or absence of calphostin C 200 nM (CM+CalC) as indicated. Immunoblotting was done with phospho-specific $p90^{Rsk}$ Ser^{381}, Thr^{573} and Thr^{359}/Ser^{363} antibodies or with an antibody that recognizes $p90^{Rsk}$ regardless of its phosphorylation state. **B:** H322 cells were transfected with a glutathione S-transferase-Bad mammalian expression vector (+Bad) or an empty vector (pcDNA). 48h after transfection, cells were treated for 30 min as indicated in A. Immunoblotting was done with phospho-specific Bad Ser^{112}, Ser^{136} and Ser^{155} antibodies or with an antibody that recognizes Bad regardless of its phosphorylation state. Actin was used as protein level loading control. Results are representative of four independent experiments.

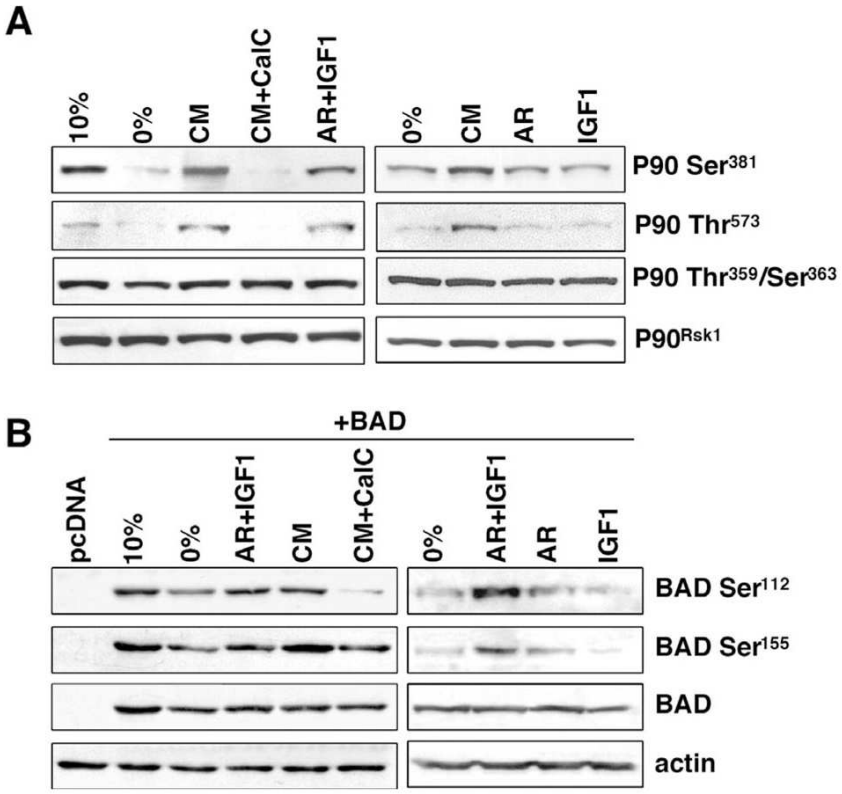

Figure 6

PKC promoted inhibition of apoptosis induced by serum deprivation by inhibiting the conformational change of Bax

Flow cytometry analysis of conformational change of Bax in H358 and H322 cells. Bax immunostaining was performed using a conformational-dependent anti-Bax antibody that recognizes Bax protein with an exposed N terminus. H358 cells (**A**) and H322 cells (**B**) were treated for 96h as indicated: with (10%) or without (0%) serum, with H358 CM (CM), and supplemented or not with calphostin C 200 nM (CalC), IGF1 1 ng/ml (IGF1) or AR 5 ng/ml (AR) or a combination of both recombinant proteins (AR+IGF1). Dotted histogram: histogram for irrelevant antibody, open histogram: histogram for untreated control cells, filled histogram: histogram for treated cells as indicated. Results shown are representative of three independent experiments.

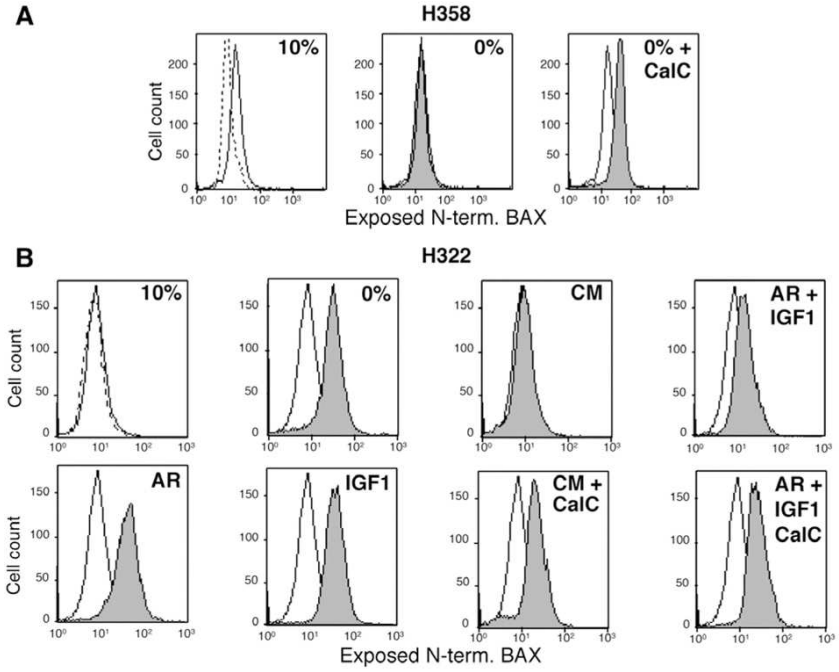

Figure 7

Subtype-specific PKC and p90^{Rsk} knockdown in H322 NSCLC cells

A: Western-blotting analysis of PKCδ, PKCζ or p90^{Rsk} in H322 cells non-transfected (−) or transfected with siRNA targeting PKCδ (δ), PKCζ (ζ), p90^{Rsk} (Rsk) or non-specific siRNA control (C). The efficiency of siRNA was analyzed 96h after transfection. Actin was used as protein level control. **B–D:** H322 cells were cultured in medium with (10 %) or without serum (0%), H358 CM (CM) or serum-free medium supplemented with AR 5 ng/ml (AR) or IGF1 1 ng/ml (IGF1) or with the both recombinant proteins (AR+IGF1) and transfected with siRNA targeting PKCδ (**B**), PKCζ (**C**), p90^{Rsk} (**D**) or non-specific siRNA control. Apoptosis was analyzed 96h after transfection after counting Hoechst stained cells. Percentages of apoptosis are expressed as the mean ± SD of at least three independent experiments (* $p<0.03$, statistically more significant than H358 CM and AR+IGF1).

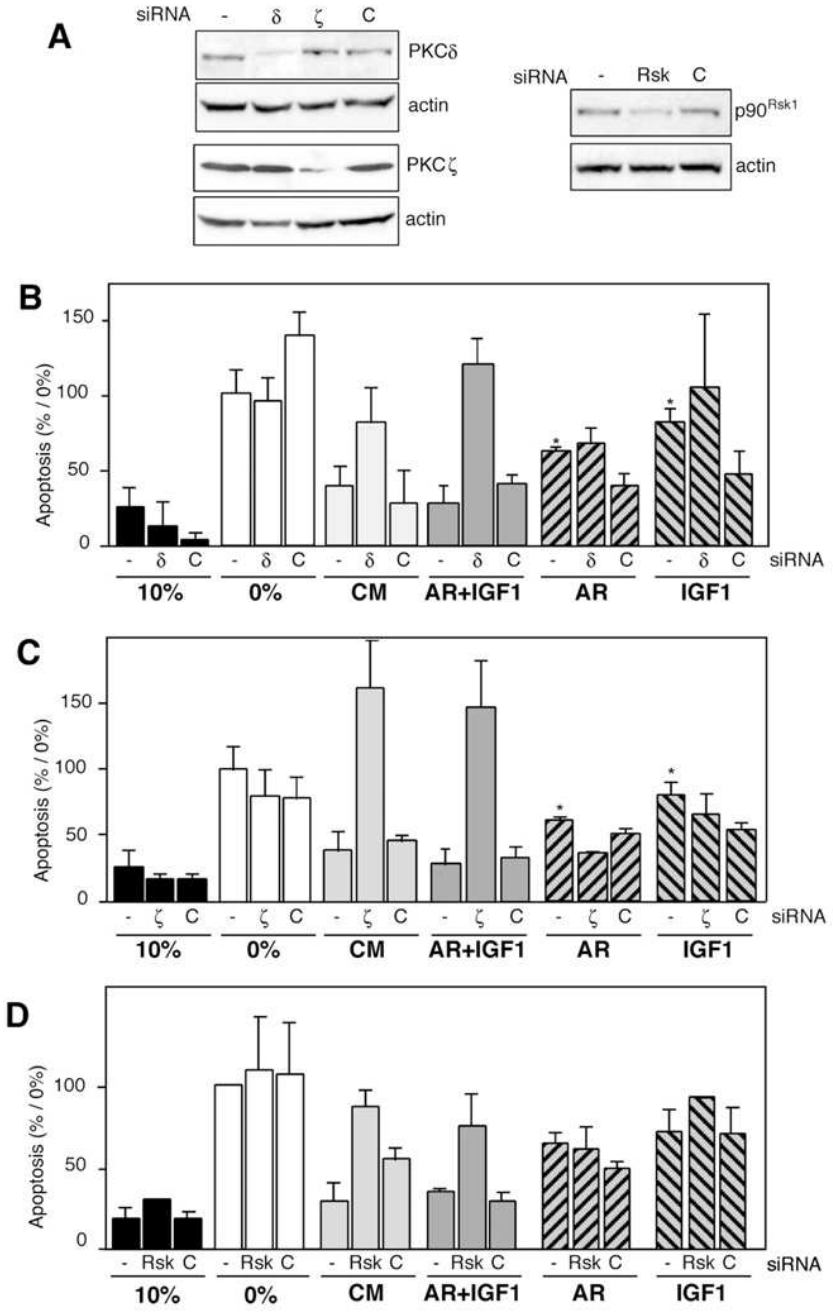

Figure 8

Overexpression of PKC or p90^{Rsk} mutants in H322 NSCLC cells

A: H322 cells, cultured in serum-free medium (0%) or H358 CM (CM), were transfected with either a vector encoding green fluorescent protein (GFP) or with an expression vector encoding the constitutively active PKCζ (ζCA) or PKCδ (δCA) mutants or the catalytically inactive form of p90^{Rsk} (Rsk2-KN). 96h after transfection, the expression of GFP, PKCζ, PKCδ or p90^{Rsk} in transfected cells was revealed by immunofluorescence and morphological aspect of Hoechst-stained cells was analyzed. Arrows indicated apoptotic nuclei. **B:** Apoptosis of transfected cells, as described in A, was analyzed after counting Hoechst counterstained cells. Percentages of apoptosis are expressed as the mean ± SD of three independent experiments.

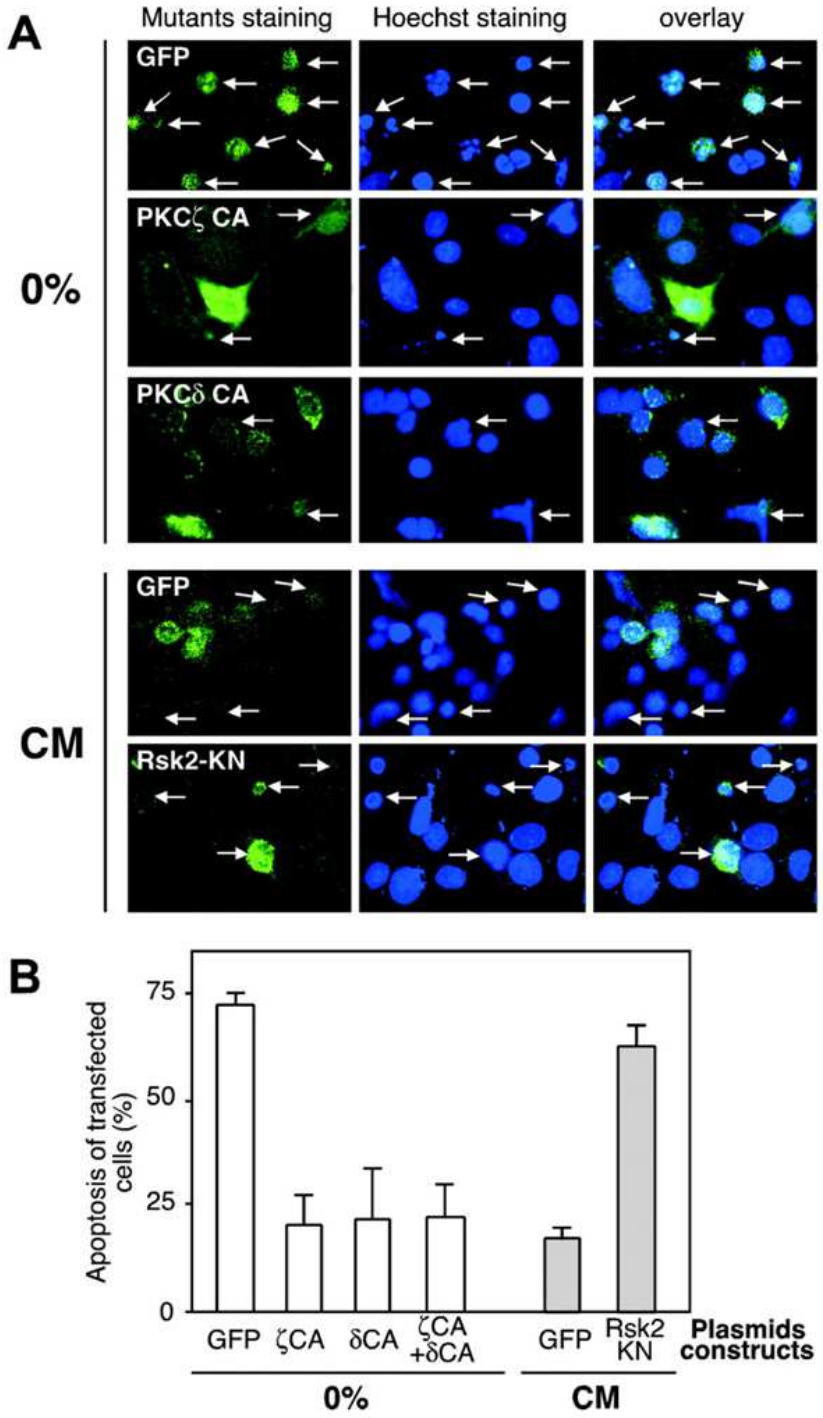

Figure 9

Schematic diagram showing the intracellular pathways for IGF1, AR or combination of the both growth factors involved in serum deprivation-induced apoptosis in NSCLC cell lines

A: Signaling pathways activated by IGF1 or AR growth factors used as single agent. IGF1 activates the IGF1 receptor and the PI_3K/Akt pathway. AR activates the EGF receptor which is linked to the MAPK/ERK and the PI_3K/Akt pathways. Both IGF1 and EGF receptors converge to the inactivation of the pro-apoptotic protein Bad. **B:** Survival pathway activated by the cooperation of IGF1 and AR growth factors. AR and IGF1, present in the H358 CM, activate both IGF1 and EGF receptors (box) (11). This cooperation induces a PKC-dependent survival pathway, independent of MAPK/ERK and PI_3K/Akt pathways. This anti-apoptotic pathway leads to Bad phosphorylation on Ser^{112} and inhibition of conformational change of the pro-apoptotic protein Bax. Inhibitors used are mentioned in blue.

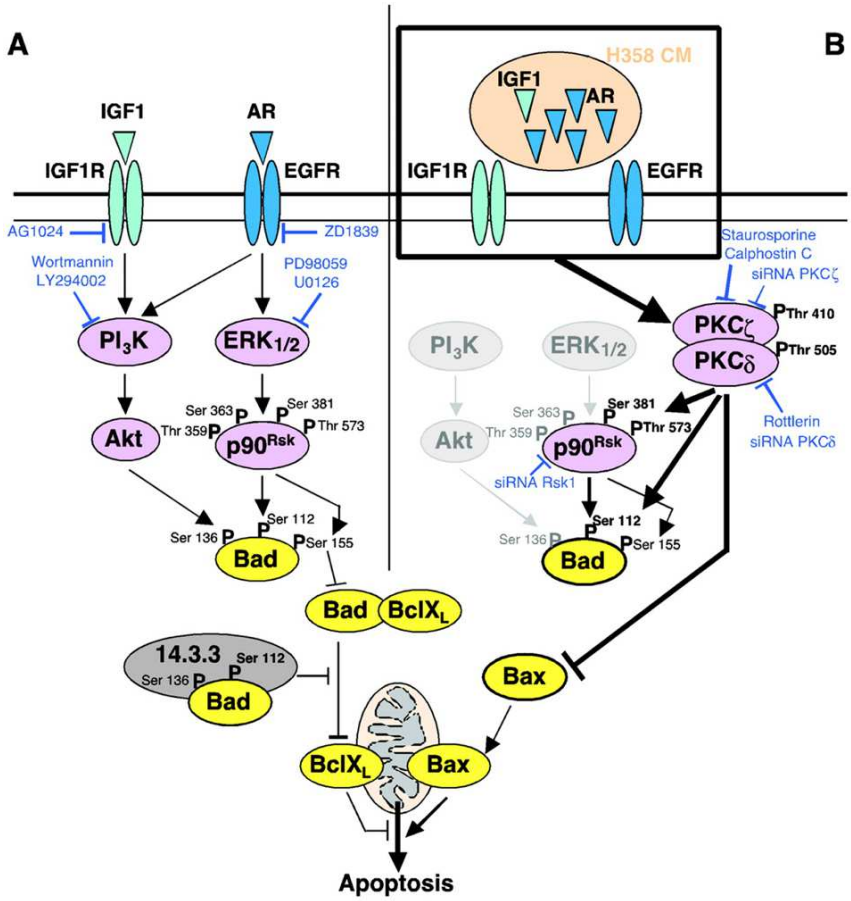

Table 1
Specific pathway inhibitors and activators used

	Pathways	Inhibitors	Concentrations
			μM
MAPK	p44/p42 (ERK1/2)	PD98059	10
		U0126	10
	p38	SB202190	10
		SB203580	10
PI3K		Wortmannin	0.2
		LY294002	10
PKA		H89	10–20
PKC		Calphostin C	0.2–0.5
		Staurosporine	0.01
	Classical PKC	Gö6976	5
		BAPTA-AM	Until 100
	Classical and novel	GF109203X	2–5
	Novel PKCδ	Rottlerin	0.1–1
	Pathways	Activators	Concentrations
			μM
PKA		dbcAMP	Until 500
PKC	Classical and novel	PMA	0.1