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Extracellular ATP regulates FoxO family of transcription factors and cell cycle progression through PI3K/Akt in MCF-7 cells

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ABSTRACT

Background: Forkhead Box-O (FoxO) transcription factors regulate the expression of many genes involved in suppression. Released nucleotides can regulate intracellular signaling pathways through membrane-bound purinergic receptors, to promote or prevent malignant cell transformation. We studied the role of extracellular ATP in the modulation of Forkhead Box O (FoxO) transcription factors and of cell cycle progression in MCF-7 breast cancer cells.
Methods: Western blot analysis, cell transfections with siRNA against Akt, immunocytochemistry, subcellular fractionation studies and flow cytometry analysis were performed.
Results: ATP induced the phosphorylation of FoxO1/3a at threonine 24/32, whereas reduced the expression of FoxO1. In addition, ATP increased the expression of the cyclins D1 and D3 and down-regulated the cell cycle inhibitory proteins p21Cip1 and p27Kip1. The use of the phosphatidylinositol 3 kinase (PI3K) inhibitor, Ly294002, and/or of siRNA to reduce the expression of the serine/threonine kinase Akt showed that these effects are mediated by the PI3K/Akt signaling pathway. ATP induced the translocation of FoxO3a from the nucleus to the cytoplasm. Also, ATP increased the number of cells in the S phase of cell cycle; this effect was reverted by the use of Ly294002 and the proteasome inhibitor bortezomib.
Conclusion: Extracellular ATP induces the inactivation of FoxO transcription factors and cell cycle progression through the PI3K/Akt pathway in MCF-7 cells.
General significance: These findings provide new molecular basis for further understanding the mechanisms involved in ATP signal transduction in breast cancer cells, and should be considered for the development of effective breast cancer therapeutic strategies.

1. Introduction

Purinergic signaling exerted by released purines and pyrimidines plays an important regulatory role in both short- and long-term processes in living cells. These extracellular nucleotides, acting through membrane-bound purinergic receptors, participate in the determination of cell fate directing cells towards proliferation, differentiation or apoptosis, promoting or preventing malignant transformation [1,2]. Many of the molecular alterations that are associated with carcinogenesis occur in cell signaling pathways responsible for regulating cell proliferation or apoptosis. Activation of P2 receptors by extracellular ATP transduces a variety of oncogenic signaling pathways including the phosphatidylinositol-3 kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK) and the transcription factors c-Fos and Jun in breast cancer cells [3–5].

Mammalian forkhead transcription factors of the class O (FoxOs) that include FoxO1, FoxO3, FoxO4 and FoxO6 have emerged as critical regulators of cellular growth and proliferation as they play an important role in tumor suppression by regulating the expression of genes involved in DNA damage repair, cell cycle arrest and apoptosis. Thus, they are considered potential targets for therapeutic strategies directed against cancer [6]. The activities of FoxOs are tightly controlled by post-translational modifications such as phosphorylation, acetylation and proteolytic degradation [7,8]. Besides, central to the regulation of FoxOs is a shuttling system, which confines these factors to either the nucleus or the cytosol. In this way, active unphosphorylated FoxO proteins usually reside in the nucleus of cells. When they are phosphorylated at several domains, they are inactivated and exported to the cytoplasm through association with 14–3–3 proteins and the nuclear transport machinery [9]. Phosphorylation of FoxO proteins not only retains these transcription factors in the cytoplasm, but also leads to ubiquitination and degradation through the 26S proteasome [6]. Activation of cell survival pathways such as phosphoinositide-3-kinase (PI3K)/Akt or Ras/ERK mitogen-activated protein kinase (MAPK) are known to phosphorylate FoxOs at conserved serine/threonine residues suppressing their transcriptional activity [10]. Loss of FoxO function

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(inactivation) has recently been associated with several human cancers, including breast cancer [7,10].

Cell proliferation depends on cell cycle progression which is controlled by cyclin-dependent protein kinases (CDKs) and cyclins that initiate phosphorylation events to allow progression through checkpoints. Progression through the G1 phase of cell cycle requires the activity of the cyclin D-dependent kinases CDK4 and/or CDK6 [11]. On the other hand, CDK inhibitors (CDKIs) are critical mediators of anti-proliferative signals inducing cell cycle arrest. There are two families of CDKIs: the Cip/Kip family which includes p21Cip1, p27Kip1, and p57Kip2; and the INK4 family comprised by p16INK4A, p15INK4B, p18INK4C, and p19INK4D. These inhibitors prevent the entry to the S phase of cell cycle [12,13]. Related to this mechanism, there is some evidence suggesting that extracellular ATP can play a mitogenic role in a number of cell types [14].

We have previously shown that extracellular ATP can modulate the MAPKs and PI3K/Akt signaling pathways and some transcription factors in the MCF-7 human breast cancer cell line [3–5]. Also, we demonstrated that ATP induced the proliferation of these cells [5]. However, the role of ATP in the proliferation of breast cancer cells remains unclear. Thus, in this work we studied if extracellular ATP regulates FoxO family of transcription factors and cell cycle progression of MCF-7 cells through the PI3K/Akt pathway.

2. Materials and methods

2.1. Materials

ATP, ADP, ATPγS, adenosine and RPMI-1640 medium were from Sigma-Aldrich Co. (St. Louis, MO, USA). Ly294002 was from EMD Chemicals, Inc. (San Diego, CA, USA). Lipofectamine 2000 transfection reagent and propidium iodide (PI) were provided by Invitrogen Corp (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Natocord (Córdoba, Argentina). Bortezomib was provided by Selleck (Munich, Germany). Monoclonal antibodies recognizing phosphorylated Akt (Ser 473), anti-cyclin D1, anti-cyclin D3, anti-p27Kip1, anti-p21Cip1,

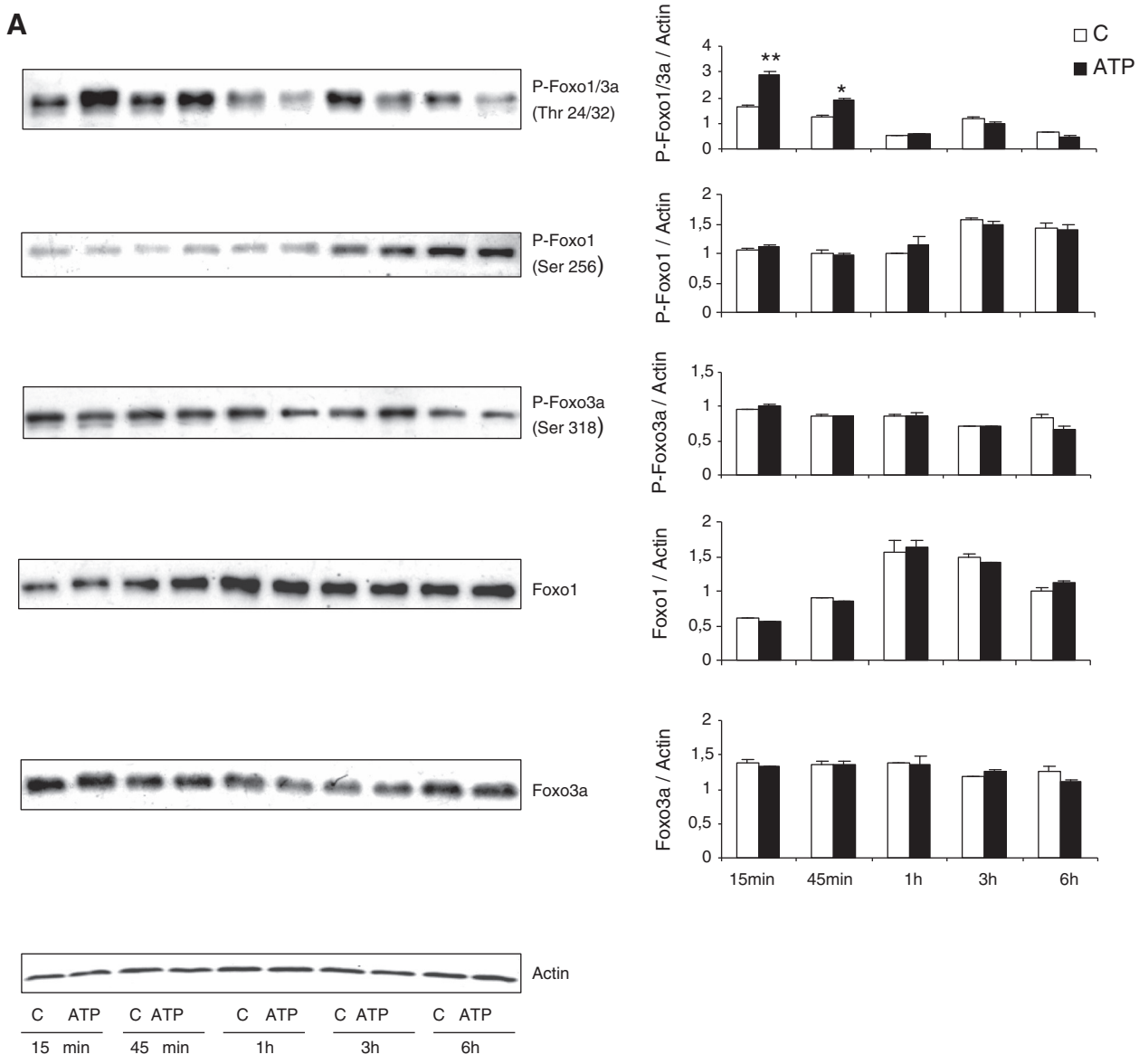

Fig. 1. Extracellular ATP induces the phosphorylation of FoxO1/3a and diminishes the expression of FoxO1 in MCF-7 cells. Serum-starved (24 h) MCF-7 cells were treated either with vehicle (C) or 5 μM ATP during 15 min–24 h. Proteins from lysates were prepared as described in Section 2.5, separated on 10% SDS-PAGE, and immunoblotted using anti-phospho FoxO1/3a (Thr24/32), anti-phospho FoxO1 (Ser256), anti-phospho FoxO3a (Ser318), anti-FoxO1, anti-FoxO3a and anti-β-actin antibodies. Densitometric analyses were performed on the immunoblots from three independent experiments; means ± SD are given. *P < 0.05, **P < 0.01 with respect to the corresponding control.

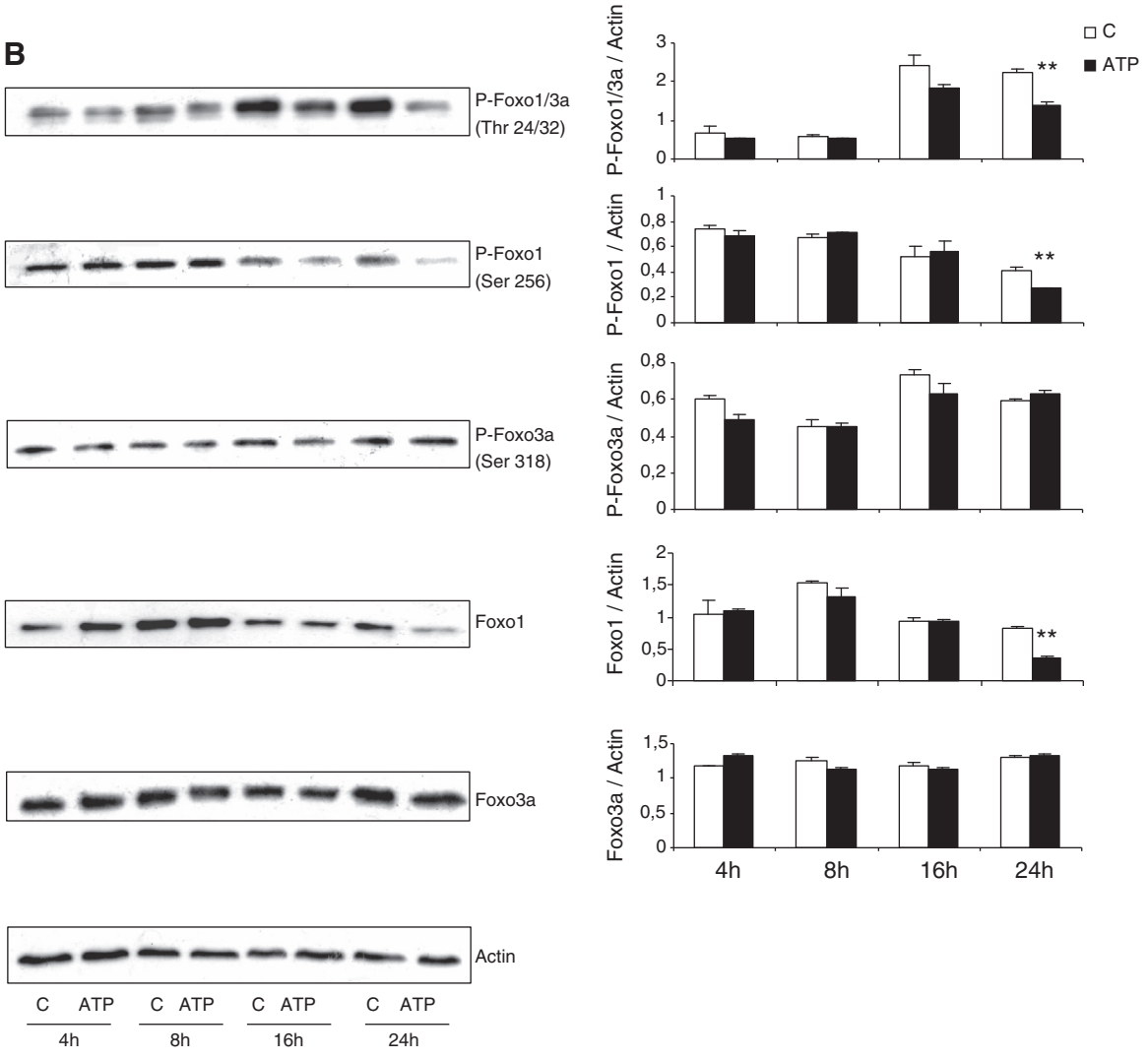

Fig. 1 (*continued*).

phosphorylated FoxO1/3a (Thr 24/32), phosphorylated FoxO1 (Ser 256), phosphorylated FoxO3a (Ser 318), anti-FoxO1, anti-FoxO3a and SignalSilence® Akt siRNA I and control siRNA were from Cell Signaling Technology (Beverly, MA, USA). Polyclonal antibodies recognizing anti-actin, anti-Akt1/2/3, polyclonal goat anti-rabbit and anti-mouse peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RNase Cocktail™ Enzime Mix was from Applied Biosystems (Carlsbad, California). Protein size markers were from Amersham Biosciences (Piscataway, NJ, USA), Immobilon P (polyvinylidene difluoride) membranes and Amersham ECL chemiluminescence detection kit were from GE Healthcare (Little Chalfont, Buckinghamshire, England). All other reagents used were of analytical grade.

2.2. Cell culture and treatment

The human breast cancer epithelial cell line MCF-7 (from the American Type Culture Collection; Manassas, VA, USA) was cultured at 37 °C in RPMI-1640 medium containing 10% FBS under humidified air (5.5% CO_2). Cultures were passaged every 2 days with fresh medium. Experimental cultures were grown to 50–70% confluence in serum-containing medium, and then cells were serum-deprived 24 h before agonist stimulation. Controls with vehicle (water) were

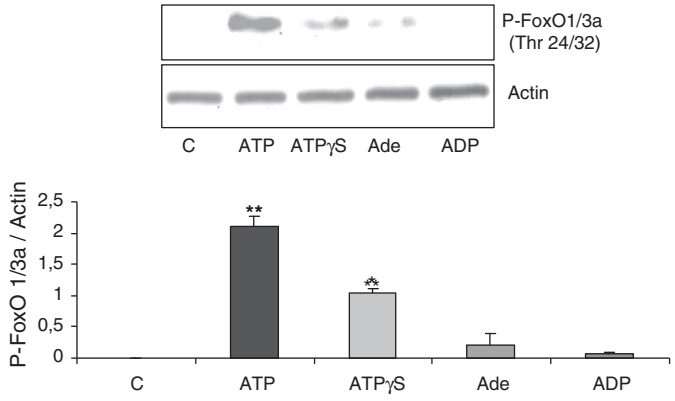

Fig. 2. Role of ATPγS, ADP and adenosine in the phosphorylation of FoxO1/3a (Thr24/32). MCF-7 cells were treated with 5 μM ATP, ATPγS, adenosine (Ade) and ADP or vehicle (C) for 15 min to compare their response on the phosphorylation of FoxO1/3a. Cell lysate proteins were immunoblotted with anti-phospho FoxO1/3a (Thr 24/32) antibody, and then the membranes were stripped and re-probed with anti-β-actin as loading control. Representative immunoblots and quantification by scanning volumetric densitometry of blots from three independent experiments are shown. Means ± SD are given. *$P < 0.05$, **$P < 0.01$, where P values refer to differences in phosphorylation of FoxO1/3a between cells in the presence or absence of agonist.

used. Where indicated, cells were pre-treated with inhibitors that were present during subsequent exposure to the agonist.

2.3. Subcellular fractionation

After cell culture and treatment (detailed in Section 2.2), MCF-7 cells were washed twice with cold PBS buffer. Then, cells were washed once with buffer A (50 mM glycerophosphate pH = 7.3, 1.5 mM EGTA, 1 mM EDTA, plus 1 mM DTT and 0.1 mM Na_3VO_4 that were added immediately before the experiment). Cells were then harvested with appropriate amount of buffer H (50 mM glycerophosphate pH = 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM Na_3VO_4, 1 mM benzamidine, 10 μg/mL aprotinin, 10 μg/mL leupeptin); and centrifuged at 100 ×g during 5 min at 4 °C. The resultant pellet was resuspended in appropriate volume of Nuclear Extraction Buffer (NEB) (10 mM HEPES-KOH pH = 7.9, 0.5% Triton X-100, 0.5 M sucrose, 0.1 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, plus fresh prepared 1 mM DTT, 0.5 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin), and homogenized with a manual homogenizer (20 strokes). This homogenate was centrifuged at 2292 ×g during 10 min at 4 °C. The supernatant was collected as cytosolic fraction; the nuclear fraction (pellet) was lysed in NEB supplemented with 0.5 M NaCl and 5% glycerol. Finally, subcellular fractions were properly prepared for SDS-PAGE and immunoblotting.

2.4. Cell transfection with siRNA against Akt

The expression of the serine/threonine kinase Akt was transiently silenced by the use of siRNA against Akt1, 2, 3. MCF-7 cells were seeded in plates at a density of 20,000 cells/cm^2 and cultured at 37 °C in RPMI-1640 medium containing 10% FBS under humidified air (5.5% CO_2). Transfections were performed using Lipofectamine 2000 reagent according to the manufacturer's instructions. siRNA against Akt1, 2, 3 as well as scrambled siRNA were used at a final concentration of 100 nM. When cells reached 30–40% confluence, their medium was renewed by adding 1.5 mL of RPMI-1640 medium containing 10% FBS to each plate. In addition, 0.5 mL of the mixtures scrambled siRNA/lipofectamine/

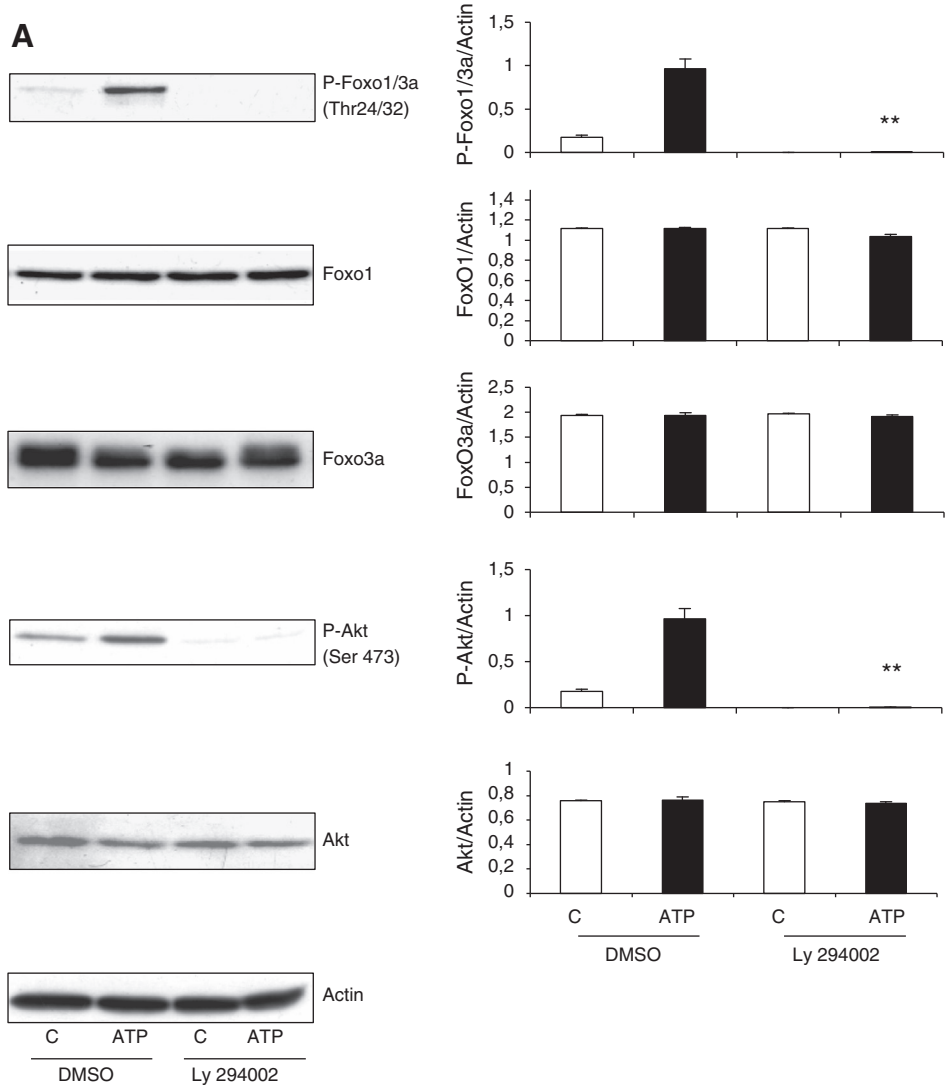

Fig. 3. Participation of the PI3K/Akt pathway in the phosphorylation of FoxO1/3a by extracellular ATP. A) MCF-7 cells were pre-incubated for 30 min with vehicle (DMSO) or Ly294002 (10 μM), an inhibitor of PI3K, and then exposed to ATP (5 μM) for 15 min followed by Western blot analysis of proteins from cell lysates using anti-phospho FoxO1/3a (Thr 24/32), anti-FoxO1, anti-FoxO3a, anti-phospho Akt (Ser 473) and anti-Akt antibodies. Blotted membranes were re-probed with anti-β-actin antibody. B) Cells were treated with 5 μM ATP or vehicle (C) for 15 min after transfection for 48 h with 100 nM siRNA against total Akt or scrambled siRNA, as described in Section 2.4. Western blot analysis was then performed using anti-phospho FoxO1/3a (Thr 24/32), anti-FoxO1, anti-FoxO3a, anti-phospho Akt (Ser 473) and anti-Akt antibodies. Blotted membranes were re-probed with anti-β-actin antibody. Representative immunoblots and the quantification by scanning densitometry of three independent experiments are shown. Means ± SD are given. *P < 0.05; **P < 0.01 where P values refer to differences in phosphorylation/expression of FoxO1/3a and Akt induced by ATP between cells in the presence or absence of inhibitor/siRNA.

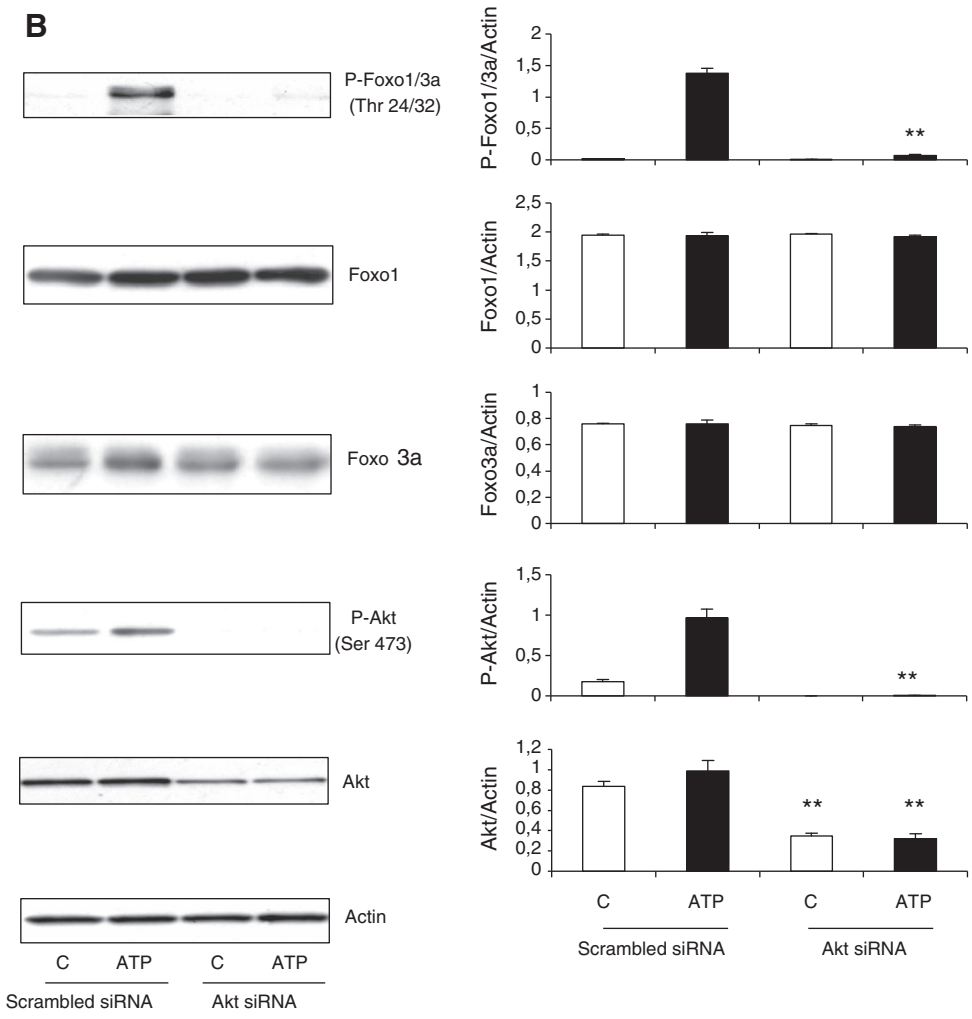

Fig. 3 (*continued*).

serum-free RPMI-1640 medium and Akt siRNA/lipofectamine/serum-free RPMI-1640 medium were added to the corresponding plate. \Cells were incubated with these mixtures at a final volume of 2 mL for 24 h at 37 °C in a CO_2 incubator. Then, medium was replaced by 2 mL of RPMI-1640 containing 10% FBS for another 24 h (transfection performed during 48 h). Finally, MCF-7 cells were starved during 24 h (2 mL serum-free RPMI-1640 medium) and then treated with ATP followed by SDS-PAGE and immunoblotting of cell extracts.

2.5. SDS-PAGE and immunoblotting

As indicated in the figure legends, MCF-7 cells were treated with ATP in the presence or absence of inhibitor. Then, they were washed with PBS buffer plus 25 mM NaF and 1 mM Na_3VO_4, and lysed in buffer containing 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 3 mM KCl, 1 mM EDTA, 1% Tween-20, 1% Nonidet P-40, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM NaF and 1 mM Na_3VO_4. The lysates were incubated on ice for 10 min, vortexed for 45 s and maintained on ice for another 10 min. After centrifugation at 14,000 ×*g* and 4 °C during 15 min the supernatant was collected and proteins were quantified by the Bradford method [10,15]. Lysate proteins dissolved in 6× Laemmli sample buffer [11,16] were separated (30 μg/lane) using SDS-polyacrylamide gels (10% acrylamide) and electrotransferred to PVDF membranes. After blocking with 5% non-fat milk in TBST buffer (50 mM Tris pH 7.2–7.4, 200 mM NaCl, 0.1% Tween-20), the membranes were incubated 90 min with the appropriate dilution of primary antibody in TBST plus 1% non-fat milk. After washing, the membranes were incubated with the appropriate dilution of horse radish peroxidase-conjugated secondary antibody in TBST plus 1% non-fat milk. Finally, the blots were developed by ECL with the use of Kodak BioMax Light film and digitalized with a GS-700 Imaging Densitomer (Bio-Rad, Hercules, CA, USA).

2.6. Stripping and re-probing of membranes

The complete removal of primary and secondary antibodies from the membranes was achieved by incubating the membranes in stripping buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS and 50 mM β-mercaptoethanol) at 55 °C for 30 min with agitation. Membranes were then washed for 10 min in TBST (1% Tween-20) and blocked, as indicated above, for 1 h at room temperature. Thereafter, membranes were ready to re-probe with the corresponding antibodies.

2.7. Immunocytochemistry

MCF-7 cells grown onto glass coverslips were fixed in methanol (at −20 °C). Non-specific sites were blocked with 5% BSA in PBS. Samples were then incubated with the appropriate primary antibody prepared in PBS, 2% BSA (1:50, 1 h, room temperature). After washing with PBS, the samples were incubated with secondary Alexa 488 conjugated antibody (1:200, 1 h, room temperature). The samples were examined using a Zeiss LSM 5 Pascal confocal laser microscope.

2.8. Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry. Cells incubated with or without ATP for 8 or 24 h were trypsinized, washed once with PBS, and fixed in absolute ethanol for at least 1 h at −20 °C. Fixed cells were washed with PBS and incubated with propidium iodide (PI) staining solution (69 μM IP, 38 mM sodium citrate and 0.7 mg/mL ribonuclease A, pH 7.4) for 30 min at 37 °C in the dark. The stained cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson). The program used for the acquisition and analysis of the samples was the CellQuest.

2.9. Statistical analysis

Data are shown as means ± standard deviation (SD). Statistical differences between groups were calculated by a two-tailed *t* test. $P < 0.01$ (**) and <0.05 (*) were considered highly statistically significant and statistically significant, respectively.

3. Results

We determined whether FoxO transcription factors were modulated by extracellular ATP in MCF-7 cells by performing time-course (15 min–24 h) studies. Western blot analyses showed that ATP maximally stimulated the phosphorylation of FoxO1/3a at the threonine residues 24/32, respectively, within 15 min of agonist exposure (Fig. 1A). A significant rate of phosphorylation was still observed after 45 min treatment with ATP (Fig. 1A). However, no statistically significant changes in the phosphorylation of FoxO1 (Ser 256) and FoxO3a (Ser 318) were observed (Fig. 1A and B). Extracellular ATP did not affect the expression of FoxO3a (Fig. 1A and B), while it diminished the expression of FoxO1 only at 24 h (Fig. 1B). Together,

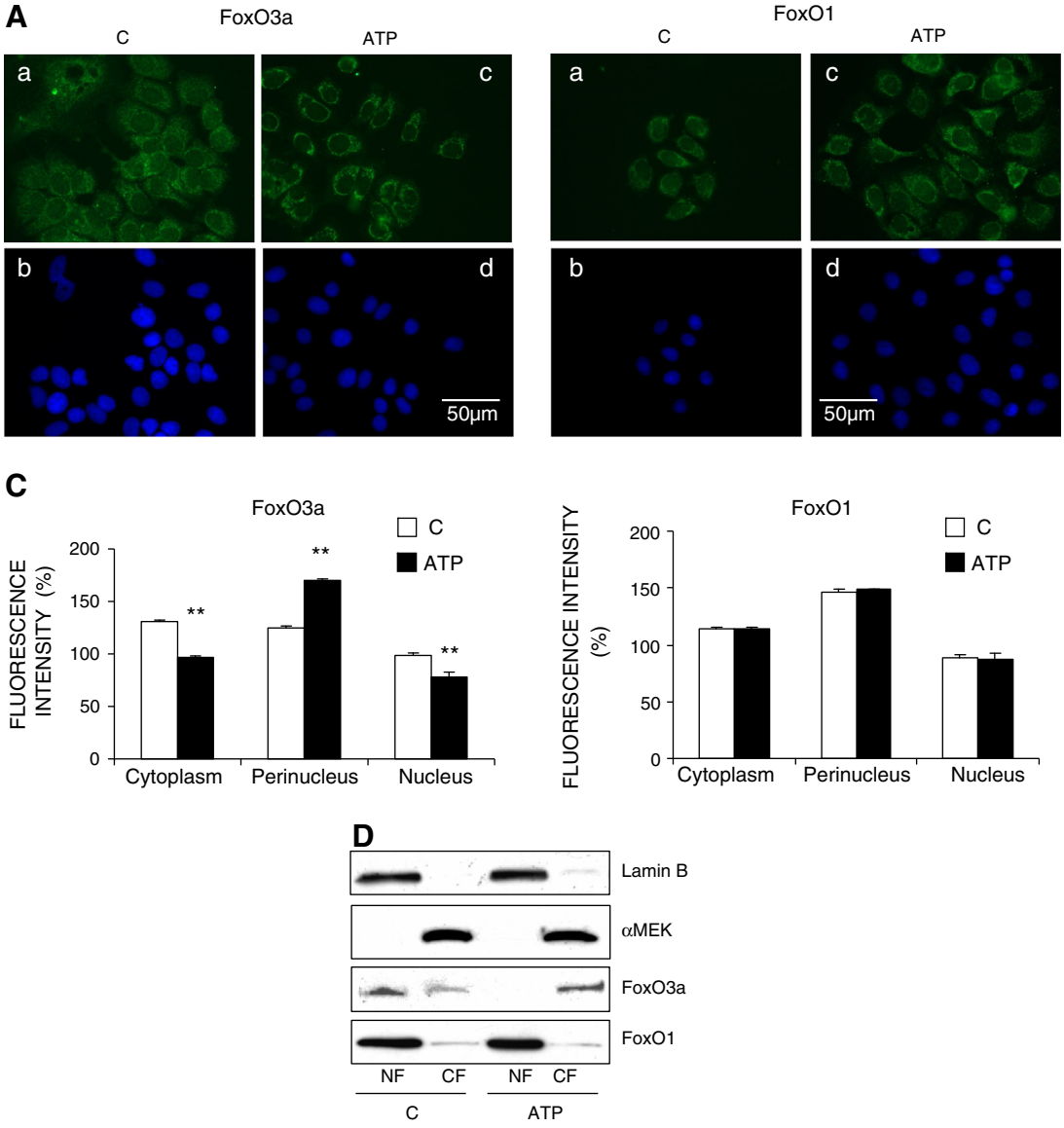

Fig. 4. Effect of ATP on the subcellular distribution of the transcription factors FoxO1 and FoxO3a in MCF-7 cells. Immunocytochemical studies were performed in MCF-7 cells treated with vehicle (C) or with 5 μM ATP for 15 min, as described in Section 2.7. Antibodies that recognize total FoxO3a (A) and FoxO1 (B) transcription factors were employed. Cell nuclei were stained with the nucleic acid stain DAPI. C) The vertical bar graphs show fluorescence quantification of nuclear, perinuclear and cytoplasmic regions of control (white bars) and ATP-treated cells (black bars). To quantify fluorescence, the summed pixel intensity was calculated by delimiting each region per cell using ImageJ software. Relative values of fluorescence were obtained and expressed as a percentage of fluorescence intensity. The results are shown as mean ± S.D. ($n = 20$). **$P < 0.01$, with respect to the control. D) Translocation of FoxO3a to the perinuclear region/cytosol was confirmed by performing a subcellular fractionation assay (Section 2.3). Briefly, cells were subject to differential centrifugation to obtain enriched nuclear (NF) and cytosolic (CF) fractions. Western blot analysis with anti-FoxO1, anti-FoxO3a, lamin B and αMEK antibodies was performed. The results shown are representative of, at least, three independent experiments.

these results suggest that FoxO1/3a is inactivated by extracellular ATP in MCF-7 cells.

Since extracellular ATP can be easily hydrolyzed into ADP and adenosine [17], the effects observed upon its addition might result from the action of these products. Western blot analyses using ATPγS (non-hydrolyzable analog of ATP), ADP and adenosine were performed to address this issue. Fig. 2 shows that the contribution of ADP and adenosine to the phosphorylation of FoxO1/3a (Thr 24/32) in MCF-7 cells is not statistically significant. However, ATPγS was a weaker inducer of FoxO1/3a phosphorylation when compared to the effect exerted by ATP.

The FoxO transcriptional factors are downstream targets of Akt and turn into transcriptionally inactive by Akt phosphorylation [10]. To address whether ATP is inactivating FoxO1/3a through the PI3K/Akt signaling pathway, Western blot analyses were performed in MCF-7 cells. Fig. 3A shows that the PI3K inhibitor, Ly294002, abolished the phosphorylation of FoxO1/3a (Thr 24/32) induced by treatment with ATP for 15 min. In addition, the inhibitor did not affect the expression of FoxO1 and FoxO3a. To test the successful inhibition of PI3K by Ly294002, the membrane was re-blotted with anti-phospho Akt (Ser 473) antibody [5]. These results were further confirmed by transiently silencing the serine/threonine kinase Akt by transfecting MCF-7 cells with siRNA against total Akt (see Section 2.4). As can be seen in Fig. 3B, the phosphorylation and expression of Akt and the levels of phosphorylation of FoxO 1/3a (Thr 24/32) were significantly reduced

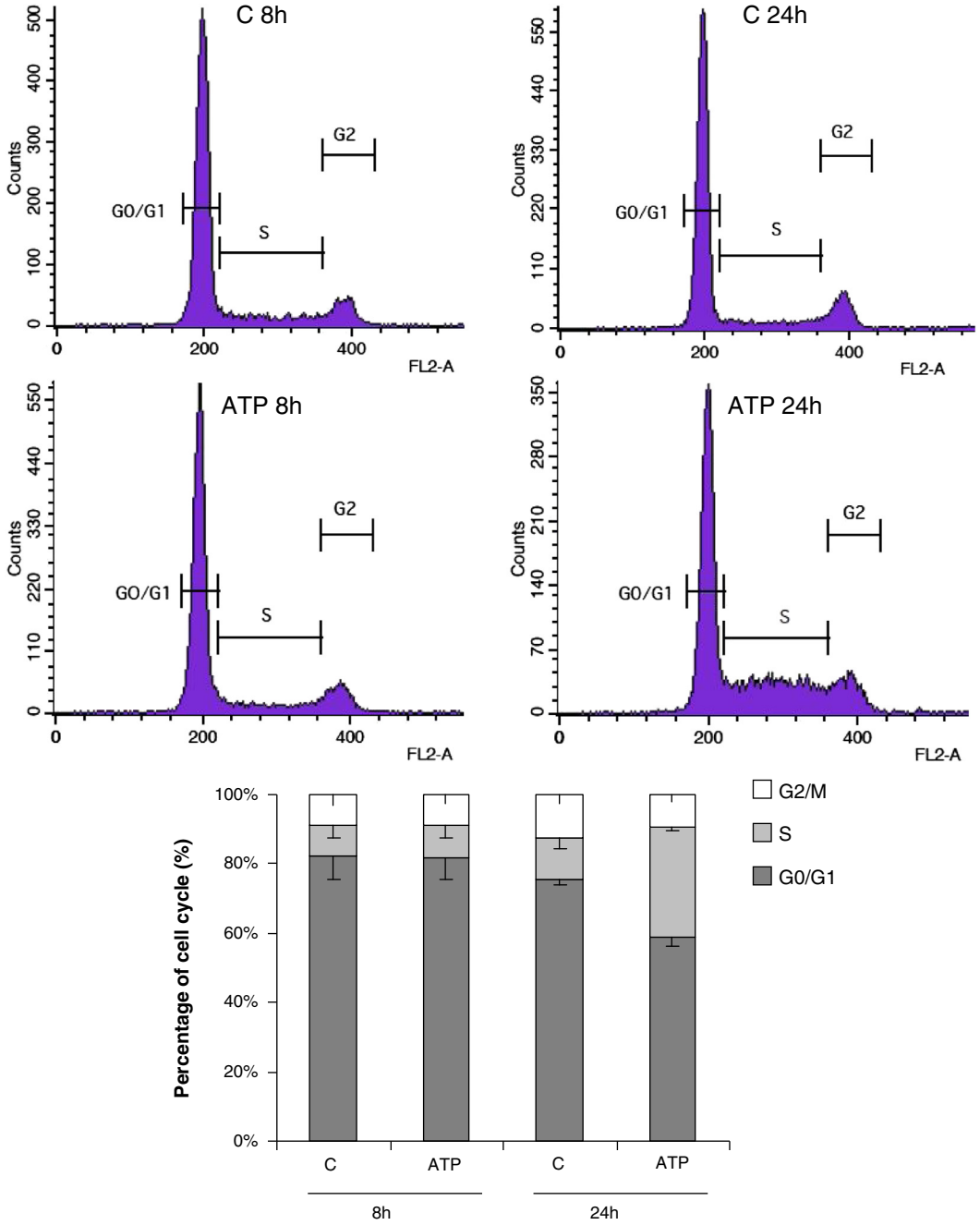

Fig. 5. Effect of extracellular ATP on cell cycle progression. MCF-7 cells were starved with serum-free RPMI-1640 medium for 24 h and then treated with 5 μM ATP (ATP) or with vehicle (C) for 8 and 24 h. Cells were then stained with propidium iodide and the distribution of cells in the cell cycle was analyzed by flow cytometry of DNA content. Representative cytometric profiles and percentages of each phase are shown. The program CellQuest was used for acquisition and analysis of FACS scans. Data represent means ± SD of three independent experiments. G0/G1: cells in G0/G1 cell cycle phases; S: cells in S cell cycle phase; G2/M: cells in G2/M cell cycle phases.

in the presence of Akt siRNA. Moreover, the transfection did not affect the expression of FoxO1 and FoxO3a. Thus, the PI3K/Akt pathway is involved in the phosphorylation of FoxO transcription factors by ATP in breast cancer cells.

Akt-mediated phosphorylation of FoxO factors reduces their binding to DNA and transcriptional activity allowing them to be exported from the nucleus. Then, cytoplasmic Akt-phosphorylated FoxOs undergo proteasomal degradation [8]. Immunocytochemical studies were performed to determine the role of ATP in the subcellular distribution of FoxO1 and FoxO3a. Fig. 4A and C shows that ATP induced a perinuclear/cytosolic distribution of FoxO3a. However, it had no effect on the cellular distribution of FoxO1, as MCF-7 cells show a clear homogeneous nuclear and cytosolic distribution of FoxO1 both in control and treated cells (Fig. 4B and C). Cellular fractionation studies further confirmed these results. Fig. 4D shows the presence of FoxO3a in nuclear and cytoplasmic fractions of control cells. However, FoxO3a was only detected in the cytoplasmic fraction after ATP exposure. In addition, no subcellular rearrangement of FoxO1 was observed between control and ATP treated cells.

FoxO transcription factors are attracting increasing interest because they can regulate the cell cycle through integration of many signaling pathways [9]. Thus, we first studied the effect of extracellular ATP on the progression of cell cycle in MCF-7 cells. To that end, cells were incubated with ATP (5 μM) or vehicle for 8 and 24 h. Then, the percentages of cells in the G0/G1, S, and G2/M phases of cell cycle were determined by flow cytometric analysis of propidium iodide stained cells as described in Section 2.8. Fig. 5 shows that ATP significantly increased the percentage of cells in S phase from 11.7% to 31.5% ($P < 0.01$) at 24 h, which was accompanied by a corresponding reduction in the percentage of cells in G0/G1 phase. These data suggest that ATP induces cell cycle progression in MCF-7 cells. We then evaluated if this effect was related to changes in the expression of cell cycle-regulatory proteins that participate in the progression from G1 to the S phase. MCF-7 cells were treated with ATP (5 μM) or vehicle (control) for 45 min–24 h followed by Western blot analysis using specific antibodies. As demonstrated in Fig. 6, extracellular ATP (45 min) markedly induced the expression of the early G1 phase cyclins D1 and D3. As the cell cycle is also controlled by specific CDK inhibitors, we studied the role of ATP in the expression of the cell cycle inhibitory proteins p27Kip1 and p21Cip1. As expected, ATP reduced the expression of p27Kip1, and to a greater extent, the expression of p21Cip1 after 6 and 24 h of exposure to the purinergic agonist (Fig. 6). Taken together, these results reveal that extracellular ATP induces cell cycle progression by up-regulation of cyclins D1 and D3 and down-regulation of the inhibitory proteins p27Kip1 and p21Cip1.

To gain insight into the signaling events that link extracellular ATP to the cell cycle machinery, we tested the participation of the PI3K/Akt signaling pathway in the regulation of the expression of cyclins D1 and D3, and p27Kip1 and p21Cip1 cell cycle inhibitory proteins. Fig. 7A shows that Ly294002 markedly reduced the expression of cyclins D1 and D3. Similar results were observed after MCF-7 cell

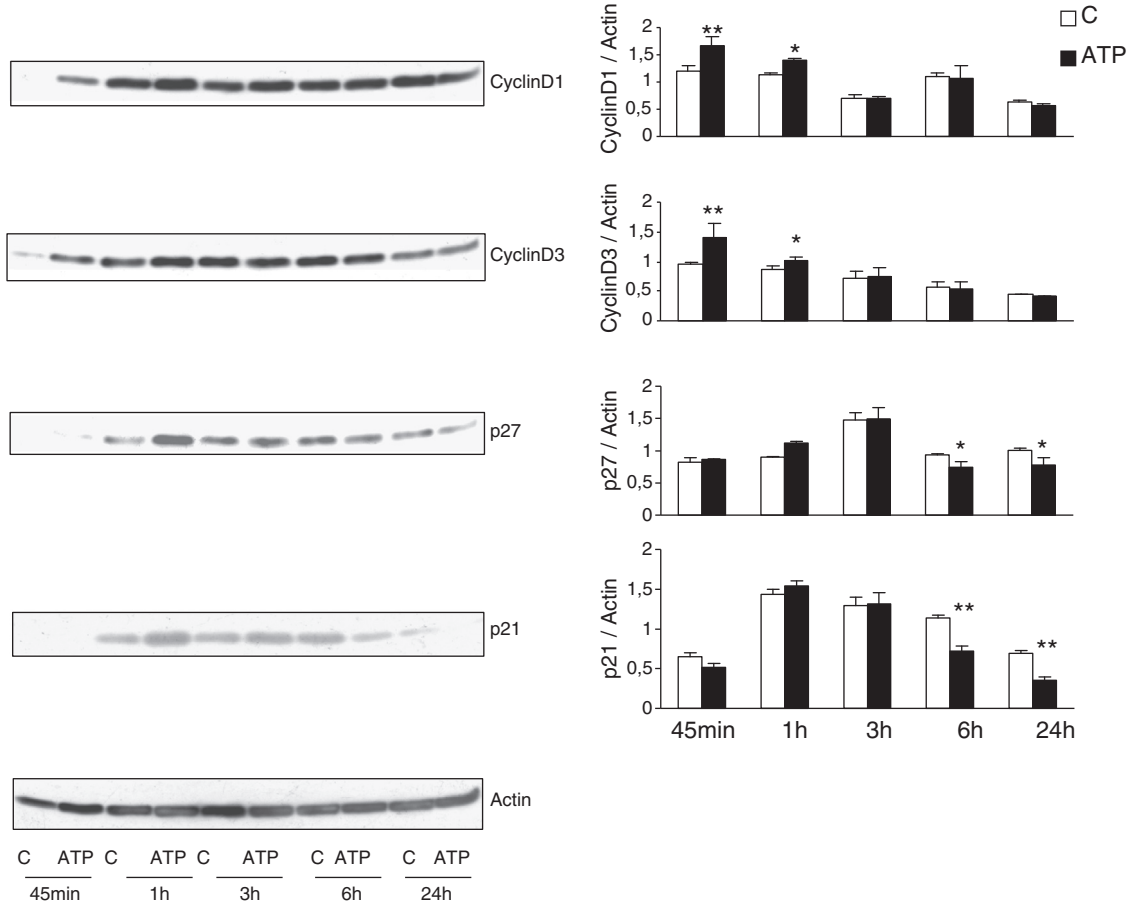

Fig. 6. Extracellular ATP induces the expression of cyclins D1 and D3 and diminishes the expression of p21 and p27. MCF-7 cells were incubated in serum-free RPMI for 24 h and then treated with vehicle (C) or ATP (5 μM) for 45 min, 1 h, 3 h, 6 h and 24 h. Lysate proteins were prepared as described in Section 2.5, separated on 10% SDS-PAGE, and immunoblotted using anti-cyclin D1, anti-cyclin D3, anti-p21Cip1 and anti-p27Kip1 antibodies. In order to evaluate the equivalence of protein content among the different experimental conditions, blotted membranes were re-probed with anti-β-actin antibody. Densitometric analyses of three independent experiments were performed; means ± SD are given. *$P < 0.05$, **$P < 0.01$ with respect to the corresponding control.

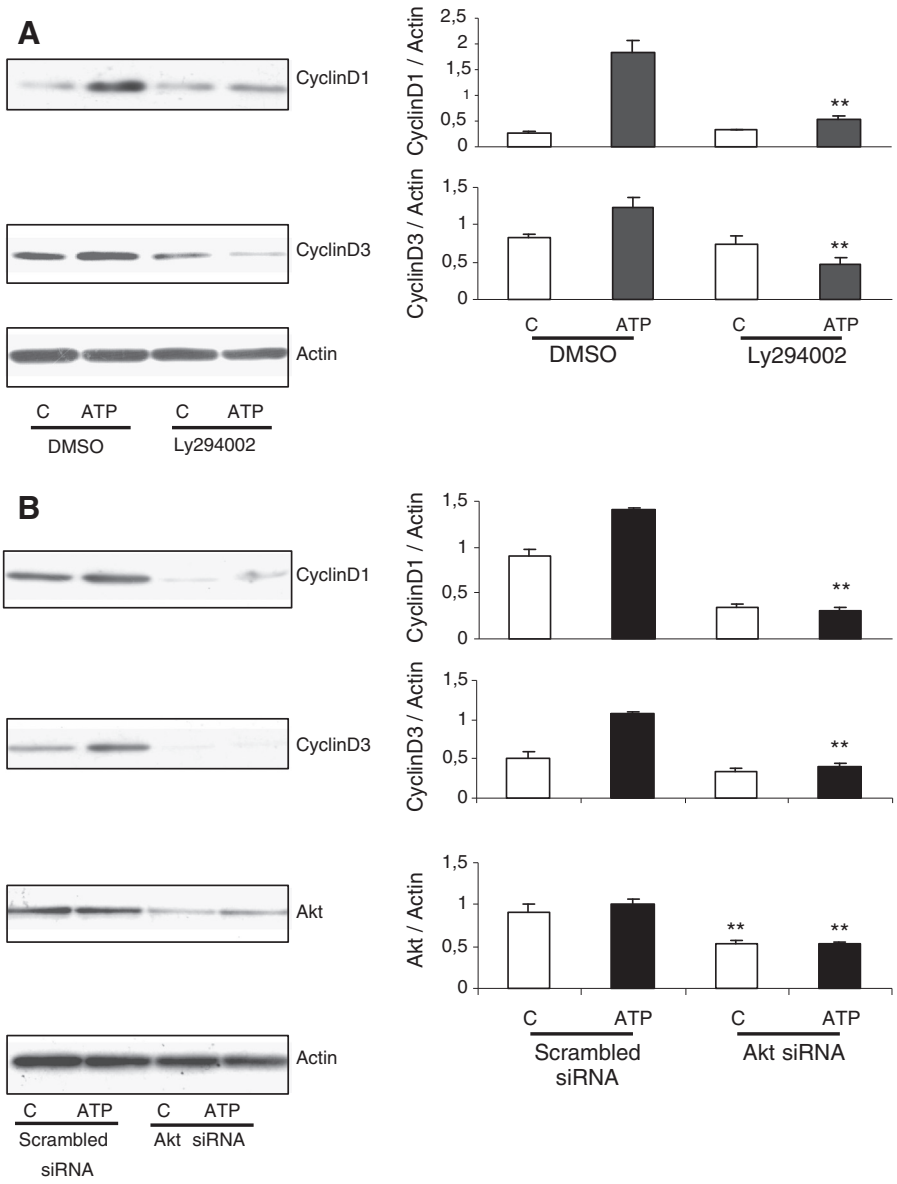

Fig. 7. Participation of the PI3K/Akt pathway in the expression of the cell cycle regulatory proteins cyclins D1 and D3. The effect of Ly294002 (A) and of siRNA against total Akt (B) on the expression of cyclins D1 and D3 was studied. MCF-7 cells were pre-incubated for 30 min with vehicle (DMSO) or Ly294002 (10 μM), an inhibitor of PI3K; or were transfected for 48 h with scrambled siRNA or Akt siRNA (100nM) as detailed in Section 2.4. Then, cells were exposed to ATP (5 μM) or vehicle (C) for 45 min followed by Western blot analysis of proteins from cell lysates using anti-cyclin D1 or anti-cyclin D3 antibodies. Blotted membranes were re-probed with Akt and anti-β-actin antibodies. Representative immunoblots and the quantification by scanning densitometry of three independent experiments are shown; means ± SD are given. *$P < 0.05$; **$P < 0.01$ with respect to the control.

transfection with Akt siRNA (Fig. 7B). Transiently silenced Akt also reversed the down-regulation of p21Cip1 and p27Kip1 cell cycle inhibitory proteins induced by extracellular ATP in MCF-7 cells (Fig. 8). These findings indicate that ATP modulation of the expression of cyclin D1, cyclin D3, p21Cip and p27Kip1 is PI3K/Akt dependent in MCF-7 cells.

Flow cytometric analysis of propidium iodide stained MCF-7 cells were performed in the presence or absence of Ly294002 to evaluate the role of the PI3K/Akt signaling pathway in the progression of cell cycle induced by extracellular ATP. As can be seen in Fig. 9, the PI3K inhibitor significantly reduced the percentage of cells in S phase from 28.9% to 8.6% ($P < 0.01$) after a 24 h treatment with ATP. This decrease was accompanied by an equivalent increment in the percentage of cells in the G0/G1 phase. These data suggest that ATP induces cell cycle progression through the PI3K/Akt pathway in MCF-7 cells.

It has been shown that FoxO transcription factors can modulate the cell cycle in many cell types [18–20]. Thus, we studied the participation of FoxOs in the regulation of cell cycle progression by extracellular ATP. To that end, flow cytometric analysis of propidium iodide stained MCF-7 cells were performed in the presence or absence of bortezomib, an in vitro inhibitor of proteasome-dependent degradation of members of the FoxO family of tumor suppressors [21]. The inhibitor conditions (pre-incubation time and dose), as determined by Western blot, were those which reversed the down-regulation of FoxO1 induced by ATP (data not shown). Fig. 10 shows that the inhibitor significantly reduced the percentage of cells in the S phase from 27.6% to 20% ($P < 0.01$) after cell treatment with ATP for 24 h. This reduction was accompanied by a proportional increase in the percentage of cells in the G0/G1 phase. These findings suggest that FoxO transcription factors negatively modulate cell cycle

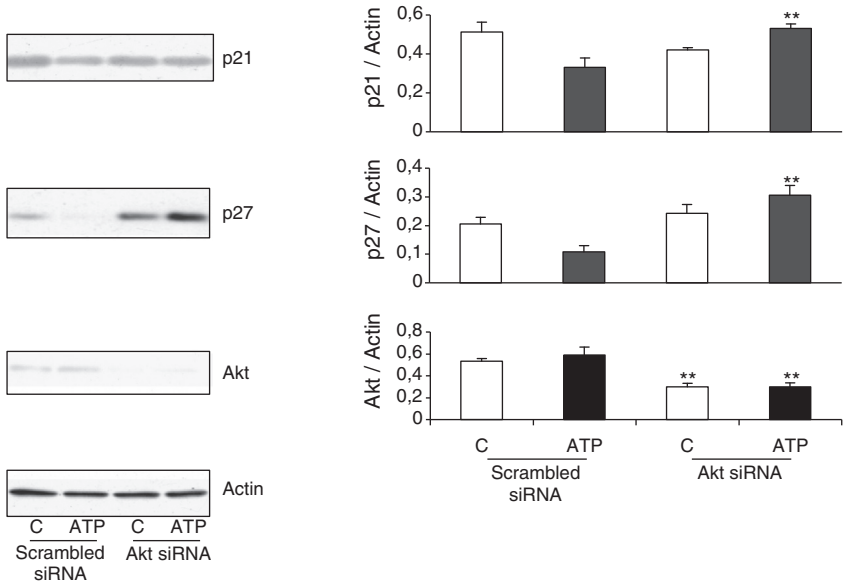

Fig. 8. Participation of the PI3K/Akt pathway in the expression of the cell cycle regulatory proteins p21Cip1 and p27Kip1. The effect of siRNA against total Akt on the expression of p21Cip1 and p27Kip1 was studied. MCF-7 cells were transfected for 48 h with scrambled siRNA or Akt siRNA (100 nM) as detailed in Section 2.4. Then, cells were exposed to ATP (5 μM) or vehicle (C) for 6 h followed by Western blot analysis of proteins from cell lysates using anti-p21Cip1 or anti-p27Kip1 antibodies. Blotted membranes were re-probed with Akt and/or anti-β-actin antibodies. Representative immunoblots and the quantification by scanning densitometry of three independent experiments are shown; means ± SD are given. *P < 0.05; **P < 0.01 with respect to the control.

progression, thus when their degradation is inhibited there is an increase in the number of cells in the S phase.

4. Discussion

The present study provides evidence to our knowledge, for the first time, on the modulation of FoxO1/3a transcription factors and cell cycle proteins through the PI3K/Akt pathway by extracellular ATP in MCF-7 cells.

Purines were long thought to be restricted to the intracellular compartment where they are used for energy transactions, nucleic acid synthesis, and a multiplicity of biochemical reactions. However, it is now clear that adenosine triphosphate and other nucleotides are abundant biochemical components of the tumor microenvironment and key players in host—tumor interaction. Moreover, ATP can directly affect tumor cell growth [22].

The FoxO transcription factors, composed of four members (FoxO1, FoxO3a, FoxO4, and FoxO6), are known to be involved in many cellular processes including apoptosis, cell cycle arrest, detoxification of reactive oxygen species, repair of damaged DNA, and glucose metabolism. Emerging evidence suggests involvement of FoxOs in diverse intracellular signaling pathways with critical roles in a number of physiological as well as pathological conditions including cancer. In addition, loss of FoxO function has been identified in several human cancers, resulting in increased cellular survival [23]. The FoxO factors are therefore tumor suppressors, and their potential use as therapeutic targets in cancer has been a matter of debate [24]. The activity of FoxO proteins is modulated by a shuttling mechanism between cell nucleus and cytoplasm, and by post-translational modifications as phosphorylation, acetylation and ubiquitination. FoxO1, FoxO3a and FoxO4 transcription factors are downstream targets of the serine/threonine kinase Akt. Phosphorylation of FoxO factors by Akt at three key regulatory sites (Thr32, Ser253, and Ser315 in the FoxO3a sequence) triggers the rapid re-localization of FoxO proteins from the nucleus to the cytoplasm [25]. As a result, their transcriptional functions are inhibited leading to their ubiquitination and degradation through the 26S proteasome [24], and contributing to cell survival, growth, and proliferation [24,26]. Therefore, we examined if FoxO transcription factors were modulated by extracellular ATP in breast cancer cells. Time-course (15 min–24 h) studies were performed to analyze FoxOs' three key regulatory phosphorylation sites by Akt. Western blot analyses revealed no statistically significant differences in the phosphorylation of FoxO1 (Ser 256) and FoxO3a (Ser 318) between control and ATP treated cells. In contrast, extracellular ATP (15–45 min) markedly induced the phosphorylation of FoxO1/3a at threonine 24/32 in MCF-7 cells. Besides, the purinergic agonist reduced the expression of FoxO1 after a longer treatment time (24 h). This down-regulation of FoxO1 could be mistakenly interpreted as a reduction in the levels of phosphorylated FoxO1/3a (Thr24/32) and FoxO1 (Ser 256).

The serine/threonine protein kinase Akt plays a pivotal role in tumorigenesis because it affects the growth and survival of cancer cells. Akt activation is a multistep process involving the phosphorylation of Ser 473 and Thr 308 residues, and the phosphorylation of these sites that can be closely correlated with the activity of Akt. These events can occur through a PI3K-dependent mechanism due to phosphatidyl-inositol triphosphate (PIP3) formation; or through a PI3K independent mechanism. We previously found, in MCF-7 cells, that extracellular ATP induces the phosphorylation of Akt at serine 473, one of the phosphorylation sites necessary for its full activation, through PI3K [5]. Thus, we studied if the phosphorylation of FoxO1/3a was mediated through the PI3K/Akt signaling pathway. As expected, our results showed that the phosphorylation level of FoxO1/3a at Thr24/32 significantly decreased after PI3K inhibition by Ly294002 and knockdown of Akt. To further prove the inactivation of FoxO1/3a transcription factors by extracellular ATP, we carried out immunocytochemical and cellular fractionation studies. Altogether, our results suggest that extracellular ATP is contributing to breast cancer cell survival, growth and proliferation through the inactivation of the transcription factors FoxO1/3a. Accordingly, studies performed in prostate cancer cells have shown loss of FoxO1 and FoxO3a activities, suggesting that they are necessary for limiting cell tumor growth [27]. In addition, inhibition of FoxO3a activity can result in enhanced prostate tumor cell growth while agents that increase FoxO3a activity prevent prostate cancer cell progression [28]. Also in breast cancer cells increased activity of FoxO3a can prevent breast cancer cell growth. There, overexpression of FoxO3a decreased the expression of estrogen receptor regulated genes and inhibited 17beta-estradiol (E2)-dependent breast

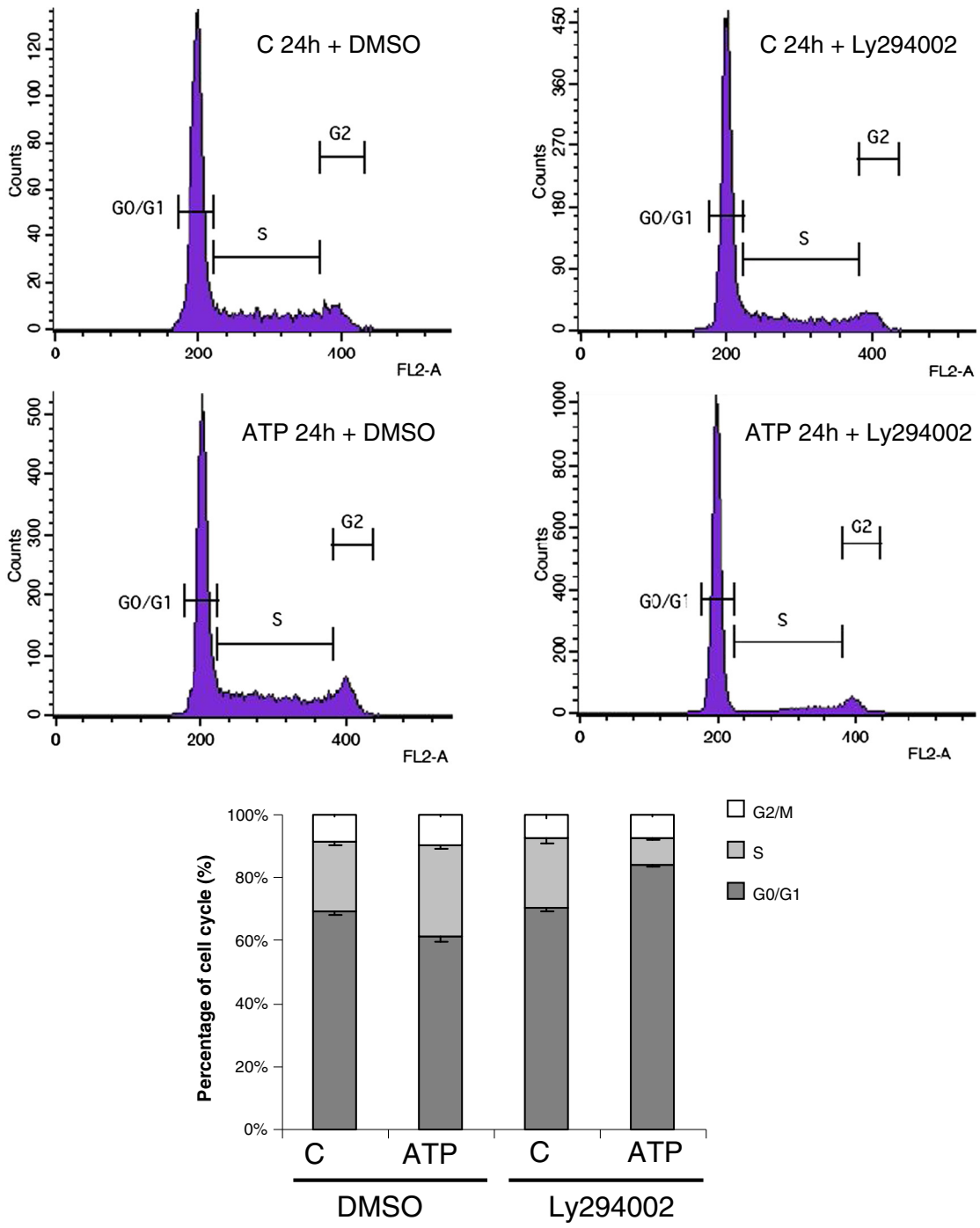

Fig. 9. Participation of the PI3K/Akt signaling pathway in cell cycle progression induced by extracellular ATP. MCF-7 cells were starved with serum-free RPMI-1640 medium for 24 h, pre-incubated with 10 μM Ly294002 or DMSO for 30 min, and finally treated with 5 μM ATP (ATP) or with vehicle (C) for 24 h. Cells were then stained with propidium iodide and the distribution of cells in the cell cycle was analyzed by flow cytometry of DNA content. Representative cytometric profiles and percentages of each phase are shown. The program CellQuest was used for acquisition and analysis of FACS scans. Data represent means ± SD of three independent experiments. G0/G1: cells in G0/G1 cell cycle phases; S: cells in S cell cycle phase; G2/M: cells in G2/M cell cycle phases.

cancer growth [29]. Therefore, FoxO proteins may represent a viable option to control tumor progression as they can function as redundant repressors of tumor growth.

Several reports show that ATP or other nucleotides inhibit or promote cell cycle progression, depending on the experimental conditions and cell types [30–34]. Previous studies in our laboratory demonstrated that extracellular ATP increased the number of viable MCF-7 breast cancer cells [5]. However, the effects exerted by ATP on breast cancer cell cycle are unknown. Therefore, we analyzed MCF-7 cell DNA contents by flow cytometry. We found that extracellular ATP increases the number of cells in the S phase and diminishes the number of cells in G0/G1 phase.

Alterations of genes involved in the regulation of cell cycle progression are frequent events in human cancers. Cell cycle progression is regulated by a group of serine/threonine kinases called cyclin-dependent kinases (CDKs) that interact with cyclins to coordinate the biochemical sequences which result in cell division. A group of small proteins called CDK-inhibitors (CDKIs) bind to and inhibit the activity of cyclin/CDK complexes, negatively regulating cell cycle progression [35]. Deregulation of key G1 phase cell cycle proteins as overexpression of cyclins

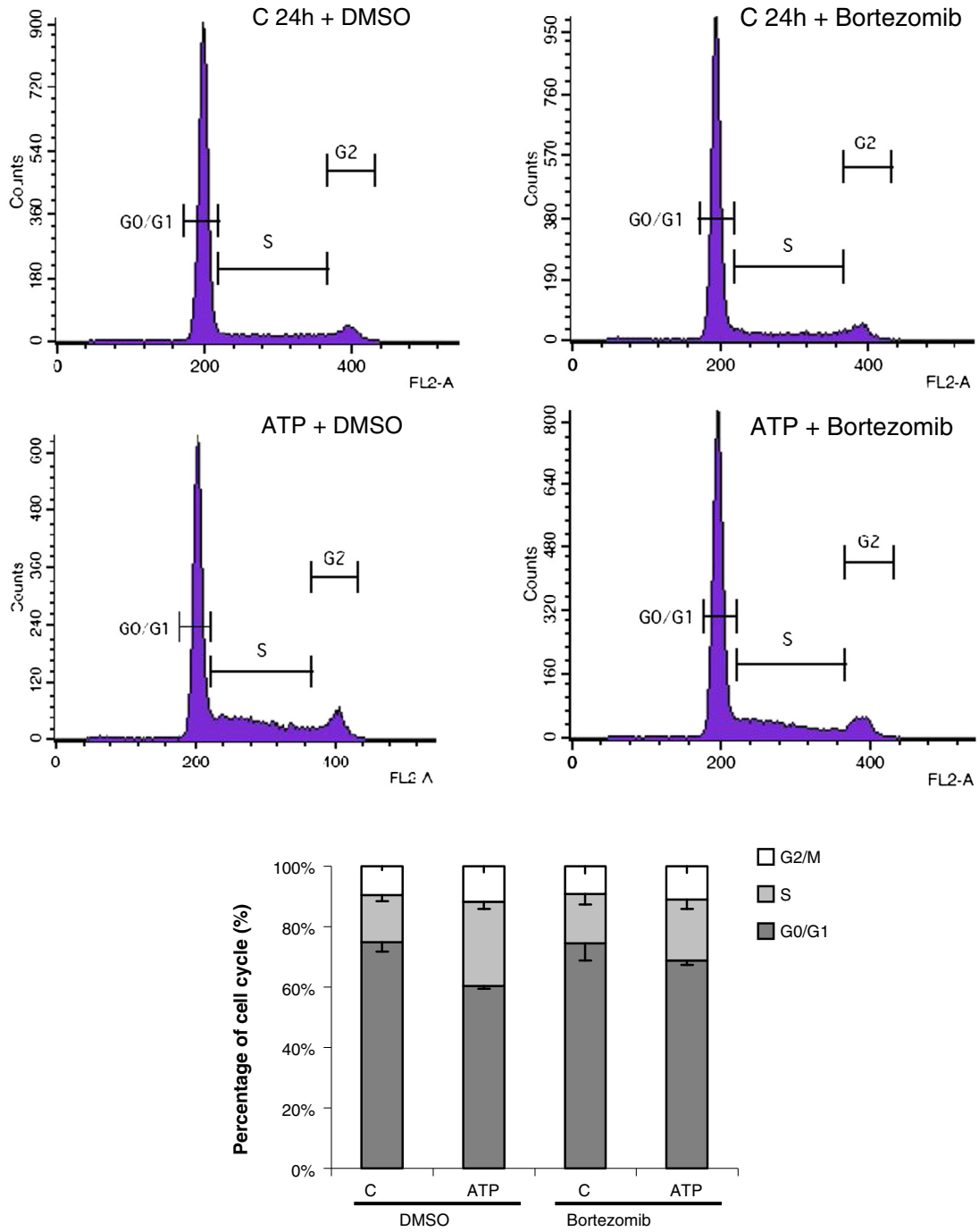

Fig. 10. Bortezomib inhibits cell cycle progression induced by extracellular ATP. MCF-7 cells were starved with serum-free RPMI-1640 medium for 24 h, pre-incubated with 10 nM bortezomib or DMSO for 60 min, and finally treated with 5 μM ATP (ATP) or with vehicle (C) for 24 h. Cells were then stained with propidium iodide and the distribution of cells in the cell cycle was analyzed by flow cytometry of DNA content. Representative cytometric profiles and percentages of each phase are shown. The program CellQuest was used for acquisition and analysis of FACS scans. Data represent means ± SD of three independent experiments. G0/G1: cells in G0/G1 cell cycle phases; S: cells in S cell cycle phase; G2/M: cells in G2/M cell cycle phases.

D1 and D3 and down-regulation of CDKIs such as p21 and p27 provides a selective growth advantage to tumor cells [36–39].

D-type cyclins (e.g. cyclins D1, D2, and D3) are regulators of CDK4 and CDK6 which mediate the growth factor-induced progression through the G1 phase of the cell cycle. Cyclin D1 plays a pivotal role as a protooncogene in a number of human malignancies including breast cancer. Moreover, overexpression of cyclin D1 in the mammary epithelium leads to the formation of tumors; also, interference of its nuclear export and proteolytic degradation has been shown to accelerate mammary carcinogenesis. Thus, cyclin D1 is a candidate molecular target for breast cancer therapy [40].

The CDK-inhibitor p21 (also called WAF1, Cip1) is the founding member of the Cip/Kip family of CDKIs, which also includes p27, and represents a downstream signal of the PI3K/Akt pathway and of p53. Higher levels of p21Cip1 expression might indicate a more indolent type of breast cancer. However, the role of p21Cip1 in breast carcinomas has been controversial in laboratory and clinical studies [41,42]. Another member of the p21 family of the CDKIs, p27Kip1, is one of the most prevalent growth inhibitors or tumor suppressors [38]. An increase in p27Kip1 protein causes proliferating cells to exit the cell cycle, whereas a decrease in p27Kip1 protein allows quiescent cells to resume proliferation. Also, loss of or low expression of p27Kip1 protein is associated

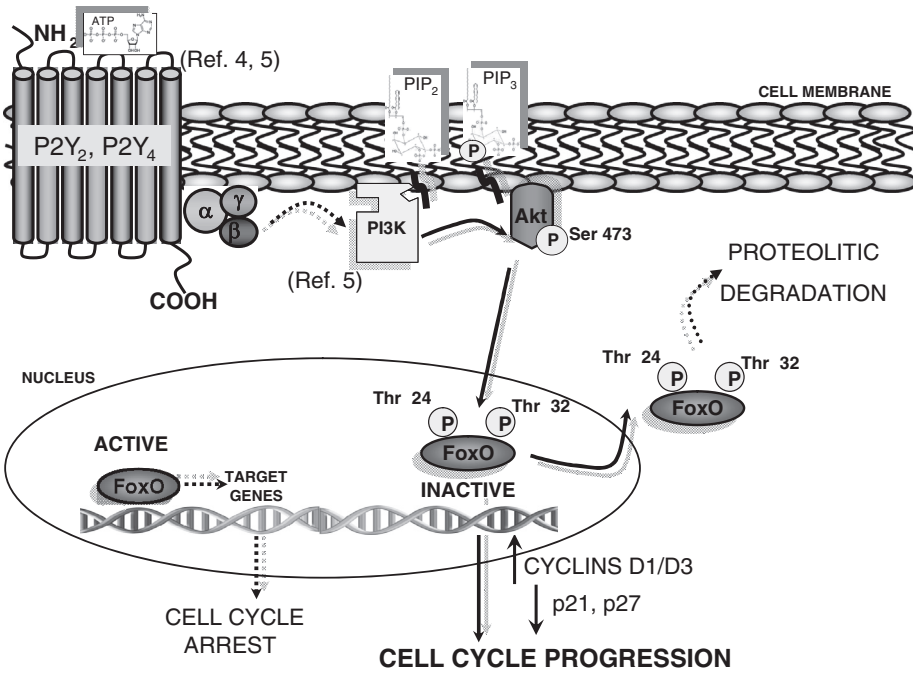

Fig. 11. Proposed model of ATP signaling through PI3K/Akt, FoxO transcription factors and cell cycle in MCF-7 cells. The scheme illustrates, on the basis of the results obtained in previous and in this study, the ATP signaling pathway through PI3K/Akt and FoxOs in breast cancer cells.

with excessive cell proliferation and with unfavorable prognosis of human tumors including breast and ovarian carcinomas [35,43]. Our studies demonstrate that ATP induces the expression of cyclins D1 and D3 in MCF-7 cells. In addition, p21 and p27 protein levels are significantly decreased by the purinergic agonist. Therefore, extracellular ATP is associated with cell cycle progression via up-regulation of cyclins D1 and D3, and down-regulation of the CDK inhibitors p21 and p27 in human breast cancer cells. These results further support the tumorigenic role of extracellular ATP in breast cancer development.

Then, to explore the potential mechanisms involved in the regulation of cell cycle proteins by extracellular ATP, we evaluated the impact of the PI3K inhibitor, Ly 294002, and/or siRNA against total Akt on the cell cycle in MCF-7 cells. Indeed, it has been found that in addition to its well-known role in various cell survival and metabolic responses, the PI3K/Akt signaling pathway plays an important function in regulating G1/S cell cycle progression. The CDKIs p21Cip1 and p27Kip1 are recognized substrates of Akt, thus hyperactivation of this lipid signaling pathway may lead to cell cycle deregulation in human cancers [44]. Particularly in breast cancer and other cell types, it has been shown that p27Kip1 is a direct target of the PI3K/Akt axis. Accordingly, it has been reported that Akt phosphorylates p27Kip1 both in vitro and in vivo in breast tumors [45–47]. On the other hand, studies have shown that blockage of Akt signaling results in apoptosis and growth inhibition of tumor cells [48]. Our data are then in keeping with these reports as both Ly294002 and siRNA against Akt could reverse the effects of extracellular ATP on the expression of the cyclins D1 and D3 and the inhibitory proteins p21Cip1 and p27Kip1, and also on cell cycle progression in MCF-7 breast cancer cells.

Besides, FoxO proteins can arrest the cell cycle by activating the cyclin-dependent kinase (CDK) inhibitors p21Cip1 and p27Kip1, and repressing the transcription of cyclins D1 and D2 [18–20,49]. Thus, the overall effect of active FoxO on cell cycle control is G1 arrest. Here, extracellular ATP induced inactivation of FoxO and cell cycle progression. The proteolytic inhibitor bortezomib could arrest MCF-7 cells in G0/G1 cell cycle phases denoting the participation of FoxO factors in cell cycle progression.

In conclusion, our results suggest that extracellular ATP, most likely through the PI3K/Akt pathway, modulates the phosphorylation, expression and subcellular localization of FoxO1/3a transcription factors and also the expression of proteins involved in the cell cycle, resulting in cell cycle progression of human breast cancer MCF-7 cells (Fig. 11). Therefore, selective antagonists of the downstream mediators PI3K/Akt, FoxO transcription factors and/or cell cycle proteins, should be considered for the development of effective breast cancer therapeutic strategies.

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