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Statins and ATP regulate nuclear pAkt via the P2X7 purinergic receptor in epithelial cells

Oras Mistafa, Johan Högberg, Ulla Stenius *

Institute of Environmental Medicine, Karolinska Institutet, Box 210, 17177 Stockholm, Sweden

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Abstract

Many studies have documented P2X7 receptor functions in cells of mesenchymal origin. P2X7 is also expressed in epithelial cells and its role in these cells remains largely unknown. Our data indicate that P2X7 regulate nuclear pAkt in epithelial cells. We show that low concentration of atorvastatin, a drug inhibiting HMG-CoA reductase and cholesterol metabolism, or the natural agonist extracellular ATP rapidly decreased the level of insulin-induced phosphorylated Akt in the nucleus. This effect was seen within minutes and was inhibited by P2X7 inhibitors. Experiments employing P2X7 siRNA and HEK293 cells heterologously expressing P2X7 and *in vivo* experiments further supported an involvement of P2X7. These data indicate that extracellular ATP and statins via the P2X7 receptor modulate insulin-induced Akt signaling in epithelial cells.

Keywords: pAkt; P2X7; Statins; Extracellular ATP; Ca^{2+}; Insulin

Statins, 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors, are potent cholesterol lowering drugs. Currently more than 25 million people worldwide are treated with statins to prevent cardiovascular disease. There is also an increasing interest in possible anti-cancer effects of statins [1,2]. Most cardiovascular effects can be attributed to their HMG-CoA-reductase-inhibiting properties, leading to low levels of cholesterol and/or intermediary metabolites affecting cell signaling [1,2]. However, HMG-CoA-reductase inhibition can hardly explain a number of rapid pleiotropic effects of statins. These effects are seen within seconds or minutes and include the binding of statins to the leukocyte LFA-1 receptor [3], activation of eNOS [4], and increased intracellular [Ca^{2+}] [5].

We have previously studied the effect of statins on Mdm2 and on insulin activated/phosphorylated Akt (pAkt) in epithelial cells [6,7], and a conspicuous finding was that statins affected nuclear levels of pAkt within 5 min [7]. Akt (protein kinase B) is an anti-apoptotic factor activated in the plasma membrane by insulin, growth factors and cellular stress, and recent studies indicate a key role for Akt in, e.g., carcinogenesis and as a target for therapeutic agents [8–12].

In this study the effect of statins was investigated in epithelial cells in an effort to understand possible anti-cancer effects of statins. We tested the possibility that statin-induced effects on nuclear pAkt are mediated by the P2X7 receptor. P2X7 is activated by extracellular purinergic nucleotides such as ATP, and the release of ATP in the blood stream leads to pleiotropic cellular effects [13] at least partially overlapping those induced by statins. These include increased intracellular [Ca^{2+}] and activation of eNOS [14]. We find that statins can decrease nuclear pAkt within minutes. This effect is inhibited by P2X7 antagonists and mimicked by ATP or other receptor agonists. Our data indicate that statins and extracellular ATP can modulate insulin-induced Akt activation via P2X7 receptors.

Materials and methods

Cell culture. Non-small cell lung cancer cells, A549, and HepG2 cells were cultured as described previously [7]. Cells were serum-starved with

* Corresponding author. Fax: +46 8 343849.
E-mail address: ulla.stenius@ki.se (U. Stenius).

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medium supplemented with 0.1% serum for 24 h (A549) or 0.5% serum for 48 h (HepG2). Human embryonic kidney (HEK) 293 cells stably expressing human P2X4 and P2X7 were kindly provided by A. Surprenant, Sheffield University, UK. HEK293 cells were grown in DMEM: F12 with 1 mM L-glutamine, 10% inactivated calf serum and 300 μg/ml G418. Cells were serum-starved with medium supplemented with 0.1% serum for 24 h. Statistical analysis was conducted using student's *t*-test. The data were presented as mean ± SD. Experiments were performed at least three times with different batches of cells. Results were considered statistically significant at $P \leqslant 0.05$.

Reagents. Pravastatin was purchased from Sigma–Aldrich (St. Louis, MO) and atorvastatin was provided by Pfizer (New York, NY). Adenosine 5′-triphosphate disodium salt (ATP), adenosine 5′-triphosphate periodate oxidized sodium salt (o-ATP), 2′(3′)-*O*-(4-benzoyl–benzoyl) adenosine 5′-triphosphate triethylammonium salt, mixed isomer (BzATP), and KN-62 were purchased from Sigma–Aldrich (St. Louis, MO). The final concentration of DMSO added to the cells was <0.2%.

Western blotting. Western blotting was performed as previously [7]. In brief the samples were subjected to SDS–PAGE and thereafter blotted onto a PVDF membrane (Bio-Rad, Hercules, CA). Some samples were subfractionated. The protein bands were subsequently probed using antibodies against Akt, Akt phosphorylated at residues Ser473 or Thr308, α-tubulin, P2X7 and Cdk2 from Santa Cruz (Santa Cruz, CA); glycogen synthase kinase 3 beta (GSK3β) phosphorylated at residue Ser9, from Cell Signaling (Beverly, MA). Proteins were visualized with ECL procedure (Amersham Biosciences, Uppsala, Sweden). The Western blot results were analyzed with NIH Image 1.62 software.

Immunocytochemical staining. Cells were fixed in 3.7% formaldehyde. After fixation the cells were stained with polyclonal antibodies against phosphorylated Akt at residue Thr308 (Santa Cruz, Santa Cruz, CA). After incubation with primary antibodies, secondary antibody conjugated with FITC (Dako, Glostrup, Danmark) was applied. No staining was detected when the primary antibodies were omitted. The nuclear staining intensity was analyzed with NIH Image 1.62 software.

Immunohistochemical staining. Female Sprague–Dawley rats were treated with pravastatin (4 mg/kg body weight) twice, 24 and 1 h before death [6]. Fixed liver sections were incubated overnight with P2X7 primary antibody (Santa Cruz, CA). Primary antibodies were visualized using the EnVision+™ peroxidase kit (DAKO). All animals received humane care, and the experimental protocol was approved by the Swedish Board of Laboratory Animals and was in accordance with National Institute of Health guidelines.

Intracellular Ca^{2+} measurement. Cells were incubated for 30 min at 37 °C with 5 μM Fura-2. Unloaded Fura-2 was removed by centrifugation at 150×*g* for 3 min. Cells were resuspended in Krebs–Ringer buffer containing 125 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM Hepes, 6 mM glucose, and 2.5 mM probenecid (pH 7.4). Fura-2-loaded cells were maintained at 25 °C for 90 min before fluorescence measurement. The absorbance was measured at 340 nm.

siRNA transfection. Cells were transfected with P2X7 small interference RNA (siRNA) (Cell signaling Technology, Beverly, MA) for 72 h according to the TranIT-TKO protocol from the manufacturer (Mirus, Madison, WI). Control siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, Santa Cruz, CA).

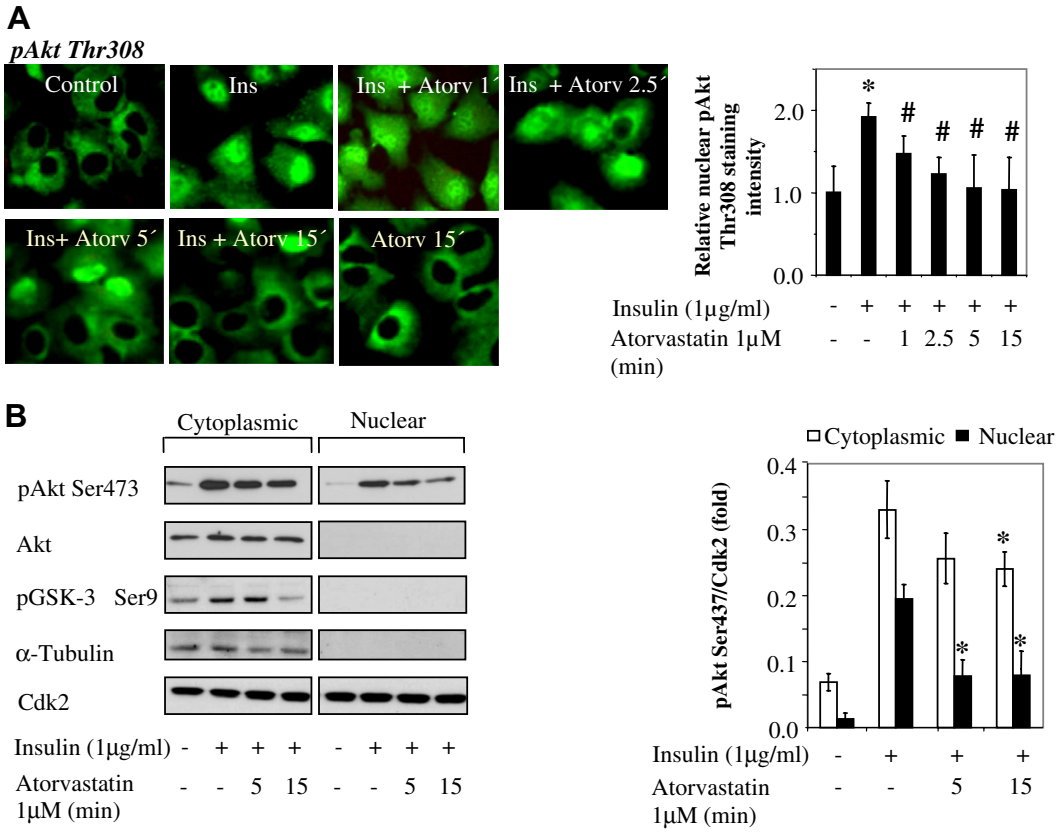

Fig. 1. Atorvastatin induce rapid changes in nuclear pAkt levels. (A, B) A549 cells were treated with insulin (1 μg/ml for 15 min) and thereafter with atorvastatin (1 μM) for times indicated. (A) Cells were stained for pAkt Thr308. The results are expressed as the relative nuclear staining intensity (mean ± SD from 20 nuclei). *Significantly different from control and # from insulin, $P \leqslant 0.05$. (B) Western blots and densitometric analysis of nuclear and cytoplasmic fractions (mean ratio ± SD from three independent experiments). *Significantly different from insulin, $P \leqslant 0.05$.