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

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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

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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).

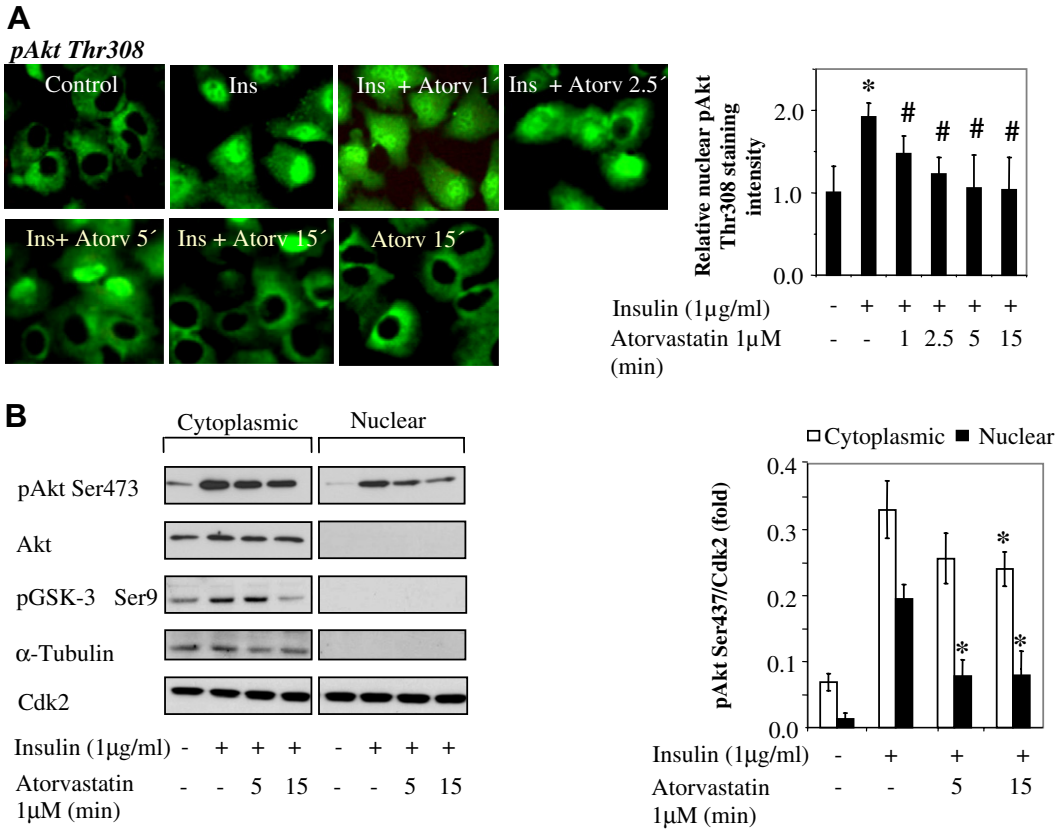

Fig. 1. Atorvastatin induce rapid changes in nuclear pAkt levels. (A, B) A549 cells were treated with insulin (1 μg/ml for 15 min) and thereafter with atorvastatin (1 μM) for times indicated. (A) Cells were stained for pAkt Thr308. The results are expressed as the relative nuclear staining intensity (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, $P \leqslant 0.05$.

Results

Statins induce rapid changes in Akt signaling

We find that insulin-induced nuclear pAkt levels significantly decreased within one minute after addition of atorvastatin (1 μM) (Fig. 1A). Similar effects were induced by pravastatin and they were not prevented by mevalonate (data not shown), a cholesterol precursor antagonizing HMG-CoA reductase inhibition [6]. As shown in Fig. 1B, the level of nuclear pAkt Ser437 was decreased without a concomitant increase of pAkt in the cytoplasmic fraction. PI3K-inhibitor LY294002 was used as a control and prevented the insulin-induced nuclear pAkt accumulation (data not shown). Low concentrations of atorvastatin (50 nM) significantly decreased the number of cells with nuclear pAkt staining (data not shown). These effects were documented employing antibodies specific for both Thr308 and Ser437 phosphorylated Akt. To determine downstream effects of atorvastatin, we analyzed pGSKβ phosphorylated at Ser9, a site phosphorylated by Akt [15]. Insulin induced pGSKβ Ser9, and atorvastatin inhibited this effect (Fig. 1B). These experiments show that nuclear pAkt is a sensitive indicator of effects induced by statins in pharmacologically relevant concentrations.

The effect of atorvastatin is inhibited by P2X7 antagonists and mimicked by ATP

P2X7 purinergic receptors have been shown to induce rapid effects on cell signaling [16]. To investigate the role of the P2X7 receptor for statin-induced effects, the P2X antagonist, oxidized-ATP (o-ATP), was tested. As shown in Fig. 2A preincubation with o-ATP abrogated the atorvastatin-induced effect on pAkt Thr308 in insulin-treated A549 cells. o-ATP alone did not have any effect on pAkt (Fig. 2A). Next, the P2X agonist, ATP, was tested. As shown in Fig. 2B the insulin-induced nuclear pAkt Thr308 accumulation was completely abolished by ATP, while ATP alone had no affect (data not shown). These data show that extracellular ATP induces similar effect as statins on nuclear pAkt. We also tested benzoyl–benzoyl ATP (BzATP), a potent P2X7 receptor agonist, and KN-62, an inhibitor of the P2X7 receptor. BzATP induced

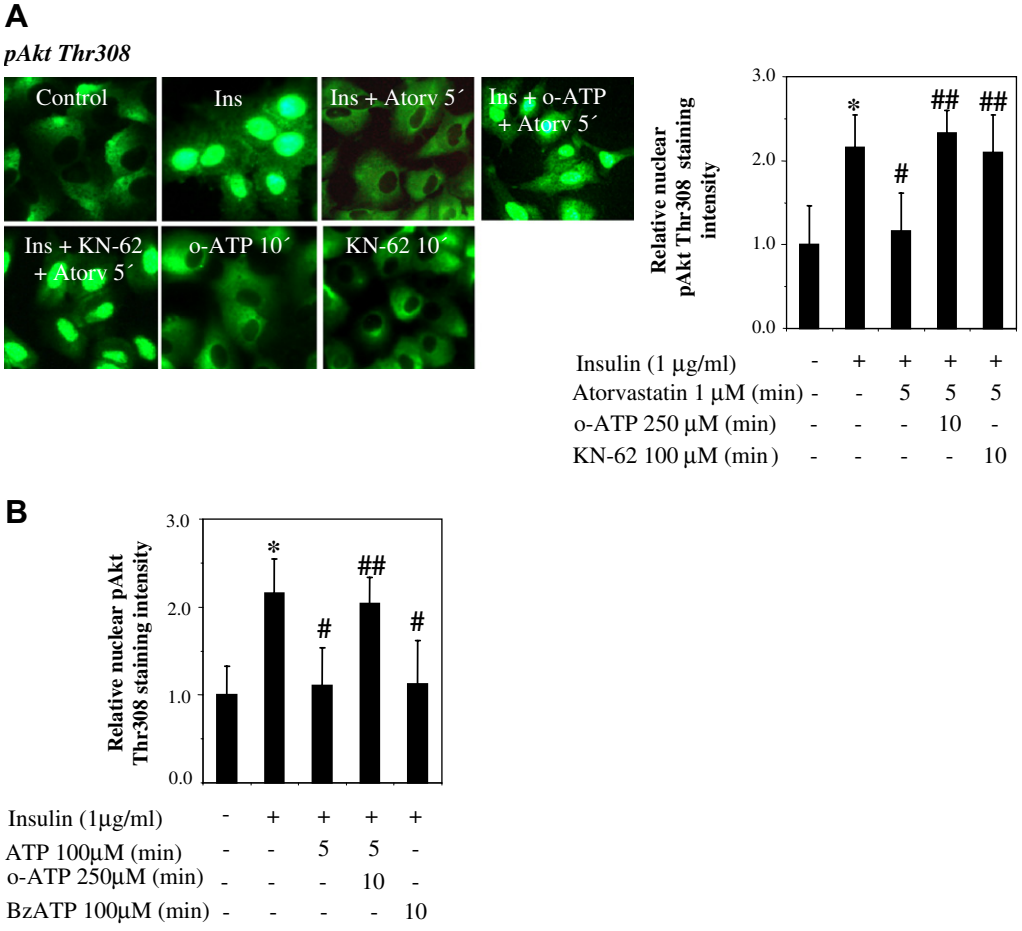

Fig. 2. P2X7 receptor agonists mimic and antagonists inhibit the effect of statin and ATP on nuclear pAkt. (A, B) Insulin-treated A549 cells (1 μg/ml, 15 min) were incubated with o-ATP (250 μM) or KN-62 (100 μM) for 10 min and thereafter with atorvastatin (1 μM), ATP (100 μM), or BzATP (100 μM) for 5 min. 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, # from insulin and ## from insulin and atorvastatin, $P \leqslant 0.05$.

the same effect on nuclear pAkt as atorvastatin or ATP (Fig. 2B), while KN-62 inhibited the statin-induced effect (Fig. 2A). ATP may activate both P2Y and P2X receptors and an agonist of P2Y receptors, UTP, was tested. However, UTP did not have any effect on nuclear pAkt (data not shown).

P2X receptors are often described as ATP-gated ion channels and we compared the effect of atorvastatin and ATP on intracellular Ca^{2+} levels. In line with previous data [5], we found that atorvastatin (10 μM) induced increased intracellular Ca^{2+} concentrations. The response to 50 μM atorvastatin was comparable to that induced by 100 μM ATP (Fig. 3A). The effect of statin was more rapid than the effect of ATP and had shorter duration. Together these data show that ATP and atorvastatin can modulate insulin signaling via P2X receptors.

A selective involvement of P2X7

Seven P2X receptors have been cloned and agonists and antagonists used here are not selective for P2X7. Employing a P2X7 antibody we documented a statin-induced nuclear localization of the antigen in cells (not shown) as well as in rat hepatocytes *in situ* (Fig. 3B). A nuclear localization of P2X7 has been observed previously [17,18], and our observation gives a direct indication that statins can affect the P2X7 receptor *in vivo*. Fig. 3B also shows that A549 cells express P2X7. To further confirm an involvement of P2X7 receptors we used HEK293 cells heterologously expressing human P2X7 or P2X4 receptors [19]. In HEK295 P2X7 cells pAkt was strongly induced by insulin, and atorvastatin decreased nuclear pAkt levels within 5 min (Fig. 4A and B). Atorvastatin did not decrease pAkt in cells expressing P2X4 (data not shown). These data corroborates the data given above and also provide evidence for an involvement of P2X7 receptor.

We also treated A549 cells with siRNA for P2X7. SiRNA decreased the level of P2X7 and the densitometric analysis shows that siRNA treatment prevented a major part of the effect of atorvastatin on pAkt. Control siRNA had no effect (Fig. 4C). Also the effect of ATP was inhibited in siRNA-treated cells (Fig. 4C). Taken together these data indicate a major, if not exclusive, role for P2X7 in the effects shown above.

Discussion

Our data show that ATP and HMG-CoA-reductase inhibiting drugs decreased insulin-induced nuclear pAkt levels via the P2X7 receptor. The P2X7 receptor has well documented functions in inflammation and in cells of mesenchymal origin [14]. P2X7 is also expressed in many epithelial cells but its function in this cell type remains largely unknown [16]. Our data indicate that P2X7 receptor activation affect insulin signaling and decrease nuclear pAkt in epithelial cells. pAkt has several cytoplasmic targets and may regulate cell growth, survival and replication [20]. The importance of nuclear pAkt has been reviewed [8] and was recently highlighted by showing that PTEN antagonize nuclear pAkt and induce apoptosis in carcinoma cells [12]. Our previous study, indicating that inhibition of pAkt by statins sensitize HepG2 and A549 cells to cytostatic

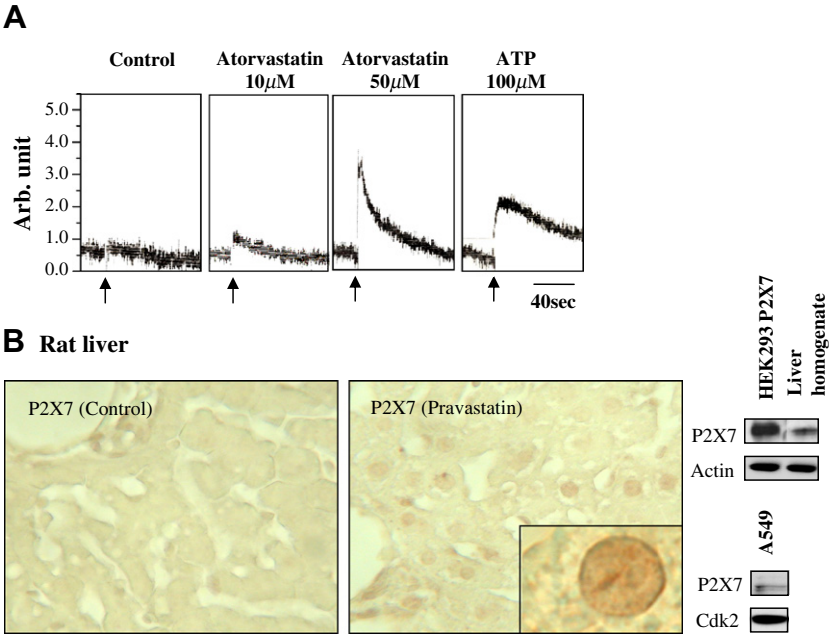

Fig. 3. Statins induce changes in [Ca^{2+}] and in the distribution of P2X7. (A) Fura-2-loaded A549 cells were treated with atorvastatin or ATP (arrows indicate addition of test substance). (B) Liver sections stained for P2X7 from rats treated with pravastatin (4 mg/kg) 24 and 1 h before sacrifice. Inset: one stained hepatocyte nucleus is shown in high magnification. Western blot showing the level of P2X7 in rat liver samples, in HEK293 cells expressing P2X7 and A549.

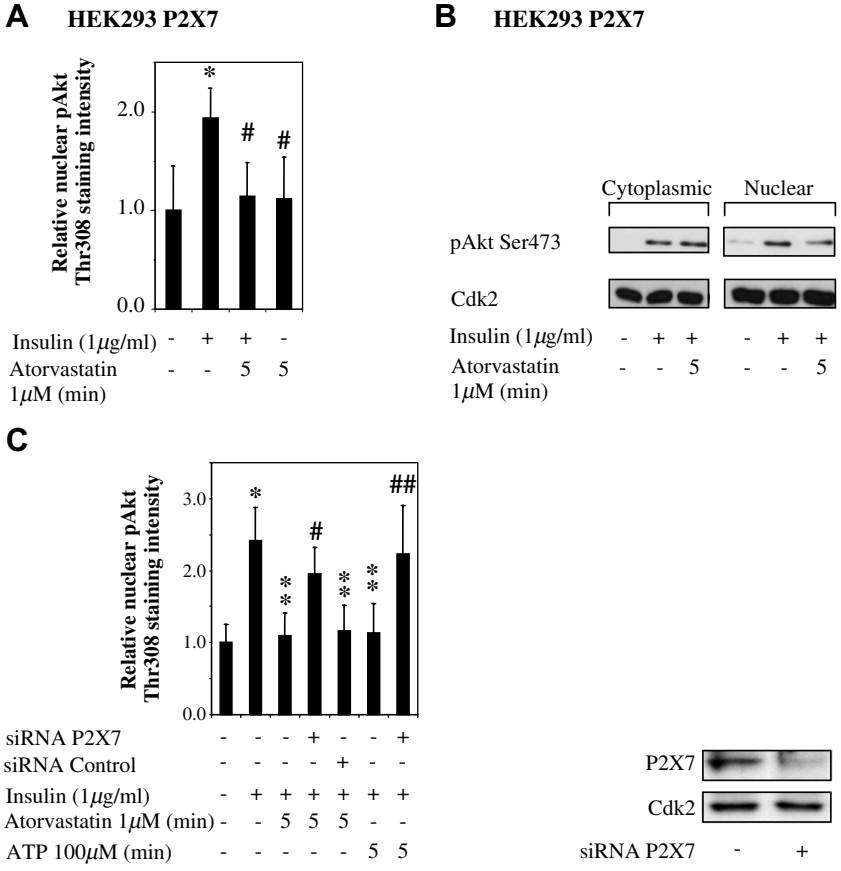

Fig. 4. Atorvastatin and ATP decrease pAkt in P2X7-expressing HEK293 cells and siRNA for P2X7 inhibit the effect of atorvastatin and ATP. (A, B) HEK293 P2X7 were incubated with insulin (1 μg/ml, 15 min) and thereafter with atorvastatin (1 μM) for times indicated. Cells were stained for pAkt Thr308. The results are expressed as the relative nuclear staining intensity (mean ± SD from 20 nuclei). *Significantly different from control, # from insulin, $P \leqslant 0.05$. (C) A549 and HepG2 cells were transfected with siRNA against P2X7 (50 nM) for 72 h and thereafter incubated with insulin (1 μg/ml, 15 min) and with atorvastatin (1 μM) or ATP (100 μM) for times indicated. The results are expressed as the relative nuclear staining intensity (mean ± SD from 20 nuclei). *Significantly different from control, ** from insulin, # from insulin and atorvastatin, ## from ATP and insulin, $P \leqslant 0.05$. Western blotting showing the level of P2X7 in siRNA P2X7 transfected cells.

drugs [7], is in line with this scenario. P2X7 may thus be an important modulator of Akt activity and its downstream targets.

The activation of P2X7 by statins may explain many pleiotropic effects of this drug type, including the effect on [Ca^{2+}], and the induction of eNOS [21]. The well documented cardiovascular effects of statins might include anti-inflammatory effects [22], and it was recently shown that P2X7 activation in endothelial cells may result in a net anti-inflammatory effect [23]. So, if it can be shown that P2X7 in endothelial cells responds to statins, it also seems possible that a statin-induced P2X7 signaling affects the atherosclerotic process.

The similarities between the ATP and statin responses and their rapid onset, as well as the lack of effect of mevalonate, argue for a HMG-CoA reductase-independent effect of statins. A remaining question is how statins activate P2X7. A receptor binding similar to that for ATP [13] seems possible but it is clear that statins do not mimic all the effects of ATP and *vice versa* (results to be published elsewhere). Another question is how P2X7 affect nuclear pAkt.

Future studies of P2X7 in epithelial cells could lead to a better understanding of possible anti-cancer effects of statins. Encouraging are studies showing that statins can prevent metastatic or fatal prostate cancer [2]. A possible involvement of P2X7 is suggested by an aberrantly expressed P2X7 in prostate epithelium undergoing malignant transformation [24], and by a proposed involvement of P2X7 in bone metastasis [25] which is frequent in fatal prostate cancer. Statins are generally regarded as safe drugs and studies of prostate cells may, e.g., lead to the development of strategies to prevent prostate cancer.

Acknowledgments

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