Atorvastatin prevents ATP-driven invasiveness via P2X7 and EHBPl signaling in PTEN-expressing prostate cancer cells

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Epidemiological studies indicate that statins, cholesterol-lowering drugs, prevent aggressive prostate cancer and other types of cancer. Employing essentially non-prostate cell lines, we previously showed that statins rapidly downregulate nuclear levels of phosphorylated Akt via P2X7, a purinergic receptor recently implicated in invasive growth. Here, we present studies on phosphatase and tensin homolog deleted on chromosome 10 (PTEN)-positive prostate cancer cells. We document an involvement of EH domain-binding protein 1 (EHBPl), previously associated with aggressive prostate cancer and insulin-stimulated trafficking and cell migration, in P2X7 signaling. We also show that EHBPl is essential for an anti-invasive effect of atorvastatin. Furthermore, EHBPl interacts with P-Rex1, a guanine nucleotide exchange factor previously implicated in invasive growth. Mevalonate did not prevent this anti-invasive effect of atorvastatin. These data indicate that atorvastatin modulates invasiveness via P2X7, EHBPl and P-Rex1. Interestingly, the interaction between EHBPl and P-Rex1 was not induced by extracellular adenosine triphosphate (ATP), the endogenous P2X7 ligand, and statins counteracted invasiveness stimulated by extracellular ATP. In support of these experimental data, a population-based genetic analysis showed that a loss of function allele in the P2X7 gene (rs3751143) associated with non-aggressive cancer, and the common allele with aggressive cancer. Our data indicate a novel signaling pathway that inhibits invasiveness and that is druggable. Statins may reduce the risk of aggressive prostate cancer via P2X7 and by counteracting invasive effects of extracellular ATP.

Introduction

Statins, 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors, are potent mevalonate and cholesterol-lowering drugs used to prevent cardiovascular disease. Several studies also indicate a chemopreventive effect of statin drugs against aggressive prostate cancer (1–3). The prostate studies provide perhaps the best support for an anticancer effect of statins in humans, but there are also reports on reduced mortality in several other cancer types (4). Possible anticancer effects of statins have been extensively studied in prostate cancer models (5). Evidence suggests a role for lowered cholesterol and androgen synthesis. Other studies emphasize the inhibiting effect of statins on the mevalonate pathway (5), which has oncogenic properties (6). The mevalonate pathway intermediates activate small guanosine triphosphate (GTP)-bound, small guanosine triphosphatase (GTPase) and PI3 kinases (7,8). This PI3 kinase activation results in the activation of Akt (9,10). Typically, we used 1 μM concentration of statins in these experiments, but we also reported pAkt depletion at 50 nM (8,10). Another prominent finding was that all translocations occurred within minutes. This time frame supported the notion that we studied effect of statins that were not dependent of HMG-CoA reductase inhibition as alterations induced by an inhibited HMG-CoA reductase are seen 2–4 h after statin addition (11).

Akt is an oncogene strongly associated with prostate cancer aggressiveness (12). It is expressed in the cytoplasm, but, as recently reviewed (13), it is also expressed in the nucleus and there are data indicating that the nuclear localization of Akt is crucial for its oncogenic activity in the prostate (14). This suggested that the statin-induced P2X7 signaling could be of importance for the prevention of aggressive prostate cancer, and perhaps for other cancer types as well. Our interest in statin-induced P2X7 signaling is also stimulated by recent data showing that P2X7 affects tumor growth (15) and invasive growth of breast cancer cells, possibly mediating effects of the natural ligand extracellular adenosine triphosphate (ATP) (16).

In an effort to link statin-induced P2X7 signaling to aggressive prostate cancer, we studied the p110β PI3 kinase (17). This PI3 kinase has been implicated in aggressive prostate cancer development (12,18) and we found that it was critical for statin-induced P2X7 signaling and pAkt depletion, presumably acting as a scaffolding protein (17).

In a search for additional support for a role of P2X7 signaling in statin-induced anticancer effects in the prostate, we hypothesized an involvement of EH domain-binding protein 1 (EHBPl). EHBPl has been associated with aggressive prostate cancer in genome-wide association study (19), but its cellular functions are poorly characterized. However, it is known that it is involved in insulin-stimulated rapid receptor trafficking and other translocations (20–23), suggesting a possible role in statin-induced and P2X7-mediated translocations. We here present a genetic study associating P2X7 activity with aggressive prostate cancer in humans. We also present cell studies showing that prostate cells respond to statins as did previously studied cell types, and we characterize an involvement of EHBPl in this response. We show that EHBPl modulates invasiveness of PTEN-positive prostate cancer cells and that statins counteracts ATP-driven invasive growth via P2X7-dependent signaling.

Materials and methods

Cell culture

The human prostate carcinoma cell lines DU145, 22RV1, LNCaP, PC3 and immortalized prostate luminal epithelial (non-tumorigenic) RWPE-1 were purchased from ATCC (Manassas, VA). DU145 cells were grown in Dulbecco’s modified Eagle’s medium, with 10% inactivated fetal bovine serum and 1 mM sodium pyruvate. 22RV1 cells were grown in RPMI-1640 from Sigma–Aldrich (St Louis, MO) supplemented with 10% fetal bovine serum. PC3 and LNCaP cells were additionally supplemented with 1 mM sodium pyruvate and 2 mM l-glutamine. LNCaP cells were additionally supplemented with 1 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid. Media was supplemented with penicillin–streptomycin. Serum-starved cells were cultured with medium supplemented with 0.1% serum for 24 h. The RWPE-1 cells were grown in keratinocyte serum-free medium, with bovine pituitary extract, human

Abbreviations: ATP, adenosine triphosphate; AR, androgen receptor; CGEMS, Cancer Genetic Markers of Susceptibility; EHBPl, EH domain-binding protein 1; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; MMP, matrix metalloproteinase; pAkt, phosphorylated Akt; PLA, proximity ligation assay; PTEN, phosphatase and tensin homolog deleted on chromosome 10; siRNA, small interfering RNA; SNP, single-nucleotide polymorphism.

This is a commonly used statin concentration, whereas clinically relevant plasma concentrations of statins are in the range of 10–100 nM (7).

We have studied statin-induced purinergic receptor-dependent signaling in hepatocytes, lung, prostate and pancreatic cells. We provided evidence for P2X7-dependent depletion of nuclear levels of activated Akt [phosphorylated Akt (pAkt)] in cells stimulated by insulin (8–10). The pAkt depletion was synchronized with rapid nuclear translocations of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), PHLP1 and PHLP2 phosphatases and proliferating cell nuclear antigen (10). Typically, we used 1 μM concentration of statins in these experiments, but we also reported pAkt depletion at 50 nM (8,10). Another prominent finding was that all translocations occurred within minutes. This time frame supported the notion that we studied effect of statins that were not dependent of HMG-CoA reductase inhibition as alterations induced by an inhibited HMG-CoA reductase are seen 2–4 h after statin addition (11).
recombinant EFG and antibiotic–antimycotic. The media and supplements were purchased from GIBCO (Grand Island, NY).

Reagents
Atorvastatin (At) was provided by 21CEC PX Pharm Ltd. BzATP, Insulin, adenosine 5′-triphosphate disodium salt (ATP), mevalonic acid lithium salt, toluidine blue and KN62 were purchased from Sigma–Aldrich. The final concentration of dimethyl sulfoxide added to the cells was <0.4%. No effect of dimethyl sulfoxide was observed.

Western blotting
Western blotting was performed as described previously (24). In brief, samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The protein bands were probed using antibodies against Akt-1, Akt-2, Akt phosphorylated at residue Ser473 or Thr308, mTOR, Actin, P2X7, α-tubulin and CDK2 from Santa Cruz Biotechnology (Santa Cruz, CA); PTEN, pGSK3β Ser9, p-mTOR Ser2448, MMP9 and MMP2 from Cell Signaling (Beverly, MA); EHBP1 was from American Diagnostica (Stamford, CT); P-Rex1 was from Sigma–Aldrich. Proteins were visualized with ECL procedure (Amersham Biosciences, Uppsala, Sweden). The results were analyzed with NIH Image 1.62 software.

Small interference RNA transfection
Cells were transfected with EHBP1, P-Rex1, P2X7 or control small interference RNA (siRNA) (Santa Cruz Biotechnology) for 40 h according to the TranIT-TKO protocol from the manufacturer (Lipofectamine™ 2000; Invitrogen, Carlsbad, CA).

Immunocytochemical staining
Cells were fixed in 3.7% formaldehyde and stained with antibodies against PTEN, pAkt Thr308, proliferating cell nuclear antigen, EHBP1 or PHLPP1. PHLPP1 was from Bethyl laboratories (Montgomery, TX). Secondary antibody conjugated with fluorescein isothiocyanate or Texas red was added (Dako, Glostrup, Denmark). The staining intensity was analyzed with NIH Image 1.62 software.

Confocal microscopy
Cells were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100 in 2% bovine serum albumin buffer. Immunostainings were performed using rabbit polyclonal P-Rex1, mouse monoclonal EHBP1 antibodies or rabbit P-Rex1 and mouse monoclonal PTEN antibodies. Secondary antibody conjugated with Alexa 488 (rabbit) and Alexa 594 (mouse). Samples were mounted in 4',6-diamidino-2-phenylindole. Fixed cells were performed with a Zeiss LSM 510 META confocal laser scanning microscope (Zeiss, Oberkochen, Germany) equipped with a x63 Plan-Aoil-immersion lens. An agar laser was excited at 488 nm and fluorescence image was recorded from 500 to 550 nm. A helium-neon laser was used for excitation at 543 nm and emission from 650 to 615 nm. Colocalization of indicated proteins was measured by Zeiss LSM imaging software in multitrack mode.

Cell invasion assay
Cell invasion assay was performed using 8 μm pore size Transwell Biocoat Control inserts (Becton Dickinson, Bedford, MA) according to the manufacturer’s instructions. The cells were stained with toluidine blue. The number of transmembrane cells was counted.

Genetic analysis of P2X7
The publicly available American Cancer Genetic Markers of Susceptibility (CGEMS) study, comprising 1172 prostate cancer cases and 1098 controls, was used (25). Among the patients, disease aggressiveness was defined by the CGEMS study as follows: patients with clinical stage T3/T4 or Gleason score of 7 or higher based on biopsy specimens were classified as having more aggressive disease, whereas the remaining patients were classified as having less aggressive disease. All participants gave written informed consent.

Proximity ligation assay
The proximity ligation assay (PLA) was performed according to the manufacturer’s protocol using the Duolink detection kit with PLA PLUS and MINUS probes for mouse and rabbit (Olink Bioscience, Uppsala, Sweden).

Statistical analysis
Association between the rs3751143 single-nucleotide polymorphism (SNP) and prostate cancer was performed in an unconditional logistic regression model assuming an additive genetic effect. In cell studies the statistical analysis was conducted using double-sided Student’s t-test. The data was presented as mean ± SD. Results were considered to be statistically significant at P < 0.05. Experiments were performed at least three times with different batches of cells.

Results
Pharmacologically relevant concentrations of atorvastatin activate P2X7 signaling in prostate cells
Our earlier studies show that statins deplete nuclear pAkt in multiple cancer cell lines (10) and this effect was now comprehensively studied in prostate cells. Figure 1A shows that among insulin-stimulated non-cancer prostate cells (RWPE-1) exposed to 10–100 nM atorvastatin there was a loss of nuclear pAkt staining in many cells. Significant effects were seen at 50 nM. We did not analyze possible changes in unphosphorylated nuclear Akt here, but in previous work we concluded that we were not able to detect such changes (8). Next, we analyzed cancer cell lines and their expression of some of the proteins associated with prostate cancer (Supplementary Figure S1A, available at Carcinogenesis Online). DU145 and 22RV1 cells express PTEN, which is needed for statin-induced nuclear pAkt depletion (10) and Pten is often lost early in prostate carcinogenesis (26). 22RV1 cells also expressed androgen receptor (AR) and is androgen responsive (27). LNCaP (10) and PC3 (28) cells, on the other hand, are PTEN negative and express high levels of pAkt or its downstream target GSK3β Ser9 (Supplementary Figure S1B, available at Carcinogenesis Online). All cells expressed P2X7 and EHBP1 (Supplementary Figure S1A, available at Carcinogenesis Online).

In non-starved DU145 cells, atorvastatin (1 μM) significantly decreased pAkt levels (Figure 1B). In starved cells, insulin increased pAkt and pGSK3β Ser9 levels (Figure 1C) and atorvastatin decreased insulin-induced pAkt levels (Figure 1C). Similar effects were seen in the Pten and AR-expressing 22RV1 cells (Figure 1D). Analysis of the nuclear fraction of DU145 cells, lacking α-tubulin, showed that nuclear levels of pAkt were decreased within 3–5 min (Figure 1E). Immunocytochemistry confirmed the western blot results showing depletion of nuclear pAkt (Figure 1F). Cell viability was studied employing 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, and atorvastatin (1–2 μM) decreased the number of viable DU145 and 22RV1 cells significantly (Figure 2A and B).

The P2X7 antagonist KN62 prevented the effect of statin (Figure 2C). As shown previously in A549 cells (10), statin induced nuclear translocations of Akt regulators PHLPP1 and PTEN (Figure 2D and E) within minutes in insulin-stimulated DU145 cells. The P2X7 agonist BzATP also translocated PTEN (Figure 2E). A rapid nuclear translocation of proliferating cell nuclear antigen was also seen after atorvastatin addition (Figure 2F). These data indicate that DU145 and 22RV1 cells respond to statin-induced P2X7 signaling in the same way and within the same time frame as did previously studied multiple cell types (8–10,17).

EHBP1 is necessary for P2X7-Akt signaling and for atorvastatin-induced inhibition of invasiveness
EHBP1 has been implicated in aggressive prostate cancer (19) and in insulin-dependent rapid trafficking (20) and we tested if EHBPI affects statin-induced P2X7 signaling in insulin-stimulated cells. We used siRNA for EHBPI, which decreased EHBPI protein levels to about half in DU145 cells (Supplementary Figure S1C, available at Carcinogenesis Online). We found that siRNA EHBPI, but not control siRNA, prevented the atorvastatin-induced nuclear pAkt depletion in DU145 cells (Figure 3A). We also used siRNA P2X7. It decreased the level of P2X7 significantly (Supplementary Figure S1D, available at Carcinogenesis Online), and as expected from previous work (8), it prevented the effect of atorvastatin on pAkt (Figure 3A). Insulin and atorvastatin enhanced the nuclear staining of EHBPI (Figure 3B). The effect of insulin was not further analyzed but is in line with previous data on insulin affecting EHBPI-trafficking functions (20). Even 50 nM atorvastatin rapidly induced nuclear staining of EHBPI (data not shown), suggesting coordination with above shown (Figure 2B–D) translocations. These data indicate that EHBPI is a downstream target of statin-induced P2X7 signaling.

P2X7 affect invasive growth (16) and we studied invasiveness in a matrigel invasion assay. As expected from previous studies on PC3
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Cells (28) atorvastatin inhibited invasiveness in DU145 (Figure 3C) and in 22RV1 (Figure 3C) cells. EHBP1 associates with proteins implicated in invasiveness (22), and we also tested if EHBP1 affected invasiveness. We found that siRNA for EHBP1, but not control siRNA, increased invasiveness and matrix metalloproteinase 9 (MMP9) expression, a marker of invasiveness, in DU145 cells (Figure 3C and D). siRNA against EHBP1 completely abrogated the effect of atorvastatin (Figure 3C). Furthermore, siRNA against P2X7 had a similar inhibitory effect (Figure 3E). These data suggest a role for EHBP1 and P2X7 in statin-modulated invasiveness.
Atorvastatin affects EHB1-regulated and mevalonate-independent invasiveness in DU145

The rapidity of EHB1 and other translocations suggested non-mevalonate depletion-dependent effects of statins in the inhibition of invasiveness of DU145 cells. However, a mevalonate depletion-dependent inhibition was shown in PC3 cells (28), and the time frame used in the invasion assay (48 h) may permit statin-induced depletion of mevalonate (5). We thus studied a possible involvement of the mevalonate pathway in DU145 cells. We found that mevalonate did not prevent, but enhanced, the inhibitory effect of atorvastatin on the invasiveness of DU145 cells (Figure 3F), indicating a lack of involvement of mevalonate depletion. In order to further explore the mechanism operating in DU145 cells, we compared the effect of atorvastatin on PC3 and DU145 cells, two cell types derived from
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PC3 cells were more invasive than DU145 or 22RV1 cells (data not shown), and in accordance with previous data (28), we found that atorvastatin effectively inhibited invasiveness of PC3 cells (Figure 3G). Expressed as % inhibition, the effect was grater in PC3 cells than in DU145 or in 22RV1 cells (c.f. Figure 3C and G). We also tested the effect of siRNA against EHBP1, and this treatment increased the invasiveness as it did in DU145 cells. However, the inhibiting effect of siRNA EHBP1 on atorvastatin-induced inhibition of invasiveness, as seen in DU145 cells (Figure 3C), was not seen in PC3 cells (Figure 3G), suggesting that statins affected invasiveness via different signaling pathways in DU145 and in PC3 cells.

**Atorvastatin-induced colocalization of P-Rex1 and EHBP1**

In PC3 cells, P-Rex1, a Rac-selective guanine nucleotide exchange factor, is ascribed a critical role for invasive growth (29) and we...
explored the possibility that P-Rex1 could explain the differences in responses between the prostate cell lines. We found that DU145 and 22RV1 cells expressed much less P-Rex1 protein than PC3 cells (Figure 4A) and P-Rex1 levels thus roughly correlated to invasiveness (c.f. ref. 29). Previous data also show that siRNA against P-Rex1 prevents PC3 invasion (29). However, this effect was not seen in DU145 cells; P-Rex1 siRNA increased invasion in DU145 cells (Figure 4B). P-Rex1 siRNA also decreased MMP2 and MMP9 levels in PC3 cells but increased these levels in the less-invasive DU145 and 22RV1 cells (Figure 4C).

P-Rex1 thus affected invasive growth in the same way as EHBP1 in DU145 and 22RV1 cells. Furthermore, similar to EHBP1 (20,30),
P-Rex1 participates in PIP3- and insulin-dependent rapid translocations of GLUT4 and in actin remodeling (31). We thus hypothesized that P-Rex1 and EHBP1 interact. Employing confocal microscopy, we found indications that P-Rex1 and EHBP1 partially interacted in the cytoplasm in response to insulin in DU145 cells. After addition of atorvastatin, a more pronounced cytoplasmic interaction was seen and also some nuclear colocalization (Figure 4D), which is consistent with the enhanced EHBP1 staining seen in the nucleus after atorvastatin addition (Figure 3B). To confirm this interaction we used PLA, which detects proximity between two targeted proteins that is <40 nm. Insulin induced similar results as seen with confocal microscopy; there were some dots in the cytoplasm but not dots in the nuclear area (Figure 4F). Insulin plus atorvastatin increased the number of cytoplasmic dots and also induced dots in the nuclear area (Figure 4F). Thus, both confocal microscopy and PLA indicated atorvastatin-induced interactions between P-Rex1 and EHBP1 in the cytoplasm and probably also in the nucleus. These data contrasted to the plasma membrane localization seen in E-selectin-stimulated PC3 cells (29).

To further document differences between the two metastatic cell lines, DU145 and PC3, we followed up a recent report showing that P-Rex1 colocalize with PTEN (32), a binding partner not available in PC3 cells. Confocal microscopy (Figure 4E) indicated interactions in the cytoplasm as a response to insulin. After atorvastatin addition colocalized PTEN-P-Rex1 was concentrated close to and in the nucleus in DU145 cells (Figure 4E). This localization was consistent with the PTEN staining shown in Figure 2E. These data also confirmed previous data on interactions between P-Rex1 and PTEN (32).

Atorvastatin prevents ATP-driven invasiveness
Extracellular ATP is an endogenous P2X7 agonist (16). In agreement with previous reports (33) we found that extracellular ATP stimulated invasiveness (Figure 5A). In addition, and in contrast to atorvastatin which decreased MMP9 and MMP2 levels (Figure 5B), ATP increased MMP9 and MMP2 levels in DU145 (Figure 5B). Similar effects of ATP on MMPs have been documented previously (33). We also found that atorvastatin counteracted the ATP effect on MMP levels in DU145 cells (Figure 5B) and prevented ATP-stimulated invasiveness (Figure 5A). To obtain additional indications of ATP having effects deviating from those induced by statins in this context, we analyzed the effects of ATP on EHBP1 and P-Rex1. We found that ATP increased the nuclear staining of EHBP1 (Figure 5C). Furthermore, ATP induced only very weak or no colocalization between EHBP1 and P-Rex1 in insulin-stimulated cells (Figure 5D), further supporting a critical role for EHBP1 and P-Rex1 in atorvastatin-induced invasive growth inhibition. Together these data indicate that atorvastatin can modulate P2X7-induced invasiveness by affecting EHBP1 and P-Rex-1.

Genetic analysis supports a role for P2X7 signaling in human aggressive prostate cancer
In an effort to further support a role for P2X7 as a possible target for statins in preventing aggressive prostate cancer, we performed a genetic analysis. For the well-characterized SNP (rs3751143) in the P2RX7 gene (34) genotype data was available from the CGEMS population. Analysis revealed a nominally significant association between
rs3751143 and prostate cancer (odds ratio = 0.86, P = 0.044). The minor allele is a loss-of-function allele (35), and when we compared less and more aggressive cases (484 cases with Gleason <7 and stage A/B versus 688 cases with Gleason ≥7 and/or stage C/D) with respect to this SNP we observed an odds ratio of 0.77 (P = 0.019), suggesting that the minor allele is associated with less aggressive disease and the major allele with aggressive disease. As far as we know, these data are the first to associate the rs3751143 SNP with cancer, but are in line with a P2X7-stimulated growth of tumor cells (15), breast cancer cells showing P2X7-stimulated invasive properties (16) and ATP-stimulated invasiveness of prostate cancer cells (33). They are also consistent with P2X7 being a target for chemopreventive effects by statins in the prostate.

**Discussion**

In this study, we show that P2X7 function associates with aggressive prostate cancer in humans, that P2X7 in prostate cells is a target for atorvastatin, and that atorvastatin induce P2X7 signaling in pharmacologically relevant concentrations (10–100 nM). Importantly, our cell studies show that atorvastatin-induced P2X7 signaling involves EHB1 and P-Rex1, and inhibits ATP-driven invasive growth. Taken together these results suggest a mechanism by which statins may prevent aggressive prostate cancer via P2X7 signaling.

EHB1 has been associated with aggressive prostate cancer in humans (19) and the involvement of EHB1 thus connects statin-induced P2X7 signaling with aggressive prostate cancer. Moreover, EHB1 connects rapid cellular effects coupled to statin-induced P2X7 signaling with our results on invasiveness. Thus, EHB1 was rapidly translocated in response to P2X7 signaling, and EHB1 knockdown both blocked the nuclear depletion of pAkt and the atorvastatin-induced inhibition of invasiveness. This connects statin-induced P2X7 signaling with the inhibited invasiveness.

Our cell data also show an involvement of P-Rex1 in atorvastatin-induced P2X7 signaling. We found that P-Rex1 interacted with EHB1 in response to atorvastatin. P-Rex1 has previously been associated with invasive growth and metastasis of prostate cancer in mice (29) and with breast cancer metastasis in humans (36), so, in analogy with EHB1, P-Rex1 is a factor linking the rapid statin-induced P2X7 signaling with aggressiveness in vivo and further support a human relevance. Thus, our data on EHB1, on P-Rex1, and on the genetic association between P2X7 function and aggressiveness argue that a role for statin-induced P2X7 signaling in aggressive prostate cancer in humans.

This conclusion is also supported by earlier indirect data suggesting a role of EHB1 in invasive growth (21–23). More recent data show that drosophila EHB1 regulates aspects of Notch signaling (37), and that Notch may play a role in metastatic prostate cancer (38). Taken together with our data they suggest that further studies of EHB1 as an inhibitor of invasiveness is warranted, and in particular as EHB1 is a target for non-toxic drugs.

Our data support and extend previous data indicating that P2X7 promotes invasive growth (16) and growth of tumor cells in mice (15). They indicate that EHB1 can negatively modulate this invasiveness, that statins and ATP affect EHB1 differently, and that EHB1 is druggable. Why the two P2X7 agonists, statins and ATP, produced contrasting effects remain to be clarified in detail. Interestingly, a recent study found that among 30 prostate genome-wide association study SNPs two associated specifically with pesticide-related prostate cancer. One SNP (rs3710647) was in the EHB1 gene and one (rs76976673) close to the PP2A gene (39). Not only EHB1 (this study) but also PP2A (10) is implicated in P2X7 signaling, so cellular studies employing pesticides might increase our understanding how xenobiotics and the endogenous ligand ATP interact with P2X7. The role of P-Rex1 binding to PTEN and the possibility that it inhibits PTEN (c.f. ref. 40) also remains to be characterized, as well as the role of nuclear pAkt depletion and the involvement of different Akt isoforms.

Our data indicate novel additional activities by statins, not dependent on HMG-CoA reductase inhibition (Figure 6). They indicate that P2X7-induced rapid translocations of both EHB1 and P-Rex1 are critical events in the modulation of invasiveness. The time frame for these translocations is consistent with the time frame documented previously for PIP3-dependent translocations (41), whereas effects related to depletion of metabolites in the mevalonate pathway are not seen until 2–4 h after statin addition (11). Strongly indicative for additional activity is also the finding that mevalonate did not prevent invasiveness.

PC3 xenographs have been used in in vivo studies of statin-induced inhibition of tumor growth (42) and a statin-related mevalonate effect has been documented in PC3 cells in vitro (28). This mevalonate effect might be explained by an epigenetically deregulated P-Rex1 in PC3 cells (43) and by the fact that their P-Rex1 levels are very high and seemingly driving an invasive growth (29). P-Rex1 is regulated both by prenylations (29) and by PIP3-dependent translocations (41) and it can be envisioned that prenylation of P-Rex1 easily becomes rate limiting for invasiveness in this highly invasive cell type (Figure 6). PC3 cells are also osteolytic and non-typical for prostate bone metastases (44), and might thus be non-typical also for studies of invasiveness modulated by statins.

PTEN is commonly lost during prostate cancer development and aggressive cancer becomes hormone-refractory upon treatment (45) and e.g. PC3 cells reflect this condition. Our data indicate that PTEN is necessary for the P2X7-mediated effects of statins, whereas AR status seems non-influential. For example, PTEN-negative but AR-responsive LNCaP cells did not respond to atorvastatin, but did so after transfection with PTEN (10). This suggests that a chemopreventive effect of statins via P2X7 may occur early in disease development. This is consistent with recent results showing reduced death in prostate cancer among men taking statin drugs at the time of diagnosis (46).

In summary, our data indicate that statins can counteract extracellular ATP-induced invasive growth in PTEN-positive cells. This inhibition is mediated by non-HMG-CoA reductase-dependent effects of statins in clinically relevant concentrations and might prevent the development of invasive growth of early lesions. Further studies of the interrelationships between statins, P2X7, EHB1, P-Rex1 and extracellular ATP may identify biomarkers reflecting preneoplastic lesions.

**Fig. 6.** Suggested events induced by statins via P2X7 and affecting invasiveness in DU145 and PC3 cells. Signaling induced by statins and ATP and mediated by P2X7 is indicated by hatched lines. Our data indicate insulin-induced interaction between PTEN and P-Rex1 in DU145 cells. Moreover, statin-induced P2X7 signaling decreases this interaction and forms a complex between P-Rex1 and EHB1. This complex formation was not seen with natural P2X7 ligand ATP and our data suggest the P-Rex1/EHB1 is critical for inhibiting the invasiveness. In PC3 cells, P-Rex1 cannot interact with PTEN and is heavily overexpressed. Previous data suggest that under these conditions statins can limit invasiveness by inhibiting prenylation via inhibition of the mevalonate pathway (28). Part of P-Rex1 might be bound to EHB1 as siRNA against EHB1 increased invasiveness (Figure 3G) and this treatment might have released P-Rex1 and supported invasiveness in PC3 cells.
that have the propensity to progress to aggressive and deadly prostate cancer and that can be targeted by chemopreventive strategies.

Supplementary material

Supplementary Figure S1 can be found at http://carcin.oxfordjournals.org/

Funding


Conflict of Interest Statement: None declared.

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Received October 3, 2013; revised January 12, 2014; accepted January 15, 2014

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