Regulation of the PTEN promoter by statins and SREBP

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Germline mutations in the tumor-suppressor gene PTEN predispose to heritable breast cancer. The transcription factor peroxisome proliferator-activated receptor-gamma (PPARγ) has also been implicated as a tumor suppressor pertinent to a range of neoplasias, including breast cancer. We previously demonstrated that lovastatin may signal through PPARγ and directly upregulate PTEN expression at the transcriptional level. In our current study, we show that simvastatin, pravastatin and fluvastatin can induce PTEN expression in a dose-dependent manner. This resulted from an increase in PTEN mRNA indicating transcriptional upregulation. In addition, we observed, for the first time, that upregulation of sterol response element-binding protein (SREBP), known to induce PPARγ expression, can increase PTEN expression. Using reporter assays, we observed that both the statins and SREBP could specifically induce PPARγ-mediated transcription. However, the statins do not appear to signal through SREBP. Furthermore, our results indicate that SREBP utilizes PPARγ’s transcriptional activity to induce PTEN transcription, whereas the statins signal through PPARγ’s protein activity to upregulate PTEN expression. Overall, our observations suggest that statins signal through another transcription factor, in a PPARγ-dependent manner, which in turn induces PTEN transcription. We, therefore, studied the full-length PTEN promoter through serial deletion reporter assays and electromobility shift assays and identified a region between −854 and −791 that binds an as-yet-unidentified transcription factor, through which the statins induce PTEN expression. Since PTEN is constitutively active, our data indicate it may be worthwhile to examine statin and SREBP stimulation as mechanisms to increase PTEN expression for therapeutic and preventative strategies in cancer, diabetes mellitus and cardiovascular disease.

INTRODUCTION

Germline mutations in PTEN, a tumor-suppressor gene on 10q23, occur in 85% of patients with the autosomal-dominant Cowden syndrome [CS (MIM 158350)] (1–3). This syndrome is reported to affect approximately one in 200 000 individuals. However, because CS is difficult to diagnose, this is generally thought to be an underestimate (4). Patients diagnosed with CS have a 25–50% lifetime risk of developing female breast cancer compared with ~13% in the general population (5,6). In addition to breast cancer, CS patients also have thyroid neoplasias, mucocutaneous lesions, fibrocystic breast disease, uterine leiomyoma and macrocephaly (6,7). Germline PTEN mutations are also associated with subsets of patients with developmental disorders. Approximately 65% of patients with Bannayan–Riley–Ravalcaba syndrome (MIM 153480), 20% of those with Proteus syndrome (MIM 176920), 50% of a Proteus-like syndrome (4) and 10–20% of autism spectrum disorder with macrocephaly (3,8,9) share germline PTEN mutations as an etiology and have all been classified as PTEN hamartoma tumor syndromes (2). Furthermore, somatic alterations in PTEN, whether by genetic or epigenetic mechanisms, play some role in the pathogenesis of a broad range of solid tumors, such as sporadic carcinomas of the breast, thyroid, endometrium and colon (4).

PTEN’s protein product, PTEN, is a dual-specificity phosphatase with both lipid and protein phosphatase activities.
(10), which elicits cell cycle arrest and apoptosis. PTEN is a constitutively expressed protein; therefore, regulation of its protein levels through transcription is key to its function. In recent years, transcriptional regulation of PTEN has been researched more extensively; however, there is still much to be understood. Several groups have shown that PTEN transcription may be regulated by early growth response-1 (Egr-1) (11), p53 (12), Sp1 (13), NF-kB (14), CBF-1 (15), USF1 (16) and c-Jun (17). In addition, in 2001, two putative binding sites for the transcription factor peroxisome proliferator-activated receptor-gamma (PPARγ) were identified ~10 kb upstream of the minimal promoter region of PTEN (18); specific binding of PPARγ was later confirmed (19). Moreover, our laboratory demonstrated that PPARγ, through activation by rosiglitazone or lovastatin, induces PTEN transcription and subsequently upregulates PTEN protein levels (20). These data were the first to suggest that a statin, more specifically lovastatin, signals through PPARγ and upregulates PTEN expression.

Statins have long been thought to have some anti-carcinogenic properties, but concrete evidence remains to be lacking. Clinically, statins are used as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) inhibitors, which downregulate cholesterol production through the mevalonate pathway. However, further research shows that this pathway can also regulate other proteins, such as Ras and Rho, that are key to cancer development (21). Lovastatin (Mevacor), simvastatin (Zocor), pravastatin (Livals) and fluvastatin (Lescol) are commonly prescribed for millions of patients to aid in lowering cholesterol levels for both the primary and secondary prevention of cardiovascular disease (22). In this study, we investigated whether four statins (simvastatin, pravastatin, fluvastatin, mevastatin), beyond lovastatin, have the ability to act as anti-carcinogenic agents by upregulating PTEN expression through PPARγ, similar to lovastatin or via other mechanisms.

RESULTS

Simvastatin, pravastatin and fluvastatin induce PTEN protein expression in a dose-dependent manner

We have recently demonstrated that lovastatin upregulates PTEN expression (20). To determine whether other statins could upregulate PTEN expression, we stimulated MCF-7 breast cancer cells with four additional statins: simvastatin, pravastatin, fluvastatin and mevastatin. The optimal concentration for increased PTEN expression was determined by performing a dose curve on the basis of their previously established IC50s in breast cancer cell lines (23). MCF-7 cells expressed a basal level of PTEN, which was unchanged when exposed to the ethanol (EtOH) vehicle control (Fig. 1; M). When MCF-7 cells were stimulated with mevastatin, we did not observe any changes in PTEN expression despite the wide range of doses centered on its IC50 (Fig. 1D; 0–25 μM). In contrast, simvastatin, pravastatin and fluvastatin induced a dose-dependent increase in PTEN expression after treatment. The greatest increase in PTEN expression occurred at 3, 30 and 18 μM for simvastatin, pravastatin and fluvastatin, respectively (Fig. 1A–C). Our previous work demonstrated that 3 μM lovastatin could stimulate ~1.8-fold induction of PTEN expression. Our current study exhibits a similar induction of PTEN with simvastatin (~1.5-fold), pravastatin (~1.4-fold) and fluvastatin (~1.6-fold).

Statin treatment induces PTEN mRNA expression

Our previous work indicated that lovastatin induces PTEN protein by upregulating PTEN transcription and, subsequently, its mRNA levels (20). To determine whether simvastatin, pravastatin, fluvastatin and mevastatin stimulation induces PTEN transcription in a similar manner as lovastatin, we examined the levels of PTEN mRNA by reverse transcriptase–polymerase chain reaction (RT-PCR) after treatment. Basal levels of PTEN transcript were observed in unstimulated MCF-7 cells or those treated with EtOH vehicle (Fig. 2A; EtOH). In contrast, cells stimulated with 3 μM simvastatin for 48 h had an ~1.7-fold increase in PTEN transcript (Fig. 2A; Sim). An ~1.8-fold induction of PTEN transcript was observed after 30 μM pravastatin stimulation (Fig. 2A; Pra). Furthermore, an ~1.8-fold induction of PTEN transcript was also observed after stimulation with 18 μM fluvastatin.

Figure 1. Simvastatin, pravastatin and fluvastatin induce PTEN protein expression in a dose-dependent manner. Cells were stimulated with simvastatin (A), pravastatin (B), fluvastatin (C) or mevastatin (D) and harvested after 48 h stimulation as indicated. Levels of PTEN and actin were detected by western blot as described in the Materials and Methods section, and relative expression of PTEN plotted against statin concentration (E). Representative blots of three individual experiments are displayed.
Statin stimulation induces PPARγ transcription.

We previously demonstrated that lovastatin induces PTEN and upregulates PPARγ-mediated transcription similar to rosiglitazone, a known synthetic PPARγ ligand (20). To determine whether simvastatin, pravastatin and fluvastatin could regulate PPARγ-mediated transcription as well, we utilized a previously described reporter assay system (20,24). This reporter assay system takes advantage of the known PPARγ response element (PPRE), which has been luciferase-tagged (24). MCF-7 cells were co-transfected with a PPRE construct and empty vector, wildtype PPARγ (WT-PPARγ) or dominant-negative PPARγ (DN-PPARγ). Twelve hours later, these cells were treated with EtOH, lovastatin, simvastatin, pravastatin, fluvastatin or mevastatin for 48 h. As expected, lovastatin stimulation induced PPARγ-mediated transcription ~100-fold over EtOH-treated MCF-7 cells (Fig. 2B; Lov-WT). Simvastatin treatment resulted in an ~80-fold induction of luciferase activity in cells transfected with WT-PPARγ, compared with EtOH-stimulated cells (Fig. 2B; Sim-WT). Treatment with pravastatin resulted in the activation of PPARγ-mediated transcription with an ~70-fold induction (Fig. 2B; Pra-WT). In addition, we observed that cells stimulated with fluvastatin had an ~120-fold induction of luciferase activity when co-transfected with WT-PPARγ, compared with EtOH-treated cells (Fig. 2B; Flu-WT). Unexpectedly, mevastatin stimulation induced PPARγ-mediated transcription ~70-fold over EtOH-treated cells (Fig. 2B; Mev-WT). In contrast, there was little activity in cells treated with any of the statins after transfection with a DN-PPARγ (Fig. 2B; DN). These results indicate that all five statins can both significantly and specifically induce PPARγ-mediated transcription. These data were both unexpected and interesting. We observed that lovastatin, simvastatin, pravastatin and fluvastatin induced PTEN expression, whereas mevastatin did not (Fig. 1). However, the reporter assay results indicate that all five statins can signal though PPARγ-mediated transcription. These results were unexpected because our previously published data demonstrated that PPARγ protein is necessary to induce PTEN (20).

We initially expected to observe all the statins induce PTEN expression signaling through PPARγ-mediated transcription; however, our mevastatin results suggest that this may not be the signaling pathway utilized. It has been previously suggested that statins can signal through sterol response element-binding protein (SREBP); however, the exact mechanisms are still being elucidated (21,25). In addition, published data indicate that SREBP can upregulate PPARγ protein expression and subsequently induce PPARγ-mediated transcription (26). Therefore, on the basis of our results and these published data, we hypothesized three potential model pathways of PTEN upregulation by statins via SREBP and PPARγ (Fig. 3). In model A, statins signal through SREBP to induce PPARγ-mediated transcription, subsequently increasing PTEN expression (Fig. 3A). However, it is entirely possible that statins may upregulate PPARγ-mediated transcription and, therefore, PTEN through a signaling pathway distinct from that of SREBP (Fig. 3B, model B). Our results from the earlier-mentioned reporter assay may indicate that statins may induce PTEN expression through a signaling pathway that utilizes PPARγ activity independent of PPARγ’s transcriptional activity (Fig. 3C, model C).

Both statins and SREBP can induce PTEN protein expression

As noted earlier, published data suggest that statins upregulate PTEN through PPARγ-mediated transcription, and that

Figure 2. Simvastatin, pravastatin and fluvastatin induce PTEN mRNA expression and PPRE-regulated transcription. Cells were stimulated and mRNA harvested as described in the Materials and Methods section. (A) PTEN mRNA levels were quantitated by densitometry and normalized against actin [EtOH vehicle control; 3 μM simvastatin (Sim); 30 μM pravastatin (Pra) and fluvastatin (Flu)]. Bars represent mean ± SEM of PTEN levels (from three individual experiments). (B) MCF-7 cells were co-transfected with PPRE-Luc and either empty vector (‘-‘, gray bars), WT-PPARγ (WT, black bars) or DN-PPARγ (DN, white bars). Cells were then stimulated with 3 μM lovastatin, 3 μM simvastatin, 30 μM pravastatin, 18 μM fluvastatin or 30 μM mevastatin for 48 h and analyzed for luciferase activity as described. Each bar represents a mean ± SEM of three individual experiments.

Statin stimulation induces PPARγ-mediated transcription

We previously demonstrated that lovastatin induces PTEN and upregulates PPARγ-mediated transcription similar to rosiglitazone, a known synthetic PPARγ ligand (20). To determine whether simvastatin, pravastatin and fluvastatin could regulate PPARγ-mediated transcription as well, we utilized a previously described reporter assay system (20,24). This reporter assay system takes advantage of the known PPARγ response element (PPRE), which has been luciferase-tagged (24). MCF-7 cells were co-transfected with a PPRE construct and empty vector, wildtype PPARγ (WT-PPARγ) or dominant-negative PPARγ (DN-PPARγ). Twelve hours later, these cells were treated with EtOH, lovastatin, simvastatin, pravastatin, fluvastatin or mevastatin for 48 h. As expected, lovastatin stimulation induced PPARγ-mediated transcription ~100-fold over EtOH-treated MCF-7 cells (Fig. 2B; Lov-WT). Simvastatin treatment resulted in an ~80-fold induction of luciferase activity in cells transfected with WT-PPARγ, compared with EtOH-stimulated cells (Fig. 2B; Sim-WT). Treatment with pravastatin resulted in the activation of PPARγ-mediated transcription with an ~70-fold induction (Fig. 2B; Pra-WT). In addition, we observed that cells stimulated with fluvastatin had an ~120-fold induction of luciferase activity when co-transfected with WT-PPARγ, compared with EtOH-treated cells (Fig. 2B; Flu-WT). Unexpectedly, mevastatin stimulation induced PPARγ-mediated transcription ~70-fold over EtOH-treated cells (Fig. 2B; Mev-WT). In contrast, there was little activity in cells treated with any of the statins after transfection with a DN-PPARγ (Fig. 2B; DN). These results indicate that all five statins can both significantly and specifically induce PPARγ-mediated transcription. These data were both unexpected and interesting. We observed that lovastatin, simvastatin, pravastatin and fluvastatin induced PTEN expression, whereas mevastatin did not (Fig. 1). However, the reporter assay results indicate that all five statins can signal though PPARγ-mediated transcription. These results were unexpected because our previously published data demonstrated that PPARγ protein is necessary to induce PTEN (20).

We initially expected to observe all the statins induce PTEN expression signaling through PPARγ-mediated transcription; however, our mevastatin results suggest that this may not be the signaling pathway utilized. It has been previously suggested that statins can signal through sterol response element-binding protein (SREBP); however, the exact mechanisms are still being elucidated (21,25). In addition, published data indicate that SREBP can upregulate PPARγ protein expression and subsequently induce PPARγ-mediated transcription (26). Therefore, on the basis of our results and these published data, we hypothesized three potential model pathways of PTEN upregulation by statins via SREBP and PPARγ (Fig. 3). In model A, statins signal through SREBP to induce PPARγ-mediated transcription, subsequently increasing PTEN expression (Fig. 3A). However, it is entirely possible that statins may upregulate PPARγ-mediated transcription and, therefore, PTEN through a signaling pathway distinct from that of SREBP (Fig. 3B, model B). Our results from the earlier-mentioned reporter assay may indicate that statins may induce PTEN expression through a signaling pathway that utilizes PPARγ activity independent of PPARγ’s transcriptional activity (Fig. 3C, model C).
SREBP may be a mediator (Fig. 3A) (26). To test this hypothesis, we utilized N-acetyl-leucyl-leucyl-norleucinal (ALLN), which inhibits SREBP catabolism, thus upregulating its expression and function. MCF-7 cells were treated with lovastatin, simvastatin, pravastatin, fluvastatin or ALLN for 48 h, and whole-cell protein lysates were examined by western blot analysis. Vehicle (EtOH)-treated cells express a basal level of SREBP and PPARγ protein (Fig. 4A, lane 1, labeled ‘-‘). Cells stimulated with lovastatin (L), simvastatin (S), pravastatin (P) and fluvastatin (F) do not significantly alter SREBP or PPARγ protein expression (Fig. 4A, lanes 2–5). In contrast, ALLN-treated cells (A) exhibit an increase in both SREBP and PPARγ protein expression as expected (Fig. 4A, lane 6).

Our current study indicates that statins can universally induce PTEN expression and suggest that SREBP can as well. Therefore, to establish that the effects we see with statin stimulation are not cell-specific, and that SREBP can induce PTEN expression, we compared our results obtained in MCF-7 (Fig. 2B, top panel) with three other breast cancer cell lines: MDA-MB-435 (second panel), MDA-MB-231 (third panel) and T47D (fourth panel). The basal level of PTEN expression varies across the cell lines, and statin and ALLN stimulation results in ~1.8-fold increased PTEN expression in all four cell lines (Fig. 4C).

**Figures**

**Figure 3.** Three potential models of statin-mediated PTEN upregulation. (A) Statins regulate SREBP levels, which induce PPARγ-mediated transcription. PTEN transcription is subsequently increased. (B) Statins and SREBP can both induce PPARγ-mediated transcription; however, their mechanisms are independent of each other. PTEN transcription is subsequently increased. (C) Statins regulate PPARγ protein activity, which regulates a transcription factor that induces PTEN transcription. SREBP also induces PTEN transcription, but through regulation of PPARγ-mediated transcription.

**SREBP induction of PPARγ-mediated transcription antagonized by statins**

ALLN upregulation of SREBP and PPARγ expressions is thought to result in an increase in PPARγ-mediated transcription (26). To determine whether upregulation of SREBP can indeed induce PPARγ-mediated transcription in our system, we performed a PPARγ reporter assay with cells treated with ALLN. In addition, we observed the effect of co-stimulation of statin/ALLN (Lov/ALLN, Sim/ALLN, Pra/ALLN, Flu/ALLN) to determine whether the agonists have an additive affect. ALLN stimulation of MCF-7 cells in the presence of WT-PPARγ significantly induced PPARγ-mediated transcription (~160-fold; Fig. 5A, ALLN-WT), which was inhibited when treated in the presence of DN-PPARγ (~20-fold; Fig. 5A, ALLN-DN). Interestingly, when MCF-7 cells were treated with ALLN in concert with the any of four statins that induced PTEN expression (lovastatin, simvastatin, pravastatin and fluvastatin), we observed an inhibition of luciferase activity compared with ALLN alone. By comparing these results (Fig. 5A) with our previous reporter assay results (Fig. 2B), we observed a significant inhibition of PPARγ-mediated transcription when MCF-7 cells are treated with a statin/ALLN combination compared with statin treatment alone. Co-stimulation of lovastatin and ALLN (Lov/ALLN) inhibited ALLN’s induction by 50% and lovastatin’s induction by 20%, whereas treating cells with simvastatin and ALLN (Sim/ALLN) inhibited their induction by 40 and 70%, respectively. Treatment of pravastatin and ALLN (Pra/ALLN) in concert inhibited their induction by 15 and 60%, respectively. In addition, co-stimulation of fluvastatin and ALLN (Flu/ALLN) inhibited fluvastatin’s induction 70% and ALLN’s induction 75%.

These observations suggest that ALLN-related upregulation of PPARγ-mediated transcription may be due to SREBP induction; however, it may also be a result of ALLN’s proteosome inhibitor activity. Therefore, to test ALLN’s role in PPARγ-mediated transcription, we performed a reporter assay (similar to that shown in Fig. 5A) with MG-132, another proteosome inhibitor (27). In contrast to ALLN, MG-132 did not produce an induction of PPARγ-mediated transcription, indicating that this is not a general proteasome inhibitor phenomenon (data not shown). These data suggest that SREBP induces PPARγ protein, resulting in increased PPARγ transcriptional activity. In addition, these data provide evidence that statins do not signal through SREBP to induce PTEN expression, thus, excluding model A (Fig. 3A). Instead, our data suggest that these two signaling pathways appear to antagonize each other.

**SREBP, but not the statins, induces PPARγ-mediated transcription to increase PTEN protein expression**

Our results suggest that statins signal down a pathway separate from SREBP, potentially as illustrated in model B (Fig. 3B). In addition, SREBP may have the ability to induce PTEN expression through the upregulation of PPARγ transcriptional activity although this observation may be artificial. In the aforementioned reporter assay system (Figs 2B and 5A), we added exogenous PPARγ protein to observe an increase in PPARγ-mediated transcription. However, in our initial western blots (Fig. 1), we observed an induction of PTEN expression after statin stimulation without the addition of exogenous PPARγ. In order to determine whether the statins and SREBP truly signal through PPARγ to induce PTEN transcription, we performed a reporter assay in the absence of exogenous PPARγ protein. MCF-7 cells were transfected with only the PPRE-Luc vector and subsequently stimulated with the four statins, ALLN or the statin/ALLN combination.
Statin stimulation alone presented no induction of PPARγ-mediated transcription, compared with EtOH, when exogenous PPARγ was not added to the system (Fig. 5B; bars 1–5). In contrast, we observed an induction of luciferase activity after ALLN stimulation (Fig. 5B; bar 6; ~40-fold). Stimulation of MCF-7 cells with ALLN in concert with the statins altered PPARγ-mediated transcription compared with ALLN alone, but remained significantly induced over statin treatment alone (Fig. 5B; bars 7–10). These data suggest that statins do not have the ability to induce PPARγ-mediated transcription when PPARγ protein levels are low, thus suggesting that PPARγ’s transcriptional activity is not a key ‘player’ in PTEN induction after statin stimulation. On the basis of these results, model C might be the most accurate representation of statin and SREBP upregulation of PTEN expression (Fig. 3C).

Rosiglitazone does not inhibit SREBP induction of PPARγ-mediated transcription

It has been previously suggested that SREBP has the ability to induce the production of natural PPARγ ligands (26). This indicates that SREBP’s ability to induce both PPARγ protein and its ligand may be a mechanism for the increase in PPARγ’s transcriptional activity. Our data demonstrate that statins can antagonize this pathway, potentially by interfering with PPARγ’s natural ligands. To test this hypothesis, we utilized rosiglitazone, a synthetic but documented PPARγ ligand. We would expect rosiglitazone to signal through the same pathway as the natural PPARγ ligands and to induce PPARγ-mediated transcription, although not hinder- ing ALLN’s induction. Therefore, we stimulated MCF-7 cells with rosiglitazone, ALLN or the combination of rosiglitazone and ALLN and analyzed PPARγ’s transcriptional activity. Rosiglitazone induced ~120-fold increase in luciferase activity, whereas ALLN induced ~160-fold increase in luciferase activity over EtOH-treated cells (Fig. 6). Interestingly, the combination of rosiglitazone and ALLN (~150-fold) did not inhibit ALLN signaling as previously observed with the four statins (Fig. 5A). This suggests that statins hinder natural PPARγ ligand signaling. In addition, these data confirm that statins do not induce PPARγ-mediated transcription, but rather, regulate a separate PPARγ protein activity to induce PTEN expression as illustrated in model C (Fig. 3C).

Statins upregulate PTEN transcription through an unknown transcription factor located between −854 and −791

Our results, consistent with our proposed model C (Fig. 3C), show that statin stimulation increases PTEN transcription in a PPARγ transcriptional activation-independent mechanism, suggesting the involvement of another transcription factor(s). To examine this in greater detail, we performed transcriptional reporter assays using serial deletions of the PTEN promoter. Seven PTEN promoter constructs were cloned into
and define the nucleotides of interest within this region, we utilized transcription factors that bind to this region. To more accurately follow this, we constructed six more serial deletion vectors over EtOH-treated cells (data not shown). Using this as a starting point, we then analyzed for luciferase activity as described. Each bar represents a mean ± SEM of three individual experiments.

Figure 5. SREBP induces PPARγ-mediated transcription, which is inhibited by the statins. (A) MCF-7 cells were transfected with PPRE-Luc. After 12 h, cells were stimulated with 15 μM ALLN alone or in combination with 3 μM lovastatin (Lov), 3 μM simvastatin (Sim), 30 μM pravastatin (Pra) or 18 μM fluvastatin (Flu) for 48 h and analyzed for luciferase activity as described. Each bar represents a mean ± SEM of three individual experiments. (B) MCF-7 cells were transfected with PPRE-Luc and stimulated with 3 μM simvastatin (Sim), 30 μM pravastatin (Pra), 18 μM fluvastatin (Flu), 15 μM ALLN or a combination of the statins with ALLN for 48 h. Cells were then analyzed for luciferase activity as described. Each bar represents a mean ± SEM of three individual experiments.

pGL3-Basic vectors: −1334 to 0, −1158 to 0, −1026 to 0, −893 to 0, −601 to 0, −453 to 0 and −203 to 0 and subsequently transfected into MCF-7 cells. Twelve hours after transfection, MCF-7 cells were treated with either EtOH or one of the statins. Luciferase activity was measured and the results indicated that only one construct, containing −893 to 0, could significantly induce PTEN transcription (~3.5-fold) over EtOH-treated cells (data not shown). Using this as a starting point, we constructed six more serial deletion vectors (Fig. 7A). These vectors were transfected into MCF-7 cells followed by protein translation, identified this region as a target for the binding of a yet-unknown transcription factor(s) (28). In order to further dissect out this region of binding, we constructed five more serial deletion probes. Figure 7D shows specific protein binding for three of the five probes, with the smallest region of interest on the PTEN promoter between −854 and −791. Overall, therefore, our data suggest that statins upregulate PTEN transcription by regulating PPARγ protein activity, which subsequently induces an as-yet-unknown transcription factor that binds to the PTEN promoter between −854 and −791. In addition, we were also able to demonstrate, for the first time, that SREBP can also induce PTEN transcription through upregulation of PPARγ-mediated transcription.

DISCUSSION

PTEN is a constitutively active dual specificity phosphatase tumor suppressor. As such, regulation of activity is determined by protein level. We have demonstrated that statins, more specifically lovastatin, simvastatin, pravastatin and fluvastatin, induce PTEN transcription through regulating PPARγ protein
activity, rather than its transcriptional activity. In addition, we subsequently isolated a small region of the PTEN promoter, \( \text{2854 to 2791} \), that the statins regulate to induce PTEN expression.

Other groups have shown that statins can increase PPAR\(\gamma\) mRNA and subsequently its protein expression \((29,30)\). Our current study indicates that statins may secondarily induce PPAR\(\gamma\) transcriptional activity independent of PTEN regulation. We demonstrated that the statins induced PTEN expression in the presence of endogenous PPAR\(\gamma\) protein levels (Figs 1 and 2A); however, additional exogenous PPAR\(\gamma\) was required for the statins to induce PPAR\(\gamma\)’s transcriptional activity (Fig. 2B). The addition of exogenous PPAR\(\gamma\) was essential because MCF-7 cells express low levels of PPAR\(\gamma\) (Fig. 4A). This indicated that PPAR\(\gamma\) protein is the limiting factor in statin regulation of its transcriptional activity, but not for the upregulation of PTEN expression. Moreover, we established that in the presence of WT-PPAR\(\gamma\), but not DN-PPAR\(\gamma\), an induction of PPAR\(\gamma\)-mediated transcription could be observed after statin stimulation (Fig. 2B). This further indicates that the statins’ ability to induce PPAR\(\gamma\) transcriptional activity is a specific effect; however, it is also clear from our work that it is not the primary pathway for PTEN induction.

We initially proposed that statins induced PPAR\(\gamma\)-mediated transcription to increase PTEN levels; however, our data demonstrate that this hypothesis was incorrect. These results were unexpected because we previously demonstrated that PPAR\(\gamma\) protein expression is necessary in order to observe the induction of PTEN expression \((20)\). Another protein that the statins have been connected with and is involved in regulating lipid metabolism is SREBP \((25)\), which has been shown to be involved in PPAR\(\gamma\)’s protein production and transcriptional activity \((26)\). This raises the possibility that statins,
PPARγ and SREBP may all play a role in modulating PTEN expression. Our results indicate that both the statins and SREBP can signal through PPARγ; however, they do so differently and independent of each other. Here, SREBP principally utilized PPARγ’s transcriptional activity, whereas statins primarily signal through a pathway independent of PPARγ’s transcriptional activity. These results are in agreement with Ravid et al.’s (31) study which demonstrated that ALLN inhibited HMG-CoA reductase degradation. The combination of statin treatment with increased SREBP activity actually antagonized the two pathways (Fig. 5B). In contrast, Mascaro et al. (32) observed a fluvastatin/ALLN synergistic effect on transcriptional activity in CHO cells. Our data indicate that SREBP has the ability to induce PTEN expression (Fig. 4A), suggesting that its upregulation of PPARγ-mediated transcription induces PTEN transcription. On the basis of these results, SREBP agonists may aid patients who demonstrate a decrease in PTEN but do not have an isolated PTEN mutation in the open reading frame; unfortunately, a clinically relevant SREBP agonist has yet to be established. However, one needs to be conscious of the idea that the combination of statins and an SREBP agonist may actually hinder the effect of each individual treatment.

Overall, our data strongly suggest that statins regulate a transcription factor(s), besides PPARγ, to induce PTEN transcription. We have demonstrated that lovastatin, simvastatin, pravastatin and fluvastatin upregulate PTEN transcription in the context of the PTEN promoter defined by a region flanking nucleotides −893 and −601 (Fig. 7B) and that an unknown transcription factor(s) binds(s) between −854 and −791 (Fig. 7D). Only two transcription factors are predicted by multiple prediction software programs to bind at this region: Sp1 [TESS (33) and Alibaba (34)] and c-Myb [TESS and TFSEARCH (35)]. Sp1 is currently thought to be a putative PTEN transcription factor, due to the full-length PTEN promoter being very GC rich; however, empiric research has yet to concretely show that it binds to any particular region of the promoter or has the ability to regulate its transcription (3,27). c-Myb has been shown to be upregulated within tumors when PTEN expression was decreased (36), indicating that it may be acting as a PTEN transcriptional repressor; however, the pathway connecting the two has yet to be determined. Thus, both Sp1 and c-Myb may be postulated to be regulators of PTEN expression; however, more in-depth studies are necessary to determine the identity of this novel PTEN transcription factor and are beyond the scope of this article.

Our data reinforce the importance and benefit that agonists which upregulate PTEN transcription may be useful to a subset of breast cancer patients. This suggests that a therapeutic tool that can regulate PTEN’s transcription would be highly effective within the subset of patients who have an identified nucleotide alteration within the PTEN promoter or in patients in whom a PTEN mutation has yet to be identified. This approach would also be useful where hemizygous deletions occur, as in sporadic neoplasias, in increasing PTEN protein levels. This study indicates that lovastatin, simvastatin, pravastatin and fluvastatin as well as an SREBP agonist would be germane to this cohort of patients. However, despite these encouraging results, we must be aware that these potential therapies may theoretically harm patients with germline intragenic PTEN mutations or those with neoplasias with somatic intragenic mutations by raising levels of mutant, as well as wildtype, protein.

MATERIALS AND METHODS

Materials
Fluvastatin, mevastatin, lovastatin, pravastatin and simvastatin were obtained from Cayman Chemical (Ann Arbor, MI, USA).

ALLN was purchased from Biomol International (Plymouth Meeting, PA, USA). Antibodies were obtained from Cascade Bioscience, Waltham, MA, USA (PTEN 6H2.1), Sigma-Aldrich (Actin) and Cell Signaling (PPARγ and SREBP1). Cell culture media was obtained from the Cleveland Clinic Media Core. M-PER mammalian protein extraction reagent was obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). All other reagents were purchased from standard commercial sources.

Cell culture and stimulation

The MCF-7 cell line was maintained at 37°C with 5% CO2 in DMEM containing 10% FBS and 100 U/ml each penicillin and streptomycin. MDA-MB-231, MDA-MD-435 and T47D breast cancer cell lines were maintained at 37°C with 5% CO2 in RPMI containing 10% FBS and 100 U/ml each penicillin and streptomycin. Cells were plated at 1.0 × 10^4 cells per well 24 h prior to the treatment. After 24 h, cells were treated as indicated in the Results section and in figure legends. Following treatments, cells were incubated for an additional 48 h prior to harvesting.

Protein extraction

MCF-7, MDA-MB-231, MDA-MB-435 and T47D cells were plated and stimulated as described earlier. At the time of harvest, media was removed and the cells were washed with PBS. Cells were then harvested into M-PER lysis buffer containing phenylmethanesulphonyl fluoride (0.75 mg/ml), benzamidine hydrochloride (0.5 mg/ml), aprotinin (2 μg/ml), pepstatin (2 μg/ml), NaOV (0.2 mM) and NaF (25 mM). Cells were incubated at room temperature with lysis buffer for 1 min before harvesting by scraping. Samples were then centrifuged at 16 000 g for 10 min at 4°C to remove cellular debris. The resulting supernatant was stored at −80°C. Protein concentration was determined using the bicoticin method (37) using BSA as a standard. Western blot

Proteins (30 μg) were prepared by the Laemmli method (38), then separated on a 10% SDS–PAGE gel and electrophoretically transferred onto nitrocellulose. Equal protein loading between conditions was confirmed by staining with Ponceau S solution. Non-specific binding was blocked by incubating the nitrocellulose blots with 5% milk in TBS-T (100 mM Tris, pH 7.0, 1 mM NaCl, 1% Tween-20) for 1 h at room temperature. Blots were then incubated with the primary antibody (1:1000 in 3% BSA) for 2 h at room temperature. Following...
the primary incubation, the blots were washed with TBS-T for 1 h with frequent changes of buffer. Blots were then incubated with the appropriate secondary antibody conjugated to horse-radish peroxidase (Promega, Madison, WI, USA) (1:2500 dilution in 5% milk) overnight at 4°C and washed with TBS-T for 1 h. Protein bands were visualized using enhanced chemiluminescence as described by the manufacturer (Amersham Pharmacia Corp., Piscataway, NJ, USA). The resultant films were then quantified using NIH-Imager densitometry software.

Reverse transcriptase–polymerase chain reaction

MCF-7 cells were stimulated as described earlier. After stimulation, cells were released by trypsin treatment and subsequently washed three times with PBS through centrifugation. Total RNA was extracted from cells, following the Gentra Versagen RNA Purification System Protocol (Minneapolis, MN, USA) and then converted to cDNA by Superscript II reverse transcriptase after DNase treatment. The resultant cDNA was subjected to multiplex PCR amplification using primers specific to PTEN exon 3 and exon 5 (F: 5′ TGAGTCTAAGCATATAACACCA 3′; R: 5′ AAAAAAGTATTGGCAACTGC 3′) and β-actin (Quantum RNA β-actin, Ambion Inc., Austin, TX, USA). Primers were allowed to anneal at 55°C for 28 cycles. The products from the PCR reactions were run on a 1% agarose gel containing ethidium bromide and visualized under a UV light.

Reporter assay

Plasmids were co-transfected into six-well cultures of MCF-7 cells with 3 μl/well of FuGene (Roche). Each well was co-transfected with 500 ng of the reporter plasmid PPRETK-LUC, 50 ng Renilla luciferase control plasmid and 100 ng of receptor expression vector (pcDNA3, WT PPARγ1 or L468A/E471A PPARγ1). Each plasmid has previously been described (20,24). After 12 h, cells were stimulated with EtOH, 3 μM lovastatin, 3 μM simvastatin, 30 μM pravastatin, 18 μM fluvastatin or 10 μM mevastatin for 48 h. PTEN promoter was PCR-amplified from normal genomic DNA and subsequently cloned into a TOPO-TA vector. DNA was PCR-amplified using 55°C as the annealing temperature for 30 cycles. All PCR amplification products were verified by direct DNA sequencing (ABI 3730xl DNA analyzer), and positive clones were subcloned into a pGL3.1-Basic vector system, Promega) and analyzed on a luminometer (Cambridge, UK). The resultant cDNA was subjected to multiplex PCR amplification using primers specific to PTEN exon 3 and exon 5 (F: 5′ TGAGTCTAAGCATATAACACCA 3′; R: 5′ AAAAAAGTATTGGCAACTGC 3′) and β-actin (Quantum RNA β-actin, Ambion Inc., Austin, TX, USA). Primers were allowed to anneal at 55°C for 28 cycles. The products from the PCR reactions were run on a 1% agarose gel containing ethidium bromide and visualized under a UV light.

Electromobility shift assay

PTEN promoter sequence was isolated through PCR amplification from normal genomic DNA. The DNA was PCR-amplified with 30 cycles at the annealing temperature of 55°C in 20 μl reactions using HotStar and Q Solution (Qiagen). Each of these products was radiolabeled with 32P-y-ATP via T4 kinase. To examine DNA–protein interaction, 1 ng of radiolabeled probe was incubated with 2 μg of either untreated or statin-treated nuclear protein extract for 20 min at room temperature with binding buffer containing 10 mM HEPES (pH 7.5), 2.5 mM MgCl2, 50 mM NaCl, 0.5 mM DTT, 4% glycerol, 1 μg/ml BSA and 2 μg poly dI/dC. Unlabeled probe in 5× molar excess was used as the specific competitor, whereas a random oligonucleotide sequence was used as the non-specific competitor. DNA–protein complexes were resolved on a 4% non-denaturing PAGE gel at 150 V for 3.5 h at 4°C and visualized using a Phospho-Imager (Amersham Biosciences, Piscataway, NJ, USA).

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