High-dose atorvastatin causes a rapid sustained increase in human serum PCSK9 and disrupts its correlation with LDL cholesterol

Greg Welder, Issam Zineh, Michael A. Pacanowski, Jason S. Troutt, Guoqing Cao, and Robert J. Konrad

University of Florida, Gainesville, FL; and the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN

Abstract Proprotein convertase subtilisin kexin type 9 (PCSK9) is a key regulator of serum LDL-cholesterol (LDL-C) levels. PCSK9 is secreted by the liver into the plasma and binds the hepatic LDL receptor (LDLR), causing its subsequent degradation. We first demonstrated that a moderate dose of atorvastatin (40 mg) increases PCSK9 serum levels, suggesting why increasing statin doses may have diminished efficacy with regard to further LDL-C lowering. Since that initial observation, at least two other groups have reported statin-induced PCSK9 increases. To date, no analysis of the effect of high-dose atorvastatin (80 mg) on PCSK9 over time has been conducted. Therefore, we studied the time course of atorvastatin (80 mg) in human subjects. We measured PCSK9 and lipid levels during a 2-week lead-in baseline period and every 4 weeks thereafter for 16 weeks. We observed that atorvastatin (80 mg) caused a rapid 47% increase in serum PCSK9 at 4 weeks that was sustained throughout 16 weeks of dosing. Importantly, while PCSK9 levels were highly correlated with total cholesterol (TC), LDL-C, and triglyceride (TG) levels at baseline, atorvastatin (80 mg) completely abolished all of these correlations.

Together, these results further suggest an explanation for why increasing doses of statins fail to achieve proportional LDL-C lowering. —Welder, G., I. Zineh, M. A. Pacanowski, J. S. Troutt, G. Cao, and R. J. Konrad. High-dose atorvastatin causes a rapid, sustained increase in human serum PCSK9 and disrupts its correlation with LDL cholesterol. J. Lipid Res. 2010, 51: 2714–2721.

Supplementary key words LDL receptor • HDL-cholesterol • proprotein convertase subtilisin kexin type 9

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Abbreviations: HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; LDLR, low density lipoprotein receptor; PCSK9, proprotein convertase subtilisin kexin type 9; TC, total cholesterol; TG, triglyceride.

G. Welder and I. Zineh contributed equally to this work.

I. Zineh and M. A. Pacanowski are currently employed by the US Food and Drug Administration (US FDA); however this work was conducted when they were employed by the University of Florida.

To whom correspondence should be addressed: e-mail: konrad_robert@lilly.com

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subject, a 49-year-old male, heterozygous for two monoallelic dominant negative PCSK9 mutations, has also been described and had an LDL-C of 16 mg/dl (33).

Statins, which are the class of drugs most widely prescribed to lower LDL-C levels, have been shown to increase the activity/nuclear translocation of sterol regulatory element-binding protein-2 (SREBP-2), a transcription factor that activates both the LDLR and PCSK9 genes (34–36). Statins were originally reported to increase PCSK9 mRNA expression (34–36), and our group hypothesized that statin treatment in humans should increase circulating PCSK9 protein levels. Utilizing a novel PCSK9 sandwich ELISA (37), we were the first to demonstrate that atorvastatin (40 mg), the most widely prescribed statin, significantly increased PCSK9 serum levels after three months of treatment (38). Since that time, at least two other laboratories have also reported that patients on statins have increased levels of circulating PCSK9 (7, 39).

These observations of statin-induced PCSK9 increases in patients offer insight into the mechanism for the non-linear statin dose-response relationship. These observations, however, are limited in that the detailed time course of statin-induced PCSK9 increases has not been described. It is not known, for instance, if PCSK9 levels plateau during statin therapy or continue to increase over time. It is possible that PCSK9 levels might increase initially and then decrease as a new steady-state level of hepatic PCSK9 secretion is reached. It is also not understood with certainty how statin treatment affects the correlation of PCSK9 levels with LDL-C levels over time or if PCSK9 levels predict treatment response to statins. The answers to these questions are not currently known because previous studies have been limited by their observational nature or sample size. In addition, previous studies have not assessed the effects of changes in PCSK9 over time or their potential role in determining statin response. Nor have the highest doses of statins been tested. As a result, in the current study, we determined 1) the detailed time course of the effect of high-dose atorvastatin on PCSK9 levels; 2) the possibility that baseline PCSK9 levels may predict the magnitude of the atorvastatin-induced LDL-C response; 3) whether atorvastatin-induced changes in PCSK9 levels correlated with atorvastatin-induced decreases in LDL-C; and 4) the effect of high-dose atorvastatin on the relationship between PCSK9 levels and LDL-C and other serum lipids over time.

MATERIALS AND METHODS

Clinical study protocol

Our clinical study design has been reported previously (40, 41). Briefly, subjects had to be at least 18 years of age and not require treatment for dyslipidemia based on ATP III guidelines. Subjects were excluded if they had a history of coronary disease or risk equivalents, were pregnant, or had liver transaminases greater than two times the upper limit of normal. In addition, subjects could not have a history of cholesterol medication use or concurrent medication or complementary medicine use that could alter cholesterol or inflammation that posed a clinically relevant interaction with atorvastatin. A total of 84 eligible subjects went through a run in control period of two weeks where no treatment was administered, followed by initiation of atorvastatin 80 mg taken once daily. Subjects were seen every 4 weeks for a total of 16 weeks on high-dose atorvastatin. Subjects were assessed for compliance and adverse effects and had research labs drawn for PCSK9 evaluation at each visit. Routine lipid levels (including total cholesterol, LDL-C, HDL-C, and TG) were assessed following a minimal 8-12 h fast at baseline and after 8 and 16 weeks on atorvastatin. PCSK9 levels were assessed at 2 weeks prior to dosing, 0-week, 4-week, 8-week, 12-week, and 16-week visits. The two PCSK9 results for each subject at the minus 2-week visit and 0-week visit (both prior to the start of atorvastatin treatment) were averaged to determine a baseline PCSK9 level. All visits were conducted at the University of Florida General Clinical Research Center (GCRC). All subjects provided written, informed consent, and the study was approved by both the University of Florida’s Institutional Review Board and the GCRC Scientific Advisory Committee (clinicaltrials.gov identifier NCT00361283).

PCSK9 ELISA

PCSK9 levels in the serum samples were measured using our recently described PCSK9 dual monoclonal antibody sandwich ELISA (37, 38, 42) with minor modifications. These observations of statin-induced PCSK9 increases were purified using an ion-exchange column followed by size-exclusion chromatography. Identity of the protein was confirmed by N-terminal sequencing, and purity was judged to be greater than 95% based on SDS-PAGE followed by Coomassie blue staining. ELISA wells were coated overnight with anti-PCSK9 monoclonal antibody at a concentration of 5 μg/ml. The following day, wells were aspirated, washed three times with assay buffer (50 mM HEPES, pH 7.40, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA), and blocked for 1 h with TBS-casein blocking buffer (Pierce). Next, 100 μl of non-His-tagged recombinant PCSK9 standards (varying concentrations of recombinant protein in assay buffer) were added to the wells as a standard curve. Afterward, serum samples were diluted 1:20 in assay buffer, added to their respective wells, and the ELISA plate was incubated for 2 h at room temperature. Following aspiration, wells were washed three times with assay buffer, and 100 μl of a 1:1000 dilution of conjugate antibody (HRP-labeled anti-PCSK9 monoclonal antibody, 1 mg/ml) were added to the wells for a 1 h incubation at room temperature. Following aspiration, wells were washed three times with TBST. After the last aspiration of TBST, 100 μl of TMB development substrate (Pierce) were added to the wells and allowed to incubate for 30 min at room temperature. The reaction was stopped with an equal volume of 2N phosphoric acid, and plates were read at 450 nm. SigmaPlot version 8.0 was used for fitting of the calibration curves. Serum samples were shipped on dry ice and stored at −70°C prior to analysis. Reproducibility of the ELISA on frozen serum samples was tested by looking at the effect of up to four freeze-thaw cycles on samples, with at least 90% recovery observed for all samples after four freeze-thaw cycles.

Immunoprecipitation and Western blotting of PCSK9

Analysis of PCSK9 levels in serum samples by immunoprecipitation and Western blotting was performed as previously described (37, 38, 42) with minor modifications. For each immunoprecipi-
and exposed to Bio-Max X-ray film (Kodak). Blots were developed with ECL reagent (Amersham), air-dried, and stored at room temperature. Following a final three washes with TBST, blots were probed with an HRP-labeled anti-sheep IgG for 1 h at room temperature. Blots were washed three times (10 min each) with TBST (10 mmol/l Tris pH 7.40, 150 mmol/l NaCl, 1 ml Tween 20/L). After washing, blots were probed with sheep polyclonal anti-human PCSK9 antibody in blocking buffer for 1 h at room temperature. Blots were blocked for 1 h at room temperature in TBS-casein blocking buffer (Pierce) containing 1 ml Tween 20/L. After blocking, blots were probed with sheep polyclonal anti-human PCSK9 antibody in blocking buffer for 1 h at room temperature. Blots were washed three times (10 min each) with TBST (10 mmol/l Tris pH 7.40, 150 mmol/l NaCl, with 1 ml Tween 20/L). After washing, blots were probed with an HRP-labeled anti-sheep IgG for 1 h at room temperature. Following a final three washes with TBST, blots were developed with ECL reagent (Amersham), air-dried, and exposed to Bio-Max X-ray film (Kodak).

**Data analysis**

Only subjects with baseline and 16-week (end of study) serum samples were included in the analysis (74 out of 84 enrolled subjects). At the 8-week time point, 72 of the 74 subjects provided adequate samples for analysis, and at the 12-week time point, 67 of the 74 subjects provided adequate samples for analysis. At all other time points (baseline, 4-week, and 16-week), data from all 74 subjects were able to be analyzed. For the PCSK9 ELISA, SigmaPlot version 8.0 was used for fitting of the calibration curves. All data were analyzed and plotted using the program FigP ( Biosoft, St. Louis, MO). Statistical analysis was performed using the same program. For Figs. 1 and 2, data were analyzed by one-way ANOVA followed by comparisons between the means using the least significant difference test. All data in Figs. 1 and 2 were expressed as the mean ± SEM. For Figs. 3 and 4, statistical analysis was performed to calculate Spearman correlation coefficients. In each case, a P value of less than 0.05 indicated statistical significance.

**RESULTS**

Fig. 1A demonstrates the effect of atorvastatin, 80 mg per day for 16 weeks, on serum TC levels. At baseline, the TC level was 180 ± 4 mg/dl. Following 8 weeks of atorvastatin treatment, there was a significant decrease in TC compared with baseline (117 ± 3 mg/dl, P < 0.01 versus baseline). This decrease in TC was sustained at the 16-week time point (118 ± 3 mg/dl, P < 0.01 versus baseline). As Fig. 1B demonstrates, atorvastatin also had a similar effect on LDL-C levels. At baseline, the LDL-C level was 101 ± 4 mg/dl. Following 8 weeks of atorvastatin treatment, there was a significant decrease in LDL-C compared with baseline (45 ± 2 mg/dl, P < 0.01 versus baseline), and this decrease in LDL-C was sustained at the 16-week time point (45 ± 2 mg/dl, P < 0.01 versus baseline). In contrast, atorvastatin had no effect on HDL-C levels with baseline, 8-week, and 16-week HDL-C being 60 ± 2 mg/dl, 58 ± 2 mg/dl, and 58 ± 2 mg/dl, respectively (Fig. 1C). As Fig. 1D shows, with regard to TG levels, the baseline TG level was 96 ± 6 mg/dl, and atorvastatin treatment resulted in significant decrease at 8 and 16 weeks (72 ± 4 mg/dl and 70 ± 5 mg/dl respectively, both P < 0.01 versus baseline).

Fig. 2A demonstrates that 80 mg/day atorvastatin treatment caused a rapid and sustained increase in circulating PCSK9 levels. At baseline, PCSK9 levels were 97 ± 4 ng/ml. After only 4-weeks of atorvastatin treatment, PCSK9 levels had increased 47% to 143 ± 5 ng/ml (P < 0.01 versus baseline). This significant increase in PCSK9 levels was maintained at the 8-week, 12-week, and 16-week time points with PCSK9 levels of 140 ± 6 ng/ml, 143 ± 7 ng/ml, and 142 ± 6 ng/ml, respectively (all P < 0.01 versus baseline).

In light of a recent report of a furin breakdown product of PCSK9 protein present in plasma migrating as a band below the PCSK9 band (39), we further investigated the atorvastatin-induced increase in PCSK9. To do this, we performed immunoprecipitation and Western blotting analyses of some representative samples included in Fig. 2A, which still had adequate volume remaining for immunoprecipitation (at least 100 μl). Results of these experiments are shown in Fig. 2B, which demonstrates that atorvastatin treatment resulted in increases in the intact PCSK9 protein band that comigrated with the recombinant PCSK9 protein standard as well as the PCSK9 propeptide band.

We next investigated the effect of atorvastatin treatment on the correlation of PCSK9 levels with serum lipid levels. Fig. 3A shows the correlation between PCSK9 and TC levels at baseline and after 16 weeks of treatment with 80 mg/day atorvastatin. At baseline, PCSK9 levels were correlated with TC (r = 0.48, P < 0.01); however, 16 weeks of atorvastatin treatment completely abolished the correlation between PCSK9 and TC (r = 0.05, P = NS). Similar results were observed with regard to the effect of atorvastatin treatment on the correlation of PCSK9 with LDL-C levels (Fig. 3B) and TG (Fig. 3C) levels. At baseline, PCSK9 levels were correlated with LDL-C (r = 0.38, P < 0.01). After 16 weeks of atorvastatin treatment, however, the correlation between PCSK9 and LDL-C levels was completely disrupted (r = 0.01, P = NS). Likewise at baseline, PCSK9 levels were directly correlated with TG (r = 0.27, P = 0.02), but 16 weeks of atorvastatin treatment abolished this direct correlation (r = 0.15, P = NS). With regard to HDL-C (Fig. 3D), there was no correlation of PCSK9 to HDL-C levels either at baseline (r = 0.21, P = NS) or 16 weeks (r = 0.19, P = NS).

In light of these results, we next examined the correlation of atorvastatin-induced changes in PCSK9 levels with changes in LDL-C levels to determine if the largest increases in PCSK9 levels predicted the largest decreases in LDL-C levels. Fig. 4A shows the correlation of percent changes in PCSK9 levels (from baseline to endpoint) to percent changes in LDL-C levels (from baseline to endpoint). Interestingly, there was a trend toward an inverse correlation, although this trend did not achieve statistical significance (r = −0.21, P = 0.06). Next we compared the baseline PCSK9 level to the absolute change in LDL-C observed (from baseline to endpoint) to determine if base-
Atorvastatin disrupts PCSK9 correlation with LDL-C. Finally, we compared atorvastatin-induced changes in PCSK9 levels to the final LDL-C achieved after 16 weeks of treatment with atorvastatin. In this case, there was a significant negative correlation (r = −0.20, P = 0.08), although this trend also did not achieve statistical significance. Finally, we compared atorvastatin-induced changes in PCSK9 levels to the final LDL-C achieved after 16 weeks of treatment with atorvastatin. In this case, there was a significant negative correlation...
increased serum PCSK9 levels after 12 weeks. In the same study, baseline PCSK9 and LDL-C concentration were significantly correlated, and the correlation was disrupted by atorvastatin treatment. Following this report, Mayne et al. reported that administration of atorvastatin significantly increased PCSK9 levels while lowering LDL-C levels (43). Cariou et al. showed that, in patients with diabetes, statin treatment increased PCSK9 levels by 32% (44). In the same study, it was also shown that the correlation between PCSK9 levels and LDL-C levels was lost following statin treatment as statins increased PCSK9 levels and decreased LDL-C levels (44). Dubuc et al. (39) demonstrated that patients treated with statins had a 45% increase in circulating PCSK9 levels and that patients treated with a statin-ezetimibe combination had a 77% increase in PCSK9. Lakoski et al. (7) demonstrated that PCSK9 levels correlated directly with LDL-C levels in a large, ethnically diverse population and that statin treatment was associated with a significant increase in circulating PCSK9 levels in both men and women.

As a result of these findings, it was not unexpected that a high dose of atorvastatin (80 mg) would cause the baseline correlation of PCSK9 to LDL-C to be lost in our current study, although it was important to confirm this effect. The positive correlation between PCSK9 and LDL-C at

$$(r = -0.25, P = 0.03),$$ which indicated that greater increases in PCSK9 levels tended to be associated with lower endpoint LDL-C levels, although the correlation itself was still relatively modest and of marginal statistical significance.

**DISCUSSION**

Our results demonstrate that high-dose atorvastatin treatment (80 mg per day) causes a rapid and sustained increase in circulating PCSK9 protein levels. After only 4 weeks of treatment, PCSK9 levels increased 47% over baseline levels, and this increase was sustained at 8-week, 12-week, and 16-week time points. Similar to what we and others have previously reported (7, 29, 37, 38), baseline PCSK9 levels were highly correlated with TC and LDL-C levels. We further demonstrated a correlation between baseline PCSK9 and TG levels. After 16 weeks of atorvastatin treatment, however, these correlations were abolished, indicating that treatment with atorvastatin had completely disrupted the relationship between PCSK9 and these lipid parameters.

Several researchers have described statin-induced increases in PCSK9 levels in humans. In a previous smaller study (38), we observed that a lower dose of atorvastatin increased serum PCSK9 levels after 12 weeks. In the same study, baseline PCSK9 and LDL-C concentration were significantly correlated, and the correlation was disrupted by atorvastatin treatment. Following this report, Mayne et al. reported that administration of atorvastatin significantly increased PCSK9 levels while lowering LDL-C levels (43). Cariou et al. showed that, in patients with diabetes, statin treatment increased PCSK9 levels by 32% (44). In the same study, it was also shown that the correlation between PCSK9 levels and LDL-C levels was lost following statin treatment as statins increased PCSK9 levels and decreased LDL-C levels (44). Dubuc et al. (39) demonstrated that patients treated with statins had a 45% increase in circulating PCSK9 levels and that patients treated with a statin-ezetimibe combination had a 77% increase in PCSK9. Lakoski et al. (7) demonstrated that PCSK9 levels correlated directly with LDL-C levels in a large, ethnically diverse population and that statin treatment was associated with a significant increase in circulating PCSK9 levels in both men and women.

As a result of these findings, it was not unexpected that a high dose of atorvastatin (80 mg) would cause the baseline correlation of PCSK9 to LDL-C to be lost in our current study, although it was important to confirm this effect. The positive correlation between PCSK9 and LDL-C at
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baseline exists under conditions where VLDL production is not inhibited by a statin. With each increasing dosage of a statin, a new steady state is reached in which hepatic LDLR expression is increased. Increased LDLR, in turn, can account for increased clearance of both LDL-C and PCSK9. It should be remembered, however, that while VLDL production in liver is decreased by the statin, production of PCSK9 is increased. Because of this, the statin-induced loss of correlation between PCSK9 and LDL-C levels could occur even if PCSK9 did not affect LDLR protein. Presumably, with each increased dose of a statin, the discrepancy in production rates of VLDL and PCSK9 would be further increased, and this makes it difficult to elucidate the significance of the loss of the direct correlation between PCSK9 and TC, LDL-C, and TG that we observed.

One of the questions we wanted to address was whether baseline levels of PCSK9 might predict the magnitude of atorvastatin-induced decreases in LDL-C. We considered the possibility that subjects with the highest baseline PCSK9 levels might have the largest LDL-C responses to atorvastatin because serum PCSK9 levels were directly correlated with LDL-C, and atorvastatin acts to decrease LDL-C through increasing LDLR protein levels, in spite of increasing PCSK9 levels. Consistent with this hypothesis, there was a modest relationship between baseline PCSK9 levels and changes in LDL-C, with relatively higher baseline PCSK9 levels tending to be associated with numerically greater decreases in LDL-C. This correlation, however, did not achieve statistical significance. Interestingly, atorvastatin-induced increases in PCSK9 were negatively correlated with endpoint LDL-C levels. This correlation was relatively modest and just managed to achieve statistical significance. In the case of all three of the above correlations, the fact that the trends either did not achieve statistical significance or just achieved statistical significance makes it difficult to draw definitive conclusions about the relationship between atorvastatin-induced changes in PCSK9 levels and LDL-C levels.

The loss in correlation may be in part due to the fact that statins increase the activity/nuclear translocation of sterol regulatory element-binding protein-2 (SREBP-2), a transcription factor that activates both the LDLR and PCSK9 genes (34–36). With PCSK9 and the LDLR thus both being increased by statin treatment, the amount of circulating PCSK9 also has more hepatic LDLR to bind to, which in turn removes it from the circulation. This aspect of the relationship between serum PCSK9 levels and hepatic LDLR levels makes it difficult to draw definitive conclusions about the relationship between atorvastatin-induced changes in PCSK9 levels and LDL-C levels.

Another question that we wanted to address was whether atorvastatin-induced changes in PCSK9 levels would correlate with the magnitude of atorvastatin-induced LDL-C decreases. We expected that subjects who had the smallest atorvastatin-induced increases in serum PCSK9 levels might also have the most significant atorvastatin-induced LDL-C lowering. This turned out, however, not to be the case. Rather, subjects that had the greatest increases in atorvastatin-induced PCSK9 levels also tended to have the largest atorvastatin-induced decreases in serum LDL-C, although this trend, similar to the previous one, did not reach statistical significance. Interestingly, atorvastatin-induced increases in PCSK9 were negatively correlated with endpoint LDL-C levels. This correlation was still relatively modest and just managed to achieve statistical significance. In the case of all three of the above correlations, the fact that the trends either did not achieve statistical significance or just achieved statistical significance makes it difficult to draw definitive conclusions about the relationship between atorvastatin-induced changes in PCSK9 levels and LDL-C levels.

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Together, our data suggest that one possible explanation for why increasing doses of statins fail to achieve proportional LDL-C lowering may be due to statin-induced...
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