Simvastatin Modulates Expression of the \textit{PON1} Gene and Increases Serum Paraoxonase
A Role for Sterol Regulatory Element–Binding Protein-2

Sara Deakin, Ilia Leviev, Sophie Guernier, Richard W. James

\textbf{Background}—The HDL-associated enzyme paraoxonase protects LDLs from oxidative stress. 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) appear to favorably influence the atherosclerotic process by different mechanisms. The present study examined the influence of simvastatin on paraoxonase expression and serum paraoxonase levels.

\textbf{Methods and Results}—Simvastatin upregulated in a dose-dependent manner the activity of the promoter of the paraoxonase gene in expression cassettes transfected into HepG2 cells. Upregulation could be blocked by mevalonate and other intermediates of the cholesterol biosynthetic pathway. Simvastatin increased nuclear factors, notably sterol regulatory element–binding protein-2, capable of binding to the paraoxonase promoter; this was also blocked by mevalonate. Sterol regulatory element–binding protein-2 upregulated promoter activity in vitro. Patients treated with statin showed a significant increase in serum concentrations and activities of paraoxonase.

\textbf{Conclusions}—The data indicate that simvastatin can modulate expression in vitro of the antioxidant enzyme paraoxonase and is associated with increased serum paraoxonase concentration and activity. It is consistent with effects of simvastatin treatment, which have the potential to influence beneficially antiatherogenic mechanisms at the HDL level. The study provides evidence for 1 molecular mechanism by which paraoxonase gene expression could be regulated. (\textit{Arterioscler Thromb Vasc Biol}. 2003;23:2083-2089.)

\textbf{Key Words:} lipoproteins \textbf{•} antioxidants \textbf{•} genes \textbf{•} coronary disease \textbf{•} transcription factors

HDLs are inversely correlated with the risk of vascular disease.\textsuperscript{1} Several antiatherogenic mechanisms have been associated with HDL, including an antioxidant function.\textsuperscript{2} Recent studies have identified the enzyme paraoxonase-1 (PON1) as a primary determinant of the antioxidant potential of HDL\textsuperscript{3}; it protects LDLs from oxidation.\textsuperscript{4} This proposal is supported by extensive in vitro data, which have demonstrated lower concentrations of lipid peroxides associated with LDL\textsuperscript{3}, and HDL\textsuperscript{5}, as well as a reduced pathobiologic influence of LDL,\textsuperscript{6} as a function of PON1 activity. More convincingly, animal models have revealed a greater degree of lipoprotein oxidation and more extensive atheroma formation in mice lacking PON1 activity,\textsuperscript{7} whereas overexpressing PON1 has a protective influence.\textsuperscript{8} These observations link the enzyme to risk of vascular disease. In this context, our recent studies have suggested that \textit{PON1} promoter polymorphisms, which strongly affect gene expression and serum PON1 levels,\textsuperscript{9} are associated with coronary disease.\textsuperscript{10} Lower-expression genotypes are associated with increased risk of disease. Previous studies, which had focused on coding-region polymorphisms of \textit{PON1}, identified the enzyme as a genetic risk factor for cardiovascular disease,\textsuperscript{11–13} although not consistently.\textsuperscript{14} A recent meta-analysis has provided confirmation of an association between genotype and risk while underlining a potential confounding factor, which is the large intrapersonal variations in serum PON1 levels, even within genotypes.\textsuperscript{15} The enzyme is entirely linked to HDL in serum, and there is a positive, albeit weak, correlation between serum HDL and PON1 concentrations.\textsuperscript{16} From this perspective, factors that increase HDL might also influence PON1. The principal lipid-lowering drugs are 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors and fibrates, which can also raise HDL. With regard to their potential effects on serum PON1, data derived essentially from population studies are somewhat inconsistent for both drugs.\textsuperscript{17–20} However, the antioxidant properties of HMG CoA reductase inhibitors might provide an indirect mechanism by which PON1 activity could be preserved.\textsuperscript{21} We have examined in greater detail the influence of an HMG CoA reductase inhibitor, simvastatin, on \textit{PON1} gene expression and thus serum concentrations/activities of the enzyme. The null hypothesis was that simvastatin would be without effect on these parameters.
Methods

Reporter-Gene Constructs

Reporter-gene constructs containing the PON1 promoter region were made by insertion of polymerase chain reaction–amplified DNA fragments into the pGL-2 Basic vector (Promega) before the firefly luciferase gene. Oligonucleotide primers AAAAAACGCTCACA-CATCCACCATTGGGG and ATCCGGATCCGGGATAGAGAAAAGGATCGATG were used for amplification of a 2900-bp DNA fragment, and primers AAAAAACGCTCAGATATTGCAAAGGGATCGATG were used for amplification of a 1000-bp DNA fragment. Human chromosomal DNA extracted from blood cells and thermostable Pfu polymerase were used in the amplification reaction. The conditions for polymerase chain reaction were those reported in the requirements of the University Hospital Ethics Commission. The number of patients necessary to observe a 10% change in serum PON1 activities with a power of 0.8 was estimated as 22 (pairwise assessment; SISA, UittenbroekDG, Binomial SISA, 1997; http://home.clara.net/sisa/binomial.htm). A fasting blood sample was obtained before initiating treatment (20 mg simvastatin, except for 2 patients who received 10 mg) and a second sample, after a mean of 6.70±1.02 weeks of treatment.

Laboratory Analyses

Serum lipid and lipoprotein analyses were performed as described previously. Serum PON1 activities and concentrations were quantified as described. Western blotting was performed as described with anti-SREBP2 (Santa Cruz Biotechnology Inc).

Statistical Analyses

Values before and after treatment were analyzed by the paired Student’s t test.

Results

Statin Treatment Increases Serum PON1 Activity and Concentration

The effects of statin treatment on serum levels of PON1 were examined in patients who were not receiving lipid-lowering therapy before entry into the study. The results are shown in Table 1. There was a significant decrease in serum cholesterol...
Simvastatin Upregulates Binding of Nuclear Factors to the PON1 Promoter

To determine whether the statin modulated nuclear factors, PON1 promoter fragments were incubated with nuclear extracts of nontransfected HepG2 cells and subjected to agarose gel electrophoresis. As shown in Figure 4A, incubation with nuclear extracts from HepG2 cells modified the electrophoretic mobility of the radiolabeled promoter fragment, and it efficiently competed with the nonlabeled promoter fragment (Figure 4A). The intensity of the band was significantly greater after incubation with nuclear extracts from simvastatin-treated cells, wherein a 3-fold increase in intensity was observed (Figure 4B and 4C). Coincubation of the cells with mevalonate abrogated the increased intensity of the gel shift band induced by simvastatin (Figure 4B and 4C).

SREBP-2 Binds to the PON1 Promoter, Interacts With Sp1, and Upregulates Promoter Activity

A final series of studies examined a role for SREBP-2 in modulation of promoter activity. Purified SREBP-2 alone was shown to modify the electrophoretic mobility of the PON1 promoter fragment (Figure 5A). Although purified SREBP-2 was capable of binding to the PON1 promoter, in the presence of Sp1 a different complex (modified electrophoretic mobility, more intensely stained) was formed (Figure 5A). This complex was supershifted with antibody to Sp1 (Figure 5A). The electrophoretic mobility of the SREBP-2+Sp1 complex corresponded to that observed for the nuclear extract from simvastatin-treated cells (Figure 5B) but differed from that formed with Sp1 alone. Western blot analysis of the HepG2 extracts confirmed an increase in concentration of the processed SREBP-2 peptide in simvastatin-treated cells compared with nontreated cells (Figure 5C).

To complement these studies, the influence of SREBP-2 on promoter activity was analyzed in HepG2 cells. The cells were cotransfected with SREBP-2 and the PON1 promoter fragment. As shown in Figure 5D, the presence of SREBP-2 increased promoter activity in a dose-dependent manner (mean 18-fold increase at the highest SREBP-2 concentration; \( P<0.0001 \)). No promoter activity and no simvastatin effect were observed when the fragment with the deleted 127 bp was transfected into the cells (Figure 5D).

Discussion

The present study provides evidence compatible with the hypothesis that the HMG CoA reductase inhibitor simvastatin influences expression of PON1. Simvastatin increased PON1
promoter activity in a dose-dependent manner, with similar stimulatory effects on PON1 promoter fragments varying in length from 2900 to 190 bp. Deletion studies confirmed that the proximal 190-bp region of the promoter was required for promoter activity and for the response to simvastatin. The statin also upregulated binding of nuclear factors to the promoter. Mevalonate blocked the effect of simvastatin.

Clinical studies revealed an increase in serum PON1 during statin treatment. The data are consistent with an effect of simvastatin at the molecular level on the PON1 gene, which could raise serum concentrations and activities of the enzyme. This would represent a beneficial impact of simvastatin on antiatherogenic mechanisms residing within HDL.

Cotreatment of transfected cells with mevalonate blocked the stimulatory effect of the statin on promoter activity. This observation associates the influence of simvastatin on PON1 with its ability to inhibit cholesterol synthesis. We also examined other intermediates of the cholesterol biosynthesis pathway. The isoprenoid lipids FP and GGP were able, to varying degrees, to reverse statin-mediated upregulation of PON1 promoter activity. However, squalene, a cholesterol biosynthetic intermediate beyond the isoprenoid lipids and committed to cholesterol synthesis, also prevented upregulation of PON1 promoter activity. This result would appear to point to decreased cellular cholesterol content as a factor regulating PON1 expression. In complementary studies with nuclear extracts, it was observed that statin treatment of nontransfected HepG2 cells modified the intensity of the electrophoretic mobility shift assay band, whereas coincuba-
tion of cells with mevalonate plus statin blocked the statin effect. These observations indicate (1) binding of nuclear factors to the PON1 promoter fragment and (2) upregulation of these factors by simvastatin. The data were consistent with the possible involvement of SREBPs, a major pathway by which cellular cholesterol content can modulate gene expression. SREBP-2 is particularly implicated in regulatory mechanisms involving cholesterol. Scanning the sequence of the PON1 promoter fragment used for the mobility assays revealed several features compatible with the involvement of SREBPs. Two regions (−104 to −95 and −138 to −130) were observed to have 70% homology to previously identified SRE sites. These findings led us to analyze the possible involvement of SREBP-2 in PON1 promoter function. We demonstrated that SREBP-2 alone formed a complex with the PON1 promoter. Moreover, together with Sp1, SREBP-2 formed a complex with the promoter fragment, which had an electrophoretic mobility comparable to that formed with nuclear extracts from HepG2 cells treated with simvastatin. SREBP-2 also upregulated promoter activity. Conversely, deletion of this proximal region from the 1000-bp promoter fragment eliminated promoter activity and the effect of SREBP-2. Of particular interest is the indication that SREBP-2 interacted with Sp1 in binding to the promoter. The SRE site at this position (−104 to −95) is contiguous with a sequence (−111 to −105) corresponding to that of Sp1. The latter is 1 of the additional transcription factors that are important for SREBP-mediated gene activation. We previously reported a polymorphism in the Sp1 recognition region (position −107) as being associated with significant variations in serum PON1 concentrations. In recent studies, we have demonstrated that the polymorphism modulates binding of Sp1 to the PON1 promoter.
Our results contrast with those of a recent study proposing that statin downregulates PON1 expression. The study also identified a regulatory region near −550 bp that mediated the statin effect, in contradiction to our data, which limit the actions of SREBP-2 to the proximal 200-bp region. Differences in the hepatic cell lines used might play a role, although the study data also contradict results from statin effects on PON1 in vivo. It was also suggested that fenofibric acid upregulated promoter activity, which also contrasts with in vivo data of fibrate effects on PON1. The observations could not be confirmed with other fibrates, however, and did not involve the peroxisome proliferator–activated receptor transcription factors, which normally mediate the lipid-oriented influence of fibrates.

Few studies have analyzed the potential effect of statins on serum PON, and they are limited to analysis of activity measurements. From an in vitro study, it was postulated that metabolites of atorvastatin could protect serum PON1 activity by an antioxidant effect. This alludes to the sensitivity of PON1 activity to oxidized substrates; the enzyme can be inactivated, for example, by oxidized LDL lipids. Serum PON1 enzyme activity is reportedly increased in familial hypercholesterolemic patients treated with simvastatin compared with nontreated patients. PON1 mass was not measured, so the precise cause of increased activity could not be determined. Moreover, there was a certain inconsistency, because increased activity was not observed for a second substrate commonly used to monitor serum paraoxonase. Nevertheless, it was speculated that increased activity might arise from an antioxidant effect, possibly owing to lowering of oxidized lipids by reduction of serum LDL. Analysis of serum PON1 concentrations in the present study allowed us to demonstrate clearly an increase in PON1 mass.

On a more speculative level, there has been considerable debate in recent years concerning the mechanisms by which statins beneficially influence the atherosclerotic process. Although lowering LDL remains the predominant factor, other pathways have been invoked. Given that PON1 is hypothesized to be a primary determinant of the antioxidant and anti-inflammatory capacity of HDL, statin-induced increases in serum concentrations would appear beneficial. Further studies will be necessary, however, to determine whether it represents an additional mechanism by which simvastatin could influence atherosclerosis, independently of lipid lowering. Although the change in serum PON1 concentrations was relatively minor (mean, 7%), we have recently shown that an increase of this order is able to significantly improve the antioxidant capacity of HDL, albeit in the experimental, in vitro setting of copper-induced oxidation.

To conclude, the present study provides evidence for upregulation of PON1 at the gene level by simvastatin in vitro and increased serum PON1 activity and concentration in patients treated with the statin. The study provides evidence consistent with a role for SREBP-2 in the molecular mechanism leading to PON1 gene activation. The effects are of potential benefit to the atherosclerotic process and thus, could reflect an additional pleiotropic effect of the statin. Further work is required to determine whether the effect encompasses other statins.

Acknowledgments

The study was supported by grants from the Swiss National Research Foundation, the Swiss Cardiology Society, the Hans Wilsdorf Foundation, and Merck Sharp & Dohme-Chibret, Switzerland. The authors are particularly grateful to Prof T. Osborne, University of California, Irvine, for supplying the SREBP plasmids. The authors acknowledge the excellent technical assistance of Marie-Claude Brulhart Meynet and Barbara Kalix and thank Dr C. Meier and his staff (Division of Endocrinology and Diabetology, University Hospital, Geneva) for help in obtaining blood samples.

References


Simvastatin Modulates Expression of the PON1 Gene and Increases Serum Paraoxonase: A Role for Sterol Regulatory Element–Binding Protein-2
Sara Deakin, Ilia Leviev, Sophie Guernier and Richard W. James

Arterioscler Thromb Vasc Biol. 2003;23:2083-2089; originally published online September 18, 2003;
doi: 10.1161/01.ATV.0000096207.01487.36

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/23/11/2083

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/