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Opposite Regulation of the Human Paraoxonase-1 Gene PON-1 by Fenofibrate and Statins

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ABSTRACT

The human paraoxonase-1 (PON-1) is a serum high-density lipoprotein-associated phosphotriesterase secreted mainly by the liver. This enzyme is able to hydrolyze toxic organophosphate xenobiotics, endogenous oxidized phospholipids, and homocysteine thiolactone. Physiologically, it is thought to protect against cardiovascular diseases. The level of *PON-1* gene expression is a major determinant of paraoxonase-1 status but little is known regarding the regulation of this gene. We identified several transcription start sites and characterized the regulation of its promoter by fibrates and statins. In HuH7 human hepatoma cells, the PON-1 secreted enzymatic activity and mRNA levels were increased by fenofibric acid (approximately 70%) and decreased by several statins (approximately 50%). Transient and stable transfection assays in HuH7 cells indicated that the modulation of the mRNA and enzymatic activity levels could be accounted for by the regulation of the PON-1 gene promoter activity by these drugs. These effects are probably not mediated by the PPARα because over-expression of this receptor decreased the fibrate effect and did not modify statins activity. The repressive effect of statins is reversed by mevalonate and 22(*R*)-hydroxycholesterol, suggesting the involvement of the liver X receptor in the mechanism. The opposite effects of fenofibrate and statins could be consistent with clinical data on homocysteine levels after hypolipidemic drug treatment. Regarding the toxicological aspects, the induction achieved with fenofibric acid, although limited, could increase organophosphate metabolism and may be relevant in certain conditions for protective treatments.

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The human paraoxonase-1 is a 354 amino acid calcium-dependent phosphotriesterase. Its name stems from its ability to metabolize paraoxon, the microsome-activated form of the pesticide parathion. Despite intensive work on the protein, the structure of the enzyme and its catalytic mechanism are still not completely elucidated (Josse et al., 1999). This enzyme is mainly expressed in the liver and is secreted in serum where it is associated with high-density lipoproteins (HDL) (Kelso et al., 1994).

Paraoxonase-1 possesses both arylesterase and organophosphatase activities and belongs to the family of phase I xenobiotic-metabolizing enzymes. Several heterocyclic compounds (lactones and thiolactones) were found to be paraoxonase-1 substrates, which include statin drugs (Billecke et al., 2000). This enzyme also metabolizes organophosphates (OPs) toxic xenobiotics such as pesticides derivatives (paraoxon, chlorpyrifos-oxon, diazoxon) and warfare nerve agents (sarin, tabun, soman, and Vx) (Davies et al., 1996; Josse et al., 1999). Paraoxonase-1 plays a protective role in case of OPs intoxication as demonstrated by mouse knock-out experiments (Shih et al., 1998) and experimental gene therapy (Cowan et al., 2001). Consistently, birds, which almost lack paraoxonase-1, are far more susceptible to OPs than mammals such as rabbits, which have high serum paraoxonase-1 levels (Primo-Parmo et al., 1996). OP poisoning may occur after exposure to agricultural pesticides (Cherry et al., 2002) or deliberate dispersion of nerve agents in a terrorist or war context. The worldwide annual number of intoxications is estimated to be above 200,000 (Maynard and Beswick, 1992). In 1995, a sarin release in the Tokyo subway caused about 5000 casualties, 12 of which were fatal.

In addition to its detoxification function, paraoxonase-1 is involved in the metabolism of endogenous substrates. This enzyme metabolizes oxidized phospholipids in high- and low-density lipoproteins (HDL and LDL) (Aviram et al., 1998), homocysteine thiolactone (Jakubowski, 2000), and platelet-

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ABBREVIATIONS: HDL, high-density lipoprotein; OP, organophosphate; LDL, low-density lipoprotein; OD, optical density; FCS, fetal calf serum; RT-PCR, reverse transcriptase-mediated polymerase chain reaction; G3PDH, glucose-3-phosphate deshydrogenase; PPRE, peroxisome proliferator responsive element; PPAR, peroxisome proliferator-activated receptor; CMV, cytomegalovirus; kb, kilobase(s); WY-14643, (4-chloro-6-[(2,3-dimethylphenyl)amino]-2-pyrimidinyl)thioacetic acid; HMG, 3-hydroxy-3-methylglutaryl; LXR, liver X receptor; LXRE, liver X receptor response element; Hcy, homocysteine; DMSO, dimethyl sulfoxide; DR, direct repeat; Apo, apolipoprotein.

activating factor (Rodrigo et al., 2001). It was shown that PON-1–deficient mice are more susceptible to atherosclerosis than wild-type littermates, and several clinical studies report that paraoxonase-1 plays a role in the physiological prevention of cardiovascular disease (see Mackness et al., 2001, and references therein).

When the human *PON-1* gene coding paraoxonase-1 was cloned, it was shown to belong to a multigene family located on chromosome 7 with the homologous *PON-2* and *PON-3* genes (Primo-Parmo et al., 1996). The products of the latter genes were characterized very recently. In contrast to *PON-1*, *PON-2* is ubiquitously expressed and is not secreted out of the cells (it is not associated with circulating HDL) (Ng et al., 2001). Like PON-1, PON-3 is mainly expressed in the liver and is found in HDL (Reddy et al., 2001). Both PON-2 and -3 seem to have antioxidant properties and protect or reverse HDL and LDL oxidation. However, they are not active against synthetic OPs such as paraoxon. Similar results were obtained with the rabbit PON gene products (Draganov et al., 2000). Compared with PON-1, the PON-2 and -3 coding regions lack an amino acid at position 105, which may account for these functional differences (Primo-Parmo et al., 1996). The *PON-1* gene was extensively studied for its genetic polymorphisms. Two main polymorphisms, located in the coding sequence, were shown to modulate the enzymatic activity toward OPs (Davies et al., 1996; Furlong et al., 2000; Cherry et al., 2002). Recently, polymorphisms in the 5′-upstream region of the gene were also reported (Leviev and James, 2000; Brophy et al., 2001). Several divergent studies were carried out to link these polymorphisms, paraoxonase-1 status, and clinical observations. It is not clear whether some polymorphisms are associated with a higher occurrence of cardiovascular disease (see Mackness et al., 2001, and references therein). However, high paraoxonase-1 expression and activity levels are clearly protective.

Because paraoxonase-1 seems to be protective in case of both OP poisoning and cardiovascular disease, it is important to investigate the factors that could influence its activity. Together with secretion mechanisms, enzymatic turnover and protein stability, the level of expression of the *PON-1* gene is a major determinant of paraoxonase-1 status. Inflammatory conditions were shown to decrease PON-1 mRNA level in vitro (addition of interleukins 6 and 1, oxidized phospholipids or tumor necrosis factor-α (Feingold et al., 1998; Van Lenten et al., 2001)). Some clinical data suggest that treatment with hypolipidemic drugs may modulate serum paraoxonase-1 activity (Tomas et al., 2000; Balogh et al., 2001). This activity is HDL-dependent, and several factors may be involved in its regulation. So far, very few studies were undertaken regarding the mechanisms of regulation of the *PON-1* gene expression itself. We therefore characterized the promoter region of the *PON-1* gene and looked for compounds that could influence its activity. Some fibrates are shown to induce *PON-1* gene expression, whereas statins have the opposite effect.

Materials and Methods

Chemicals. Fenofibric acid was a kind gift of Dr. A. Edgar (Laboratoires Fournier, Daix, France). Simvastatin, pravastatin and fluvastatin were generous gifts from Merck Sharpe and Dohme, Bristol-Myers Squibb, and Novartis laboratories, respectively. WY-14643 was obtained from Calbiochem (Meudon, France). Other chemicals were obtained from Sigma (Saint-Quentin Fallavier, France).

Cell Culture. The human hepatoma cell line HuH7 was maintained at 37°C in an atmosphere containing 5% CO_2. Dulbecco's modified Eagle's medium was supplemented with 10% fetal calf serum (Invitrogen, Cergy-Pontoise, France), 100 U/ml penicillin (Diamant, Puteaux, France), 100 U/ml streptomycin (Invitrogen), and 0.5 mg/ml amphotericin B (Bristol-Myers Squibb, Princeton, NJ).

Primer Extension Assay. A 30 nucleotide primer (5′-GGTAAGAAGACTGGTGGTTCCTGAAGAGTG-3′), corresponding to bases +42 to +72 of the human *PON-1* gene (the +1 position corresponds to the A of the ATG start codon) was end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Biosciences, Orsay, France) using T4 polynucleotide kinase and purified on a Sephadex G-50 spin column. Twelve, 25, or 50 μg of human liver total RNA were hybridized overnight at 50°C with 5×10^6 cpm of the labeled oligonucleotide in a hybridization buffer (0.01 M Tris-HCl, pH 8.3, 1 mM EDTA, and 0.15 mM KCl). After ethanol precipitation and resuspension in 11 μl of water, the oligonucleotide was extended for 90 min at 42°C with 20 units of avian myeloblastis virus reverse transcriptase (Finnzymes, Saint-Quentin Fallavier, France) according to the manufacturer's instructions (in 25 μl of 25 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 50 mM KCl, 2 mM dithiothreitol, 1 mM dNTP) in the presence of 0.15 μg of actinomycin D. Rnase A (10 μg) was added to the mixture to stop the reaction. After a phenol-chloroform extraction and ethanol precipitation, the extension products were fractionated on a 6% polyacrylamide, 7 M urea gel and analyzed by autoradiography. The size of the extended fragments was determined by comparison with a sequence ladder obtained with a T7 sequencing kit (Amersham Biosciences) run on the same gel.

S1 Nuclease Protection Assay. Two oligonucleotides, 5′-GCGCAATCAGCTTCGCCATGGTCGGGGATAGACAA AGGGATCGATGGGCG-3′ (probe A) and 5′-GCGCAGACACCGACGGGCTAGGAGGCTCTGCTGCCTGCAGCCGCAGCCCTGCTGGGGCACGGCCGATTGGCCCG-3′ (probe B), corresponding, respectively, to bases + 18 to −31 and −29 to −102 of the human *PON-1* gene (the + 1 position corresponds to the A of the start codon) were 5′-end-labeled using [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Biosciences) and T4 polynucleotide kinase. The unincorporated nucleotides were separated from the labeled oligonucleotide by precipitation with 2 M ammonium acetate. Human liver total RNA (50 μg) and 300,000 cpm of the labeled oligonucleotide were hybridized in 100 μl of 3 M NaCl, 0.5 M HEPES, pH 7.5, and 1 mM EDTA, pH 8.0, by heating at 100°C for 5 min and then at 65°C overnight. S1 nuclease digestion was carried out at 37°C for 1 h using 300 U of S1 nuclease (Roche, Meylan, France) according to the manufacturer's instructions. The reaction was stopped by adding 3 μl of 0.5 mM EDTA, pH 8.0, and 10 μg of tRNA. Size analysis of the protected fragments was performed on a sequencing gel, as described above.

PON-1 Enzymatic Activity. The secreted paraoxonase-1 arylesterase activity was measured in HuH7 cell culture medium using phenylacetate as substrate following a method adapted from Deakin et al. (2001). This activity is expressed as ΔOD_{270}/min. Owing to the presence of paraoxonase-1 in fetal calf serum (FCS), FCS was heated 1.5 h at 56°C, resulting in the loss of FCS-associated paraoxonase-1 arylesterase activity (data not shown). The tested compounds were incubated with the cells for 24, 48, or 72 h in medium containing standard FCS to allow full cell growth. This medium was then cleared, cells were washed with PBS (Invitrogen, Cergy Pontoise, France) and new medium containing the heated serum was added. Paraoxonase-1 activity was measured 24h later. This protocol avoids the interaction of the tested drugs with the enzymatic activity itself (Billecke et al., 2000). These results were normalized to the protein content of the cells and expressed as (ΔOD_{270}/min − blank/protein content). Blanks were obtained with naive (i.e., not in contact with cells) medium containing heated serum.

Northern Blots. Total RNA preparation was performed with the RNA Easy Midi Kit (QIAGEN, Les Ulis, France). Poly(A^+) mRNA were purified with the Oligotex mRNA Purification Kit (QIAGEN). Northern blots were performed as already described (Morel and

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Barouki, 1998), using 3 μg of poly(A$^+$) mRNA. The probe used to detect PON-1 mRNA is a 283-base fragment of the 3′-untranslated region of the PON-1 mRNA starting immediately after the stop codon (this fragment is nonhomologous with PON-2 and PON-3 mRNAs). It was cloned from the mRNAs of HuH7 cells by reverse transcriptase-mediated polymerase chain reaction (RT-PCR). This probe allowed a specific quantification of the endogenous *PON-1* gene expression. PON-1, glucose-3-phosphate deshydrogenase (G3PDH) and actin probes were labeled with the Megaprime DNA labeling kit (Amersham Biosciences) according to the manufacturer's instructions. Hybridization was performed for 20 h using Rapid-hybrid buffer (Amersham Biosciences) according to the manufacturer's instructions. The membrane was washed 30 min at 65°C with 2× standard saline citrate, 0.1% SDS, and 30 min at 65°C with 0.5× standard saline citrate, 0.1% SDS. Quantifications were performed with a PhosphorImager and the ImageQuant software (Amersham Biosciences).

LightCycler Real-Time PCR. Primers were used to amplify a 78-base fragment of the human PON-1 gene mRNA (positions +1035 to +1112; GenBank accession number NM_000446; forward primer, 5′-GATTGGCACAGTGTTTC-3′; reverse primer, 5′-CCTCAGTTTCTATGGCA-3′). This sequence located in the 3′-untranslated region is specific to PON-1 mRNA (it is not homologous with PON-2 and PON-3 mRNAs). Results were normalized to the G3PDH mRNA content. G3PDH primers were used to amplify a 136-base fragment of the human G3PDH gene mRNA (positions +517 to +653, GenBank accession number NM_002046; forward primer, 5′-AGCAATGCCTCCTGCACCACCAAC-3′; reverse primer, 5′-CCGGAGGGGCCATCCACAGTCT-3′). Total RNA was extracted with the RNA Easy Midi Kit (QIAGEN). Reverse transcription was performed on each RNA sample (10 μg) using oligo dT and the Prostar First Strand RT-PCR kit (Stratagene, Amsterdam, Netherlands) in a final reaction volume of 50 μl, according to the manufacturer's instructions. Real-time PCR was performed with 2.5 μl of the cDNA solution, diluted 1/10, in a final volume of 20 μl containing SYBR-Green I dye (Roche), 0.5 μM of both primers, and 4 or 3 mM $MgCl_2$ for PON-1 and G3PDH PCRs, respectively. Reactions were transferred to glass capillaries and analyzed in a Lightcycler (Roche) according to the manufacturer's instructions, using fluorescence detection for SYBR-Green I with an excitation wavelength of 470 nm and an emission wavelength of 530 nm. PCR cycles proceeded as follows; for PON-1, denaturation, 5 s at 95°C; annealing, 15 s at 52°C; extension, 10 s at 72°C; for G3PDH, denaturation, 5 s at 95°C; annealing, 10 s at 59°C; and extension, 7 s at 72°C. Melting curve analysis showed the specificity of the amplifications.

For each PCR reaction, the crossing point (i.e., the maximum of the second derivative of the cycle-by-cycle fluorescence curve) was calculated by the Lightcycler software. In each experiment, the initial cDNA content of the samples was extrapolated from a standard curve. The standard curves were obtained as follows; the amplified DNA sequences of *PON-1* and *G3PDH* genes were subcloned using the TOPO-TA kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed with logarithmically increasing quantities of these plasmids (10^0 to 10^8 copies per assay). There was a linear relation between the crossing points (expressed as a cycle number) and the log of plasmid copy number ($R^2 = 0.98$ for PON-1; $R^2 = 0.99$ for G3PDH). Thus, for each sample, crossing points were related to a template copy number, and the final result was expressed as the ratio [assessed PON-1 copy number]/[assessed G3PDH copy number].

Plasmids. Clones containing DNA fragments of the 5′-region of the human *PON-1* gene were generous gift of Prof. R. James (Hôpital Universitaire, Genève, Switzerland). The sequence of the *PON-1* gene is accessible under GenBank accession number AC004022 (BAC clone GS1–155M11). This gene sequence displays polymorphisms (Leviev and James, 2000; Brophy et al., 2001). After sequencing, we selected the clone corresponding to the most frequent allele. Two plasmids derived from the luciferase reporter vector pGL3 (Promega, Charbonnières, France) named pPON1000-FL and pPON2500-FL were subsequently constructed. They contained, respectively, 1009 bp [−1013, −4] (the +1 position corresponds to the A of the start codon) and 2531 bp [−2535, −4] of the *PON-1* gene 5′-region upstream of the firefly luciferase reporter gene. Five deleted promoters, containing, respectively 813 bp [−817, −4], 647 bp [−651, −4], 487 bp [−491, −4], 435 bp [−439, −4], and 190 bp [−194, −4] of the *PON-1* gene 5′-region (derived from pPON1000-FL) were also subcloned into the pGL3 reporter vector. The pPPRE-FL plasmid was a kind gift from Dr. C. Massaad (INSERM U488, Le Kremlin-Bicêtre, France); it contains the firefly luciferase reporter gene driven by two peroxisome proliferator responsive element (PPRE) consensus sequences (DR1).

The *Renilla reniformis* luciferase reporter plasmid pαglob-RL was used as an internal control of the transfection efficiency (Morel and Barouki, 1998). The pPPARα and pBK-CMV, a mouse peroxisome proliferator-activated receptor (PPARα) expression vector and its empty parent plasmid used as a control, respectively, were generous gifts from Dr. B. Staels (INSERM U325, Lille, France). Additional experiments with a human PPARα expression vector showed similar results (data not shown).

Transfection Experiments. Transient transfection experiments were performed in HuH7 cells in the same conditions as described previously (Morel and Barouki, 1998). Briefly, 1 day before the transfection, cells (0.5×10^6 cells/6-cm dish) were seeded into the usual culture medium. The firefly and *R. reniformis* luciferase expression vectors (2 and 0.25 μg, respectively) and the vector expressing PPARα (0.25 μg) were introduced into the cells by the calcium phosphate coprecipitation technique followed 4 h later by a 2-min glycerol shock; 18 h later, cells were exposed to chemicals that were added to the culture medium. After a 24- or 48-h incubation, cells were homogenized for enzymatic assays. Dual luciferase assay (firefly and *R. reniformis*) was performed with a Promega kit according to the manufacturer's instructions. *R. reniformis* luciferase activity was used to normalize the transfection efficiency in all culture dishes. Blanks were obtained by assaying luciferase activity in mock-transfected cells. Results were expressed as (firefly luciferase-blank)/(*R. reniformis* luciferase-blank).

HuH7 stably transfected clones were obtained as described below. One day before the transfection, the cells (1.5×10^6 cells/10-cm dish) were seeded into the usual medium containing fetal calf serum. The reporter plasmid (2 μg) and the pSV2neo plasmid (0.5 μg) were introduced into the cells using Fugene 6 (Roche) according to the manufacturer's recommendations and medium was changed 6 h later. Two days later, the cells were resuspended, seeded in four plates, and allowed to grow for 24 h before the addition of 600 μg/ml of the neomycin analog, G418 (Invitrogen). The medium containing G418 was changed every 3 days. Two to 4 weeks later, the surviving cells were harvested and pooled for luciferase assay. Cells were continuously propagated in medium containing G418. Luciferase assay was conducted as above, and results were normalized to the protein content of cell lysates.

Statistics. Student's two-tailed *t* tests were performed using the Statview software (Abacus Concepts, Inc.).

Results

Characterization of the Human *PON-1* Gene Promoter. Several clones containing DNA fragments of the 5′-region of the human *PON-1* gene were sequenced. As reported recently, this gene sequence displays polymorphisms mainly at position −107 and at positions −126, −160, −830, and −907 (Leviev and James, 2000; Brophy et al., 2001). In our experiments, we selected the clone corresponding to the most frequent allele.

To characterize the transcription initiation site(s), we performed primer extension analysis and S1 nuclease protection ("S1 mapping") experiments using human liver total RNA. As shown in Fig. 1A, the primer extension analysis revealed five transcription start sites at position −18, −24, −27, −61, and

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−97 (the base immediately preceding the A of the ATG translation start site being labeled with the −1 position). The two start sites at positions −61 and −97 seem to be predominant.

To confirm this observation, S1 nuclease protection assay was undertaken. Two oligonucleotide probes were used that, respectively, overlap the three proximal transcription start sites determined by primer extension analysis (probe A, region −31; +18) and the two distal ones (probe B, region −102; −29). Five transcription start sites were found at positions consistent with those observed in the primer extension analysis (data not shown). The two distal transcription starts were again shown to be predominant. The full-length (fully protected) probe A (50 bases) accounts for 72 ± 7% (n = 3) of the signal, indicating the presence of predominant transcription start sites upstream of the −31 position. These start sites are those located at positions −61 and −97.

As shown below, the 1-kb DNA genomic region located immediately 5′ of the ATG start codon is able to drive the transcription of a reporter gene. The promoter sequence displays several transcription start sites (Fig. 1B), which is often observed in TATA-less gene promoters.

Effect of Fenofibric Acid on Paraoxonase-1 Arylesterase Activity. The analysis of the promoter sequence of the *PON-1* genes revealed the presence of several AGGTCA-like sequences, which indicates that it could possibly be regulated by nuclear receptors [for example, activated PPARα can bind the DR1 sequence (Staels et al., 1998)]. In addition, owing to the role of PON-1 in cardiovascular disease prevention and to the availability of protective drugs, we tested several such drugs for their potential inducing effect on the *PON-1* gene. Two main classes of drugs were tested: fibrates and statins. Moreover, both classes have been shown to increase the levels of HDL-associated Apo AI and to be PPARα activators (Staels et al., 1998; Martin et al., 2001).

Paraoxonase-1 has been shown to be secreted in HuH7 cells (Deakin et al., 2001). Paraoxonase-1 arylesterase activity was measured in the culture medium of treated and control HuH7 cells. As shown in Fig. 2A, treatment of HuH7 cells for 24 or 48 h with 250 μM fenofibric acid, the pharmacologically active form of fenofibrate, resulted in an increase of paroxonase-1 arylesterase activity in the culture medium reaching about 50%.

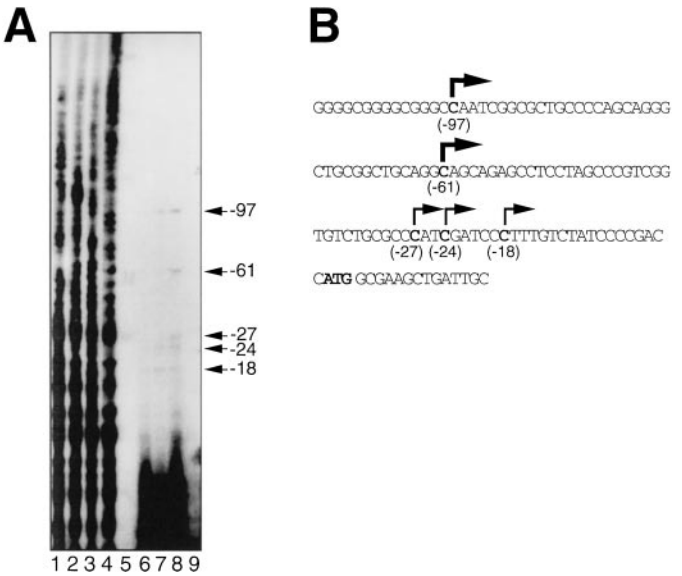

Fig. 1. Determination of the *PON-1* gene transcription initiation sites. A, primer extension analysis was performed as described under *Materials and Methods*. Lanes 1 to 4, Maxam-Gilbert sequence used as DNA ladder; lane 5, molecular weight markers; lanes 6 to 8, 12, 25, and 50 μg of human liver RNA, respectively; lane 9, 50 μg of tRNA (control). The positions of the presumed transcription start sites are mentioned on the right (the base immediately preceding the A of the ATG translation start site being labeled with the −1 position). The figure shows one experiment typical of three different experiments. B, DNA sequence of the 5′- region of the *PON-1* gene showing the different transcription start sites as gathered from primer extension and S1 nuclease protection experiments. The main sites are indicated in bold.

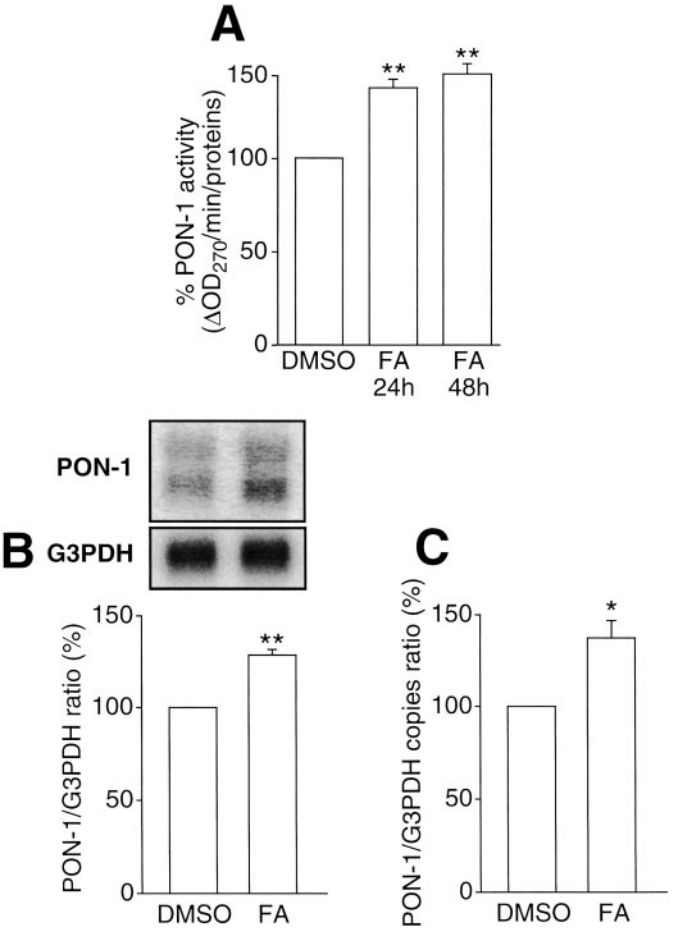

Fig. 2. Fenofibric acid increases paraoxonase-1 arylesterase activity and PON-1 mRNA level. HuH7 cells were treated with 250 μM fenofibric acid (FA) or with the solvent vehicle alone [DMSO, 0.1% (v/v)]. A, after 24 or 48 h of treatment, the medium was replaced with medium containing heated serum for 24 h. Paraoxonase-1 enzymatic activity was assayed in the culture medium as described under *Materials and Methods*. Results were expressed as paraoxonase-1 activity ΔOD_{270}/min/protein content (mean ± S.E.M., $n \geq 6$). The histogram shows induction percentage. For each group (24 or 48 h), 100% corresponds to the ratio in cells treated with DMSO alone. Statistically significant difference from these controls is marked with ** ($p < 0.01$). B, after 48-h treatment, mRNAs were extracted and Northern blot analysis was performed as described under *Materials and Methods*. The PhosphorImager picture shows a typical experiment. The *G3PDH* gene was used as a normalizing control. The histogram shows the mean ± S.E.M. (n = 4) of the quantification ratio PON-1/G3PDH; 100% corresponds to the ratio in cells treated with DMSO alone. Statistically significant difference from these controls is marked with ** ($p < 0.01$). C, after 48-h treatment, mRNAs were extracted and Lightcycler real time PCR analysis was performed as described under *Materials and Methods*. The histogram shows the mean ± S.E.M. (n = 6) of the quantification ratio PON-1/G3PDH; 100% corresponds to the ratio in cells treated with DMSO alone. Statistically significant difference from these controls is marked with * ($p < 0.05$).

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Effect of Fenofibric Acid on PON-1 mRNA levels. Figure 2B shows a typical Northern blot analysis: the presence of two distinct bands can be explained by polyadenylation variations and the width of each band could be related to the existence of several transcription start sites. The expression of PON-1, PON-2, and PON-3 mRNA has been shown by RT-PCR in HuH7 hepatoma cell line (data not shown), but it occurs at a low level. The probe used for Northern blots allowed a specific quantification of PON-1 mRNA (see *Materials and Methods*).

Northern blot and real-time PCR analysis showed that treatment of HuH7 cells with 250 μM fenofibric acid for 48 h resulted in a somewhat limited (30–40%) but significant increase of PON-1 mRNA levels (Figs. 2, B and C). A 24-h induction showed a slightly lower induction (data not shown).

Effect of Fenofibric Acid on PON-1 Promoter Activity. The effect of fenofibric acid on the PON-1 gene promoter activity was then tested using transient transfection assays of the pPON1000-FL and pPON2500-FL plasmids in HuH7 cells. As shown in Fig. 3A, fenofibric acid significantly increased the expression of the reporter gene. This induction seemed to be dose-dependent and reached a plateau (30% at 100 μM, 70% at 250 and 350 μM) for the 1-kb PON-1 promoter. A lower induction (about 50%) was observed for the

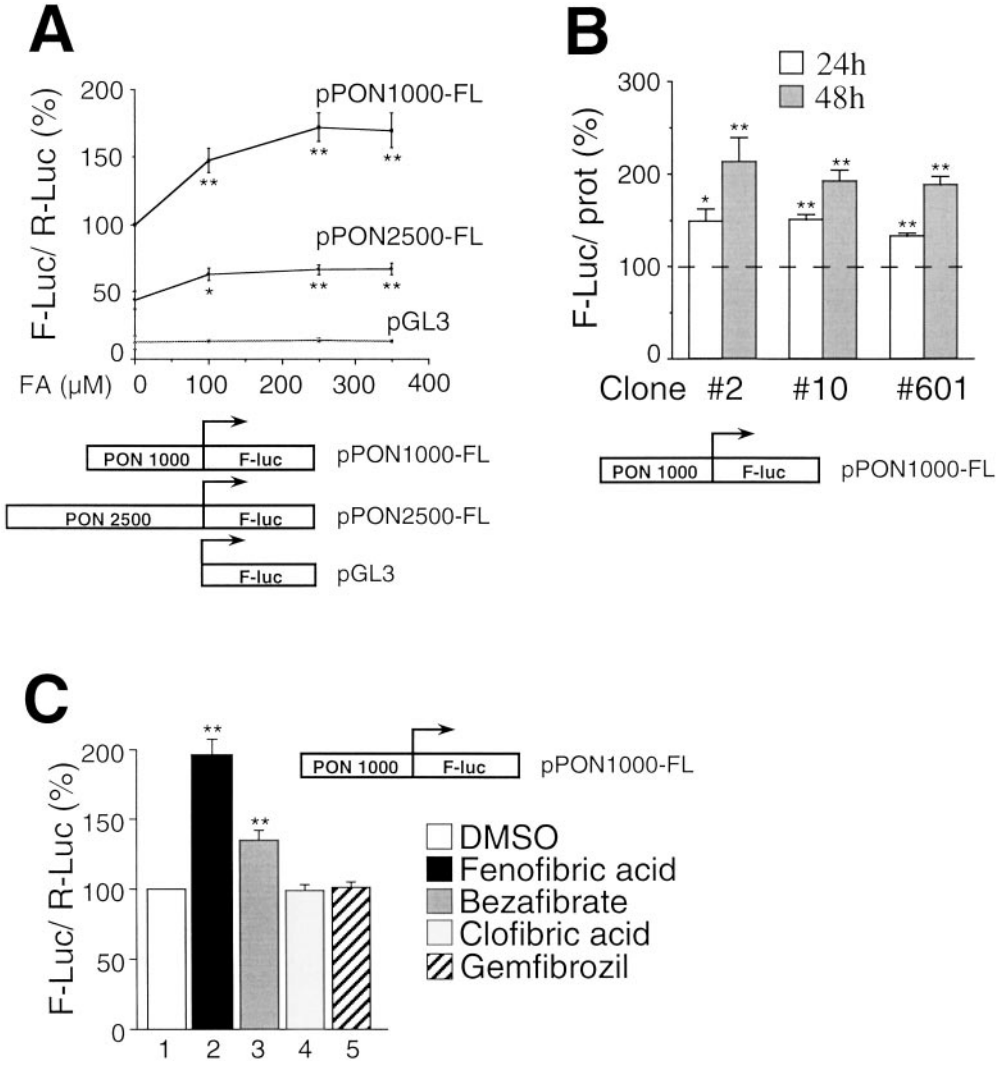

Fig. 3. Fenofibric acid increases the activity of the PON-1 gene promoter. A, HuH7 cells were transiently transfected with either the pPON1000-FL, pPON2500-FL, or pGL3 (empty vector) plasmid and cotransfected with pαglob-RL as an internal control. Cell cultures were treated with the indicated amount of fenofibric acid (FA) or with the solvent vehicle alone (DMSO 0.1% v/v) for 24h. Firefly and *R. reniformis* luciferases were assayed as described under *Materials and Methods*. Results were expressed as firefly luciferase activity/*R. reniformis* luciferase activity. Results are mean ± S.E.M. ($n \geq 8$); 100% corresponds to the firefly/*R. reniformis* ratio in cells transfected with pPON1000-FL and treated with DMSO alone. For each plasmid, the statistically significant differences to the DMSO are marked with * ($p < 0.05$) and ** ($p < 0.01$). B, two distinct HuH7 clones (nos. 2 and 10) expressing the firefly luciferase reporter gene as the result of the stable transfection of pPON1000-FL were treated with the solvent vehicle alone [DMSO, 0.1% (v/v)] or 250 μM fenofibric acid for 24 or 48 h. A pool of clones was also used (no. 601). Firefly luciferase and protein content were assayed as described under *Materials and Methods*. Results were expressed as Firefly luciferase activity/protein content (mean ± S.E.M., $n \geq 8$). For each group (the two clones and the pool), 100% corresponds to the cells treated with DMSO alone (represented by a horizontal line) and statistically significant differences to these controls are marked with * ($p < 0.05$) and ** ($p < 0.01$). C, HuH7 cells were transiently transfected with pPON1000-FL and pαglob-RL as an internal control. Cells were treated with 250 μM of the indicated fibrate or with the solvent vehicle alone [DMSO, 0.1% (v/v)] for 24 h. Firefly and *R. reniformis* luciferases were assayed as described under *Materials and Methods*. Results were expressed as firefly luciferase activity/*R. reniformis* luciferase activity. Results are mean ± S.E.M. ($n \geq 6$); 100% corresponds to the firefly/*R. reniformis* ratio in cells treated with DMSO alone; statistically significant differences from this control are marked with ** ($p < 0.01$).

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2.5-kb promoter. To confirm these results, various stably transfected HuH7 clones expressing luciferase under the control of the 1-kb PON-1 promoter were also tested (Fig. 3B). Similar inductions were observed after a 24-h treatment and a >2-fold induction was observed after 48 h.

We also tested the effect of different fibrates (commercial drugs) on the PON-1 gene promoter activity (Fig. 3C). Fenofibric acid provided the most efficient induction on promoter activity (Fig. 3C, bar 2). Under the same conditions, bezafibrate (bar 3) slightly increased the promoter activity (35% induction). Clofibric acid (bar 4) and gemfibrozil (bar 5) did not significantly modify the PON-1 gene promoter activity.

To investigate the mechanism involved in the induction elicited by fenofibric acid, we tested whether PPARα, a receptor known to be activated by fibrates, was involved (Fig. 4A). Cells were cotransfected with pPON1000-FL and a PPARα expression vector (or the parent empty vector pBK-CMV). The cotransfection of the pBK-CMV vector did not significantly modify

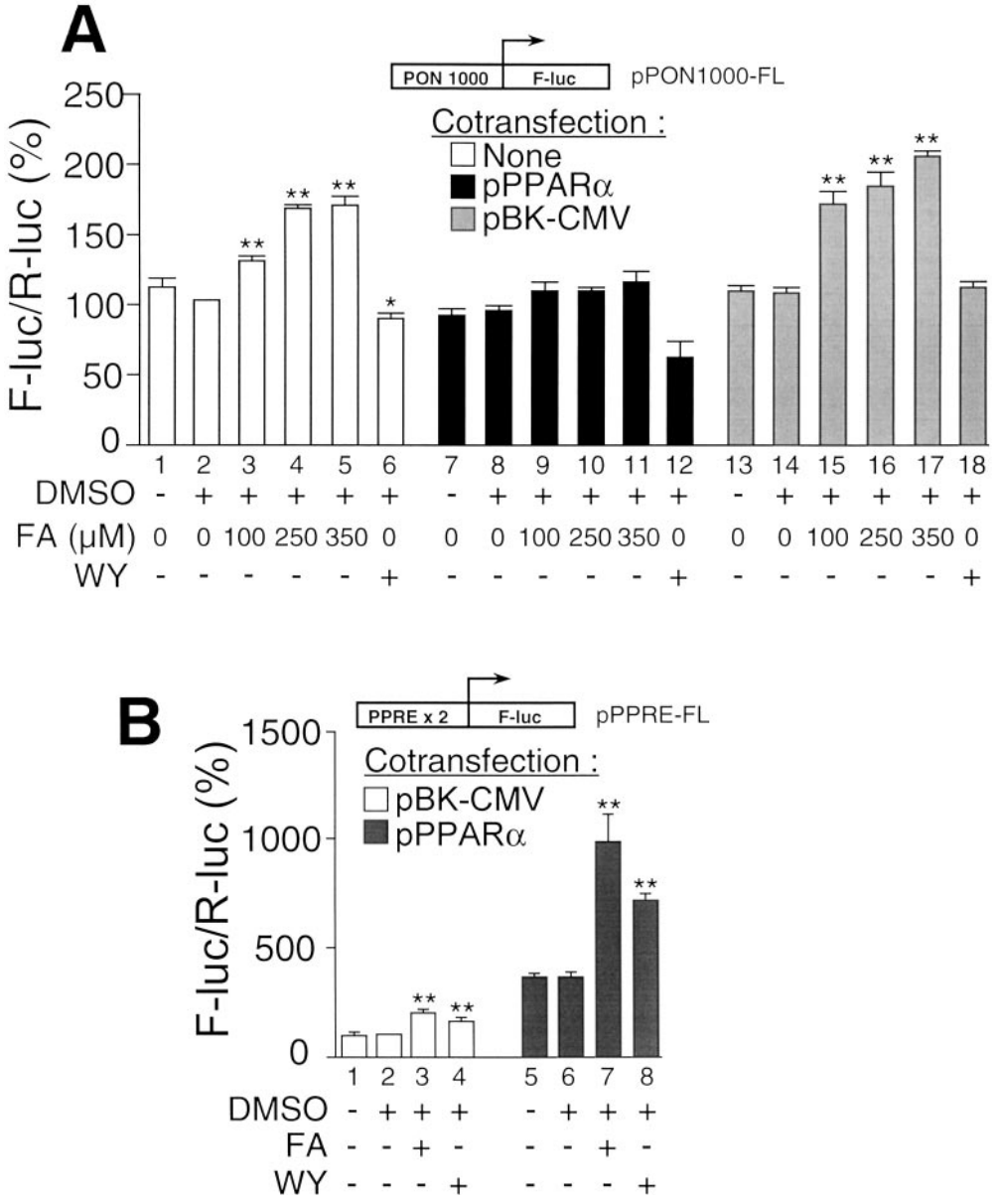

Fig. 4. Effect of PPARα expression and PPARα agonists on the activity of the PON-1 gene promoter. HuH7 cells were transfected with the indicated plasmids. Cell cultures were treated as indicated and harvested 24 h after the treatments. Firefly and *R. reniformis* luciferases were assayed as described under *Materials and Methods*. Results were expressed as Firefly luciferase activity/*R. reniformis* luciferase activity, and are represented as mean ± S.E.M. ($n \geq 8$). A, cells were transfected with the pPON1000-FL and pαglob-RL plasmids and cotransfected with the PPARα expression plasmid [pPPARα or the corresponding empty vector (pBK-CMV) as indicated]. Cells were left untreated (bars 1, 7, and 13) or treated with the solvent vehicle alone [DMSO, 0.1% (v/v)], the indicated amount of fenofibric acid (FA), or 250 μM of the PPARα agonist WY-14643 (WY); 100% corresponds to the firefly luciferase/*R. reniformis* luciferase ratio in cells treated with DMSO alone and not cotransfected. For each group (bars 1 to 6, 7 to 12, and 13 to 18), statistically significant differences with the DMSO control are indicated by * ($p < 0.05$) and ** ($p < 0.01$). B, as a positive control, cell cultures were transfected with the pPPRE-FL (includes a promoter containing two consensus PPRE) and pαglob-RL plasmids and cotransfected with the PPARα expression plasmid [pPPARα or the corresponding empty vector (pBK-CMV) as indicated]. Cells were left untreated or treated with the solvent vehicle alone [DMSO, 0.1% (v/v)], 250 μM fenofibric acid, or 250 μM of the PPARα agonist WY-14643, as indicated, for 24 h; 100% corresponds to the firefly luciferase/*R. reniformis* luciferase ratio in cells cotransfected with the empty control expression vector and treated with DMSO alone. For each group (bars 1 to 4 and 5 to 8), statistically significant differences with the DMSO control are indicated by ** ($p < 0.01$).

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either the basal activity of the PON-1 gene promoter or the effect of fenofibric acid. The expression of PPARα slightly decreased the promoter activity (compare bars 2 and 8) and, surprisingly, when the expression vector was cotransfected, the inducing effect of fenofibric acid on the PON-1 gene promoter was abolished (compare bars 3–5 with bars 9–11). Treatment of the cells with WY-14643 (250 μM), a specific ligand of PPARα, slightly repressed the PON-1 gene promoter activity in transient transfection experiments (bar 6). The same effect was observed in stable transfected HuH7 clones (data not shown). The cotransfection of the empty vector pBK-CMV did not modify the effect of WY-14643. When pPPARα was cotransfected, the PPARα ligand WY-14643 decreased the reporter activity by 40% (bar 12). These data suggest that the positive effect of fenofibric acid on the PON-1 gene promoter is not mediated by PPARα in our experimental model. Furthermore, PPARα expression seems to prevent the fenofibric acid-inducing effect and to repress the basal activity of the promoter, especially in presence of its ligand WY-14643.

As a positive control, we used the pPPRE-FL plasmid, which includes a promoter containing two consensus PPREs (Staels et al., 1998). As shown in Fig. 4B, treatment of the pBK-CMV-cotransfected cells with fenofibric acid and WY-14643 resulted in a significant induction (bars 3 and 4). When the pPPARα expression vector was cotransfected, a 3-fold increase of the basal expression level was observed (compare bars 2 and 6). Treatments with either fenofibric acid or WY-14643 resulted in strong inductions of the PPRE promoter activity (3.5-fold for fenofibric acid and 3-fold for WY-14643, bars 7 and 8). These controls showed that our experimental system was functional (PPARα expression, agonist effect of fenofibric acid and WY-14643 on PPARα and *trans*-activation of PPRE sequences by PPARα).

Because fibrates may activate other isoforms of PPARs, especially at the concentrations used in our experiments, we also cotransfected PPARβ or PPARγ. The expression of either PPARβ or PPARγ did not influence the inducing effect of fenofibric acid on the PON-1 gene promoter, whereas, in the same conditions, PPARα expression antagonized this effect (data not shown).

The results shown here were obtained with mouse PPAR expression vectors. However, a control experiment using a human PPARα expression led to the same conclusion: the cotransfection of this vector also abolished the inducing effect of fenofibric acid (data not shown). This suggests that the PPARα expression effect is not species-dependent.

Effect of Statins on Paraoxonase-1 Arylesterase Activity. Statins are the drugs used most frequently in cardiovascular disease prevention. In addition, some statins were shown to be paraoxonase-1 enzymatic substrates (Billecke et al., 2000). Because several xenobiotic metabolizing enzyme genes are induced by their substrates, we tested the effect of several statins on *PON-1* gene expression: pravastatin, simvastatin, and fluvastatin.

As shown in Fig. 5A, treatment of HuH7 cells with either 100 μM pravastatin, 10 μM simvastatin, or 10 μM fluvastatin for 48 h resulted in a decrease of paroxonase-1 arylesterase activity in the culture medium. This decrease reached 25% for pravastatin, 30% for fluvastatin, and 40% for simvastatin (compare bars 3–5 with the control lane 1). It should be noted that in our cellular model, statins were not cytotoxic at the doses used in the study (the protein content was not significantly affected; data not shown). Treatment of the cells for 72 h enhanced the inhibitory effect of statins only slightly (data not shown). These unexpected results are opposite to those observed with fibrates (see above). Statins are 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors. To determine whether this pharmacological effect was involved in the mechanism of paraoxonase-1 arylesterase activity decrease, we used mevalonate, the product of HMG-CoA reductase activity. The simultaneous treatment of cells with mevalonate and statins completely reversed the in-

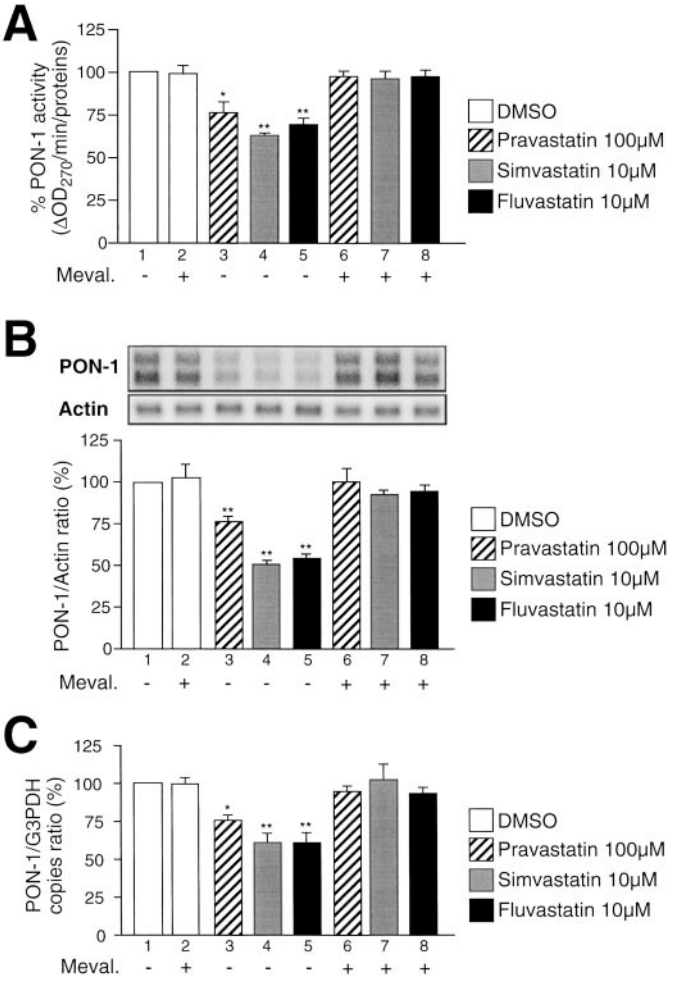

Fig. 5. Statins decrease paraoxonase-1 arylesterase activity and PON-1 mRNA level. HuH7 cells were treated with different statins as indicated or with the solvent vehicle alone [DMSO, 0.1% (v/v)] for 48 h. In addition, cells were treated or not with 3 mM mevalonate (Meval.). A, after treatment, the medium was replaced with medium containing heated serum for 24 h. Paraoxonase-1 enzymatic activity was assayed in the culture medium as described under *Materials and Methods*. Results were expressed as paraoxonase-1 activity ΔOD_{270}/min/protein content (mean ± S.E.M., $n \geq 6$); 100% corresponds to the ratio in cells treated with DMSO alone. Statistically significant differences from these controls is marked with * ($p < 0.05$) or ** ($p < 0.01$). B, mRNAs were extracted and Northern blot analysis was performed as described under *Materials and Methods*. A PhosphorImager picture shows a typical experiment. The actin gene mRNA was used as a normalizing control. The histogram shows the mean ± S.E.M. ($n \geq 3$) of the quantification ratio of the PON-1 and actin gene mRNAs; 100% corresponds to the ratio in cells treated with DMSO alone. Statistically significant differences to this control are marked with ** ($p < 0.01$). C, mRNAs were extracted and Lightcycler real-time PCR analysis was performed as described under *Materials and Methods*. The histogram shows the mean ± S.E.M. ($n = 4$) of the quantification ratio quantification ratio PON-1/G3PDH; 100% corresponds to the ratio in cells treated with DMSO alone. Statistically significant differences from this control are marked with ** ($p < 0.01$) and * ($p < 0.05$).

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hibitory effect of statins on paraoxonase-1 activity (bars 6–8). Mevalonate alone did not modify this activity (bar 2). These data show that the repressive effect of statins on the paraoxonase-1 activity seems to be mediated by the inhibition of HMG-CoA reductase and that modifying the level of downstream products of the mevalonate pathway could account for the observed effects.

Effect of Statins on PON-1 mRNA Levels. Northern blot and real-time PCR analysis showed that treatment of HuH7 cells with either 100 μM pravastatin, 10 μM simvastatin, or 10 μM fluvastatin for 48 h resulted in a 30 to 50% decrease of PON-1 mRNA levels (Figs. 5, B and C, compare bars 3–5 with the control bar 1). The simultaneous addition of mevalonate completely reversed the inhibitory effect of statins on PON-1 mRNA level (compare bars 6–8 with the control bar 2). Mevalonate alone did not influence PON-1 mRNA levels (bar 2). These effects are consistent with those observed regarding paraoxonase-1 arylesterase activity. These results suggest that the regulation of the *PON-1* gene itself determines paraoxonase-1 activity level in our model: the variations of mRNA level and secreted enzymatic activity are correlated ($\rho = 0.91$, $n = 8$, data not shown).

Effect of Statins on PON-1 Promoter Activity. Because statins induced a decrease of paraoxonase-1 activity and a down-regulation of PON-1 mRNA levels, the effect of these drugs on the PON-1 promoter activity was assayed using transfection assays with the pPON1000-FL plasmid in HuH7 cells. Figure 6A shows that statins significantly decreased PON-1 promoter activity in transient transfection experiments. This effect was dose-dependent and occurred at doses as low as 10^{-7} M. It reached 40 to 60% for pravastatin (100 μM), simvastatin (10 μM), or fluvastatin (10 μM) as shown in Figs. 6A and 7A. It should be noted that in the same experiments, fenofibric acid had an inducing effect similar to that described above (data not shown). Stably transfected HuH7 clones expressing luciferase under the control of the PON-1 promoter were also tested. Inhibitions were observed after 24 and 48 h of treatment (Fig. 6B). In the same experiments, fenofibric acid had an inducing effect. In addition, we tested the effect of statins in serum-free culture medium because serum could contain paraoxonase-1, which could hydrolyze statin-like compounds. The absence of serum indeed enhanced the statins inhibitory effect, but only at the lowest concentration (data not shown). Two hypotheses were tested to investigate the mechanism responsible for statins effect as described below.

First, because statins inhibit HMG-CoA reductase, it is possible that a deficit in mevalonate was involved. As shown in Fig. 7A, the simultaneous addition of mevalonate completely reversed the inhibitory effect of each tested statin on the PON-1 gene promoter. However, mevalonate alone did not significantly modify the promoter activity. These data are consistent with those shown above for mRNA levels and paraoxonase-1 activity. They suggest a role for the intracellular level of mevalonate or a down-stream product of mevalonate in the modulation of the promoter activity. In addition, statins were shown to inhibit LXR activity (Forman et al., 1997). We tested 22(*R*)-hydroxycholesterol, an oxysterol downstream product of mevalonate that activates LXRs (Forman et al., 1997). When HuH7 cells were cotreated with statins and 22(*R*)-hydroxycholesterol, the repressive effect of statins was almost fully reversed in the case of pravastatin (100 μM) and completely reversed for both simvastatin and fluvastatin (10 μM; Fig. 7A). As a positive control of the LXR pathway in our experimental model, a reporter gene driven by a synthetic promoter containing three DR4 sequences (LXRE sequences, typically activated by LXRs) was used. This promoter was activated 2-fold by 22(*R*)-hydroxycholesterol and strongly repressed by simvastatin (data not shown). In preliminary experiments, LXRα or LXRβ expression activated this synthetic promoter and caused a strong response to 22(*R*)-hydroxycholesterol treatment (data not shown). In the same preliminary experiments, LXRα or LXRβ expression did not activate the 1-kb PON-1 gene promoter. The endogenous LXR level may be sufficient to cause the maximum activation of the promoter.

The second hypothesis was the possible involvement of PPARα. Indeed, because PPARα expression and activation repress the PON-1 gene promoter activity (especially when induced with fenofibric acid; see Fig. 4); because statins repress this promoter and have recently been shown to activate PPARα (Martin et al., 2001), we asked whether the repressive effect of statins was mediated by PPARα. Cells were cotransfected with pPON1000-FL and the PPARα expression vector (or the parent empty vector pBK-CMV). As observed previously, the plasmid-driven expression of PPARα seems to have a slight repressive effect on the activity of the PON-1 gene promoter (Fig. 7B, compare bars 1 and 3). The effect of statins themselves was not significantly modified by the cotransfection of either the PPARα expression vector or the empty vector (Fig. 7B, compare bars 4 and 6, 7 and 9, 10 and 12). Thus, in our study, we have no evidence that the repressive effect of statins on the *PON-1* gene is mediated by PPARα. As a positive control, we used the pPPRE-FL reporter vector. As shown in Fig. 7C, pravastatin, simvastatin, and fluvastatin activated the PPRE-containing promoter activity in cells cotransfected with the PPARα expression vector (bars 5–8) but not with the empty vector pBK-CMV (bars 1–4). These results confirm the hypothesis that statins are PPARα activators.

Mapping of Responsive Elements within the Promoter Sequence. Five deletions of the promoter were constructed and used in transfection assays. Reporter constructs containing these deletions were used to define the roles of the putative responsive elements in the regulation of the *PON-1* gene by the two drugs. The relative basal activities of these deleted promoters were somewhat variable (Fig. 8). The effects of fenofibric acid and simvastatin were also tested (Fig. 8). Regarding fenofibric acid induction, all the deleted promoters were still responsive to the drug without significant modulation in the intensity of the effect. This suggests that the regulatory elements are located within the proximal promoter (containing the first 194 base pairs).

Regarding the statin repressive effect, at least one regulatory sequence was identified. The repression of the three shorter deleted promoters (−491, −439, and −194 base pairs) by simvastatin was weaker than that of the three larger ones (−1014, −817, and −651). The repressive effect is significantly reduced ($p < 0.01$) between the inhibition obtained with the −651 construct and construct −491, −439, and −194. A regulatory element at least partially mediating the statin effect is thus located between the position −651 and −491. The data described above regarding the LXR agonist 22(*R*)-hydroxycholesterol suggests that the LXRE element (between positions −552 and −537, see Table 1) located within this sequence could be involved. However simvastatin still significantly represses the

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−194-bp proximal promoter that lacks putative LXRE sequences. Therefore, the inhibition of the LXR pathway may not account alone for the entire repressive effect of statins on the PON-1 gene promoter.

Discussion

Because paraoxonase-1 was shown to detoxify several xenobiotic substrates and protect against atherosclerosis, it was of interest to investigate the regulation of this gene. Therefore, we characterized for the first time the PON-1 gene promoter region, which displays several transcription start sites and putative regulatory elements. In the present study, we tested the two main classes of cardiovascular protection drugs: fibrates and statins.

In HuH7 human hepatoma cells, fenofibric acid treatment increased secreted paraoxonase-1 arylesterase activity and mRNA levels. Further investigations showed that this drug stimulated the activity of the PON-1 gene promoter up to 2-fold in our cellular model at concentrations consistent with previous studies and clinical data (Staels et al., 1998; Vu-Dac et al., 1998). This induction was confirmed with several batches of cells and fenofibric acid. To evaluate the specificity of this effect, we tested other fibrate compounds that, like fenofibrate, are known to activate PPARα (Staels et al., 1998); these compounds were either poor or ineffective inducers. Thus, the induction of the *PON-1* gene expression by fenofibric acid does not seem to be a class effect. A recent clinical study reported that gemfibrozil treatment resulted in a very slight increase of serum paraoxonase-1 activity (Balogh et al., 2001). Our data show that gemfibrozil has no inducing effect on the gene promoter in HuH7 cells, in contrast to fenofibric acid. Recently, fenofibrate was shown to decrease plasma paraoxonase-1 activity in rats (Beltowski et al., 2002), suggesting a species difference. Opposite regulations in humans and rodents have been described for other genes [transaminase (Edgar et al., 1998) and Apo AI (Vu-Dac et al., 1998)] and, in some cases, have been shown to be related to promoter sequence differences. Moreover, owing to several divergent observations, it is difficult to compare human and rodent models for the effects of hypolipidemic drugs (reviewed in Krause and Princen, 1998). Therefore, a clinical study assessing serum paraoxonase-1 activity in patients treated with fenofibrate could be interesting.

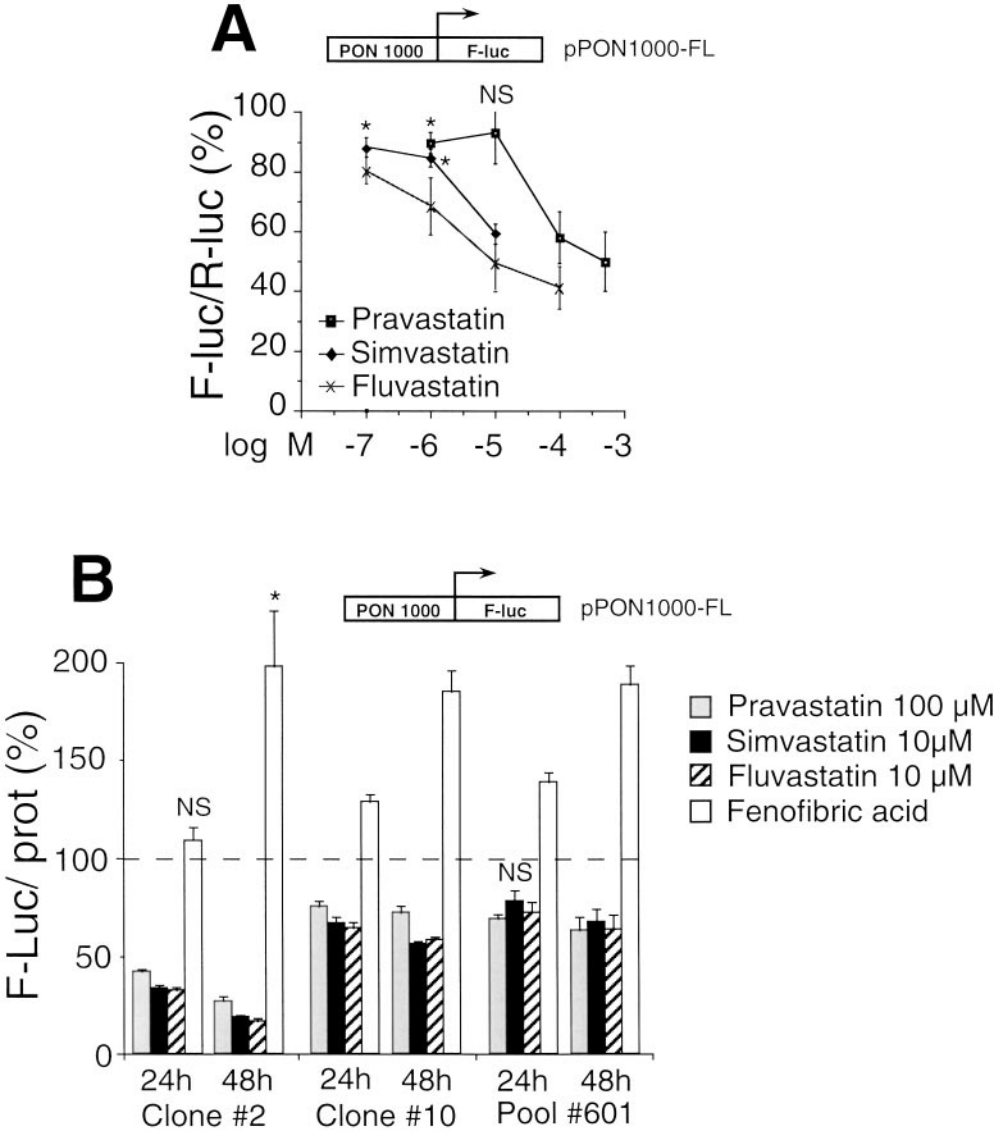

Fig. 6. Statins repress the activity of the PON-1 gene promoter. A, HuH7 cells were transiently transfected with the pPON1000-FL plasmid and cotransfected with pαglob-RL as an internal control. Cell cultures were treated with various amounts of the indicated statins or with the solvent vehicle alone (DMSO 0.1% v/v) for 48h. Firefly and *R. reniformis* luciferases were assayed as described under *Materials and Methods*. Results were expressed as Firefly luciferase activity/*R. reniformis* luciferase activity, mean ± S.E.M. ($n \geq 6$); 100% corresponds to the firefly luciferase/*R. reniformis* luciferase ratio in cells treated with DMSO alone, and differences from this control are statistically significant ($p < 0.01$) except for data marked with * ($p < 0.05$) or NS (not significant). B, two distinct HuH7 clones (nos. 2 and 10), expressing the firefly luciferase reporter gene as the result of the stable transfection of pPON1000-FL, were treated with the same statins as above at the indicated concentrations, 250 μM fenofibric acid (as a positive control), or the solvent vehicle alone [DMSO, 0.1% (v/v)], for 48h. A pool of clones (no. 601) was also used. Firefly luciferase and protein content were assayed as described under *Materials and Methods*. Results were expressed as firefly luciferase activity/protein content (mean ± S.E.M., $n \geq 6$). For each group (the two clones and the pool), 100% corresponds cells treated with DMSO alone [0.1% (v/v), represented by a horizontal line]. In each group, differences with DMSO are statistically significant ($p < 0.01$), except for bars marked with * ($p < 0.05$) or NS (not significant).

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We tested whether PPARα was involved in the fenofibric acid-elicited induction of the *PON-1* gene expression, as in the case of Apo A genes (Staels et al., 1998). Unexpectedly, we observed that PPARα expression prevented the induction mediated by fenofibric acid, suggesting that the inducing effect does not involve this receptor. Various mechanisms could account for the PPARα-mediated repression: fibrate binding, hijacking of retinoid X receptor α (which forms an heterodimer with PPARα), or activation of another as yet unknown repressive pathway. In preliminary experiments, retinoid X receptor α cotransfection did not modify the regulation of the PON-1 gene promoter activity described above (data not shown). Our results suggest that regulatory elements responsible for the fibrate induction are located within the proximal promoter. Their precise identification requires further investigation.

The other class of drugs used in this study was statins. One study reports a modest (12%) increase of serum paraoxonase-1 activity in simvastatin-treated patients (Tomas et al., 2000). Our results are not consistent with this observation.

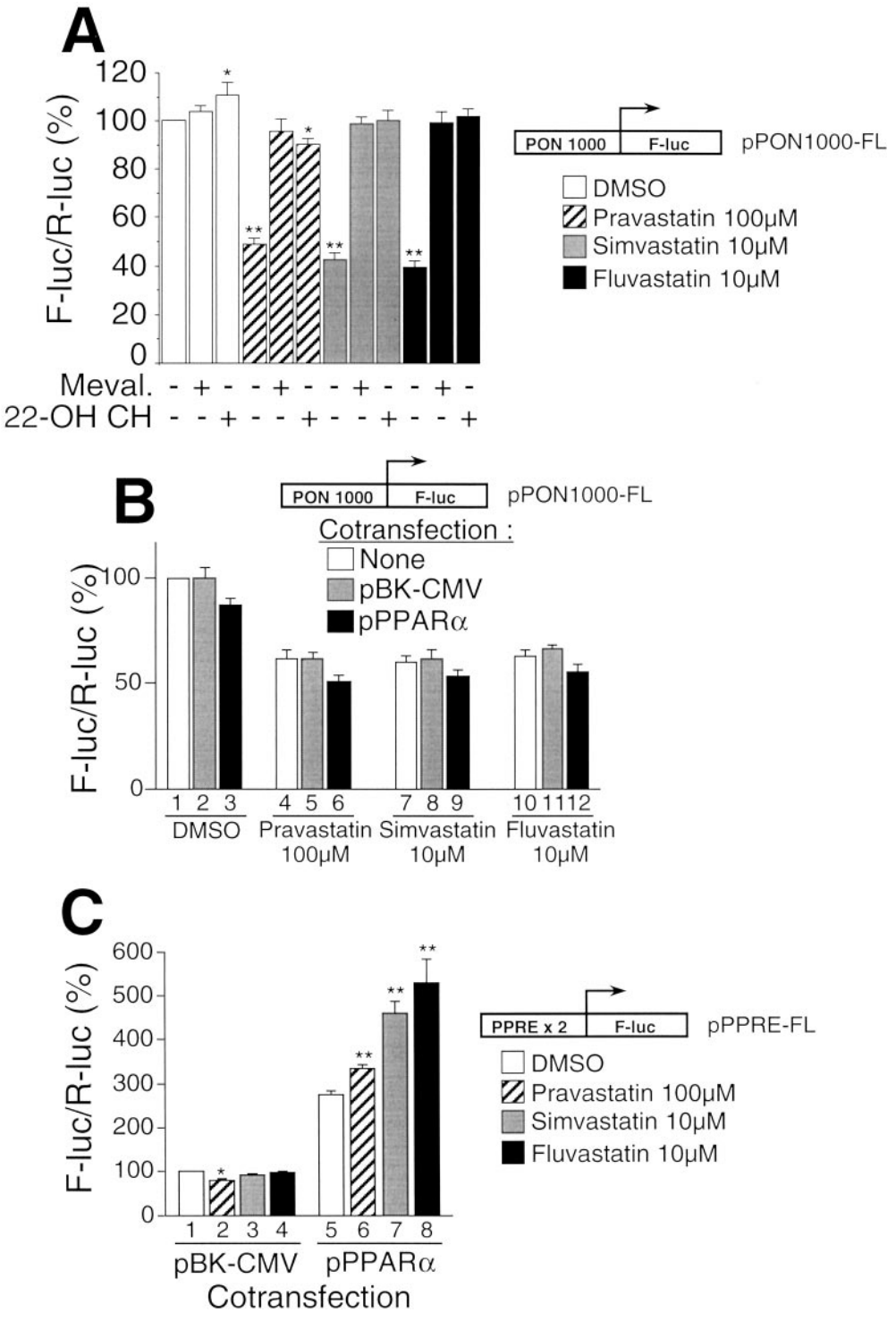

Fig. 7. The repressive effect of statins is not enhanced by PPARα expression. HuH7 cells were transiently transfected with the indicated plasmids. Cell cultures were treated with the different statins as indicated or with the solvent vehicle alone [DMSO, 0.1% (v/v)] for 48 h. Firefly and *R. reniformis* luciferases were assayed as described under *Materials and Methods*. Results were expressed as firefly luciferase activity/*R. reniformis* luciferase activity and are represented as mean ± S.E.M. ($n \geq 6$). A, HuH7 cells were transiently transfected with the pPON1000-FL plasmid and the pαglob-RL plasmid as an internal controls. Cell cultures were treated or not with 3 mM mevalonate (Meval.) or 10 μM 22-OH hydroxycholesterol (22-OH CH); 100% corresponds to the firefly luciferase/*R. reniformis* luciferase ratio in cells treated with DMSO alone. Statistically significant differences to this control are marked with ** ($p < 0.01$). B, cells were transfected with the pPON1000-FL and pαglob-RL plasmids and cotransfected with the PPARα expression plasmid pPPARα or the corresponding empty vector (pBK-CMV) as indicated; 100% corresponds to the firefly luciferase/*R. reniformis* luciferase ratio in cells treated with DMSO alone and not cotransfected. For each treatment, differences with the noncotransfected condition are not statistically significant. C, as a positive control, cell cultures were transfected with the pPPRE-FL (includes a promoter containing two consensus PPRE) and pαglob-RL plasmids. Cells were cotransfected with the PPARα expression plasmid (pPPARα) or the corresponding empty vector (pBK-CMV) as indicated; 100% corresponds to the firefly luciferase/*R. reniformis* luciferase ratio in cells cotransfected with the empty control expression vector and treated with DMSO alone. For each group (lanes 1 to 4 and 5 to 8), statistically significant differences with the DMSO control are spotted by * ($p < 0.05$) and ** ($p < 0.01$).

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Indeed, in our experimental model, statins caused a significant decrease of the secreted paraoxonase-1 arylesterase activity and mRNA levels. Moreover, transient and stable transfection experiments showed that statins repressed the activity of the PON-1 gene promoter at concentrations consistent with clinical data (Corsini et al., 1999). This discrepancy could be explained by different factors: 1) physiologically, serum paraoxonase-1 activity is likely to depend on genes other than *PON-1* itself, including apolipoproteins (Staels et al., 1998; Deakin et al., 2001; Martin et al., 2001); 2) because oxidized LDLs repress the *PON-1* gene (Van Lenten et al., 2001), the decrease of the oxidized LDL level observed after statin treatment (Tomas et al., 2000) could compensate for the *PON-1* gene down-regulation. Although the magnitude of the repression is not the same with the different statins we used, our data suggest that it is a class effect; simvastatin is the most active repressor. Little is known about the mechanisms that are involved in the regulation of gene expression by statins. They were shown to antagonize LXR (Forman et al., 1997) and our study suggests that this mechanism could be involved in the regulation of the *PON-1* gene because 1) cotreatment with mevalonate reversed the inhibitory effect of statins on paraoxonase-1 activity and mRNA levels; 2) the oxysterol 22(*R*)-hydroxycholesterol, a downstream product of mevalonate, completely abolished the repression of the promoter by statins; 3) 22(*R*)-hydroxycholesterol is a typical agonist of LXR; 4) the LXR pathway is functional in our cellular model; 5) at least one LXRE sequence is found in the PON-1 gene promoter used in transfections; 6) deleted promoters lacking this sequence are significantly less sensitive to simvastatin. The possible involvement of LXR in the regulation of PON-1 would be in agreement with its known role in the regulation of lipid-metabolizing enzymes. Yet, an LXR-mediated mechanism cannot account alone for the repressive effect of statin, and further studies are required to identify an additional pathway. Our results suggest that the latter would involve regulatory elements located within the proximal promoter. In the case of the *Apo AI* gene, statins act through the activation of PPARα (Martin et al., 2001) and, in HuH7 cells, we have shown that statins can indeed induce the activity of a PPRE-driven promoter only in the presence of PPARα. Yet, although PPARα seems to be a repressor of the PON-1 gene promoter, our results do not support the hypothesis that a statin-PPARα interaction is involved in the repression of the *PON-1* gene by statins.

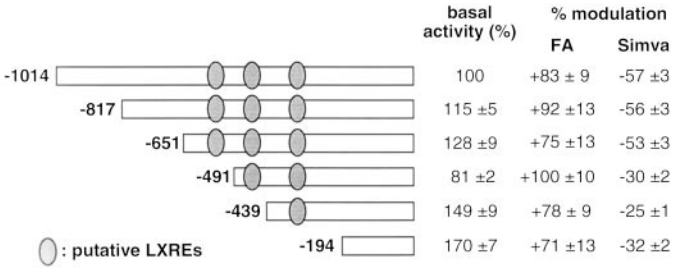

Fig. 8. Mapping of the promoter using deleted fragments. HuH7 cells were transiently transfected with the plasmids containing deleted promoters whose length in base pairs is indicated. In addition, the location of putative LXRE sequences (see Table 1) within the different deleted promoters are shown. Cell cultures were treated with 250 μM fenofibric acid or 10 μM simvastatin as indicated or with the solvent vehicle alone [DMSO, 0.1% (v/v)] for 24 or 48 h, respectively. Firefly and *R. reniformis* luciferases were assayed as described under *Materials and Methods*. Results were expressed as firefly luciferase/*R. reniformis* luciferase luciferase activity, and are shown as mean ± S.E.M. ($n \geq 6$). For basal activities, 100% corresponds to the firefly luciferase/*R. reniformis* luciferase ratio in cells transfected with the 1-kb promoter. Regarding the effects of both drugs, results are expressed as percentage modulations of the basal activities for each construction.

TABLE 1

Putative LXREs in the PON-1 gene promoter

The DNA sequence was searched to find elements fitting the DR4/LXRE consensus T(G/A)A(C/A)C(T/C)nnnnT(G/A)A(C/A)C(T/C) (Lu et al., 2001). Three sequences were identified in the 1-kb promoter used in transfection experiments. Their fitting with the consensus sequence are indicated as well as their positions in the promoter sequence (the +1 position corresponds to the A of the start codon).

Sequences	Consensus Fit	Position
TGTACTtgagTGAACT	11/12	−552;−537
TAGCCTggtgAGAACA	9/12	−469;−454
AAAGCTtgacTGTCCT	9/12	−331;−316

Several mechanisms contributing to cardiovascular disease have been identified among which a deficit in serum paraoxonase-1 activity (reviewed in Mackness et al., 2001). Paraoxonase-1 activity is related to another marker of cardiovascular disease: elevated homocysteine (Hcy) plasma levels. Hcy can be converted into Hcy-thiolactone, which can damage proteins by homocysteinylation and could be involved in the pathology of vascular diseases (Jakubowski, 1999). Because Hcy-thiolactone can be converted into its parent form Hcy by paraoxonase-1 (Jakubowski, 2000), an increase in paraoxonase-1 activity could raise the level of homocysteine and detoxify Hcy-thiolactone. The regulations of the *PON-1* gene reported here are consistent with clinical data showing the different effects of cardiovascular disease-preventing drugs on plasma homocysteine levels. Indeed, fenofibrate and bezafibrate were shown to increase Hcy levels, whereas gemfibrozil had no effect (Westphal et al., 2001) and high-dose statins led to a decrease (Luftjohann et al., 2001). This suggests that the induction of the *PON-1* gene could explain in part the benefit of fenofibrate regarding its use in cardiovascular disease prevention. Conversely, the regulation of the *PON-1* gene itself by statins is rather unexpected. The well-established clinical benefit of statins is likely to involve *PON-1*–independent mechanisms. Together with the hydrolysis of toxic endogenous compounds (oxidized phospholipids and homocysteine thiolactone), probably accounting for its antiatherogenic capacity, paraoxonase-1 is also a xenobiotic metabolizing enzyme that detoxifies OPs. These molecules can produce several forms of toxicity, including acute intoxication (caused by the inhibition of central and peripheral acetylcholinesterases) and/or a specific syndrome of lasting delayed peripheral neuropathy (Maynard and Beswick, 1992). Moreover, long-term low-level exposure has been associated with impaired neurobehavioral performance, possibly involving targets other than acetylcholinesterases (Ray and Richards, 2001; Cherry et al., 2002). This may occur either because of limited and repeated exogenous contacts

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(pesticide workers) or because of high-dose acute intoxications (some OPs are stocked in adipocyte tissues and released slowly). Regarding the protective activity of paraoxonase-1 against OP poisoning, the present study suggests that treatment with fenofibrate could improve the metabolism of OPs, preventing them, at least partially, from reaching their toxicological targets. In conclusion, this work allowed us to identify at least one inducer and one class of repressors of the *PON-1* gene. Several evidences suggest that fenofibric acid could be a candidate drug for the treatment of OPs intoxication: 1) the protective role of paraoxonase-1 against OP-poisoning in vivo is clearly established [knock-out mice (Shih et al., 1998), purified paraoxonase-1 injections (Li et al., 1995), or gene therapy (Cowan et al., 2001)]; 2) a limited variation (60%) of paraoxonase-1 activity can influence significantly OP intoxication severity (Cowan et al., 2001); 3) fenofibrate is a well-characterized drug, used in long-term treatments without major adverse effects (Staels et al., 1998); 4) the stimulation of an endogenous gene through drug administration is more advisable than gene therapy or even direct injection of exogenous enzymes owing to unresolved practical problems. The use of new drugs to treat OP intoxication, especially in the case of long-term, low-dose exposure, is needed to avoid side effects of classical antidotes.

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