

# A Common Mutation in Paraoxonase-2 Results in Impaired Lactonase Activity<sup>\*S</sup>

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Paraoxonases (PONs) are a family of lactonases with promiscuous enzyme activity that has been implicated in multiple diseases. PON2 is intracellularly located, is the most ubiquitously expressed PON, and has the highest lactonase activity of the PON family members. Whereas some single-nucleotide polymorphisms (SNPs) in PON1 have resulted in altered enzymatic activity in serum, to date the functional consequences of SNPs on PON2 function remain unknown. We hypothesized that a common PON2 SNP would result in impaired lactonase activity. Substitution of cysteine for serine at codon 311 in recombinant PON2 resulted in normal protein production and localization but altered glycosylation and decreased lactonase activity. Moreover, we screened 200 human lung samples for the PON2 Cys<sup>311</sup> variant and found that *in vivo* this mutation impaired lactonase activity. These data suggest that impaired lactonase activity may play a role in innate immunity, atherosclerosis, and other diseases associated with the PON2 311 SNP.

The paraoxonase (PON)<sup>3</sup> family consists of three members: PON1, PON2, and PON3 (1–3). PON1 was the first family member described and was named for its ability to degrade the organophosphate paraoxon. PON1 was subsequently found to inhibit low density lipoprotein oxidation and be important for cardiovascular disease (4). In addition, analysis of PON1 single-nucleotide polymorphisms (SNPs) has linked PON1 to the pathogenesis of numerous other human disorders including atherosclerosis, diabetes, cerebrovascular disease, Alzheimer disease, amyotrophic lateral sclerosis, organophosphate susceptibility, and Parkinson disease (5–11). Despite these numer-

ous association studies, the mechanism(s) underlying the role of PON in disease pathogenesis remains to be fully determined, in part because of uncertainty regarding the endogenous or natural substrate of PON.

However, the native enzyme activity of PON was recently found to be as a lactonase (12), suggesting that despite its known enzymatic promiscuity for other substrates including esters and phosphotriesters, its endogenous substrates are lactones. Importantly, all three PON family members have conserved their lactonase activity, but individual PON family members have widely disparate enzymatic activities toward other substrates. One specific lactone-containing molecule that PONs can degrade is the bacterial quorum-sensing molecule, *N*-3-oxododecanoyl homoserine lactone (3OC12-HSL) (13–16). 3OC12-HSL is an acyl-homoserine lactone that *Pseudomonas aeruginosa*, a common cause of hospital-acquired infections and an important cause of pulmonary morbidity and mortality in cystic fibrosis, uses to control biofilm formation and virulence factor production (17, 18). Of the three PON family members, PON2 has the greatest lactonase activity toward 3OC12-HSL (13, 19). In contrast to PON1 and PON3, PON2 is not present in serum, and it has minimal arylesterase and paraoxonase activity. Human airway epithelial cells degrade 3OC12-HSL (19, 20), and murine PON2-deficient airway epithelial cells have an impaired ability to inactivate 3OC12-HSL (19). In addition, we have recently found that PON1-transgenic *Drosophila melanogaster* are protected from organophosphate poisoning and *P. aeruginosa* lethality (21). PON2 activity in airway epithelial cells may represent a novel antibacterial defense mechanism against invading pathogens that utilize acyl-homoserine lactones for quorum sensing and virulence regulation.

Genetic variation in human genes can have a substantial impact on host responses, and SNP analysis may be used in anticipating responses and outcomes or in adjusting drug dosing. In this study we asked whether a common PON2 SNP, a serine to cysteine amino acid change at codon 311 in PON2 (2), alters PON2 lactonase activity for 3OC12-HSL. This SNP is fairly common in the general population and is strongly conserved evolutionarily. We chose this PON2 SNP because PON2 has the greatest lactonase activity of PON family members, phylogenetic analysis suggests that PON2 is the oldest PON family member (11), and several PON1 SNPs are in linkage

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<sup>3</sup> The abbreviations used are: PON, paraoxonase; SNP, single-nucleotide polymorphism; 3OC12-HSL, *N*-3-oxododecanoyl homoserine lactone; PNGase F, peptide-*N*-glycosidase F; Endo H, endoglycosidase H; ALS, amyotrophic lateral sclerosis; CHO, Chinese hamster ovary; ER, endoplasmic reticulum.

disequilibrium with PON2 SNPs. We found that recombinant PON2 Cys<sup>311</sup> exhibits an impaired ability to inactivate 3OC12-HSL and that airway epithelial cells from humans homozygous for Cys/Cys at PON2 amino acid position 311 also have an impaired ability to degrade 3OC12-HSL.

## EXPERIMENTAL PROCEDURES

**Recombinant PON2 Cys<sup>311</sup> or Ser<sup>311</sup> Expression**—Chinese hamster ovary (CHO) cells were used for transfection studies because these cells have no endogenous PON or lactonase activity (13). CHO cells were cultured as monolayers in plastic dishes and transfected with plasmids expressing hPON2 Cys<sup>311</sup> or Ser<sup>311</sup> using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol, and allowed to express for 24–48 h.

**3OC12-HSL Degradation by Airway Epithelia and CHO Cells**—For epithelial cell lysate experiments, epithelia were first washed with cold phosphate-buffered saline (containing calcium and magnesium), and then 50  $\mu$ l of lysis buffer (50 mM Tris·HCl, pH 6.9, 150 mM NaCl, 10  $\mu$ M leupeptin, 10  $\mu$ M aprotinin, 1  $\mu$ M pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml benzamidine) was added to the apical surface. After 20 min of rocking in lysis buffer at 4 °C, the cells were scraped free from the membrane with a pipette tip and lysed by sonication (10 pulses, pulse duration of  $\sim$ 1 s) (Branson Sonifier 250, Danbury, CT). The cellular debris was cleared from the lysate by centrifugation (4500  $\times$  g for 30 s at 4 °C). The relative protein concentrations were determined by the Bio-Rad protein assay (500-0006). Lysate preparations (10–20%) were diluted in phosphate-buffered saline (containing calcium and magnesium) and incubated in the presence of 3OC12-HSL (RTI International, Research Triangle Park, NC) at 37 °C. 3OC12-HSL in acidified ethyl acetate was dried under a nitrogen gas stream and then dissolved in phosphate-buffered saline (containing calcium and magnesium) to achieve a final concentration of 10  $\mu$ M 3OC12-HSL. At various time points, 6- $\mu$ l aliquots were collected, added to 100  $\mu$ l of ethyl acetate, and stored in airtight glass vials at  $-20$  °C.

**3OC12-HSL and 3OC12-HSL Quantitative Assay**—3OC12-HSL was obtained from RTI International. 3OC12-HSL was measured in a quantitative bioassay as previously described using *Escherichia coli* MG4 (pKDT17) (22).

**Tunicamycin Treatment of Transfected CHO Cells**—PON2 chemical deglycosylation was performed by first transfecting CHO cells with a plasmid expressing PON2 Cys<sup>311</sup> or Ser<sup>311</sup>. 30 min later, tunicamycin was added to the cell culture medium at a final concentration of 0.5  $\mu$ g/ml. 36–48 h later cell lysates were harvested, and SDS-PAGE was performed for PON2.

**Deglycosylation of PON2 with Peptide-N-glycosidase F and Endoglycosidase H Digestion**—Peptide-N-glycosidase F (PNGase F) (Sigma) digestion was performed according to the manufacturer's directions. Briefly, cell lysates were collected in lysis buffer (50 mM Tris·HCl, pH 7.5, 138 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM NaF, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and aprotinin) and incubated with PNGase F (10 units) for 4 h at 37 °C. The reaction was stopped by incubating at 100 °C for 5 min. Deglycosylation was then assessed with SDS-PAGE. Endoglycosidase H (Endo H) (Sigma) digestion was

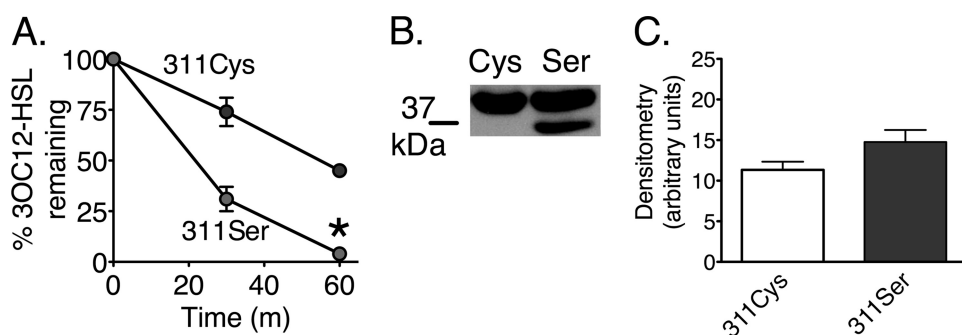
performed according to the manufacturer's directions. The cell lysates were incubated with reaction buffer and denaturation solution and heated for 5 min at 100 °C. Following cooling of the sample, Endo H was added to the samples and incubated for 4 h at 37 °C. Digestion was then determined using SDS-PAGE.

**Western Blot Analysis**—CHO cell lysates were made by incubation in lysis buffer (50 mM Tris·HCl, pH 7.5, 138 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM NaF, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and aprotinin) with 1% Triton X-100 for 20 min at 4 °C on a rocker. The cells were scraped from the tissue culture plates and transferred to a microcentrifuge tube. Sonication was performed for 10 s, and cell debris was cleared by centrifugation at 4,500  $\times$  g for 30 s at 4 °C. The lysates were then combined with loading buffer and separated by SDS-PAGE. Protein was then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA). Polyvinylidene difluoride membranes were blocked either overnight at 4 °C or for 2 h at room temperature in 5% bovine serum albumin in phosphate-buffered saline. The membranes were then incubated with the PON2 antibody (1:1000; rabbit, polyclonal anti-human PON2; Orbigen, San Diego, CA) for 2 h at room temperature and then washed three times in 1 $\times$  TTBS (137 mM NaCl, 2.7 mM KCl, 2.5 mM Tris, 0.05% Tween 20). Secondary antibodies were conjugated to horseradish peroxidase (Amersham Biosciences) and used at 1:10,000 for 1 h. Following three washes with 1 $\times$  TTBS, immunoreactive bands were detected with SuperSignal solution (Pierce) and exposed to film.

**Construction of N-Linked Glycosylation Mutants**—Site-directed mutagenesis was performed with the Stratagene QuikChange<sup>TM</sup> kit according to the manufacturer's standard protocol (Stratagene, Cedar Creek, TX). Double-stranded DNA template was prepared by a standard megaprep protocol (Qiagen). Mutant strand synthesis was performed with the following primers for N226Q (5'-GATTCAGCAAATGGGATCCAGATTTACCTGATGATAAG-3'), for N254Q (5'-GTTTTGGAAAAACACACTAATATGCAGTTAACTCAGTTGAAG-3'), for N269Q (5'-TGGATACACTGGTGGATCAGTTATCTATTGATCCTCC-3'), for N323Q (5'-ACTACAGTTTATGCCAACCGGGTCTGTTCTCCAAG-3'), for N254A (5'-GTTTTGGAAAAACACACTAATATGGCTTTAACTCAGTTGAAG-3'), and for N323A (5'-ACTAGTTTATGCCAACCGTGGTCTGTTCTCCAAG-3'). The reaction products were then treated with the restriction endonuclease DpnI to enrich for multiply mutated single-stranded DNA. This reaction mixture was then transformed into XL10-Gold<sup>®</sup> Ultracompetent cells and spread on Luria-Bertani ampicillin agar plates (containing 80 ng/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) and 20 mM isopropyl  $\beta$ -D-thiogalactopyranoside). The colonies were picked, and site-specific mutations were confirmed by DNA sequencing (DNA Facility, University of Iowa).

**Immunofluorescence Localization of PON2 in Transfected CHO Cells**—CHO cells were plated on collagen-coated four-well chamber slides and transfected with a plasmid encoding either human PON2 Cys<sup>311</sup> or Ser<sup>311</sup>. At 36 h following transfection, the cells were fixed with 1% paraformaldehyde in methanol, blocked in 2% bovine serum albumin in Super-

## PON2 311 SNP Impairs Lactonase Activity



**FIGURE 1. Recombinant PON2 Cys<sup>311</sup> has impaired lactonase activity.** A, 3OC12-HSL degradation by CHO cell lysates following transfection with PON2 Cys<sup>311</sup> or PON2 Ser<sup>311</sup>. *n* = 3–5 experiments/group. Lysates (20% volume) of CHO cell cultures were incubated with 10  $\mu$ M 3OC12-HSL and after 0, 30, or 60 min of incubation the lysate samples were obtained for measurement of 3OC12-HSL inactivation. The data are expressed as the percentages of active 3OC12-HSL remaining compared with initial levels. The data are the means  $\pm$  S.E. \*, *p* < 0.05. B, CHO cells were transfected with PON2 Cys<sup>311</sup> or Ser<sup>311</sup> and 48 h later lysed for gel electrophoresis. Subsequently, Western immunoblot analysis for PON2 was performed as described under "Experimental Procedures." C, densitometric analysis of PON2 Cys<sup>311</sup> and Ser<sup>311</sup> protein levels in CHO cell lysates at 48 h following transfection. ImageJ software was used for densitometric analysis of PON2 immunoreactive bands. GAPDH levels were used for normalization. *n* = 3. The data are the means  $\pm$  S.E.

Block (Pierce), and stained with anti-human PON2 antibody (rabbit polyclonal, Orbigen) and a monoclonal antibody specific for protein-disulfide isomerase to localize the endoplasmic reticulum (Assay Designs, Ann Arbor, MI). Confocal images were acquired with a Bio-Rad MRC-1024 laser scanning confocal system mounted on a Nikon E600 microscope.

**Primary Airway Epithelial Cell Culture Model**—Primary human airway epithelial cells were isolated from the trachea and bronchi of human donor lungs. The isolated cells were seeded onto collagen-coated, semi-permeable membranes (0.6-cm<sup>2</sup> Millicell-HA; Millipore, Bedford, MA) and grown at the air-liquid interface as previously described (23). After genotypic identification of PON2 SNPs in donor fibroblasts, primary or passage 1 cryopreserved airway epithelial stocks were thawed and expanded for the described studies. All of the experiments were performed on well differentiated human airway epithelia (more than 14 days after seeding of cells). The samples were collected with the approval of the University of Iowa Institutional Review Board.

**Identification of PON Polymorphisms and Determination of Genotypic Frequencies**—DNA samples were genotyped for the presence of several common coding and noncoding polymorphisms, including PON2 Cys<sup>311</sup>  $\rightarrow$  Ser. PCR primers for amplifying the sequences containing the SNP were designed using Primer 3. The PCR primers are listed in the [supplemental materials](#). Sense and antisense probes used for fluorescence polarization-single base extension detection of SNPs were designed using Primer Premier. The sense and antisense probes for genotyping the PON2 Cys<sup>311</sup>  $\rightarrow$  Ser SNP are: 5'-CTG TAG TCA CTG TAG GCT TCT CA-3' and 5'-CCG CAT CCA GAA CAT TCT AT-3', respectively. Each genotyping reaction consisted of a polymerase chain reaction followed by a single base pair extension reaction using either the sense or antisense probe and dideoxynucleotides labeled with carboxytetramethylrhodamine (TAMRA) or R110 for the alternative alleles. The fluorescence signal was detected by fluorescence polarization using the analyst HT (Molecular Devices, Sunnyvale, CA), and genotypes

were determined based on the plot of TAMRA *versus* R110 signal values.

**Statistical Analysis**—All of the experiments were performed in at least triplicate, and the data are presented as the means  $\pm$  S.E. of the mean (S.E.). Comparisons between two groups were made with Student's *t* test.

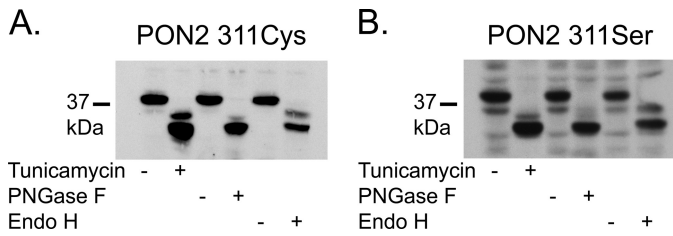
## RESULTS

**Cysteine Substitution at Amino Acid Position 311 Impairs PON2 Lactonase Activity**—We cloned PON2 and asked whether mutating the 311 amino acid, from a serine to cysteine, would affect PON2 lactonase activity. Lysates from CHO cells expressing both PON2 Cys<sup>311</sup> and

Ser<sup>311</sup> inactivated 3OC12-HSL over time, but PON2 Cys<sup>311</sup> lysates had an impaired ability compared with PON2 Ser<sup>311</sup> lysates (Fig. 1A). These data suggest that cysteine substitution at amino acid 311 impairs PON2 function through mechanisms other than alternative splicing, linkage disequilibrium, or promoter differences.

To test whether the PON2 Cys<sup>311</sup> variant expresses similar PON2 protein levels as PON2 Ser<sup>311</sup>, PON2 immunoblotting was performed in CHO cell lysates 48 h following transfection. Fig. 1B shows that in PON2 Cys<sup>311</sup> samples we observed a single immunoreactive band migrating slightly higher than 37 kDa, a molecular mass consistent with PON2. In PON2 Ser<sup>311</sup> samples, we also observed a similarly sized upper band as in PON2 Cys<sup>311</sup>, but noted an additional band with greater electrophoretic mobility several kDa lower than the upper PON2 band. Densitometric analysis and quantification showed no difference in total PON2 protein levels between PON2 Cys<sup>311</sup> and Ser<sup>311</sup> groups (Fig. 1C). These findings suggest that the 311 amino acid change does not alter PON2 protein stability. The smaller, second band could result from alternative splicing (although this seems highly unlikely to occur using cDNA) (2), from PON2 aggregation (24), or most likely from differential post-translational modification or proteolysis.

**An Altered Core Glycosylation Pattern Occurs in the PON2 Cys<sup>311</sup> Variant**—PON2 has four putative *N*-linked glycosylation sites at asparagine residues. Purified PON1 is ~15.8% carbohydrate by weight (25). To investigate the glycosylation of PON2 and determine whether PON2 Cys<sup>311</sup> and Ser<sup>311</sup> are differentially glycosylated, we first expressed PON2 Cys<sup>311</sup> or Ser<sup>311</sup> in CHO cells and then treated with tunicamycin, an antibiotic from the bacterium *Streptomyces iisosuperficus* that inhibits GlcNAc phosphotransferase, one of the initial steps required for glycoprotein synthesis. Two days later, cell lysates were harvested, and Western blot analysis showed that, in PON2 Cys<sup>311</sup> samples, the predominant immunoreactive band following tunicamycin treatment had a greater electrophoretic mobility compared with the PON2 band in untreated cells (Fig. 2A). This finding is consistent with *N*-linked core glycosylation

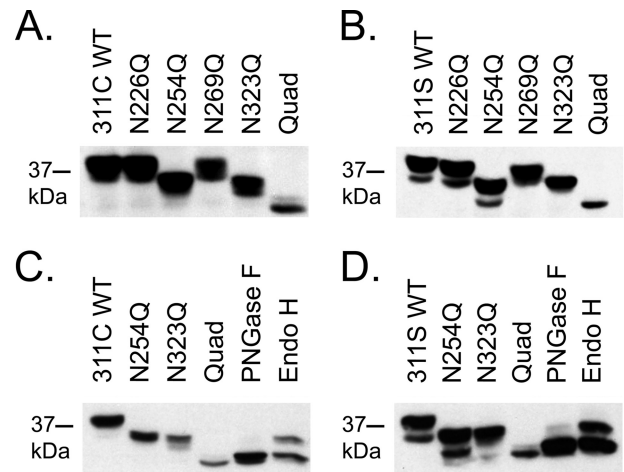


**FIGURE 2. Effect of chemical and enzymatic deglycosylation of PON2.** CHO cells were transfected with a plasmid expressing human PON2 Cys<sup>311</sup> (A) or PON2 Ser<sup>311</sup> (B). Tunicamycin was added shortly after transfection, and cell lysates were harvested 48 h later. In separate experiments, enzymatic deglycosylation with either PNGase F or Endo H, as described under "Experimental Procedures," was also performed on cell lysates 36–48 h following transfection.

of PON2. Similar findings were observed with PON2 Ser<sup>311</sup> (Fig. 2B). After tunicamycin treatment there was no difference in the appearance of the PON2 band between Cys<sup>311</sup> and Ser<sup>311</sup> variants. This suggests that the lower band in PON2 Ser<sup>311</sup> samples represents differential or incomplete glycosylation compared with the upper band in PON2 Cys<sup>311</sup> and Ser<sup>311</sup>.

Because tunicamycin has a number of effects on the cell including induction of the unfolded protein response, we also treated cell lysates with PNGase F at 48 h following transfection. PNGase F is an enzyme that cleaves *N*-linked glycoproteins between the innermost GlcNAc and asparagine residues. *N*-Linked deglycosylation with PNGase F caused both PON2 Cys<sup>311</sup> and Ser<sup>311</sup> migration patterns to change as observed with tunicamycin treatment, and after PNGase F treatment there was no difference between the Cys and Ser variants (Fig. 2). PON2 was also sensitive to treatment with Endo H, an endoglycosidase that cleaves the chitobiose core of high mannose and oligosaccharides from *N*-linked glycoproteins. When proteins are correctly processed through the endoplasmic reticulum and Golgi complex, they become resistant to Endo H. Therefore sensitivity to Endo H indicates the presence of proteins that have not been processed beyond the ER. We observed qualitatively similar results when comparing Endo H, tunicamycin, and PNGase F treatments in both PON2 Cys<sup>311</sup> and Ser<sup>311</sup> samples (Fig. 2). From these results, we conclude that PON2 undergoes *N*-linked core glycosylation, and incomplete glycosylation is likely responsible for the additional band observed when PON2 Ser<sup>311</sup> is expressed in CHO cells.

**PON2 Undergoes *N*-Linked Core Glycosylation at Asparagine Residues 254 and 323**—Based on the two different bands of glycosylated PON2, we predicted that at least two of the four putative *N*-linked glycosylation sites are glycosylated in PON2. To determine which asparagine residues of the Asn-Xaa-(Ser/Thr) consensus sequence are *N*-glycosylated in PON2 Cys<sup>311</sup> and Ser<sup>311</sup> variants, putative *N*-linked glycosylation sites were disrupted by substituting glutamine for asparagine individually and also in combination (quadruple mutant). Western blotting for PON2 revealed one immunoreactive band in wild-type PON2 Cys<sup>311</sup> and the N226Q and N269Q mutants. Cell lysates from the N254Q and N323Q mutants also showed one PON2 immunoreactive band, but with a greater electrophoretic mobility than wild-type PON2 Cys<sup>311</sup> (Fig. 3A). Lysate from the quad mutant had a PON2 immunoreactive band with a smaller molecular mass than either N254Q or N323Q. This shows that,



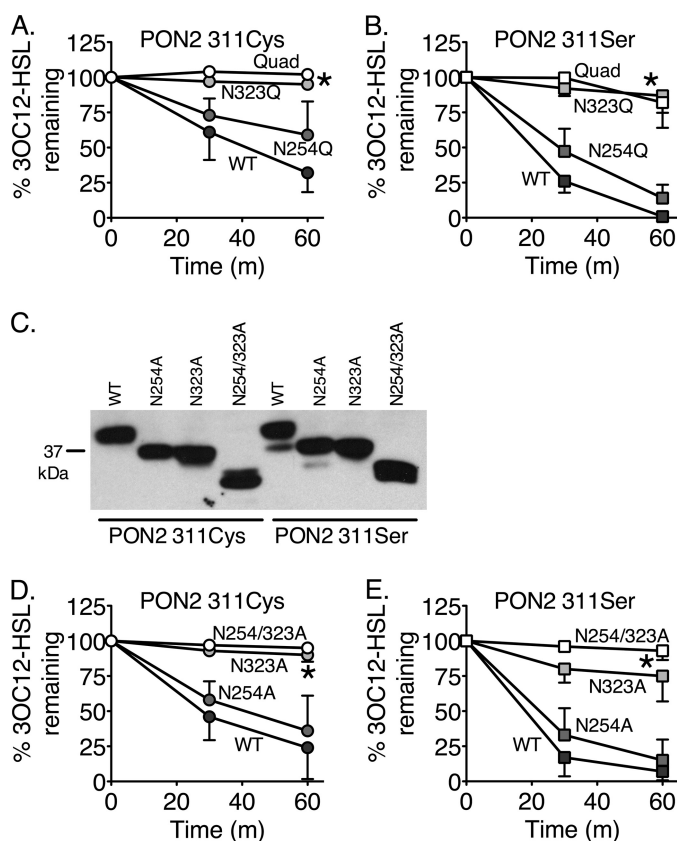
**FIGURE 3. Effect of asparagine to glutamine site-specific mutations in predicted *N*-linked glycosylation sites of PON2.** CHO cells expressing wild-type (WT) and glutamine site-specific glycosylation mutants of PON2 Cys<sup>311</sup> (A) and PON2 Ser<sup>311</sup> (B) were lysed, electrophoresed, and analyzed by Western blot analysis for PON2 immunoreactive bands. PON2 Cys<sup>311</sup> (C) and PON2 Ser<sup>311</sup> (D) immunoblots following site-directed mutagenesis and PNGase F or Endo H treatment are shown.

for PON2 Cys<sup>311</sup>, two of the four putative *N*-linked glycosylation sites undergo oligosaccharide addition: asparagine residues 254 and 323.

We next hypothesized that the upper band in PON2 Ser<sup>311</sup> also represents glycosylation at both asparagine 254 and 323, whereas the lower electrophoretic mobility band could be glycosylation at either one of these asparagine residues or a mixture of individually glycosylated PON2 proteins. Alternatively, PON2 Ser<sup>311</sup> could use different asparagine residues for core glycosylation. Similar to the PON2 Cys<sup>311</sup>, only asparagine 254 and 323 were glycosylated (Fig. 3B). The immunoreactive band in both PON2 Cys<sup>311</sup> and Ser<sup>311</sup> quad mutants had a similar molecular mass compared with PON2 treated with PNGase F or Endo H (Fig. 3, C and D). These substitution experiments allowed us to investigate which site is glycosylated in the lower band of PON2 Ser<sup>311</sup>. The N254Q mutant had an unglycosylated band as well as an intermediate glycosylated band, suggesting that the lower PON2 Ser<sup>311</sup> band is glycosylated at asparagine 254. This is confirmed by the finding that the N323Q substitution results in a single intermediate band (Fig. 3B). Substitution at asparagine 323 caused loss of the upper band, but the lower band remained in place. These data show that the upper band in PON2 Cys<sup>311</sup> and Ser<sup>311</sup> is glycosylated at asparagine 254 and 323, whereas the lower band in PON2 Ser<sup>311</sup> is only glycosylated at asparagine 254.

**Glycosylation at Asparagine Residue 323 Is Required for PON2 Lactonase Function**—We hypothesized that differential glycosylation of PON2 Cys<sup>311</sup> and Ser<sup>311</sup> accounts for the differences in lactonase activity between the 311 variants, so we initially asked whether glycosylation is important for PON2 inactivation of 3OC12-HSL. Lysates from CHO cells transfected with the PON2 Cys<sup>311</sup> or Ser<sup>311</sup> with the N254G mutation had preserved 3OC12-HSL lactonase activity (Fig. 4, A and B). We expected that if the lower electrophoretic band, only glycosylated at asparagine 254, in wild-type PON2 Ser<sup>311</sup> accounts for its enhanced 3OC12-HSL inactivation, then

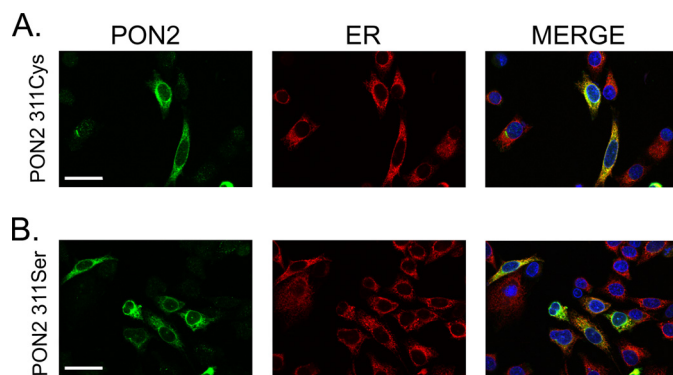
## PON2 311 SNP Impairs Lactonase Activity



**FIGURE 4. Effect of mutating N-linked glycosylation sites on lactonase activity.** A and B, PON2 Cys<sup>311</sup> (A) or PON2 Ser<sup>311</sup> (B) transfected CHO cell lysates from either wild-type (WT), N254Q, N323Q, or N226/254/269/323G (Quad) mutants were tested for 3OC12-HSL inactivating ability. Lysates (20% volume) of CHO cell cultures were incubated with 10  $\mu$ M 3OC12-HSL and after 0, 30, or 60 min of incubation the lysate samples were obtained for measurement of inactivation of 3OC12-HSL. The data are expressed as the percentages of active 3OC12-HSL remaining compared with initial levels. \* denotes  $p < 0.05$  for lactonase activity between wild-type samples and N323Q and quad mutant. C, CHO cells expressing wild-type and alanine site-specific glycosylation mutants of PON2 Cys<sup>311</sup> and PON2 Ser<sup>311</sup> were lysed, electrophoresed, and analyzed by Western blot analysis for PON2 immunoreactive bands. D and E, PON2 Cys<sup>311</sup> (D) or PON2 Ser<sup>311</sup> (E) transfected CHO cell lysates from either wild-type, N254A, N323A, or N254A/N323A mutants were tested for 3OC12-HSL inactivating ability. Lysates (20% volume) of CHO cell cultures were incubated with 10  $\mu$ M 3OC12-HSL; after 0, 30, or 60 min of incubation the lysate samples were obtained for measurement of inactivation of 3OC12-HSL. The data are expressed as the percentages of active 3OC12-HSL remaining compared with initial levels. The data are the means  $\pm$  S.E. \* denotes  $p < 0.05$  for lactonase activity between wild-type samples and N323A and double mutant.

glycosylation at asparagine 323 must inhibit PON2 lactonase activity. In contrast, we found that the N323Q mutant had impaired lactonase activity, showing that glycosylation at asparagine 323 is important for PON2 function. Similarly, lysates from CHO cells transfected with the PON2 Cys<sup>311</sup> or Ser<sup>311</sup> quad mutant (N226Q/N254Q/N269Q/N323Q) were unable to degrade 3OC12-HSL, also showing that glycosylation might be important for PON2 3OC12-HSL inactivation.

Mutation of asparagine residues to glutamine could have other effects on PON2 structure and activity besides disrupting N-linked glycosylation. Therefore, we constructed additional mutants of the PON2 Cys<sup>311</sup> and Ser<sup>311</sup> variants by replacing asparagine with alanine individually at positions 254 and 323, as well as a double mutant, N254A/N323A. Compared with the glutamine mutants, we found similar patterns of PON2 immu-



**FIGURE 5. Immunohistochemical localization of PON2 Cys<sup>311</sup> and Ser<sup>311</sup> variants.** CHO cells were transiently transfected with a plasmid encoding human PON2 Cys<sup>311</sup> or Ser<sup>311</sup>. 24 h later cells were fixed, stained with human anti-PON2 and an ER-specific antibody, and examined with confocal microscopy. A, PON2 Cys<sup>311</sup>-expressing CHO cells. B, PON2 Ser<sup>311</sup>-expressing CHO cells. Scale bar, 30  $\mu$ m.

noreactive bands in the N254A, N323A, and N254A/N323A mutants (Fig. 4C). In addition, the N323A and N254A/N323A mutants had an impaired ability to inactivate 3OC12-HSL (Fig. 4, D and E). From these data we conclude that: 1) PON2 undergoes N-linked core glycosylation at asparagine residues 254 and 323; 2) the upper band in PON2 Ser<sup>311</sup> is glycosylated at asparagine 254 and 323, whereas the lower band is only glycosylated at asparagine 254; 3) glycosylation at asparagine 323 is required for PON2 lactonase activity; and 4) differential glycosylation between the PON2 Cys<sup>311</sup> and Ser<sup>311</sup> variants does not account for greater lactonase activity by PON2 Ser<sup>311</sup>.

**PON2 Localizes to the Endoplasmic Reticulum**—Our data show that PON2 is Endo H-sensitive and does not undergo complex glycosylation,<sup>4</sup> suggesting that PON2 resides in the ER. Moreover, recent work by Horke *et al.* (26) demonstrated PON2 localization to the ER in endothelial cells. To test for changes in PON2 cellular localization as a mechanism of disrupted lactonase activity between PON2 Cys<sup>311</sup> and Ser<sup>311</sup> variants, immunohistochemical staining for PON2 was performed in CHO cells following transfection with either PON2 Cys<sup>311</sup>- or Ser<sup>311</sup>-expressing plasmids. A similar pattern of staining for both PON2 Cys<sup>311</sup> and Ser<sup>311</sup> was observed with the most intense staining corresponding to an ER location (Fig. 5).

**Genotypic Analysis of Human Airway Donor Samples for PON2 S311C SNP**—To test whether the PON2 311 SNP also affects lactonase activity *in vivo*, we asked whether naturally occurring PON2 mutations at position 311 would affect the ability of primary human airway epithelial cells to degrade 3OC12-HSL. We first isolated DNA from 200 human donor lung samples and performed genotyping by polymerase chain reaction allele-specific oligonucleotide hybridization assays for the PON2 S311C polymorphism and a number of other common PON1, PON2, and PON3 allelic variants including coding (PON1 Q192R, PON1 L55M, PON3 C133A, and PON3 G99A) and promoter (PON1 G-907C) polymorphisms (supplemental Table S1). For PON2, we found that 62% of human donor samples were homozygous for Ser/Ser, 35% heterozygous for Ser/Cys, and 3% homozygous for Cys/Cys at amino acid 311 (Fig.

<sup>4</sup> D. A. Stoltz, T. J. Recker, and J. Zabner, unpublished data.

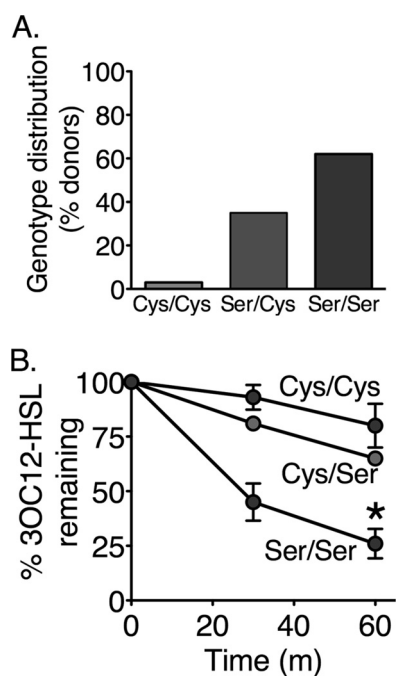


FIGURE 6. Impaired airway lactonase activity in humans homozygous for cysteine at amino acid 311 in PON2. *A*, percentage of human subjects with genotype of Cys/Cys, Cys/Ser, or Ser/Ser at amino acid position 311 in PON2. *B*, 3OC12-HSL degradation by human airway epithelia.  $n = 5$  human donor samples for Ser/Ser, 2 for Ser/Cys, and 4 for Cys/Cys. Lysates (20% volume) of human airway epithelial cell cultures were incubated with  $10 \mu\text{M}$  3OC12-HSL, and after 0, 30, or 60 min of incubation the lysate samples were obtained for measurement of 3OC12-HSL inactivation. The data are expressed as the percentages of active 3OC12-HSL remaining compared with initial levels. The data are the means  $\pm$  S.E. \*,  $p < 0.005$  comparing lactonase activity between Cys/Cys and Ser/Ser donor epithelia.

6A). These findings are consistent with the HapMAP-CEU European SNP data base showing 57% of individuals homozygous for Ser/Ser, 38% heterozygous for Ser/Cys, and 5% homozygous for Cys/Cys.

*Human Airway Epithelial Cells from PON2 Cys/Cys<sup>311</sup> Donors Have an Impaired Ability to Inactivate 3OC12-HSL*—To test whether differences in the common PON2 311 variant affect airway epithelia inactivation of 3OC12-HSL, human airway epithelial cells from four individuals homozygous for cysteine, five homozygous for serine, and two heterozygous for cysteine/serine at amino acid position 311 were grown at the air-liquid interface. We chose these donors based upon their PON2 311 SNP and similarities in other described SNPs for PON1 and PON3. Lysates from airway epithelial cells homozygous for serine degraded 3OC12-HSL with  $\sim 25\%$  active 3OC12-HSL remaining after 60 min of exposure (Fig. 6B). In contrast, airway epithelial cell samples from donors homozygous for cysteine had an impaired ability to inactivate 3OC12-HSL, and samples from heterozygotes (Ser/Cys) demonstrated an intermediate phenotype for 3OC12-HSL inactivation compared with Ser/Ser and Cys/Cys samples (Fig. 6B). These data show that the common PON2 311 polymorphism alters the endogenous ability of human airway epithelia to degrade 3OC12-HSL.

## DISCUSSION

The human PON2 S311C polymorphism is common and has been linked to several disorders including coronary artery dis-

ease, abnormal plasma lipoprotein levels, ischemic stroke, and Alzheimer dementia (2, 7, 27–31), but the mechanisms underlying these potential associations remain unknown. The serine allele is ancestral, has relatively little variation across human populations (Single Nucleotide Polymorphism database (dbSNP) data), and is strongly conserved in evolution. We hypothesized that the cysteine for serine substitution at amino acid position 311 in PON2 would disrupt PON2 lactonase activity. We found that the PON2 Cys<sup>311</sup> variant had an impaired ability to inactivate 3OC12-HSL. More importantly, human airway epithelial cells from Cys/Cys homozygous individuals had an impaired ability to degrade 3OC12-HSL, compared with cells from Ser/Ser homozygous individuals. The impaired lactonase activity by Cys/Cys airway epithelia was not due to changes in promoter activity, alternative splicing, or linkage disequilibrium with other PON SNPs or genes because transfection of CHO cells with a plasmid expressing PON2 Cys<sup>311</sup> also showed similar results as primary human airway epithelia.

Linkage studies of monogenic diseases have resulted in the identification of genes responsible for the pathogenesis of several diseases including cystic fibrosis and Huntington disease. In contrast, association studies of complex genetic diseases often result in the identification of SNPs that may represent false positives or linkage to other genetic mutations. This becomes particularly troubling when the SNPs are found in proteins whose function are poorly understood, when the effect of the SNP on protein function is not known, and when there is no obvious physiologic connection between the gene and the pathogenesis of the studied disease.

The identification of a physiologic consequence of a SNP becomes important in understanding association studies. A great example is the work of Richter and Furlong (32) in their studies determining the “PON1 status” of an individual. These investigators use a functional genomic approach and are able to directly measure, from plasma samples, the PON1 function of an individual based upon hydrolysis of both paraoxon and diazoxon. By using this method, any polymorphisms that might affect PON1 function are taken into account. In addition, hydrolysis of diazoxon quantifies the PON1 levels of an individual, allowing for comparisons in PON1 function to be determined. This assay allows for accurate identification of PON1 phenotype, predicts PON1 E192R status (Glu/Glu, Glu/Arg, or Arg/Arg), and can account for any other yet unidentified SNPs or those changes that may be secondary to a second SNP in linkage disequilibrium with the SNP of interest.

This functional genomic approach was utilized by Jarvik *et al.* (33) to study the effects of PON1 polymorphisms on risk of vascular disease, an association that has been inconsistently linked. Examination of PON1 192 or 55 genotypes alone failed to predict the risk of vascular disease of the carotid arteries. However, when genotype status was combined with the rates of substrate analysis, as described above, vascular disease risk was predicted accurately, thus demonstrating the importance of including a functional analysis in association studies. A similar approach was used to test organophosphate hydrolysis by subjects with sporadic amyotrophic lateral sclerosis (ALS) (34). As with vascular disease, discrepant results regarding a linkage

## PON2 311 SNP Impairs Lactonase Activity

between PON1 polymorphisms and ALS exist. Wills *et al.* (34) reported an increased frequency of the PON1 Arg/Arg<sup>192</sup> variant in ALS patients but similar levels of organophosphate hydrolysis by plasma samples from control and ALS patients. These findings were important because they suggested that if a linkage does exist between the PON1 192 SNP and ALS, etiologies other than organophosphate toxicity are responsible for the association, including disturbances in metabolism of other yet unidentified toxic substances or an effect secondary to linkage disequilibrium with either additional PON variants or other proteins.

Application of this functional genetic analysis for PON2 is more difficult because PON2 is primarily an intracellular enzyme, as opposed to PON1, which is found in the serum, and no simple assay exists to detect a functional consequence of the PON2 311 SNP in human samples. Furthermore, the natural substrate and *in vivo* physiological function of PON2 are not yet fully elucidated. However, by performing genetic analysis of donor human lung samples and testing 3OC12-HSL degradation by human airway epithelia, we were able to show that the Cys allele at PON2 311 impairs lactonase activity. The *in vivo* physiological consequence of this effect remains to be determined, but our previous data showing that PON2 deficiency in airway epithelial cells impairs the ability of these cells to inactivate 3OC12-HSL suggest that despite the intracellular location of PON2, it may be important in the host response to quorum sensing-dependent bacteria.

Differing molecular masses of PON2 immunoreactive bands between the PON2 311 alloenzymes were observed, so we investigated the *N*-linked glycosylation pattern of PON2. In our study, tunicamycin, Endo H, and PNGase F treatment of PON2 Cys<sup>311</sup> and Ser<sup>311</sup> showed that PON2 undergoes *N*-linked core oligosaccharide addition and that following deglycosylation PON2 Cys<sup>311</sup> and Ser<sup>311</sup> immunoreactive bands appeared similar in molecular mass. Using directed evolution, Harel *et al.* (35) reported a crystal structure of PON1 and suggested that of the four corresponding PON1 asparagine residues, 254 and 323 are most likely core glycosylated because of their location on surface loops. Asparagine 226 and 269 are in the central tunnel of the  $\beta$ -propeller and are predicted not to be glycosylated. These predictions are in agreement with our findings. We found that PON2 Cys<sup>311</sup> has a single molecular mass protein glycosylated at asparagine residues 254 and 323, whereas PON2 Ser<sup>311</sup> had one isoform identical to PON2 Cys<sup>311</sup> (glycosylated at asparagine 254 and 323) and another with a smaller molecular mass only glycosylated at 323. Based upon our findings and others (15, 36, 37), it appears that differential glycan processing occurs between PON family members and that differential *N*-linked glycosylation occurs between the PON2 311 alloenzymes. Furthermore, even though we showed that glycosylation is important for PON2 3OC12-HSL degradation, it does not explain the differences seen in PON2 Cys<sup>311</sup> lactonase activity.

Although we hypothesized that glycosylation at asparagine 323 would inhibit PON2 activity (based upon the differential glycosylation pattern and lactonase activity between PON2 Cys<sup>311</sup> and Ser), this was not the case. Several possible explanations exist for both differential glycosylation and lactonase activity between PON2 Cys<sup>311</sup> and Ser<sup>311</sup>. First, although

unlikely, the Cys<sup>311</sup> mutation may affect PON2 function, which in turn affects the glycosylation pathway of other proteins. Second, changes in glycosylation and PON2 function may be completely unrelated. Finally, whereas differential glycosylation may not account for the impaired ability of the PON2 Cys<sup>311</sup> airway epithelia to degrade 3OC12-HSL, the two findings might be related. We hypothesize that the most likely underlying mechanism common to both findings is due to differences in disulfide bond formation. This might result from changes in which cysteine residues undergo disulfide bond formation, changing the number of disulfide bonds formed, or changes in which intermolecular disulfide bonds between cysteine residues of different PON2 molecules occur. For example, the free cysteine residue at position 284 in PON1 is required for its lactonase activity (38), and disruption of PON1 disulfide-linked Cys residues 41 or 352 impaired catalytic activity (39). Similarly, we found that treatment of both PON2 Cys<sup>311</sup> and Ser<sup>311</sup> with the reducing agent dithiothreitol (10 mM) impaired lactonase activity (data not shown). These data also suggest that disulfide bonds are important for PON2 activity. Alternatively, an altered protein structure or differences in substrate binding to the PON2 active site might account for the phenotype of PON2 Cys<sup>311</sup> mutants. Therefore, it appears that the altered glycosylation profile observed between the PON2 311 alloenzymes is a marker of altered lactonase activity but is not the mechanism for this difference.

In summary, we found that human airway epithelia homozygous for PON2 Cys<sup>311</sup> have an impaired ability to inactivate 3OC12-HSL. There have been few successes to date in identifying the specific etiologic variant influencing a common disease when an association has been confirmed (40), mostly because of inadequate functional assay testing. This is the first time for PON2 that a functional consequence for the 311 SNP has been demonstrated in humans. Moreover, these findings suggest three important speculations. First, infections with quorum sensing-dependent bacteria should have a differential phenotype in patients with the PON2 Cys<sup>311</sup> variant. Second, diseases that associate genetically with this PON2 311 SNP may have an infectious etiology. Third, some of these diseases may be caused by an as yet unidentified metabolic pathway that requires lactonase activity.

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