Antioxidative properties of paraoxonase 2 in intestinal epithelial cells

Louis-Philippe Précourt,1 Valérie Marcil,2 Thierry Ntimbane,1 Rame Taha,1 Jean-Claude Lavoie,2 Edgard Delvin,3,4 Ernest G. Seidman,3,5 Jean-François Beaulieu,5 and Emile Levy1,5

1Department of Nutrition, Research Centre, CHU-Sainte-Justine, Université de Montréal, Montreal, Quebec, Canada; 2Research Institute, McGill University, Montreal, Quebec, Canada; 3Department of Pediatrics, Research Centre, CHU-Sainte-Justine, Université de Montréal, Montreal, Quebec, Canada; 4Department of Biochemistry, Research Centre, CHU-Sainte-Justine, Université de Montréal, Montreal, Quebec, Canada; and 5Canadian Institutes for Health Research Team on the Digestive Epithelium, Department of Anatomy and Cellular Biology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Quebec, Canada

Submitted 3 February 2012; accepted in final form 19 June 2012

Précourt LP, Marcil V, Ntimbane T, Taha R, Lavoie JC, Delvin E, Seidman EG, Beaulieu JF, Levy E. Antioxidative properties of paraoxonase 2 in intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 303: G623–G634, 2012. First published June 28, 2012; doi:10.1152/ajpgi.00039.2012.—Paraoxonase (PON) family members seem central to a wide variety of human illnesses, but appreciation of their antioxidative function in the gastrointestinal tract is in its infancy. The major objective of the present work is to highlight the role of the ubiquitously expressed PON2 in the small intestine. With use of pLKO lentiviral vector containing short hairpin RNA (shRNA) lentivirus, PON2 expression was knocked down in intestinal Caco-2/15 cells, where antioxidative status, lipid peroxidation, and degree of inflammation were evaluated. As a consequence of PON2 inactivation in the epithelial cells, we observed 1) imbalanced primary and secondary antioxidative responses, characterized by increased superoxide dismutases and decreased catalase, 2) high concentrations of H2O2 and malondialdehyde, along with low glutathione-to-glutathione disulfide ratio, 3) upregulation of TNF-α, IL-6, and monocyte chemotactic protein-1 gene expression after induction of oxidative stress, and 4) raised level of the activation of transcription factor NF-κB, which was likely implicated in exacerbation of the inflammatory activation. These results suggest that PON2 is involved in the antioxidative and anti-inflammatory response in intestinal epithelial cells.

paraoxonase; small intestine; oxidative stress; inflammation; lipid peroxidation

IN NORMAL PHYSIOLOGICAL CONDITIONS, reactive oxygen species (ROS) are neutralized by numerous endogenous and nutritional antioxidant molecules. Thus antioxidants appear vital for maintaining an oxidative balance (14). A decrease in antioxidant defenses or an excess in ROS can lead to major damage to lipids, DNA, and proteins. Oxidative stress has been recognized as an important contributing factor for many diseases, such as cancer, atherosclerosis, cystic fibrosis, Alzheimer’s disease, and Parkinson’s disease (6, 18, 41).

In the gastrointestinal tract, oxidative stress and inflammation are important processes highly implicated in many disorders (13). The intestinal mucosa is continuously exposed to oxidants from ingested food and metabolically active flora (20). Inflammatory bowel diseases (IBD), including Crohn’s disease (CD) and ulcerative colitis (UC), are generally thought to have multifactorial etiologies, such as genetic predisposition, immune system dysregulation, oxidative stress, and various environmental factors. Nutritional antioxidants and endogenous antioxidant enzymes can contribute to the prevention of loss of intestinal homeostasis by controlling ROS levels. Oxidative stress is even thought to be directly involved in the etiology of IBD (22).

Paraoxanes (PONs) are relatively newly identified antioxidant enzymes that consist of three members (PON1, PON2, and PON3), located near each other on the long arm of chromosome 7 (7q21.3–q22.1) (34). All members are believed to be powerful antioxidants, and research has particularly focused on their atheroprotective potential (31, 42). PON1, mainly synthesized by the liver, is by far the most studied member of the three-gene family because of its association with HDL particles in the blood circulation and its protective effects against LDL oxidation and atherosclerosis development (39). PON3 is similar to PON1 in terms of function and location, as both are associated with HDL and show the capacity to delay LDL oxidation in vitro (24). PON2 is expressed in all human tissues and is not associated with HDL in the blood circulation. It appears to be cell-based and, thus, is a good candidate for preventing oxidative stress locally within cells (16). Despite the growing interest in PON2, there is little information about its functions and characteristics in the gastrointestinal system.

PONs have been identified throughout the digestive tract in humans. Moreover, oxidative stress and inflammation selectively affect the expression of PONs, which suggests their potential implication in IBD (33). PON1 activities have been found to be reduced in the serum of patients with active CD (4) and UC (3). PON1 and PON3 gene expression was also decreased in colonic samples obtained from CD and UC patients (36). Moreover, PON1 and PON2 polymorphisms have been shown to protect against the development of IBD (37). PON2 may be particularly instrumental in fighting the potentially proinflammatory flora and prooxidant diet that challenge the intestinal epithelium, since the addition of purified PON2 to permeabilized intestinal Caco-2/15 cells protected against iron/ascorbate (Fe/Asc)-induced oxidative stress (23). However, whether PON2 is an antioxidant or anti-inflammatory player in the digestive tract remains unclear. To demonstrate the physiological and biological functions of PON2 in the intestine, molecular strategies were applied to knock down PON2 expression in the human intestinal Caco-2/15 cell line.

The major objective of the present work is to highlight the role of the ubiquitously expressed PON2 in the small intestine by generating a reliable intestinal model of PON2 knockdown (PON2 KD). We thus evaluated how the inactivation of PON2...
influences intestinal cell integrity by assessing differentiation and tight junction markers, expression and activity of the classical endogenous antioxidant enzymes, extent of oxidative stress, and inflammatory response. We also attempt to evaluate the status of intestinal PON2 expression in patients with CD. In fact, the involvement of PON2 in the antioxidative defense and anti-inflammatory response in the intestinal cell would suggest a potential role for PON2 in pathophysiological conditions such as IBD.

**MATERIALS AND METHODS**

**Cell culture.** Caco-2/15 cells were grown at 37°C with 5% CO2 in MEM, as described previously (21, 23, 26, 27, 33). PON2 gene expression was silenced by infection of these intestinal cells with lentivirus by a procedure also described in our previous studies (21, 23, 26, 27, 33).

**Generation of a stable shRNA-expressing Caco-2/15 cell line.** Exponentially growing 293FT cells were transiently infected with pGFP-V-RS vectors (Origene Technologies, Rockville, MD), allowing...

---

### Table 1. Primers used for PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forwar d (5'-3')</th>
<th>Reverse (5'-3')</th>
<th>Amplicon, bp</th>
<th>Annealing Temperature, °C</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>AGAAGGCTGGGGCTGATT</td>
<td>GGGGCACTGCGAACGCTCTT</td>
<td>256</td>
<td>58</td>
<td>21</td>
</tr>
<tr>
<td>PON1</td>
<td>GCTAATGGACTGGCGTTTCA</td>
<td>ACTGAGCAGCAGTGGGTC</td>
<td>242</td>
<td>58</td>
<td>38</td>
</tr>
<tr>
<td>PON2</td>
<td>TGATCTAGCAATATGAGTCA</td>
<td>GTCTCCTGCGACGATAGGTA</td>
<td>212</td>
<td>58</td>
<td>25</td>
</tr>
<tr>
<td>PON3</td>
<td>TTCTTGACCTGGCTGGACT</td>
<td>GCATGACATGCGCACTCTT</td>
<td>216</td>
<td>58</td>
<td>30</td>
</tr>
<tr>
<td>SOD-1</td>
<td>AGGGGATGACATGATTTTCA</td>
<td>AGATGTCGACAGTCTCCAC</td>
<td>217</td>
<td>58</td>
<td>23</td>
</tr>
<tr>
<td>SOD-2</td>
<td>CAGCTGCTCAGACTCTGAG</td>
<td>TGACGCGACCATGAGATTTT</td>
<td>198</td>
<td>58</td>
<td>22</td>
</tr>
<tr>
<td>SOD-3</td>
<td>GCTGACATGGCTGGCTGGAG</td>
<td>AGATGTCGACAGTCTCCACT</td>
<td>181</td>
<td>58</td>
<td>30</td>
</tr>
<tr>
<td>G-Red</td>
<td>CATGGGAGTGGTGGGAGGAAAGAT</td>
<td>AAACCCGACGAGTCTTT</td>
<td>205</td>
<td>58</td>
<td>27</td>
</tr>
<tr>
<td>G-Px</td>
<td>TCGCTCTGAGCGCAAACCG</td>
<td>AGAGGCTGCCAAATGAGG</td>
<td>151</td>
<td>58</td>
<td>28</td>
</tr>
<tr>
<td>CAT</td>
<td>GCCTGAGGAGGACCAATTTATCTTT</td>
<td>GAATGTCGACAGTCTCCACT</td>
<td>203</td>
<td>58</td>
<td>23</td>
</tr>
<tr>
<td>IL-6</td>
<td>GAGGACATGGGAGAAGAAGAA</td>
<td>TGGGAGTGGTGGTGTTGGT</td>
<td>274</td>
<td>60</td>
<td>38</td>
</tr>
<tr>
<td>IL-8</td>
<td>CTGGCGGCAACACAGAAAATTA</td>
<td>ATTCGAGTGCCAGACCTCCACT</td>
<td>238</td>
<td>58</td>
<td>37</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TGCTTGTTCCTGACGCTTCT</td>
<td>ATCCGAAATGAGACCTGCCCC</td>
<td>602</td>
<td>58</td>
<td>38</td>
</tr>
<tr>
<td>MCP-1</td>
<td>GCCAGACGTACGCTGGTTATAT</td>
<td>GAATTGCGGCTGGTCTGGT</td>
<td>201</td>
<td>58</td>
<td>35</td>
</tr>
<tr>
<td>Occludin</td>
<td>GAAGAGACACCTGGTGGAGGGCC</td>
<td>GAAGAGACATGCTGGTCTGGT</td>
<td>229</td>
<td>58</td>
<td>30</td>
</tr>
<tr>
<td>Villin</td>
<td>CAAGACAGGCTCACTCAGCAA</td>
<td>TGCTCATGAGGACGCTGCTGG</td>
<td>203</td>
<td>58</td>
<td>27</td>
</tr>
</tbody>
</table>

**Quantitative PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
<th>Gapdh</th>
<th>Pon2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON2</td>
<td>TGATCATGCAATGAGAATCTT</td>
<td>TGGACTCAGGACGCTGCTGG</td>
<td>108</td>
<td>21</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AAACCGCTCGACGAGTCTTT</td>
<td>TGGACTCAGGACGCTGCTGG</td>
<td>108</td>
<td>21</td>
</tr>
</tbody>
</table>

PON1, PON2, and PON3, paraoxonase 1, 2, and 3; G-Red, glutathione reductase; G-Px, glutathione peroxidase; MCP-1, monocyte chemoattractant protein-1; SOD, superoxide dismutase; CAT, catalase.

**Fig. 1.** Paraoxonase 2 (PON2) knockdown (KD) in Caco-2/15 cells. Cells were infected with lentiviruses containing anti-PON2 short hairpin RNA (shRNA) and then trypsinized, harvested, and treated with Fe/ascorbate. A and B: semiquantitative PCR and Western blot analysis of gene and protein expressions, respectively. Cells with pLKO.1 empty vector (Mock) and cells that were not infected with lentivirus by a procedure also described in our previous studies (21, 23, 26, 27, 33). Values are means ± SE of 3 independent experiments, each performed in triplicate. **P < 0.01 vs. CTRL.
ing stable delivery of the short hairpin RNA (shRNA) expression cassette against PON2 into host cells via a replication-deficient lentivirus. Upon transient transfection of the plasmids into the packaging cell line, replication-deficient viruses were obtained and used to infect target Caco-2/15 cells. At 48 h postinfection, cells were transferred to flasks and grown in Dulbecco’s modified Eagle’s medium containing 1 μg/ml puromycin (Sigma-Aldrich) for 7 days to establish stable shRNA-expressing Caco-2/15 cells. Thereafter, cells were cultivated until 10 days postconfluence and used only following the validation of suppression of the gene of interest by semiquantitative PCR and Western blot assays. The medium was refreshed every 2nd day (26–28, 33).

PON2 KD and cell integrity. Caco-2/15 cells were plated at a density of 1 × 10⁶ cells/well in a six-well plate containing 2 ml of MEM + 1% penicillin-streptomycin, 1% MEM nonessential amino acids, and Polybrene (4 μg/ml). They were supplemented with 10% decomplemented FBS and then infected with 3 ml of lentiviral stock solution. After 3 days of incubation, cells were trypsinized 1:3 in a six-well plate, and selection was started with addition of puromycin (2 μg/ml) to establish a stable PON2 KD. When cells reached confluence, they were allowed to differentiate for 10–12 days before treatments. The degree of PON2 KD was monitored by semiquantitative PCR and Western blot assay before each experiment. Caco-2/15 cells infected with pLKO.1 empty vector (Mock) served as controls throughout the experiments, since comparable PON2 gene and protein expression has been noted compared with noninfected cells.

Cell integrity was monitored by assessment of gene and protein expression of villin and sucrase (as markers of cell differentiation) (26), as well as occludin and transepithelial resistance (TER) as indicators of cell permeability and tight junction reliability (12).

Human ileal specimens. Endoscopic biopsies from the ileum were obtained from CD patients and healthy human subjects admitted to the Gastroenterology/Hepatology/Nutrition Unit of Hôpital Sainte-Justine for evaluation of gastrointestinal symptoms and screening colonoscopy. The biopsies were taken from macroscopically and histologically normal mucosa. Importantly, clinically indicated supplemental examination did not reveal significant disease in healthy controls, who had colonoscopy with normal macro- and microscopic findings. Biopsies were immediately frozen in liquid nitrogen until they were processed. The study was approved by the Ethics Committee of our institution, and a written informed consent was obtained from each subject before the procedure.

![Fig. 2. PON2 knockdown and cell integrity.](http://ajpgi.physiology.org/ by 10.220.33.6 on June 28, 2017)
Induction of oxidative stress and inflammation. To induce oxidative stress, Caco-2/15 cells were incubated with a mixture of 200 μM iron (Fe; Sigma) and 2 mM ascorbate (Asc; Sigma) for 4 h. Furthermore, inflammation was induced by addition of 100 μg/ml LPS (Sigma) to cells for 24 h. Cells were previously supplemented for 18–20 h with MEM, 1% penicillin-streptomycin, and 1% MEM nonessential amino acids without FBS (33).

RNA isolation and reverse transcription. Total RNA was extracted from differentiated Caco-2/15 cells and intestinal biopsies using TRIzol reagent (Invitrogen), and its amount was determined by spectrophotometer. Moloney murine leukemia virus reverse transcriptase (Invitrogen) was used to obtain cDNA according to the manufacturer’s instructions. Reverse transcription lasted 50 min at 37°C, and a quantity of 3 μg total RNA was added in each reaction.

Semi-quantitative PCR. cDNA was amplified by PCR using a Taq polymerase (Feldan Bio, Quebec, QC, Canada) according to the manufacturer’s instructions. Briefly, 20–40 cycles of amplification were used at 95°C for 30 s, annealing temperature for 30 s, and 72°C for 30 s. Primers and specific conditions are listed in Table 1. PCR was performed using the UNO II thermocycler (Biometra). Amplicons were visualized on standard ethidium bromide-stained agarose gels. The number of amplification cycles corresponds to the linear portion of the exponential phase, as determined in preliminary experiments. Fold induction was calculated using GAPDH as a housekeeping gene, and quantification was determined with the software UN-SCAN-IT gel 6.1.

Quantitative PCR. Quantitative PCRs were performed using the Quantitect SYBR Green kit (Applied Biosystems, Foster City, CA) in a Step One Plus Real-Time PCR System (Applied Biosystems). The RT-PCRs were carried out in 96-well plates with a final volume of 25 μl/well; 12.5 μl of SYBR Green mix (2×) were added to a well containing 25 pmol of the forward and reverse primers and 0.5 μg of cDNA template in a total of 12.5 μl of diethylpyrocarbonate-H2O. Subsequently, negative controls without cDNA were prepared. The reaction of amplification was carried out using 40 cycles. To normalize the different cDNA sample amounts, the housekeeping gene GAPDH was employed as a reference gene. Primers are listed in Table 1. The analyses were performed in triplicate for each gene and for GAPDH in the same plate. The relative mRNA fold changes for GAPDH in the same plate. The relative mRNA fold changes between the animal groups were calculated using the cycle threshold (2−ΔΔCt) method (25).

Western blot analysis. Caco-2/15 cells were collected in mammalian protein extraction reagent (Thermo Fisher Scientific, Rockford, IL) containing a mixture of antiproteases (Roche Diagnostics, Laval, QC, Canada). Cells were sonicated to ensure proper homogenization, and the Bradford assay (Bio-Rad, Mississauga, ON, Canada) was used to determine the protein concentration of each sample. Proteins were denatured in sample buffer containing SDS and β-mercaptoethanol, separated on a 7.5% SDS-polyacrylamide gel, and electroblotted onto nitrocellulose membranes. Nonspecific binding sites of the membranes were blocked using defatted milk proteins, and one of the following primary antibodies was added: rabbit polyclonal anti-PON2 (42 kDa, 1:2,000 dilution; Invitrogen), chicken polyclonal anti-PON3 (35 kDa, 1:4,000 dilution; HyperOmics Farma, Montreal, QC, Canada), mouse monoclonal anti-villin (94 kDa, 1:2,000 dilution; BD Biosciences, Mississauga, ON, Canada), rabbit polyclonal anti-occludin (59 kDa, 1:5,000 dilution; Abcam, Cambridge, MA), goat polyclonal anti-NF-κB p65 (65 kDa, 1:10,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-IκBα (39 kDa, 1:5,000 dilution; Cell Signaling, Beverly, MA), rabbit anti-nuclear factor erythroid-2-related factor 2 (Nrf2, 68 kDa, 1:2,000 dilution; Abcam), rabbit anti-peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α, 92 kDa, 1:5,000 dilution; Abcam), and mouse monoclonal anti-β-actin (42 kDa, 1:250,000 dilution; Sigma). The relative amount of primary antibody was detected with species-specific horseradish peroxidase-conjugated secondary antibody (Jackson Laboratory, Bar Harbor, ME). Expression of β-actin protein was
determined to confirm equal loading. Molecular size markers (Fermentas, Glen Burie, MD) were simultaneously loaded on gels. Blots were developed and the protein mass was quantitated using an HP Scanjet scanner equipped with a transparency adapter and the UNSCAN-IT gel 6.1 software.

Oxidative stress markers. Lipid peroxidation was estimated by measuring the release of free malondialdehyde (MDA) from Caco-2/15 in the culture medium by HPLC (8). Proteins were first precipitated with a 10% sodium tungstate solution (Sigma). The protein-free supernatants then reacted with an equivalent volume of 0.5% (wt/vol) thiobarbituric acid solution (Sigma) at 95°C for 60 min. After cooling to room temperature, the pink chromogene [(thiobarbituric acid) 2-MDA] was extracted with 1-butanol and dried over a stream of nitrogen at 37°C. The dry extract was then resuspended in a potassium dihydrogen phosphate-methanol mobile phase (70:30, pH 7.0) before MDA determination by HPLC with fluorescence detection, as previously described (33).

The ratio of reduced glutathione (GSH) to glutathione disulfide (GSSG) was determined after exposure to Fe/Asc using the Bioxytech GSH/GSSG-412 kit (OxisResearch, Portland, OR). Caco-2/15 cells were washed twice with ice-cold PBS, harvested in 5% metaphosphoric acid (wt/vol), and centrifuged at 1,000 g at 4°C for 10 min. The supernatants were divided into two aliquots, one for the measurement of total GSH and the other for measurement of GSSG, which was mixed with thiol-scavenging 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate. Samples were immediately stored at −80°C. For sample analysis, chromogen 5,5′-dithiobis-(2-nitrobenzoic acid), the enzyme glutathione reductase (G-Red), and NADPH were added, and absorbance was recorded every 30 s for 3 min at 412 nm. Total GSH and GSSG concentrations were calculated using a calibration curve.

Measurement of $H_2O_2$. An aliquot of 100 μl of culture medium from PON2 KD Caco-2/15 or Mock cells was added to 900 μl of FOX reagent (for 100 ml: 270 μl of concentrated H2SO4, 5 mM xylenol orange, 12.5 mM FeCl2, and 40 mM butylated hydroxytoluene in 80% methanol) and incubated at room temperature for 30 min. Absorbance was recorded at 560 nm, and $H_2O_2$ concentrations were determined using a calibration curve.

Endogenous antioxidant enzyme activities. Caco-2/15 cells were harvested in hypotonic lysis buffer (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF). Total SOD activity was determined as described by McCord et al. (30). Briefly, superoxide radicals ($O_2^-$) were generated by the addition of xanthine and

![Fig. 4](http://ajpgi.physiology.org/) Primary antioxidative response in Caco-2/15 cells with PON2 knockdown. Cells were infected with lentiviruses containing anti-PON2 shRNA and then trypsinized, harvested, and treated with Fe/Asc. A–C: semiquantitative PCR analysis of SOD-1, SOD-2, and SOD-3 gene expression. D: total SOD activity. Values are means ± SE of 3 independent experiments, each performed in triplicate. **$P < 0.01$ vs. Mock.
xanthine oxidase, and oxidation of the SOD assay cocktail was followed using a spectrophotometer at 550 nm for 5 min. The same reaction was repeated with addition of the sample, and the SOD assay cocktail was less oxidized because of the SOD activity in the sample. Total SOD activity was then calculated.

The assay for catalase (CAT) activity was adapted from the protocol reported by Jiang et al. (17) with measurement of xylenol orange oxidation at 560 nm in the presence of ferrous ions. Briefly, 100 µM H$_2$O$_2$ was added, and absorbance was monitored at 560 nm, which gave a reference value. Samples were then incubated on ice with the same H$_2$O$_2$ concentrations and allowed to react for 5 min. Because of the CAT activity in the sample, xylenol orange was less oxidized, since H$_2$O$_2$ had been hydrolyzed. CAT activity was then calculated using a standard curve.

For glutathione peroxidase (G-Px) activity, aliquots of cell homogenates were added to a PBS buffer containing 10 mM GSH, 0.1 U of G-Red, and 2 mM NADPH with 1.5% H$_2$O$_2$ to initiate the reaction. Absorbance was monitored every 30 s at 340 nm for 5 min.

For G-Red activity, cell homogenates were added to a PBS buffer containing 2 mM NADPH and 10 mM GSSG to initiate the reaction. Absorbance was monitored every 30 s at 340 nm for 5 min.

**Statistical analysis.** Values are means ± SE. Data were evaluated by ANOVA using Prism 5.01 (GraphPad Software), and differences between the means were assessed post hoc using Tukey’s test.

**RESULTS**

Validation of PON2 KD in Caco-2/15 cells. To define the roles of PON2 in the oxidative stress and inflammatory response at the intestinal level, Caco-2/15 cells were infected with lentiviruses containing anti-PON2 shRNA to generate a model of intestinal cells in which PON2 expression is largely reduced. Caco-2/15 cells were allowed to differentiate for 10–12 days, and PON2 expression was compared with that in cells that were not infected with lentiviruses (CTRL) and in Mock cells. Gene expression analyzed by semiquantitative PCR revealed a marked (~75%) reduction in PON2 transcript in anti-PON2 shRNA-transfected cells compared with Mock and CTRL cells (Fig. 1A). Western blot experiments confirmed the major PON2 KD at the protein level, as a reduction of >80% was noted in anti-PON2 shRNA-transfected cells com-
pared with Mock and CTRL cells (Fig. 1B). Since no significant differences were noted between CTRL and Mock cells, the latter were employed as controls for the subsequent experiments.

**PON2 KD and cell integrity.** The effects of PON2 KD on cell viability and monolayer integrity were examined. As assessed by Trypan blue exclusion, cell viability was not affected (data not shown). Furthermore, under these normal conditions, the gene and protein expression of villin was analyzed as a marker of cell differentiation and that of occludin as a marker of tight junctions and mucosal barrier function (9), and no significant differences were observed between Mock and PON2 KD cells (Fig. 2). Finally, sucrase activity (40.4 ± 5.0 vs. 38.8 ± 4.7 μmol·min⁻¹·g protein⁻¹) and TER (1,982 ± 177 vs. 2,201 ± 2,178 Ω·cm²) were not different between PON2 KD and Mock cells, respectively. Overall, these results did not disclose any significant perturbation in mucosal barrier function and cell differentiation, which indicates that the RNA interference mechanism did not exert cytotoxic effects on Caco-2/15 cells. However, occludin expression was of particular interest after Fe/Asc-induced oxidative stress, since tight junctions are known to be affected markedly in such conditions (7). In our experiments, occludin expression was significantly reduced in Fe/Asc-treated PON2 KD cells compared with Mock cells (Fig. 2, C and D), which suggests a vulnerability of Caco-2/15 cell line with PON2 KD to oxidative stress.

**Gene expression of PON1 and PON3 in Caco-2/15 cells with PON2 silencing.** PON1 and PON3 expression was analyzed in anti-PON2 shRNA-transfected cells to verify if the other members of the PON family could compensate for the major PON2 reduction. No marked differences were observed in PON1 and PON3 gene expression, as well as in PON3 protein expression, between PON2 KD and Mock groups, as Fe/Asc-induced oxidative stress remained without a significant impact (Fig. 3). PON1 protein expression could not be detected in Caco-2/15 cells, as reported in our previous investigation (33).

**PON2 KD and antioxidative response.** The antioxidative response of endogenous antioxidant enzymes was analyzed to...
determine whether it was modified after PON2 KD in Caco-2/15 cells and whether the oxidative balance was disturbed.

When the primary antioxidative response of SOD enzymes was assessed, SOD-2 gene expression remained unchanged (Fig. 4B), but SOD-1 (Fig. 4A) and SOD-3 (Fig. 4C) mRNA expression was upregulated in PON2 KD cells following treatment with Fe/Asc. Total SOD activity was also strongly induced in prooxidative conditions, but with similar patterns in Mock and PON2 KD groups (Fig. 4D).

The secondary antioxidative response in PON2 KD Caco-2/15 cells was also examined. G-Px gene expression was slightly and equally increased in Mock and PON2 KD groups after Fe/Asc treatment (Fig. 5A). However, G-Px activity was strongly upregulated in both groups upon induction of oxidative stress (Fig. 5B). Interestingly, CAT gene expression was reduced in PON2 KD cells, independently of oxidative stress induction (Fig. 5C). CAT activity was also decreased in the PON2 KD group in normal conditions but was similarly reduced in both groups upon induction of oxidative stress (Fig. 5D).

The G-Red enzyme is an important part of the antioxidative response for the maintenance of an oxidative balance. G-Red mRNA expression was upregulated in PON2 KD cells after Fe/Asc-induced oxidative stress (Fig. 6A), but the activity was not altered (Fig. 6B).

**Oxidative stress markers in Caco-2/15 cells with PON2 KD.** The extent of lipid peroxidation in PON2 KD cells was assessed by determining levels of MDA in the culture medium. MDA levels were significantly increased in Mock and PON2 KD groups after exposure to Fe/Asc compared with nonexposed cells. However, the rise in MDA levels was significantly more important in PON2 KD cells (Fig. 7A).

Given that primary and secondary antioxidative responses were altered in our study, H2O2 levels were also measured in the culture medium to verify if the oxidative balance was maintained in PON2 KD cells. H2O2 levels were elevated in the PON2 KD group, even without Fe/Asc treatment (Fig. 7B).

We finally turned to the redox potential, since reduced GSH-to-GSSG ratio is considered a powerful cellular sensor of oxidative stress. After Fe/Asc-induced oxidative stress,
GSH-to-GSSG ratio was lower in PON2 KD than Mock cells (Fig. 7C).

Inflammatory response in Caco-2/15 cells with PON2 KD. Since inflammation and oxidative stress are two closely linked phenomena, we analyzed the modulation of gene expression of various proinflammatory components as a possible consequence of PON2 silencing. In a first step, we could observe that, without treatment, TNF-α mRNA was significantly higher in PON2 KD than Mock cells (Fig. 8A). Induction of oxidative stress by Fe/Asc strongly stimulated TNF-α gene expression in PON2 KD and Mock cells, but the impact was more pronounced in PON2 KD cells (Fig. 8A). Gene expression of IL-6 and monocyte chemoattractant protein-1 (MCP-1) was augmented only after the induction of oxidative stress in the PON2 KD cells (Fig. 8, B and C), whereas IL-8 gene expression was increased similarly in both groups (Fig. 8D).

NF-κB activation in LPS-challenged Caco-2/15 cells with PON2 KD. Since the expression of various inflammatory genes was induced in PON2 KD cells, it was relevant to evaluate the status of NF-κB, a critical transcription factor that mediates inflammatory pathways. Its activation was determined after a challenge with LPS. We examined the protein expression of NF-κB and its inhibitory molecule IκB, capable of preventing the translocation of NF-κB to the nucleus for the transcription activation of proinflammatory target genes (33). A marked decreased level of IκB characterized PON2 KD and Mock cells following LPS treatment (Fig. 9A) without changes in NF-κB protein expression (Fig. 9B), but there were no significant statistical differences between the two groups. However, calculation of the NF-κB-to-IκB ratio indicated an enhanced activation NF-κB in PON2 KD cells (Fig. 9C).

Transcription factors involved in regulation of the antioxidative and inflammatory response in Caco-2/15 cells with PON2 KD. We evaluated the protein expression of the transcription factors PGC-1α and Nrf2, which are involved in the regulation of cellular antioxidant status. Little change was observed in Nrf2 in response to Fe/Asc treatment (Fig. 10A), whereas a significant decrease was noted in PGC-1α protein expression (Fig. 10B).

Intestinal PON2 mRNA levels in patients with CD and healthy subjects. Evaluation of the relative gene expression of ileal PON2, determined by quantitative RT-PCR, showed that PON2 mRNA was significantly overexpressed in the nondiseased mucosa of CD patients compared with healthy controls (Fig. 11).

DISCUSSION

PON2 was shown to be widely expressed throughout the digestive tract and affected by oxidative stress and inflammatory stimuli (33). Moreover, the addition of purified PON2 to permeabilized intestinal Caco-2/15 cells decreased lipid peroxidation after induction of oxidative stress (23), while some polymorphisms of PON2 constitute a protective factor with regard to IBD development (37). However, the antioxidant and anti-inflammatory functions of PON2 in the intestine have remained poorly defined. In the present investigation, we succeeded in achieving a reliable model of PON2 KD in the human intestinal Caco-2/15 cell line that ablated the expression of PON2 gene and protein by >80%. Importantly, PON2 silencing did not affect cell integrity, viability, tight junctions,
and differentiation, as the expression of villin, sucrase, and occludin and TER were not affected. However, oxidative balance seemed to be lost in PON2 KD cells, given the rise in SOD activity and the fall in CAT activity. As a result, H₂O₂ levels were found to be increased in the culture medium of PON2 KD cells. Cells with PON2 KD were also more susceptible to oxidative stress, as demonstrated by a concomitant higher MDA level and a reduced GSH-to-GSSG ratio. The inflammatory response was exacerbated in PON2 KD cells, since TNF-α, IL-6, and MCP-1 expression was increased after Fe/Asc-induced oxidative stress compared with Mock cells. The proinflammatory transcription factor NF-κB was also overactivated after LPS challenge.

After generation of the PON2 KD in intestinal Caco-2/15 cells, experiments were undertaken to ensure that cellular viability and integrity were not altered by the lentiviral genetic manipulation. To this end, various markers of differentiation (i.e., sucrase and villin) and mucosal barrier function (i.e., occludin and TER), which represent typical components of the mature small intestinal enterocytes, were employed in our study. PON2 KD did not result in significant changes in sucrase, a microvillar hydrolase considered the most reliable indicator of intestinal cell differentiation in vitro (40), or in villin, the major cytoskeletal protein of the brush border in epithelial cells of the digestive tract (5, 26). Similarly, no differences between Mock and PON2 KD cells were observed in expression of occludin, a tetra-transmembrane protein that, with other structural components, seals the space between adjacent cells by forming tight junctions (9). These junctional complexes are well known to function not only as a control for the paracellular diffusion of ions and certain molecules, but also as vital structures able to control signal transduction and cell integrity (1). Finally, no divergences were recorded in TER, which reflects the uniform presence of tight junctions and barrier function. Overall, our results show that the differentiation process and integrity of Caco-2/15 cells were not affected by PON2 reduction, which allows us to conclude that our intestinal cellular model is reliable. Besides, in Fe/Asc-treated PON2 KD cells, we observed a decline in expression of occludin, which may result in loss of cohesion of the tight junction structure, leaky barrier, and enhanced paracellular permeability, allowing increased exposure to pathogenic bacteria and invasion of the intestinal tissue by toxic luminal antigens, including endotoxins.

Fig. 10. Transcription factors involved in regulation of antioxidative and anti-inflammatory responses in Caco-2/15 cells with PON2 knockdown. Cells were infected with lentiviruses containing anti-PON2 shRNA and then trypsinized, harvested, and treated with Fe/Asc. A and B: Western blot analysis of protein expression of nuclear factor erythroid-2-related factor 2 (Nrf2) and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α). Values are means ± SE of 3 independent experiments, each performed in triplicate. *P < 0.05, **P < 0.01 vs. Mock, unless otherwise shown.

Fig. 11. PON2 gene expression in human tissues. Quantitative RT-PCR analysis of PON2 was performed in normal and noninflamed ileal biopsies from 6 control subjects and 3 patients with Crohn’s disease (CD). GAPDH was used as internal loading control. Values are means ± SE. ***P < 0.0006 vs. controls.
found in intestinal tissue in humans and mice (29, 31), but conflicting data have been noticed (33). In the current investigation, when PON1 and PON3 expression was analyzed in response to PON2 KD, it remained invariable, which points out the lack of compensation for the major PON2 reduction.

Similarly, the expression of other endogenous antioxidant enzymes was expected to compensate for the relative absence of PON2, since the oxidative balance has to be preserved to maintain intestinal homeostasis. This means that the various endogenous antioxidant enzymes have to be regulated in a coordinated manner. SOD enzymes are responsible for the primary antioxidative response and catalyze the dismutation of O$_2^-$ to oxygen and H$_2$O$_2$, which is further converted, in a secondary antioxidant response, to H$_2$O$_2$ with the help of GSH-Px and CAT (2). In PON2 KD cells, SOD-1 and SOD-3 gene expression was upregulated compared with Mock cells after induction of oxidative stress. However, a similar increase in total SOD activity was found in both groups. Secondary antioxidant G-Px gene expression and activity were upregulated similarly in both groups when cells were subjected to oxidative stress. In contrast, CAT expression and activity were strongly downregulated in PON2 KD cells, even in normal conditions. These results suggest that PON2 KD cells have an imbalanced secondary antioxidant response, which could lead to H$_2$O$_2$ accumulation and loss of homeostasis. Accordingly, measurement of H$_2$O$_2$ concentration in normal conditions revealed a significant rise in PON2 KD cells compared with Mock cells. Moreover, estimation of lipid peroxidation in the culture medium (since levels in cell lysates gave similar information) disclosed significantly higher levels of MDA in PON2 KD than Mock cells after Fe/Asc-induced oxidative stress. These results suggest that an imbalanced antioxidative response occurs in PON2 KD cells, leading to H$_2$O$_2$ accumulation and making the cells more at risk from oxidative stress. This is particularly true given the decline in GSH-to-GSSG ratio, an indicator of the cellular oxidative state (38), despite upregulation of G-Red gene expression, possibly as a compensatory mechanism to repulse oxidative stress in vain.

Oxidized lipids can also induce proinflammatory genes, such as TNF-α and MCP-1, via NF-κB activation (11). Given that major changes in our model have been observed in the anti-oxidative response, the inflammatory response was analyzed. In PON2 KD cells, induction of oxidative stress by Fe/Asc significantly increased TNF-α gene expression compared with Mock cells. Even in basal conditions, TNF-α gene expression was also significantly higher in PON2 KD cells, suggesting that PON2 KD makes intestinal epithelial cells more prone to inflammation. Similarly, gene expressions of IL-6 and MCP-1 were also increased in PON2 KD cells in response to Fe/Asc. These results demonstrate that PON2 silencing predisposes to the development of an exacerbated inflammatory response. Macrophages isolated from PON2-deficient mice that were treated with LPS also displayed increased TNF-α and IL-1β gene expression (31).

The NF-κB transcription factor regulates genes that are critical for inflammation and immunity. The upregulation of various proinflammatory genes in our study suggests an activation of NF-κB. Indeed, when PON2 KD cells were challenged with LPS, the NF-κB-to-IκB ratio was elevated in PON2 KD cells compared with Mock cells. These results strengthen the role of PON2 in the prevention of inflammation.


