Mouse Macrophage Paraoxonase 2 Activity Is Increased Whereas Cellular Paraoxonase 3 Activity Is Decreased Under Oxidative Stress

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Objective—To determine whether paraoxonases (PONs) are expressed in macrophages and to analyze the oxidative stress effect on their expression and activities.

Methods and Results—We demonstrated the presence (mRNA, protein, activity) of PON2 and PON3 but not PON1 in murine macrophages, whereas in human macrophages, only PON2 was expressed. Under oxidative stress as present in mouse peritoneal macrophages (MPMs) from apoE-deficient (E0) mice as well as in C57BL6 mice, MPMs that were incubated with buthionine sulfoximine, with angiotensin II, with 7-ketocholesterol, or with oxidized phosphatidylcholine, PON2 mRNA levels and lactonase activity toward dihydrocoumarin significantly increased (by 50% to 130%). In contrast, PON3 lactonase activity toward lovastatin was markedly reduced (by 29% to 57%) compared with control cells. The supplementation of E0 mice with dietary antioxidants (vitamin E, pomegranate juice) significantly increased macrophage PON3 activity (by 23% to 40%), suggesting that oxidative stress was the cause for the reduced macrophage PON3 activity. Incubation of purified PON2 or PON3 with E0 mice MPMs resulted in reduced cellular lipid peroxides content by 14% to 19% and inhibition of cell-mediated LDL oxidation by 32% to 39%.

Conclusions—Increased macrophage PON2 expression under oxidative stress could represent a selective cellular response to reduce oxidative burden, which may lead to attenuation of macrophage foam cell formation. (Arterioscler Thromb Vasc Biol. 2003;23:468-474.)

Key Words: macrophages ■ oxidative stress ■ paraoxonase ■ antioxidants ■ pomegranate juice

Paraoxonases (PONs) 1, 2, and 3 are members of a multigene family. These genes share 65% identity at the amino acid level, but all PON2 and PON3 cDNAs sequenced to date lack the three nucleotides residues of codon 106, which are present in PON1.1-3 PON1 is an esterase associated in serum with HDL.4 Recent studies in PON1 knockout mice5 and PON1 overexpressing mice6 revealed that PON1 acts as an antiatherogenic agent. In vitro studies demonstrate that PON1 protects against oxidative stress7-10 by hydrolyzing specific oxidized lipids in lipoproteins,7,8 macrophages,9 and atherosclerotic lesions.10 Human and rabbit PON3 are also HDL associated11,12 and rabbit PON3 is more efficient than rabbit PON1 in protecting LDL against oxidation.12 Under oxidative stress, which is associated with enhanced atherosclerosis,13 PON1 is inactivated,14,15 and antioxidants preserve its activity.14 Serum PON1 levels and activities are lower in patients with cardiovascular heart disease compared with healthy subjects16-18 and also in rabbits and mice fed an atherogenic diet.19,20 PON1 and PON3 mRNA are predominantly expressed in liver, whereas PON2 mRNA is found in different tissues,21 including human endothelial and aortic smooth muscle cells,22 but its presence in macrophages has not been analyzed. PON2 protein is not detectable in HDL22 and, therefore, was thought to function within cells. PON2 overexpression in HeLa cells was shown to lower intracellular oxidative state and these cells were less able to oxidize LDL.22 PON2 and PON3 can modulate oxidative stress, but it is unknown whether these proteins are inactivated when cells are oxidatively stressed or whether expression levels are regulated in response to PON inactivation. Macrophages may be a model to address these questions because they play a key role in early atherogenesis,23,24 and under oxidative stress their lipids are oxidized.25-27 These “oxidized macrophages” possess increased capability to oxidize LDL25-27 and to take up oxidized LDL,9 leading to foam cell formation.

The aim of the present study was to determine the expression profile and activities of PONs in macrophages,
and to analyze the effect of oxidative stress on macrophage PON expression and activities.

Methods

Mouse Studies

ApoE-deficient (E\(^0\)) mice, which are hypercholesterolemic under oxidative stress and develop accelerated atherosclerosis, were compared with age- and gender-matched controls (C57BL6 mice). All mice received a chow diet. For the vitamin E and pomegranate juice (PJ) consumption experiments, 12 female 5-week-old E\(^0\) mice were equally distributed into 3 groups. Two groups received either vitamin E (40 mg/kg/day) or PJ (0.3 μmol polyphenols/kg/day) in their drinking water for 2 months. The placebo group received only water.

Cells

J774 A.1 Macrophage-Like Cell Line

J774 A.1 cells were obtained from ATCC (Rockville, MD) and maintained in DMEM containing 5% FCS, penicillin (100 U/mL), streptomycin (100 μg/mL), and glucose (2 mmol/L), that is, supplemented DMEM.

Mouse Peritoneal Macrophages (MPMs)

MPMs were harvested 4 days after intraperitoneal injection of thioglycolate. The cells (2×10\(^7\)/mouse) were washed with PBS at 1000g for 10 minutes, diluted to 10\(^6\)/mL in supplemented DMEM, plated, and incubated (5% CO\(_2\), 95% air) at 37°C. After 2 hours, nonadherent cells were removed, and the monolayer was further incubated.

Isolation of E\(^0\) Mice Arterial Macrophages

E\(^0\) mice were sacrificed and their entire aortas were removed and placed in Hanks’ balanced salt solution. The macrophage fraction was separated by discontinuous density gradient of metrizamide.

Human Monocyte-Derived Macrophages (HMDMs)

HMDMs were separated from blood of healthy volunteers. The cells were plated at 10\(^5\) /mL in RPMI medium containing 10% FCS. After 2 hours of incubation at 37°C, nonadherent cells were removed, and RPMI containing 10% autologous serum was added. Macrophages were used 8 days after plating.

Preparation of Cell Lysate and Cell Fractionation

Cell Lysate

Cells (2×10\(^7\)) were washed with PBS and pelleted (1000g for 10 minutes at 4°C). Pellets were suspended in 0.5 mL of RIPA buffer (1× PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/L phenylmethyl sulfonyl fluoride [PMSF], and 100 μmol/L leupeptin).

Cell Fractionation

Membrane (microsomal) and cytosol fractions were prepared, and membranes were suspended to 10\(^6\) cell equivalents/mL in RIPA buffer. Aliquots were removed from cell lysates, membrane, and cytosol fractions for protein determination.

Preparation of Oxidized L-α-1-Arachidonyl-2-Palmitoyl-Phosphatidylcholine (Ox-PAPC)

PAPC (5 mg) was dissolved in 1 mL of 10 mmol/L Tris buffer (pH 7.4) to form multilamellar liposomes. The PAPC liposomes were oxidized by incubation for 4 hour at 37°C under oxygen stream. The extent of PAPC oxidation was measured by the thiobarbituric acid reactive substances (TBARS) and the lipid peroxides assays.

Ox-PAPC contained 275 nmol of MDA equivalents/mL and 1770 nmol lipid peroxides/mL, respectively.

Macrophage Lipid Peroxides

MPMs (2×10\(^7\)) were washed with PBS and lipid extracted with hexane/isopropanol (3:2, v:v). The hexane phase was evaporated under nitrogen, and the amount of lipid peroxides was determined.

Macrophage PON Activities Measurements

Cells (2×10\(^7\)) were washed, scraped into Tris buffer (25 mmol/L Tris/Cl, pH 7.6, 1 mmol/L CaCl\(_2\)), and pelleted (1000g for 10 minutes at 4°C). The pellets were resuspended with 2 mL of Tris buffer and sonicated for 2×20 seconds at 80w on ice, and the protein concentration was determined.

Enzyme activities were measured using 200 μL of sonicate (200 to 300 μg protein) per mL assay mixture. PON and lactonase activities were measured using paraoxon and DHC as substrates, respectively. The absorbance was monitored at 0 and 5 minutes after substrates addition. Lactonase activity using LST was determined by high-performance liquid chromatography after cell sonicates incubation with LST for 4 hours. Measurements were as follows: 1 U of PON activity = 1 nmol of paraoxon hydrolyzed/minute and 1 U of lactonase activity = 1 μmol of DHC hydrolyzed/minute or 1 pmol of LST hydrolyzed/minute.

Purification of PON1, PON2, and PON3

Human PON2 (hPON2) was purified from HEK293 cells stably transfected with hPON2 cDNA, and its identity was confirmed at the Protein Sequencing Core, University of Michigan, (Ann Arbor, Mich). hPON1 and rabbit PON3 (rPON3) were purified as previously described.

Macrophage PONs Protein Assay

Western blot analysis was performed as previously described. The primary antibody was rabbit antihuman PON2 diluted (1:1000, v:v). This antibody is polyclonal and was raised against PON2-specific peptide H-LKEEKPRARELRISRGFDLA by Research Genetics, Inc, (Huntsville, Ala). This antibody cross-reacts also with rPON3 and with mouse peptides of the expected molecular size of PON2 and PON3 but not with human PON1. The secondary antibody was horseradish peroxidase-conjugated antirabbit IgG, diluted 1:4000 v:v. The membranes were developed using the ECL Western blotting kit (Amersham). Purified hPON1, hPON2 and rPON3 were used as positive controls.

PON2 and PON3 mRNA Expression by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from macrophages with Tri-reagent. cDNA was generated from 1 μg of total RNA using RT and an oligo DT primer. RT products were subjected to PCR amplification using gene specific primers (Life Technologies).

The PCR program used for mouse and human PONs was as follows: 1 minute at 94°C, 30 cycles for PON3 and GAPDH, or 40 cycles for PON1 and PON2 (30 seconds at 94°C, 30 seconds at 57°C, 1 minute at 68°C), and 7 minutes at 68°C. The cDNA products were separated on 1% agarose gel containing ethidium bromide.

Macrophage-Mediated LDL Oxidation

Human LDL was isolated from plasma of normolipidemic subjects. MPMs (2×10\(^7\)) were incubated with LDL (100 μg of protein/mL) in the presence of 5 μmol/L CuSO\(_4\) for 5h at 37°C. Cell-mediated oxidation of LDL was then determined by the TBARS assay.

Statistical Analysis

Statistical analysis was performed using the Mann–Whitney test in comparing means from 3 to 4 experiments, whereas ANOVA was used when more than 2 groups were compared. Results represent mean±SD.
Results

PON 2 and PON3 But Not PON 1 Are Expressed in Macrophages

RT-PCR analysis revealed PON2 and PON3 mRNA in several types of macrophages, including MPMs from Balb C mice, J-774 A.1 murine macrophage-like cell line, and arterial macrophages derived from E0 mice aortas (Figure 2A). The MPM cDNA bands were confirmed as mouse PON2 and PON3 by sequencing.

Analysis of HMDMs demonstrated expression of PON2 but not PON3 mRNA (Figure 2A). PON1 mRNA was not detected in either mouse or human macrophages (data not shown). Western blot analysis (Figure 1B) was performed using rabbit antihuman PON2 antibody or rabbit preimmune serum. The antibody reacted with purified hPON2 and rPON3 but not with hPON1 and detected bands of mouse peptides of the expected molecular size of PON2 and PON3 in lysate of MPMs and J774 A.1. Furthermore, we demonstrated that PON2 and PON3 were located in microsomes but not in cytosol of murine macrophages. In lysate from HMDMs, however, only a PON2 protein band was observed. All samples that reacted with the antihuman PON2 antibody (except for hPON1 and MPM lysate) were analyzed in the gel that was incubated with the preimmune serum. In this gel, no bands could be detected, indicating the antibody specificity. PON2 and PON3 protein expression correlates with the mRNA expression pattern.

To determine whether macrophage PON2 and PON3 proteins were functional, we measured the enzymatic activities of known PONs in MPM sonicate with the following substrates: paraoxon, DHC, and LST. No PON activity (PON1 specific) was detected, in accordance with the lack of PON1 mRNA in these macrophages. Lactonase activity with LST could be attributed to PON3, whereas DHC hydrolysis is catalyzed by both PON2 and PON3. The purified hPON2 and rPON3 preparations used in this study had lactonase activity (DHC) of 49 U/mL or 114U/mL, respectively, but only rPON3 hydrolyzed 79% of LST (5 μg/mL) after 4 hours of incubation. Lactonase activity (DHC) was found in both HMDMs and MPMs (0.110±0.008 versus 0.090±0.017 U/mg cell protein). In contrast, only MPM hydrolyzed LST (85.5±3.5 U/mg cell protein), whereas in HMDMs this activity was absent, suggesting that in human macrophages only PON2 activity is present.

Lactone hydrolysis could be catalyzed also by carboxylesterases. To demonstrate that macrophage lactonate activities with DHC and LST are specific for the respected PONs, we used EDTA to chelate calcium ions (required for the enzymatic activities of PONs) and PMSF (to inhibit the carboxylesterases). The addition of EDTA (10 mmol/L) but not of PMSF (2 mmol/L) to MPM sonicate resulted in a marked inhibition (90%) of the lactonate activity with DHC (from 0.090±0.017 to 0.009±0.001 U/mg cell protein) and a 77% inhibition of lactonate activity with LST (from 85.5±3.5 to 19.7±2.1 U/mg cell protein). Furthermore, on incubation of MPM sonicate for 2 hours at 25°C with the antihuman PON2 antibody (diluted 1:500) PON3 lactonase activity (LST) was reduced by 52% (from 85.5±3.5 to 41±2 U/mg cell protein), and PON2+PON3 lactonase activity (DHC) was reduced by 63% (from 0.090±0.017 to 0.034±0.005 U/mg cell protein), indicating that these activities could be attributed to PONs and not to other enzymes.

PON2 and PON3 mRNA Levels and Activities in E0 Mice Macrophages: Ex Vivo Studies

During atherosclerosis progression in E0 mice, increased oxidative stress in serum and macrophages has been demonstrated. To examine whether oxidative stress affected macrophage PON2 and PON3 expression and activities, we isolated MPM from 2- and 4-month-old E0 mice. In 2-month-old E0 mice MPMs, PON2 mRNA levels were increased (∼1.5 fold) compared with control (C57BL/6) mice MPMs (Figure 2A), whereas PON3 mRNA levels were similar (Figure 2B) in both strains. By 4 and 7 months of age, PON2 mRNA levels in E0 mice MPMs were further increased (≥2
DHC was similar in both E⁰ and control mice at 2 months of age (Table 1) but was increased by 60% in E⁰ MPMs at 4 months of age (Table 1). The reduction in macrophage PON3 activity was not the result of PON3 secretion because no lactonase activity (LST) could be detected in the medium obtained from E⁰ MPM.

To test if the reduction of PON3 lactonase activity in E⁰ macrophages is caused by oxidative stress, 6-week-old E⁰ mice were supplemented for 2 months with PJ (0.3 μmol of total polyphenols/kg/d), or vitamin E (40 mg/kg/d), which have been shown previously to reduce macrophage oxidative stress and atherosclerosis in E⁰ mice.³⁷,³⁸ Both PJ and vitamin E consumption by E⁰ mice decreased MPM lipid peroxides content by 37% and 33%, respectively, (from 40±2 to 25.2±3.0 and 26.8±3.0 nmol/mg cell protein, respectively). MPM PON3 lactonase activity (LST) was increased by 23% or 26% on PJ and vitamin E supplementation (from 47.0±2.5 to 57.9±1.2 and 59.1±1.7 U/mg cell protein, respectively), indicating that PON3 inactivation in E⁰ MPM was caused by oxidative stress. Lactonase activity (DHC) in the placebo group and in the supplemented mice was similar (data not shown), which might be the result of reduced PON2 activity and increased PON3 activity.

**Macrophage PON2 and PON3 mRNA Levels and Activities Under Oxidative Stress: In Vitro Studies**

To directly assess the effect of oxidative stress on macrophage PON2 and PON3 expression and activities, C57BL6 mice MPMs were incubated with 0.05 mmol/L of buthionine sulfoximine (BSO), a specific inhibitor of glutathione synthesis,³⁹ or with Angiotensin II (Ang II; 10⁻⁷ mol/L; Table 2). In the BSO- treated cells, reduced glutathione (GSH) content was decreased by 90% (from 10±0.2 to 1.0±0.1 nmol/mg cell protein). Macrophage lipid peroxides content increased on incubation with BSO or Ang II (49% and 39%, respectively).

In parallel, BSO- and Ang II-treated macrophages exhibited reduced PON3 lactonase activity (LST; 30% and 41%, respectively). In contrast, lactonase activity (DHC) was markedly increased on macrophage treatment with BSO or with Ang II (52% or 39%, respectively).

To analyze macrophage-oxidized lipids, which could be responsible for the observed changes in PONs activities, we incubated control MPMs with 7-ketocholesterol (7-KETO, 20 μmol/L), or with Ox-PAPC (15 μg/mL; Table 2). This resulted in increased MPM lipid peroxides content (30% and 60%, respectively) and in substantial reduction of PON3 mRNA levels (LST, 30% and 27%, respectively). In contrast, lactonase activity (DHC) was increased on PON2 incubation with ox-PAPC or with 7-KETO significantly increased lactonase activity (DHC) by 40% or 14%, respectively, compared with control cells. In all oxidative systems (BSO-, Ang II-, 7-KETO-, and Ox-PAPC–treated macrophages), PON2 mRNA levels were significantly higher (2.3-, 2.0-, 1.9-, and 2-fold, respectively) whereas PON3 mRNA levels were similar compared with untreated control cells (Table 2).

**Effect of PON2 and PON3 on Macrophage Oxidative Stress**

As oxidative stress selectively affected PON2 (activation) and PON3 (inactivation), we next questioned the abilities of...
purified PON2 and PON3 to protect macrophages against oxidative stress. E10 mice MPMs were incubated with hPON2 or rPON3 (0.2 lactonase [DHC] units/mL) for 20 hours at 37°C. Both PON2 and PON3 resulted in a significant reduction in macrophage lipid peroxides content by 19% and 14%, respectively (Figure 3A) and inhibited cell-mediated LDL oxidation by 39% and 32%, respectively (Figure 3B).

Discussion

Atherosclerosis is associated with oxidized lipids accumulation in arterial macrophages, which affects their atherogenic properties.9,25,27 PON1 mass was detected in atherosclerotic lesions,31 PON2 was detected in human endothelial and smooth muscle cells,22 but no data are available yet on expression of PONs in macrophages and on their possible role in atherogenesis. We hypothesized that macrophages possess PONs and that their expression and activities may be regulated by oxidative stress. Indeed, in the present study we clearly demonstrated for the first time the presence of PONs (mRNA, protein, and enzymatic activities) in macrophages. PON2 and PON3 were found in murine macrophages, whereas, in human macrophages only PON2 was present. No PON1 was found in human and mouse macrophages. In MPMs, both PON2 and PON3 were associated with the membrane fraction. Similarly, PON2 protein was recently shown to be present in the plasma membrane of PON2-overexpressing cells.32

Next, we demonstrated that mouse macrophage PON2 and PON3 expression and activities were affected differently by oxidative stress. We performed ex vivo as well as in vitro studies, which showed that under oxidative stress PON2 expression and enzymatic activity increased, whereas PON3 expression did not change, but PON3 activity decreased. In the ex vivo studies, E10 mice MPMs had significantly higher PON2 mRNA levels but similar PON3 mRNA levels compared with control C57BL6 mice. In the in vitro studies we induced oxidative stress in macrophages with the GSH synthesis inhibitor BSO or with Ang II, which also resulted in a significant increase of PON2 but not PON3 mRNA levels. Oxidized lipids were shown to reduce hepatic PON1 expression15 but not PON3 expression in HepG2 cells.11 In the current study, treatment of MPMs with 7-KETO or Ox-PAPC did not affect PON3 mRNA levels but increased PON2 expression. The increase in PON2 mRNA levels may be the cell response to oxidative stress, as was shown for other cellular antioxidant enzymes.32

In all systems studied (E10 MPMs, BSO-, Ang II-, 7-KETO-, and Ox-PAPC–treated cells), we found increased PON2 lactonase activity (with DHC), which could be explained with the increased PON2 expression and/or higher resistance to inactivation. In contrast, PON3 lactonase activity (with LST) decreased in parallel to the extent of the oxidative stress. Oxidized lipids were shown to inactivate both serum and hepatic PON1.14,15 In the present study we

| Table 2. Effect of Oxidative Stress Induced by BSO, Ang II, 7-Ketocholesterol, and Oxidized Phospholipids on PON2 and PON3 mRNA Levels and Activities in MPM |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Lipid Peroxides Content, nmol/mg cell protein | PON3, Lactonase Activity (LST), U/mg cell protein | PON2 + PON3, Lactonase Activity (DHC), U/mg cell protein | PON2 mRNA, PON2/GAPDH | PON3 mRNA, PON3/GAPDH |
| Control | 10.9±0.1 | 71.0±3.0 | 0.193±0.023 | 1.5±0.2 | 0.97±0.01 |
| + BSO | 16.2±0.3* | 49.7±2.0* | 0.293±0.025* | 3.4±0.3* | 0.91±0.02 |
| + Ang II | 15.1±0.3* | 41.6±1.6* | 0.268±0.056* | 2.9±0.3* | 1.02±0.10 |
| + 7-KETO | 14.3±0.5* | 49.7±2.0* | 0.219±0.025* | 2.8±0.4* | 0.99±0.12 |
| + Ox-PAPC | 17.4±0.3* | 51.8±1.6* | 0.282±0.056* | 2.9±0.4* | 1.03±0.20 |

Results are given as mean±SD of three different experiments.

C57BL6 mice-MPM were incubated for 20 hours at 37°C without (Control) and with 0.05 mmol/L of BSO, with 10−7 mol/L of Ang II, with 20 μmol/L of 7-KETO, or with 15 μg/mL of Ox-PAPC. The amount of protein and lipid peroxides, as well as lactonase activities toward DHC or LST were measured in the cell (107/mL) sonicate. Densitometric analysis of PON2 and PON3 mRNA bands intensity was normalized to that of GAPDH.

*p<0.01 vs Control.
demonstrated that PON3 was similarly inactivated in E0 MPMs or on incubation of macrophages with 7-KETO or with Ox-PAPC. Moreover, antioxidants has been shown to preserve PON1 activity,14 and we observed similar effect on macrophage PON3 activity after supplementation of E0 mice with PJ and vitamin E.

The physiological role of cellular PON2 and PON3 is not yet known, but recent studies indicate that PON2 and PON3 may act as potent cellular antioxidants.11,22 Pretreatment of cultured aortic endothelial cells with supernatants from HeLa cells overexpressing PON3 prevented the formation of mildly oxidized LDL.11 PON2 overexpression was shown to lower the intracellular oxidative state of HeLa cells treated with hydrogen peroxide or with oxidized phospholipids,22 and these cells were less able to oxidize LDL.22 Similarly, in the presented study we demonstrated that PON2 and PON3 reduced oxidative stress in macrophages from E0 mice. It is possible that these enzymes hydrolyze oxidized phospholipids in the macrophage plasma membrane, leading to reduction in cellular lipid peroxides content. PON1 was also shown to reduce the content of macrophage lipid peroxides, and it possesses the capability to hydrolyze cholesteryl linoleate hydroperoxides in oxidized LDL and in atherosclerotic lesions in addition to core aldehydes in oxidized phosphatidylcholine.7,10,43,44 The antiatherogeneity of PON1 was recently demonstrated45 by its capacity to decrease macrophage cholesterol biosynthesis.

All three PONs may act selectively in the arterial wall and in the circulation. PON2 can protect against oxidation in arterial cells, such as endothelial cells, smooth muscle cells, and macrophages. In HMDMs only PON2 is expressed, suggesting that PON2 may be more suited than PON3 to act against macrophage oxidative stress. As for PON3, its absence in human macrophages suggests that in humans (but not in mice) it may act (like PON1) only in the circulation.

In the present study we have demonstrated for the first time the presence of PON2 and PON3 in mouse macrophages. Under oxidative stress, macrophage PON2 but not PON3 mRNA levels and activity significantly increased, whereas PON3 activity was significantly reduced. The exact role of cellular PONs in macrophage foam cell formation under oxidative stress during the development of atherosclerosis may be further elucidated by the use of PON2 and PON3 transgenic knockout and knockin animal models, as well as by studies of PON2 and PON3 transfected cells.

References


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