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Paraoxonase 1-treated oxLDL promotes cholesterol efflux from macrophages by stimulating the PPARγ–LXRα–ABCA1 pathway

Souade Ikhlef[1], Hicham Berrougui[1,2], Olivier Kamtchueng Simo[1] and Abdelouahed Khalil[1,3]

1 Research Centre on Aging, CSSS-IUGS, Sherbrooke, Canada
2 Department of Biology, University Sultan My Slimane, Beni Mellal, Morocco
3 Department of Medicine, Geriatrics Service, Faculty of Medicine and Biological Sciences, University of Sherbrooke, Canada

Correspondence
A. Khalil, Health Campus, FMSS, 3001 12th North Avenue, Sherbrooke, Quebec, Canada J1H 4N4.
Fax: +1 819 564 5320
Tel: +1 819 821 8000 ext. 70148
E-mail: a.khalil@usherbrooke.ca

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Here, we investigate the mechanism through which paraoxonase 1 (PON1) may regulate cholesterol efflux. Pretreatment of oxLDL with PON1 (oxLDL–PON1) contributed to the formation of LysoPC. In J774 macrophages, oxLDL–PON1 increased cholesterol efflux by more than 47% compared to oxLDL alone. oxLDL–PON1 significantly increased mRNA and protein expression of ABCA1 and ABCG1, as well as of PPARγ and LXRα compared to oxLDL alone. Intraperitoneal injection of oxLDL–PON1- or LysoPC-treated J774 macrophages significantly increased the fecal elimination of macrophage-derived cholesterol in these mice. Our results suggest that PON1 stimulates cholesterol efflux via a mechanism that involves oxidized phospholipid hydrolysis.

Keywords: cholesterol efflux; LysoPC; PON1; reverse cholesterol transport

The inverse relationship between high-density lipoprotein (HDL) plasma levels and the risk of cardiovascular disease is well established. HDL has antioxidant and anti-inflammatory activities. However, the most atheroprotective effect of HDL has been mainly attributed to their key role in reverse cholesterol transport (RCT) by which excess cholesterol from peripheral cells is transported to the liver for excretion [1]. The first critical and natural rate-limiting step of macrophage-derived foam cell RCT involves the efflux of cellular cholesterol to circulating HDL particle [2,3], a process that occurs via three pathways. The first pathway involves the ATP-binding cassette transporters ABCA1 and ABCG1, which mediate cholesterol efflux in a unidirectional manner to lipid-poor apoA-I and other HDL subfamily members, respectively [4–6]. The second involves scavenger receptor class B type I (SR-BI)-mediated bidirectional free cholesterol exchanges that depend on the cholesterol gradient. This pathway mediates cholesterol efflux to a wide variety of cholesterol acceptors [3]. The third is aqueous diffusion by which cholesterol molecules spontaneously desorb from the plasma membrane, diffuse through the aqueous phase, and become adsorbed on acceptor particles by collision [2].

Apo-AI is the major protein component of HDL and plays a critical role in cholesterol metabolism. ApoA-I reacts specifically with ABCA1 to generate nascent HDLs, which are then enriched with esterified cholesterol by LCAT (activated by apoA-I) to form mature spherical HDL particles [7]. However, a number of other HDL-associated proteins may play a significant role in the regulation of this antiatherogenic activity of HDL, including paraoxonase 1 (PON1) [8].

Abbreviations
ABCA1, ATP-binding cassette A1; ABCG1, ATP-binding cassette G1; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HDL, high-density lipoprotein; LXR, liver X receptor; NEM, N-ethylmalemide; PON1, paraoxonase 1; RCT, reverse cholesterol transport.

PON1 is mainly associated with HDL in human serum [9], and its association with cardiovascular risk, oxidative stress [10,11], and inflammation has been extensively studied. It has been reported that PON1 is involved in the protection of low-density lipoprotein (LDL) and HDL from oxidation by oxygen free radicals [12] and in the regulation of HDL anti-inflammatory activity [13]. Recent studies, including ours, have suggested that PON1 may be involved in the regulation of macrophage-derived cholesterol efflux to HDL [8,14–16]. Rosenblat *et al.* [14] have also shown that PON1 stimulates macrophage-derived cholesterol efflux and have proposed that this effect is mediated by an increase in HDL binding to cells. We have shown that PON1 stimulates macrophage-derived cholesterol efflux to HDL via an apoA-I-like mechanism [8]. Furthermore, the stimulatory effect of PON1 on cholesterol efflux has been associated with the capacity of PON1 to hydrolyze oxidized lipids, which may increase HDL binding to macrophages and upregulates the ABCA1 transporter [15,16]. However, the mechanism by which PON1 upregulates ABCA1 expression remains to be determined.

The ABCA1 transporter plays a pivotal role in HDL formation. It promotes cellular cholesterol efflux and maintains cellular cholesterol homeostasis [17]. A mutation of ABCA1 has been shown to abolish cholesterol efflux and increase the risk of developing atherosclerosis [18]. ABCA1 is regulated both at the transcriptional level via liver X receptor (LXR)/retinoid X receptor-mediated induction of the ABCA1 gene [19] and at the post-translational level via changes in the turnover of the ABCA1 protein [20]. ABCA1 expression is induced by PPARγ through a transcriptional cascade mediated by the nuclear receptor LXRα [21]. The activation of the PPARγ–LXRα–ABCA1 pathway by natural or synthetic agonists upregulates ABCA1 expression, enhances cell cholesterol efflux, and reduces the development of the atherosclerosis process. The goals of the present study were to investigate the mechanism by which PON1 upregulates ABCA1 expression and to determine its effect on the stimulation of both *in vitro* macrophage-derived cholesterol efflux and *in vivo* RCT process.

Materials and methods

Chemicals

Methanol, chloroform, n-butanol, ethanol, n-isopropanol, toluene, hexane, ethyl acetate, aqueous ammonia, sodium dodecyl sulfate (SDS), Tween 20, and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Fisher Scientific (Montreal, Canada). 2xTY medium, Tris/HCl, potassium bromide, sodium chloride, Tergitol®, imidazole, calcium chloride, sodium hydroxide, glycine, glycerol, bromophenol blue, β-mercaptoethanol, 8-(4-chlorophenylthio)adenisine3′:5′-cyclic monophosphate (cAMP), 2-chloro-5-nitro-N-phenyl-benzamide (GW9662), geranylgeranyl pyrophosphate ammonium salt (GGPP), 4,4′-diisothiocyanotostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS), and L-α-lysophosphatidylcholine (LysoPC) were from Sigma-Aldrich (St. Louis, MO, USA) T0070907 and SR9243 from Selleckchem (Boston, MA, USA). Acrylamide bis-acrylamide was from Amresco (Solon, OH, USA). ApoA-I was from Calbiochem (Ontario, Canada). TEMED and ammonium persulfate were from Bio-Rad (Saint-Laurent, Canada). RIPA buffer was from Cell Signaling Technology (Danvers, MA, USA). Dialysis membranes were from Spectrum Medical Industries Inc. (Waltham, MA, USA). Origami B competent cells and BugBuster® His-bind® purification kits were from Novagen (Madison, WI, USA). Ni-NTA agarose was from Qiagen (Montreal, Canada). Primers were from Integrated DNA Technologies, Inc. (San Diego, CA, USA). Cholesterol, [1, 2-^{3}H (N)] (^{3}H-cholesterol), and Western Lightning® Plus-ECL were from Perkin Elmer (Montreal, Canada). Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin (BSA), fetal bovine serum (FBS), sodium pyruvate, PBS, and penicillin/streptomycin were from Wisent (St-Bruno, Canada).

Cell cultures

J774 macrophages (ATCC) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 10% pyruvate sodium, and 100 U·mL^{-1} of penicillin/streptomycin and were maintained in a 5% CO_2 atmosphere at 37 °C.

Animals

Male wild-type C57BL/6 mice were obtained from Charles River Laboratories (Saint-Constant, Canada). The experimental protocol was approved by the Institutional Animal Care and Use Committee at the School of Medicine of the University of Sherbrooke. The mice were handled in strict accordance with Canadian Council on Animal Care guidelines. They were given standard chow diet and water *ad libitum*. Two weeks after their arrival, on the day of the experiment, the mice were weighed and were placed in individual cages for the duration of the experiment (48 h).

Expression and purification of human recombinant PON1 (hrPON1)

Recombinant PON1 was produced in *Escherichia coli* and was purified as previously described by Gaidukov *et al.* [22], with some modifications [23]. Briefly, and described by Loued *et al.* [23], competent Origami B® cells were

transformed with the pET32b plasmid (G3C9-8His) encoding the PON1 sequence and a histidine tag. The cells were grown in 2xTY medium for 8 h at 30 °C then for 12 h at 20 °C. PON1 expression was induced by adding 11 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation, and were resuspended and lysed using BugBuster® kits. Precipitated proteins were eluted on a Ni-NTA resin column. The elution buffer consisted of 50 mM Tris/HCl, 1 mM $CaCl_2$, 50 mM NaCl, 0.1% Tergitol®, and 250 mM imidazole (pH 8.0). Fractions containing PON1 activity were pooled and were extensively dialyzed at 4 °C against 50 mM Tris/HCl (pH 8.0) storage buffer supplemented with 1 mM $CaCl_2$. The pooled fractions were stored in aliquots at − 80 °C. The purity of the enzyme was verified by SDS/PAGE.

Measurement of human recombinant PON1 paraoxonase activity

PON1 paraoxonase activity was measured by recording the increase in absorbance at 412 nm using paraoxon (O, O-diethyl-O-p-nitrophenylphosphate; Sigma) as the substrate as previously described by Jaouad *et al*. [24]. Briefly, 10 μL of hrPON1 was mixed with 250 μL of 100 mM Tris/HCl buffer (pH 8.0) containing 2 mM $CaCl_2$ and 5.5 mM paraoxon at 25 °C. The rate of 4-nitrophenol release was measured at 412 nm, and enzymatic activity was calculated using a molar extinction coefficient of 13 893.75 M^{-1} cm^{-1}. One unit of paraoxonase activity was defined as 1 nmol 4-nitrophenol formed per minute.

Collection of blood and preparation of lipoproteins

Blood samples were collected in EDTA vacuum tubes after overnight fasting. The plasma was collected by low speed centrifugation (1000 ***g***), and the LDL was isolated in the presence of 0.4 $mg{\cdot}mL^{-1}$ of EDTA by ultracentrifugation using a Beckman Optima TLX ultracentrifuge equipped with a TLA-100.4 rotor as previously described by Sattler *et al*. [25]. The LDL was dialyzed extensively overnight at 4 °C against 10^{-2} M sodium phosphate buffer (pH 7). LDL concentrations were measured using protein assay kits (Bio-Rad) and were expressed as total protein concentrations.

Induction of LDL lipid peroxidation

LDL oxidation was induced by exposure to oxygen free radical species generated by irradiating LDL dissolved in 0.01 M sodium phosphate buffer (pH 7) using a ^{60}Co gamma cell 220 (Atomic Energy of Canada, Chalk River, Canada) as previously described [26]. The dose rate of the ^{60}Co gamma cell was 0.13 $Gy{\cdot}s^{-1}$ as determined using a Fricke (ferrous sulfate) dosimeter [27]. The main oxygen free radical species produced were hydroxyl ($^{\cdot}OH$) and superoxide anion ($O_2^{\cdot -}$) radicals with yields of 2.8×10^{-7} and 3.4×10^{-7} $mol{\cdot}J^{-1}$, respectively [28]. The maximum total radiation dose applied was equal to 450 Gy. LDL oxidation was confirmed by measuring conjugated dienes (differential absorbance at 234 nm) and plateaued at 450 Gy.

Extraction and quantification of LysoPC

LysoPC formation in oxidized LDL (oxLDL) and after a 4-h incubation of oxLDL with PON1 was determined as previously described by Loued *et al*. [23]. Briefly, total lipids were extracted using the Folch method [29]. Samples (100 μL) were separated using a Schimadzu liquid chromatography instrument equipped with a quaternary solvent gradient delivery pump, an evaporative light scattering detector (ELSD), and a Licospher Si 60 column (5 μm, 125 mm × 4 mm i.d.; Agilent Technologies, Saint-Laurent, Canada) at 50 °C using a two-solvent gradient elution protocol. Mobile phase A consisted of a chloroform:methanol:30% aqueous ammonia solution (60/34.5/0.5). Mobile phase B consisted of chloroform:methanol:30% aqueous ammonia solution (80/19.5/0.5). Mobile phase A was increased from 0% to 100% over 14 min, maintained at 100% for 10 min, and decreased to 0% over 14 min. Mobile phase B (100%) was then used until the end of the 40-min run. A flow rate of 1 $mL{\cdot}min^{-1}$ was used to separate the various lipid classes. The amount of LysoPC ($\mu g{\cdot}mL^{-1}$) was calculated using an external calibration curve.

Measurement of *in vitro* cholesterol efflux

J774 macrophages (1×10^6 $cells{\cdot}mL^{-1}$) were radiolabeled with 2 $\mu Ci{\cdot}mL^{-1}$ of [^{3}H]-cholesterol in DMEM supplemented with 1% FBS, 1% BSA, 10% sodium pyruvate, and 100 $U{\cdot}mL^{-1}$ of penicillin/streptomycin for 24 h. To confirm that LysoPC enhances cholesterol efflux, [^{3}H]-cholesterol-loaded macrophages were preincubated for 12 h with different concentrations of LysoPC. To confirm that LysoPC, formed when PON1 hydrolyzes oxidized lipids in oxLDL, enhances cholesterol efflux, [^{3}H]-cholesterol-loaded macrophages (control) or [^{3}H]-cholesterol-loaded macrophages were preincubated for 12 h in the presence of cAMP (0.3 mM), oxLDL (100 $\mu g{\cdot}mL^{-1}$) pretreated with PON1 (50 $\mu g{\cdot}mL^{-1}$) (oxLDL–PON1), or LysoPC. To confirm that the LysoPC produced by oxLDL–PON1 mediates ABCA1-dependent cholesterol efflux, [^{3}H]-cholesterol-loaded macrophages were preincubated with oxLDL–PON1 in the presence of ABCA1 signaling pathway antagonists [ABCA1 inhibitor (DIDS), LXRα inhibitors (GGPP and SR9243), or PPARγ inhibitors (GW9662 and T0070907)]. In all experiments, the macrophages were washed three times following the preincubation phase and were then incubated with 50 $\mu g{\cdot}mL^{-1}$ of apoA-I for 4 h to induce

ABCA1-dependent cholesterol efflux. The supernatants were collected and were centrifuged to eliminate detached macrophages. The adherent macrophages were washed three times to eliminate excess [^{3}H]-cholesterol. They were then lysed in 0.1 M NaOH and were sonicated to release intracellular [^{3}H]-cholesterol. Counts per minute (cpm) in the supernatants and cell lysates were determined using a liquid scintillation counter. Cholesterol efflux (radiolabeled cholesterol released from cells) was calculated using the following formula: [radioactivity (cpm) in supernatant/radioactivity (cpm) in cells + medium] × 100.

Assaying *in vivo* reverse cholesterol transport

Briefly, J774 macrophages were radiolabeled with [^{3}H]-cholesterol as previously described [30]. The radiolabeled J774 macrophages were washed and incubated for 12 h in fresh medium containing 1% FBS and 1% BSA for equilibration and then incubated for 6 h with: (a) medium (Control), (b) medium containing 100 $\mu g \cdot mL^{-1}$ of oxLDL + PON1 or (c) 80 μM of LysoPC. They were then washed with PBS and resuspended in DMEM. Cell viability was superior to 98 % according to Trypan blue exclusion. About 100μL of cells suspension (~ 3.5×10^6 dpm) was injected intraperitoneally. Following, animals were individually housed in metabolic cages with free access to food and water. Blood was drawn from the mandibular vein at 6, 24, and 48 h after ^{3}H-cholesterol-radiolabeled macrophages peritoneal injection. Blood was centrifuged and the plasma was immediately stored at 4 °C until the radioactivity was measured. The mice were euthanized 48 h after the intraperitoneal injections, and their livers were harvested and stored at − 20 °C until used. Feces were collected throughout the 48-h period and were stored at 4 °C until the cholesterol and bile acids were extracted. Total liver [^{3}H]-cholesterol was extracted from 50 mg pieces of liver using the Folch method.

Fecal [^{3}H]-cholesterol and [^{3}H]-bile acids were extracted from 100 mg of feces homogenized in 2 mL of distilled water and 2 mL of ethanol. The homogenate (1 mL) was combined with 1 mL of ethanol and 200 μL of NaOH, saponified at 95 °C for 2 h, and cooled to room temperature. The mixture was extracted three times with 4.5 mL hexane. The extracts containing the [^{3}H]-cholesterol were pooled, evaporated, and resuspended in toluene. The radioactivity was counted using a liquid scintillation counter. To extract ^{3}H-bile acids, the aqueous portion of the homogenate was acidified with concentrated HCl and was extracted three times with 4.5 mL of ethyl acetate. The extracts were pooled, evaporated, and resuspended in ethyl acetate. The radioactivity was counted using a liquid scintillation counter.

Macrophage RCT was assessed by monitoring [^{3}H]-cholesterol mobilization from labeled intraperitoneally injected macrophages to the plasma, liver, and feces. The results are expressed as the percentage of radioactivity recovered in the plasma, liver, and feces. The plasma volume was estimated at 3.85% of body weight.

Western blotting

Macrophages were lysed in RIPA buffer. Identical amounts of protein were separated on 10% SDS/PAGE gels and were transferred to poly(vinylidene difluoride) (PVDF) membranes. The membranes were blocked with 5% fat-free powdered milk in TBST (15 mM Tris/HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) and were incubated with ABCA1, ABCG1, PPARγ, LXRα, or β-actin primary antibodies (Abcam, Cambridge, MA, USA). The membranes were washed three times with TBST and were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG H&L or goat anti-rabbit IgG H&L (Abcam). Immunoreactive bands were visualized using the ECL Western blotting system (GE Healthcare, Baie-d'Urfé, Canada).

Quantitative RT-PCR

Total RNA from treated J774 macrophages was extracted using EZ-10 Spin Column Genomic RNA Minipreps kits (Bio Basic Inc., Markham, Canada) according to the manufacturer's protocol. Two micrograms of RNA was transcribed using Reverse Transcriptase Superscript II (Invitrogen, Carlsbad, CA, USA) [8]. ABCA1, ABCG1, PPARγ, and LXRα gene expressions were normalized to the corresponding amount of β-actin. The primer sets (IDT DNA Technology, Coralville, IA, USA) are listed in Table S1. The qPCR assays were performed using the Stratagene MX3005P system (Agilent Technologies) and Brilliant II SYBR Green QPCR Master Mix (Agilent). The qPCR assays were performed with 25 ng of template cDNA. Samples were incubated at 95 °C for 10 min and then for 40 cycles using the following conditions: 95 °C for 40 s, 56 °C for 40 s, and 72 °C for 40 s. All assays were run in triplicate for each replicate, and the average values were used for quantification purposes. The relative quantities of target genes were determined using the $\Delta\Delta C_t$ method. Briefly, the Ct (threshold cycle) values of target genes were normalized to an endogenous control gene (β-actin) ($\Delta C_t = C_{t\ target} - C_{t\ \beta\text{-actin}}$), and were compared to a calibrator ($\Delta\Delta C_t = \Delta C_{t\ sample} - \Delta Ct_{\ calibrator}$). Relative expression (RQ) was calculated using a sequence detection system (MXPRO and QPCR software; Agilent) and the formula $RQ = 2^{-\Delta\Delta Ct}$.

Statistical analysis

Values are expressed as means ± SEM. Each experiment was repeated at least in triplicate. Mean values were compared using one-way ANOVA. *P*-values ≤ 0.05 were considered significant. Statistical analyses were performed using GRAPHPAD PRISM 6.0 (GraphPad Software®, Inc., La Jolla, CA, USA).

Results

PON1 hydrolyzes oxLDL to form LysoPC

LDLs were oxidized by exposure to oxygen free radical species produced by γ radiolysis of water at 450 Gy. This radiation dose generated the highest LDL oxidation rate as measured by conjugated diene formation (result not shown). OxLDL were then incubated for 4 h with a physiological concentration of PON1 (50 $\mu g \cdot mL^{-1}$) [31]. Incubating PON1 with oxLDL caused a significant increase in LysoPC levels compared to oxLDL alone (43.8% increase, $P < 0.0001$) (Fig. 1A). The inactivation of PON1 by incubation with N-ethylmalemide (NEM) or exposure to γ-ray-induced free radicals [23] significantly reduced the capacity of PON1 to increase LysoPC formation in the presence of oxLDL (Fig. 1A).

Cholesterol efflux is enhanced in LysoPC-treated macrophages

LysoPC is a major lipid component of oxLDL that can activate various cells. To determine whether the LysoPC formed by the hydrolytic action of PON1 on oxLDL can activate macrophages, we studied the capacity of purified LysoPC to stimulate cholesterol efflux. J774 macrophages were radiolabeled with 2 $\mu Ci \cdot mL^{-1}$ of [^{3}H]-cholesterol and were incubated with increasing concentrations of LysoPC (0–80 μM). These LysoPC concentrations are in the same range as the physiological concentration of free LysoPC in the plasma (60–100 μM) [32] and far below plasma albumin-associated LysoPC (0.2 mM) [33]. Our results showed that purified LysoPC significantly stimulated cholesterol efflux from J774 macrophages to apoA-I in a LysoPC concentration-dependent manner (Fig. 1B).

OxLDL–PON1 stimulates macrophage-derived cholesterol efflux

We investigated whether LysoPC formed by the hydrolytic action of PON1 (Fig. 1A) can stimulate cholesterol efflux from macrophages and explored the cholesterol efflux pathway involved. We used J774 macrophages because they express very low levels of ABCA1 in basal conditions and because cAMP upregulates ABCA1 expression in these macrophages [34]. Stimulating J774 macrophages with cAMP induced a significant increase in cholesterol efflux, whereas incubating them with oxLDL significantly reduced cholesterol efflux by 18.09% compared to the control ($P < 0.002$) (Fig. 1C). Interestingly, pretreating oxLDL with PON1 (oxLDL–PON1) before adding it to the radiolabeled J774 macrophages reversed the effect of oxLDL alone and induced a significant increase in cholesterol efflux from J774 macrophages (47.18% increase in cholesterol efflux compared to oxLDL alone, $P < 0.0001$) (Fig. 1C).

To further explore the mechanisms by which PON1 stimulates cholesterol efflux, we measured cholesterol efflux via the ABCA1 transporter and used DIDS as an ABCA1 inhibitor. Incubating oxLDL–PON1 with J774 macrophages in the presence of DIDS significantly reduced ABCA1-dependent cholesterol efflux (47.41% reduction of cholesterol efflux compared to oxLDL–PON1, $P < 0.0001$) (Fig. 1C). It is well known that ABCA1 expression is induced by PPARγ via a transcriptional cascade mediated by the nuclear receptor LXRα [21]. Given this, we investigated the effects of two other inhibitors of the ABCA1 signaling pathway, GW9662 and GGPP, which are PPARγ and LXRα antagonists, respectively [35,36]. Adding these inhibitors caused a significant reduction in ABCA1-dependent cholesterol efflux [28.22% for GW9662 and 18.70% for GGPP ($P < 0.001$)] compared to oxLDL–PON1 alone (Fig. 1C).

OxLDL–PON1 stimulates cholesterol efflux by upregulating ABCA1/ABCG1 synthesis

To determine whether oxLDL–PON1 regulates ABCA1 synthesis, we measured ABCA1 protein expression by J774 macrophages before and after the incubation with oxLDL–PON1. cAMP-induced ABCA1 stimulation was used as a control (Fig. 2A). As expected, oxLDL induced a slight but nonsignificant reduction in ABCA1 protein expression. However, pretreating oxLDL with PON1 caused a 58.51% increase in ABCA1 protein expression compared to oxLDL alone ($P < 0.0001$). OxLDL–PON1-induced ABCA1 protein expression was comparable to that induced by cAMP (Fig. 2A). Interestingly, the stimulatory effect was significantly reduced in the presence of the ABCA1 inhibitor DIDS and the two signaling pathway antagonists GW9662 and GGPP (Fig. 2A,B).

The effect of oxLDL–PON1 on ABCA1 expression was also measured in the presence of two potent antagonists of LXRα and PPARγ, respectively SR9243 and T0070907 [37,38]. Our results show that the stimulatory effect of oxLDL–PON1 toward ABCA1 expression is significantly reduced in the presence of SR9243 or T0070907 (Fig 2C). Interestingly, inactivation of PON1 with NEM also affects the capacity of oxLDL–PON1 to stimulate the ABCA1 protein expression (Fig 2C).

Unlike ABCA1, ABCG1 is a protein transporter involved in cholesterol efflux to mature HDL (HDL_2

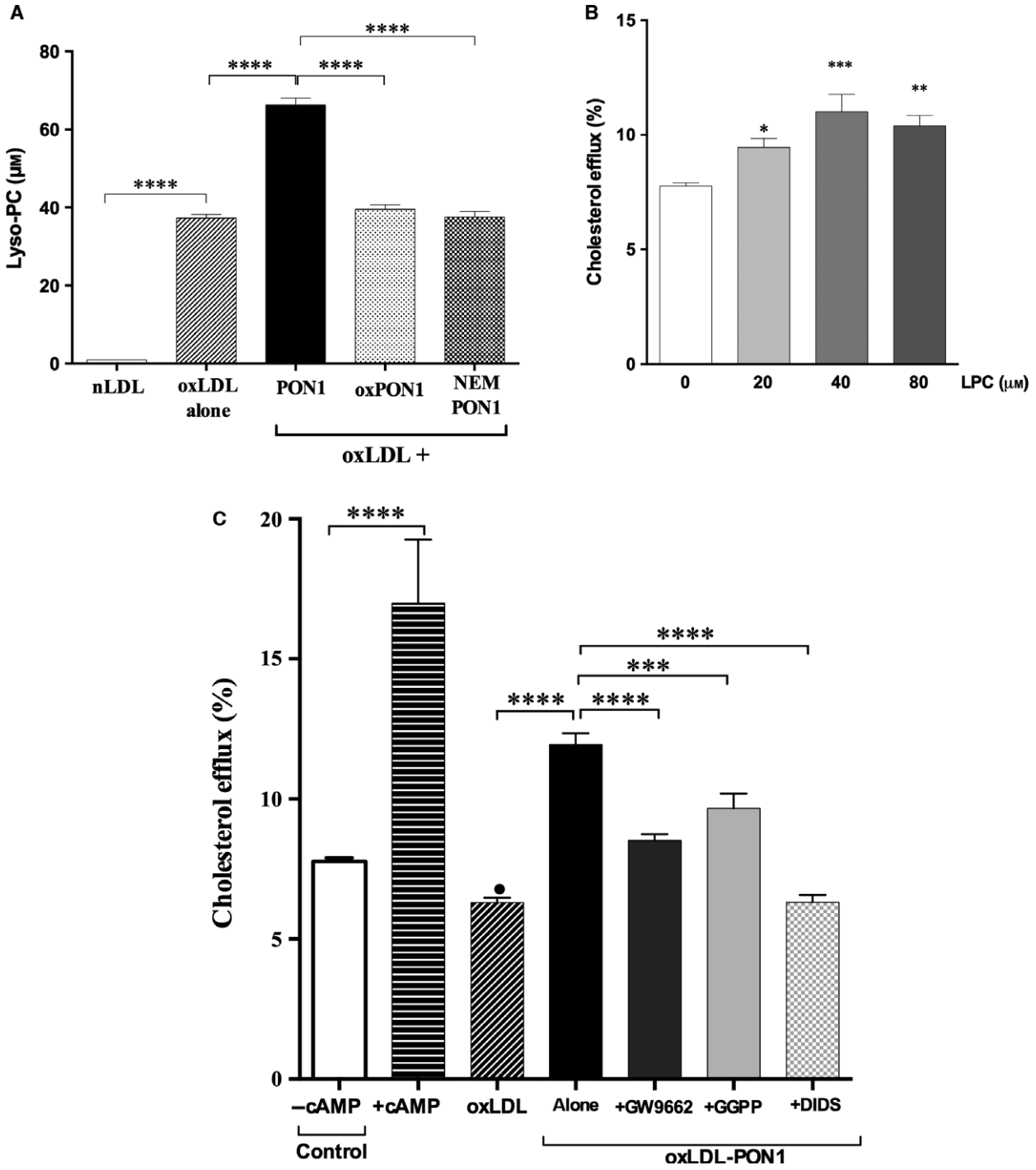

Fig. 1. LysoPC and oxLDL–PON1 stimulate ABCA1-dependent cholesterol efflux. (A) OxLDL (100 $\mu g \cdot mL^{-1}$) was incubated in the presence of PON1 (50 $\mu g \cdot mL^{-1}$). HPLC evaporative light scattering was used to detect LysoPC. PON1 was inactivated by exposure to oxygen free radicals (oxPON1) or NEM. J774 macrophages (1×10^6 cells$\cdot mL^{-1}$) were loaded with [^{3}H]-cholesterol (2 $\mu Ci \cdot mL^{-1}$) for 24 h and were incubated 12 h with (B) increasing concentrations of LysoPC (0–80 μM) or (C) cAMP (0.3 mM), oxLDL (100 $\mu g \cdot mL^{-1}$), oxLDL–PON1 [oxLDL (100 $\mu g \cdot mL^{-1}$) were oxidized and were then incubated for 4 h with hrPON1 (50 $\mu g \cdot mL^{-1}$) at 37 °C], or oxLDL–PON1 in the presence of PPARγ, LXRα, or ABCA1 inhibitors [GW9662 (20 μM), GGPP (10 μM), and DIDS (400 μM), respectively]. Data are expressed as means ± SEM. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.0001$. •$P < 0.002$ oxLDL compared to the control.

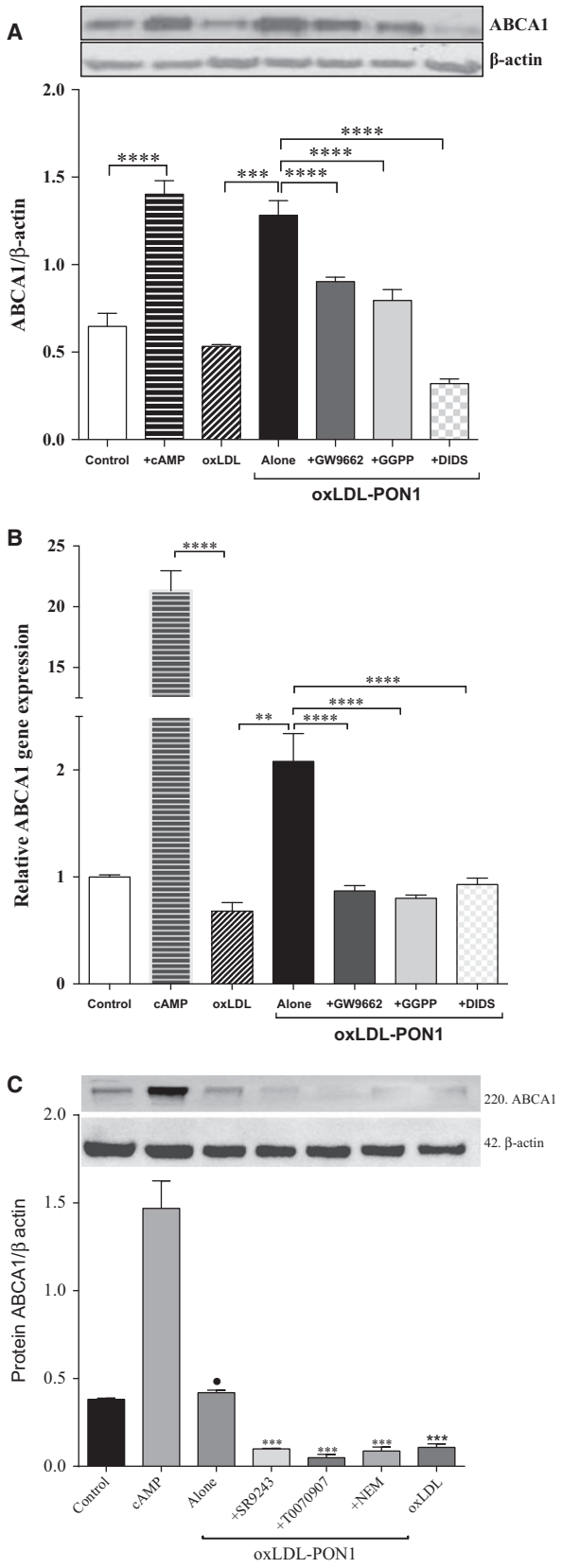

Fig. 2. PON1 hydrolyzes oxLDL and regulates ABCA1 protein and RNA expression. J774 macrophages (1×10^6 cells·mL^{-1}) were incubated for 12 h with cAMP (0.3 mM), oxLDL (100 μg·mL^{-1}), oxLDL–PON1 [oxLDL (100 μg·mL^{-1}) and were then incubated for 4 h with hrPON1 (50 μg·mL^{-1}) at 37 °C), or oxLDL–PON1 + GW9662 (20 μM), GGPP (10 μM), or DIDS (400 μM). ABCA1 protein and RNA expression were determined by (A) western blot and (B) RT-PCR, respectively. (C) Macrophages were incubated with cAMP, oxLDL–PON1 alone or in the presence of LXRα inhibitor (SR9243 1 μM), PPARγ inhibitor (T0070907, 1 μM), NEM (10 μM), or oxLDL. Data are expressed as means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.0005$, $^{****}P < 0.0001$.

and HDL_3) and not to nascent lipid-poor apoA-I. While we did not study the effect of oxLDL–PON1 on cholesterol efflux via the ABCG1 pathway, we did measure the effect of oxLDL–PON1 on ABCG1 expression by J774 macrophages. Our results showed that ABCG1 protein expression by J774 macrophages decreased by 17.72% ($P < 0.03$) in the presence of oxLDL, whereas oxLDL pretreated with PON1 increased ABCG1 protein expression by 51.80% ($P < 0.0001$). As with ABCA1, the stimulatory effect of oxLDL–PON1 was slightly but significantly reduced in the presence of PAPRγ and LXRα antagonists [11.67% and 10.94% reduction, respectively ($P < 0.001$)] (Fig. 3A). ABCG1 RNA expression was also significantly increased when the macrophages were incubated with oxLDL–PON1 and this effect was inhibited in the presence of PPARγ and LXRα antagonists (Fig. 3B).

OxLDL–PON1 stimulates ABCA1 expression through the PPARγ/LXRα signaling pathway

To determine whether PPARγ and LXRα are involved in the upregulation of ABCA1 and ABCG1 expression when macrophages are incubated with oxLDL–PON1, RT-PCR and western blot analyses were performed. OxLDL–PON1 upregulated the expression of the PPARγ and LXPα genes (Fig. 4A,C). This upregulation was significantly reduced in the presence of GW9662 and GGPP. OxLDL–PON1 also had a significant stimulatory effect on PPARγ and LXRα protein expression compared to oxLDL alone (Fig. 4B,D). This effect was reduced in the presence of PPARγ and LXRα inhibitors (Fig. 4B,D).

Pretreating macrophages with oxLDL–PON1 enhances RCT *in vivo*

Lastly, we performed an RCT assay to determine whether the high cholesterol efflux induced *in vitro* by

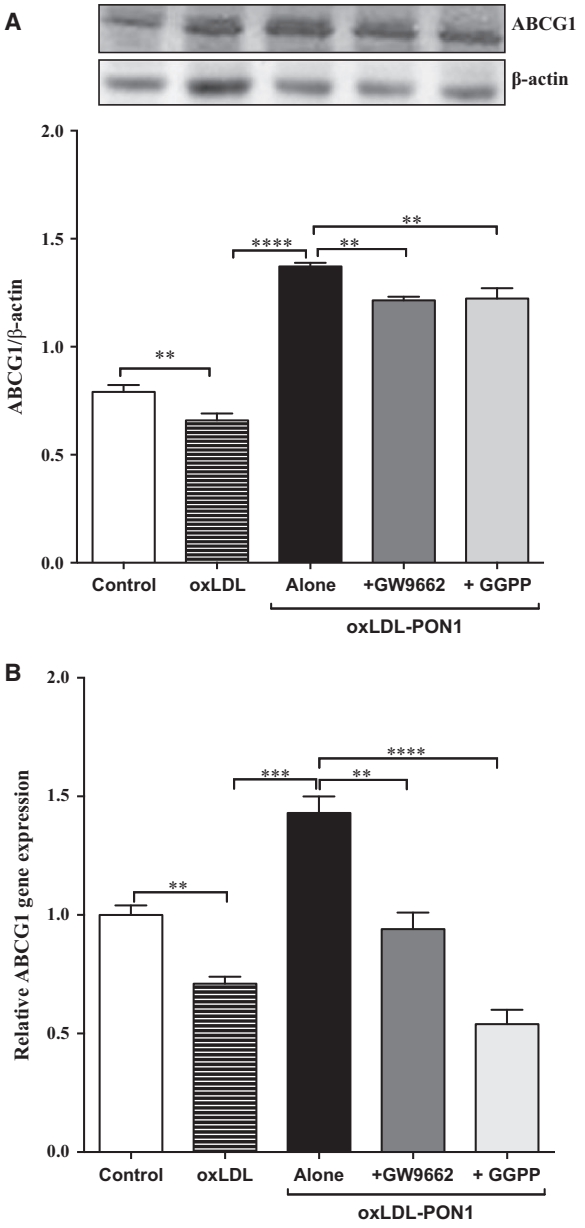

Fig. 3. PON1 hydrolyzes oxLDL and regulates ABCG1 and SR-BI expression. J774 macrophages were incubated for 12 h with oxLDL (100 μg·mL^{-1}), oxLDL–PON1 [oxLDL (100 μg·mL^{-1}) were oxidized and were then incubated for 4 h with hrPON1 (50 μg·mL^{-1}) at 37 °C], and oxLDL–PON1 + GW9662 (20 μM) or GGPP (10 μM). ABCG1 protein and RNA expression were determined by (A) western blot and (B) RT-PCR, respectively. Data are expressed as means ± SEM. (C) SR-BI protein expression was determined by western blot. Each experiment was repeated at least in triplicate. Data are expressed as means ± SEM. $^{**}P < 0.0024$, $^{***}P = 0.0001$, $^{****}P < 0.0001$.

oxLDL–PON1 contributes to cholesterol homeostasis *in vivo* by upregulating the transport of cholesterol from peripheral cells to the liver and its excretion into bile and feces. [^{3}H]-cholesterol-loaded macrophages were preincubated for 12 h with oxLDL (control), oxLDL–PON1, or purified LysoPC and were injected into the peritoneal cavities of the mice. [^{3}H]-cholesterol counts in plasma were measured and were expressed as a percentage of the total labeled cholesterol injected. [^{3}H]-cholesterol levels in the plasma of oxLDL–PON1 mice were significantly higher at all time points (6, 24, and 48 h) than those of control mice (72.17 to 63.98% higher, $P < 0.0001$ at times 24 and 48 h) (Fig. 5A). Interestingly, pretreating the macrophages with LysoPC before injecting them in the mice increased [^{3}H]-cholesterol levels in the plasma compared to a pretreatment with oxLDL–PON1 (Fig. 5A).

[^{3}H]-cholesterol counts in the liver of oxLDL–PON1 and LysoPC mice were 72.07% ($P < 0.001$) and 79.04% ($P < 0.02$) higher, respectively, after 48 h than in control mice (Fig. 5B). Pretreating the macrophages with oxLDL–PON1 or LysoPC prior to injection increased the fecal excretion of macrophage-derived cholesterol by 64.39% ($P < 0.005$) and 84.46% ($P < 0.04$), respectively, compared to control mice (Fig. 5C). The excretion of cholesterol by oxLDL–PON1 and LysoPC mice was significantly higher in terms of both sterols and biliary acids (Fig. 5C).

To determine whether the stimulation of RCT when macrophages were preincubated with LysoPC is due to an upregulation the PPARγ–LXRα–ABCA1 pathway, we used two potent inhibitors of LXRα and PPARγ, respectively SR9243 and T0070907. Our results show that treating macrophages with LysoPC in the presence of PPARγ and LXRα inhibitors prior to injecting them into mice reduced significantly the [^{3}H]-cholesterol levels in the plasma at all time points (6, 24, and 48 h) when compared to those of macrophages preincubated with LysoPC alone or control (without LysoPC) (about 36.65% reduction for both SR9243 and T0070907 when compared to LysoPC, $P < 0.001$) (Fig. 6A). Similar results were obtained for the measurement of [^{3}H]-cholesterol levels in the liver; that is a reduction of [^{3}H]-cholesterol when macrophages were treated with LysoPC in the presence of SR9243 and T0070907 (Fig. 6B). Moreover, pretreating the macrophages with LysoPC in the presence of SR9243 and T0070907 prior to intraperitoneal injection reduced also the fecal excretion of macrophage-derived cholesterol (sterols and biliary acids) by about 69% when compared to macrophages treated with LysoPC alone ($P < 0.001$) (Fig. 6C).

Discussion

Like apoA-I, which is the main apoprotein of HDL and which plays an important role in initiating

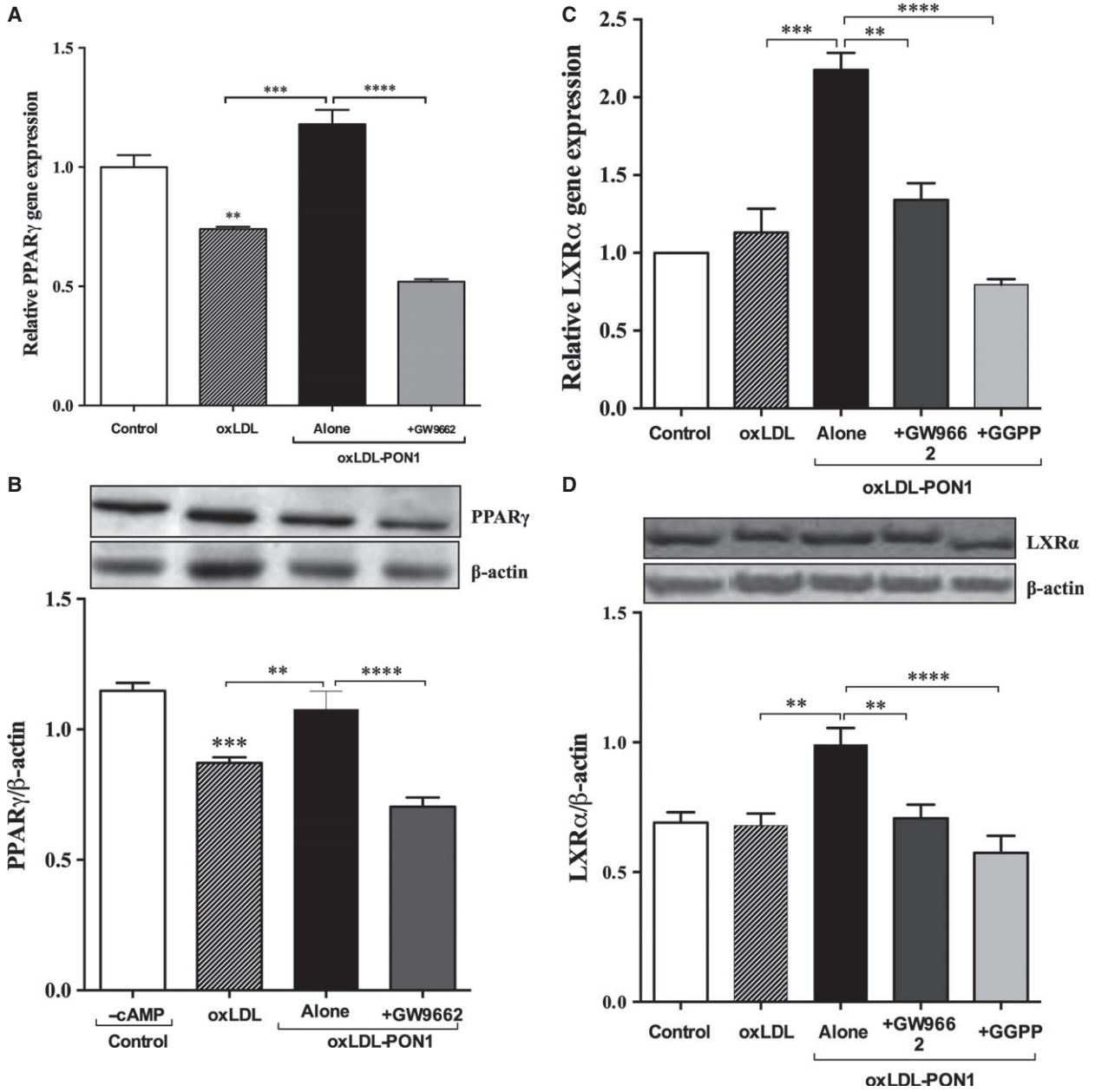

Fig. 4. OxLDL–PON1 regulates LXRα and PPARγ protein and RNA expression. J774 macrophages were incubated with oxLDL (100 μg·mL^{-1}) or oxLDL that had been pretreated for 4 h with PON1 (oxLDL–PON1; 100–50 μg·mL^{-1}) alone or in the presence of PPARγ or LXRα inhibitors [GW9662 (10 μM) and GGPP (20 μM), respectively]. (A, B) PPARγ and (C, D) LXRα protein and RNA expression were analyzed by western blot and RT-PCR, respectively. Each experiment was repeated at least in triplicate. Data are expressed as means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.0005$, $^{****}P < 0.0001$.

cholesterol efflux from macrophages, PON1 is one of many other HDL proteins that may play a significant role in regulating cholesterol homeostasis [8,14,39]. Various studies, including ours, have shown that PON1 promotes cholesterol efflux by acting as an independent acceptor of free cholesterol from macrophages via the ABCA1 pathway and by stimulating cholesterol efflux to apoA-1 and HDL [8,14,39]. However, the mechanism involved in the *in vitro* stimulation of cholesterol efflux and its impact on the enhancement of RCT *in vivo* remain to be clarified.

Our results showing that incubating oxLDL with PON1 contributes to a significant increase in LysoPC formation were in agreement with previous studies

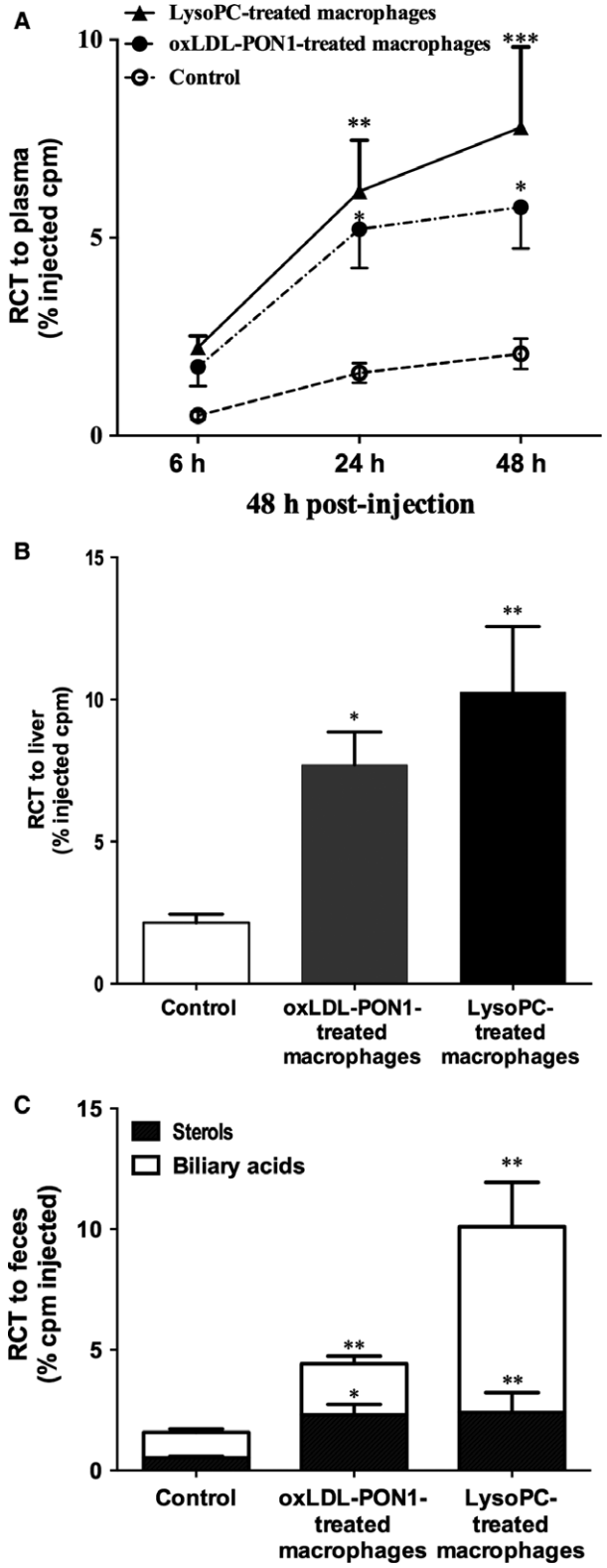

Fig. 5. Preincubating J774 macrophages with oxLDL–PON1 or LysoPC increases RCT. [^{3}H]-cholesterol-loaded J774 macrophages alone (control) or incubated with oxLDL (100 μg·mL^{-1}), oxLDL–PON1 (100–50 μg·mL^{-1}), or LysoPC (80 μM) were intraperitoneally injected in the mice. [^{3}H]-cholesterol plasma levels were measured at 0, 6, 24, and 48 h postinjection. Feces were collected continuously from 0 to 48 h postinjection. (A) Time course of ^{3}H-cholesterol distribution in plasma, (B) ^{3}H-cholesterol recovery in the liver after 48 h, (C) ^{3}H-cholesterol recovery in feces as sterols and biliary acid. Data are expressed as means ± SEM versus the control. n = 5 mice/group, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0005$.

suggesting that PON1 possesses phospholipase-A2-like activity that allows it to hydrolyze oxidized phospholipids at the sn*2* position, generating LysoPC and free oxidized fatty acids [23,40,41]. Marathe *et al.* raised doubts about the PLA2-like activity of plasma PON1 and attributed it to contamination with PAF-AH [42]. It is worth noting that, in the present study, we used recombinant PON1, which should not be contaminated with PAF-AH. Moreover, inactivating PON1 with NEM or oxygen free radicals, which block or oxidize the only free SH group of the active site of PON1, respectively, caused a significant reduction in the capacity of PON1 to form LysoPC when incubated with oxLDL. Rosenblat *et al.* [16] also showed that PON1 hydrolyzes oxidized lipids in macrophages to form LysoPC and associated this effect with the PON1 lactonase activity. However, regardless of the mechanism by which PON1 hydrolyzes oxidized lipids, our results confirmed that this hydrolytic activity of PON1 contributes to the formation of LysoPC in oxLDL.

LysoPC is a major lipid component of atherogenic lipoproteins and is reported to have a proatherogenic function, notably by inducing the production of several growth factors and the expression of chemoattractant genes [43]. On the other hand, LysoPC can also play an important antiatherogenic role by, in part, increasing the local synthesis of nitric oxide in the initial phases of the formation of atherosclerotic plaques, leading to vasodilation [44,45]. The antiatherogenic properties of LysoPC may also include its capacity to regulate macrophage-derived cholesterol homeostasis [46,47]. Our results showed that LysoPC stimulates macrophage-derived cholesterol efflux to apoA-I in a LysoPC concentration-dependent manner. The increase in macrophage-derived cholesterol efflux in the presence of oxLDL–PON1 could thus be explained by the stimulatory effect of LysoPC formed by the hydrolysis of oxLDL by PON1.

Macrophage-derived cholesterol efflux is mediated via four pathways (ABCA1, ABCG1, SR-BI, and cholesterol diffusion). ABCA1 mediates the transport of cholesterol and phospholipids to lipid-free apolipoproteins, principally apoA-I, and is the first and rate-limiting pathway for cholesterol efflux. We

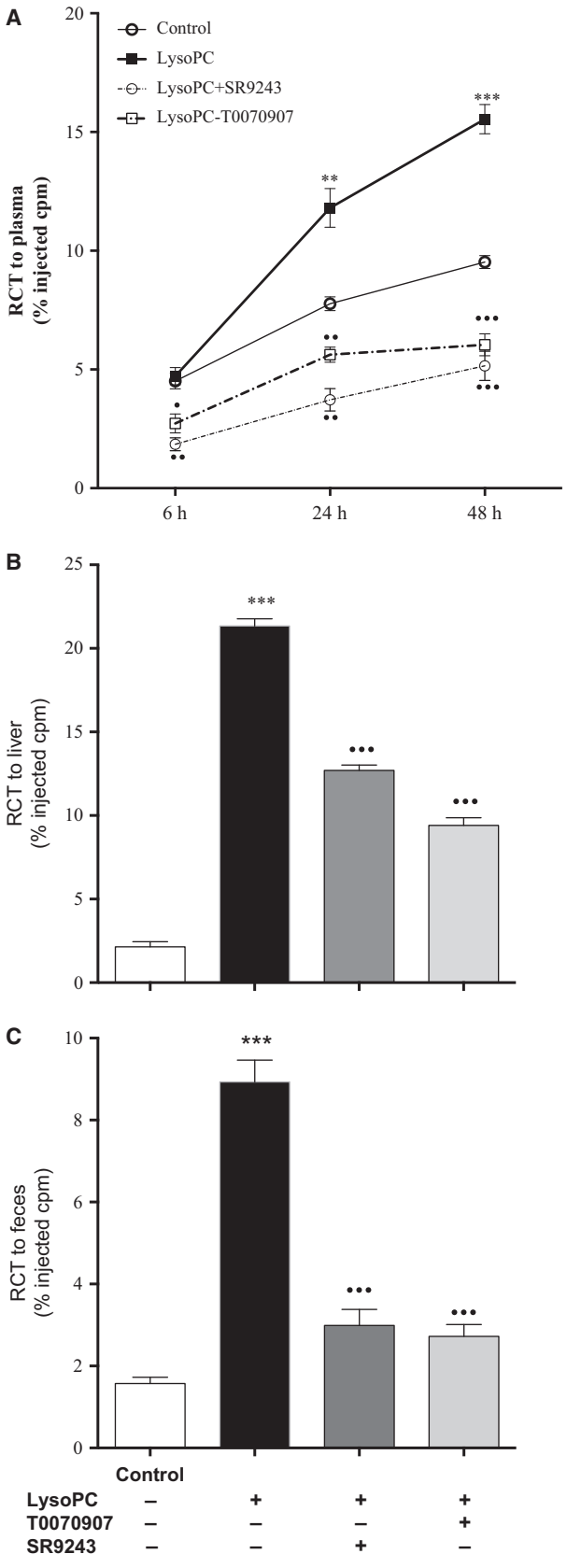

Fig. 6. Inhibition of ABCA1 expression on macrophages decreases RCT. [^{3}H]-cholesterol-loaded J774 macrophages alone (control) or incubated with LysoPC (80 μM), LysoPC and LXRα inhibitor (SR9243, 1 μM) or LysoPC and PPARγ inhibitor (T0070907, 1 μM) and were then intraperitoneally injected into mice. [^{3}H]-cholesterol plasma levels were measured at 0, 6, 24, and 48 h postinjection. Feces were collected continuously from 0 to 48 h postinjection. (A) Time course of ^{3}H-cholesterol distribution in plasma, (B) ^{3}H-cholesterol recovery in the liver after 48 h, (C) ^{3}H-cholesterol recovery in feces (sterols and biliary acid). Data are expressed as means ± SEM versus the control or versus LysoPC alone. $^{**}P < 0.01$, $^{***}P < 0.0005$ compared to control and $P < 0.01$ and $P < 0.0005$ compared to LysoPC.

used J774 macrophages and lipid-free apoA-I as cholesterol acceptors to study the mechanism by which oxLDL–PON1 stimulates cholesterol efflux. This allowed us to investigate cholesterol efflux via the apoA-I–ABCA1 interaction. Our results showed that oxLDL–PON1 significantly increases ABCA1-dependent cholesterol efflux, while DIDS significantly inhibits cholesterol efflux. This suggested that LysoPC formed during the hydrolysis of oxLDL by PON1 stimulates cholesterol efflux via the ABCA1 pathway. Rosenblat *et al.* showed that purified PON1 stimulates cholesterol efflux by increasing HDL binding to macrophages [14]. This has been explained by the hydrolysis of cell surface phospholipids by PON1 to form LysoPC, which in turn increases the apo-AI–ABCA1 binding responsible for higher cholesterol efflux [14]. We recently showed that purified human PON1 enhances HDL-mediated cholesterol efflux [8] and that this process is associated with an upregulation of the ABCA1 transporter [8]. While the mechanism of this upregulation has not been investigated, we hypothesized that it could involve the LysoPC formed by the hydrolytic activity of PON1 [39]. Our results, which are consistent with those of Hou *et al.*, who reported that the increase in LysoPC-induced cholesterol efflux is due to an upregulation of ABCA1 transporter [47], showed that incubating oxLDL–PON1 with macrophages induces a significant increase in ABCA1 mRNA and protein expression and that this effect is significantly reduced in the presence of DIDS, an ABCA1 inhibitor. OxLDL alone (without PON1), and despite the formation of LysoPC, do not stimulate the expression of ABCA1. This discrepancy could be attributed to an increase in the uptake of oxLDL by J774 macrophages for the formation of foam cells [48]. Preincubation of oxLDL with PON1, and due to the capacity of PON1 to hydrolyze lipid peroxides [49], might reduces the oxidative damages within oxLDL and therefore reduces their uptake by macrophages.

Nevertheless, our results are consistent with findings from other studies, even showing a reduction in the expression of ABCA1 on J774 macrophages in the presence of oxLDL [50,51].

The transcriptional regulation of ABCA1 is mediated by LXRα, which in turn is regulated by PPARγ. The regulation of the PPARγ–LXRα–ABCA1 pathway is thus critical for cholesterol efflux from peripheral cells [52]. Interestingly, our results showed that incubating oxLDL–PON1 with macrophages upregulates the mRNA and protein expression of both LXRα and PPARγ and that this effect is inhibited in the presence of their respective inhibitors (GW9662 and GPPP). The upregulation of ABCA1 in the presence of oxLDL–PON1 is also significantly reduced in the presence of LXRα and PPARγ antagonists. Given that LysoPC is the major product of PON1 hydrolytic activity in the presence of oxLDL and that LysoPC can stimulate the PPARγ–LXRα–ABCA1 pathway, this strengthens the notion that oxLDL–PON1 promotes cholesterol efflux by the activation of the PPARγ–LXRα–ABCA1 pathway in J774 macrophages and that this effect is due to the LysoPC formed by the hydrolytic activity of PON1. Previous study of Delerive *et al*. showed that oxLDL induced the activation of PPARα on human coronary endothelial cells in a phospholipase A2-dependent manner [53]. In the present study, we did not investigate the effect of oxLDL–PON1 on PPARα expression on macrophages; however, our results confirm that PON1, due to its PLA2-like activity, hydrolyzes oxLDL to induce the activation of PPARγ.

ABCG1 is another macrophage-derived cholesterol transporter that, unlike ABCA1, is thought to facilitate cholesterol efflux to HDL rather than to free apoA-I and lipid-poor apoA-I [54]. Vaughan and Oram reported that the ABCA1 and ABCG1 transporters act sequentially to eliminate excess of cholesterol from cells, with ABCA1 acting first by transporting cholesterol to lipid-poor apoA-I [55]. Our results showed that ABCG1 mRNA and protein expression by J774 macrophages also increased when the macrophages are incubated with oxLDL–PON1. Furthermore, the stimulatory effect of oxLDL–PON1 on ABCG1 mRNA and protein expression was significantly reduced in the presence of LXRα and PPARγ inhibitors. Interestingly, unlike ABCA1 mRNA expression, which is regulated in a number of cell types by signaling mechanisms independent of both LXR isotypes, ABCG1 mRNA expression is exclusively under the transcriptional control of LXRα [56]. This provides support for the notion that the upregulation of ABCA1 and ABCG1 by J774 macrophages that have been incubated with oxLDL–PON1 is principally due to the activation of the PPARγ–LXRα pathway and that this effect is due to the LysoPC formed by the hydrolytic activity of PON1.

The oxidation of LDL is a critical early event in the pathogenesis of atherosclerosis [57]. OxLDL is present in human atheroma [58] and is the proximal source of lipid that accumulates in the cells of atherosclerotic lesions [57]. Mackness *et al*. showed that PON1 levels are higher in the artery lesions and suggested that this is a protective response to oxidative stress associated with the development of atherosclerosis [59]. Moreover, Cohen *et al*. reported that PON1 levels and activity are higher in atherosclerotic plaque and suggested that PON1 decreases plaque atherogenicity by reducing the levels of specific oxidized lipids [60,61]. The coexistence of PON1 in its active form and oxLDL in the atherosclerotic plaque may promote the formation of oxLDL–PON1 complexes that initiate the hydrolysis of oxidized phospholipids and the formation of LysoPC as proposed in the present study.

The antiatherogenic activity of PON1 is attributed principally to its antioxidant and anti-inflammatory activities [13,24,62]. However, several other studies have indicated that PON1 plays a significant role in stimulating macrophage-derived cholesterol efflux, which may be critical in reducing atherosclerotic plaque formation [8,14,39]. There is increasing evidence that PON1 is involved in regulating cholesterol efflux and in maintaining cholesterol homeostasis at the cellular level [13,24,62]. Our results pointed to a mechanism by which PON1 stimulates macrophage-derived cholesterol efflux and suggested that this effect is mediated by the capacity of PON1 to form LysoPC.

Reverse cholesterol transport measurements showed that pretreating macrophages with oxLDL–PON1 before injecting them into the peritoneal cavities of the mice induces a significant increase in fecal excretion of macrophage-derived cholesterol, which is consistent with the *in vitro* findings. The RCT process is composed of three key steps, the efflux of cholesterol from macrophages to plasma apoA-I and HDL, the transport of cholesterol from plasma HDL to the liver, and the excretion of HDL-derived cholesterol from the liver to bile. Our results suggested that PON1, by stimulating macrophage cholesterol efflux, increases cholesterol transport to bile for excretion. Rosenblat *et al*. showed that the intraperitoneal injection of PON1 in mice increases the antiatherogenic activity of HDL and reduces the cholesterol content of peritoneal macrophages [63]. We injected macrophages that were enriched with ABCA1/ABCG1 by preincubating them with oxLDL–PON1. Inversely, preincubation of

macrophages with oxLDL–PON1 in the presence of LXRα or PPARγ inhibitors and injecting them into mice affects significantly the RCT process. This highlighted the fact that PON1 may stimulate RCT *in vivo* by increasing the expression of membrane cholesterol transporters and that this effect is largely due to the hydrolytic activity of PON1, which causes the overexpression of membrane cholesterol transporters by activating the LXRα–PPARγ pathway. Several studies have shown that the activation of this pathway helps protect against atherosclerosis. This led to the development of several synthetic LXRα agonists. While Breevoort *et al*. reported that RCT is due to the functional activity of HDL rather than macrophage-derived cholesterol efflux [64], our results showed that an increase of cholesterol transporters on macrophages significantly increases the fecal excretion of macrophage-derived cholesterol. Our results are in disagreement with the results of Naik *et al*. advancing that treating J774 macrophages with potent LXR agonists prior injecting them into mice has no effect on RCT [65]. However, Wang *et al*. showed that injecting macrophages obtained from ABCA1-deficient mice reduced significantly the RCT [66], which is in agreement with our results and strengthen the importance of macrophages ABCA1 levels in the RCT process. Furthermore, the discrepancy between our results and those of Naik *et al*. or Breevort *et al*. [64,65] could be attributed to the fact that LysoPC and oxLDL–PON1 activate additional pathways influencing RCT independent of PPARγ–LXRα–ABCA1 pathway.

In summary, we showed that PON1 contributes to the hydrolysis of oxidized phospholipids in oxLDL at the sn*2*-position to form LysoPC. Purified LysoPC and LysoPC formed by the hydrolytic activity of PON1 stimulated macrophage-derived cholesterol efflux to apoA-I in a LysoPC concentration-dependent manner. LysoPC stimulated cholesterol efflux by activating the LXRα–PPARγ–ABCA1 pathway, causing the overexpression of the ABCA1 and ABCG1 transporters on macrophages. Increased ABCA1/ABCG1 expression and macrophage-derived cholesterol efflux contribute to cholesterol homeostasis by increasing the fecal excretion of macrophage-derived cholesterol. The question that remains to be answered is whether a cholesterol efflux mechanism involving PON1 exists *in vivo*? There is some evidence, including the coexistence of PON1 and oxLDL in atherosclerotic lesions, to suggest that such a mechanism does indeed exist *in vivo*. However, further studies are required to show that PON1 can maintain cholesterol homeostasis by stimulating RCT.

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Author contributions

AK conceived and supervised the study; AK and SI designed experiments; SI and OKS performed experiments; AK, SI and HB analyzed data; AK, SI and HB wrote the manuscript.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Sequences of primers for real-time quantitative PCR.