Reduction of atherosclerosis in apolipoprotein E knockout mice by activation of the retinoid X receptor

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A common feature of many metabolic pathways is their control by retinoid X receptor (RXR) heterodimers. Dysregulation of such metabolic pathways can lead to the development of atherosclerosis, a disease influenced by both systemic and local factors. Here we analyzed the effects of activation of RXR and some of its heterodimers in apolipoprotein E −/− mice, a well established animal model of atherosclerosis. An RXR agonist drastically reduced the development of atherosclerosis. In addition, a ligand for the peroxisome proliferator-activated receptor (PPAR)γ and a dual agonist of both PPARα and PPARγ had moderate inhibitory effects. Both RXR and liver X receptor (LXR) agonists induced ATP-binding cassette protein 1 (ABC-1) expression and stimulated ABC-1-mediated cholesterol efflux from macrophages from wild-type, but not from LXRα and β double −/−, mice. Hence, activation of ABC-1-mediated cholesterol efflux by the RXR/LXR heterodimer might contribute to the beneficial effects of rexinoids on atherosclerosis and warrant further evaluation of RXR/LXR agonists in prevention and treatment of atherosclerosis.

Retinoid X receptors (RXRs) are ubiquitously expressed nuclear receptors that heterodimerize with a number of other receptors (e.g., thyroid hormone receptor, vitamin D receptor, retinoic acid receptors, etc.; refs. 1 and 2). The elucidation of RXRs biological activity has advanced significantly through the characterization of rexinoids, high-affinity selective synthetic ligands for RXRs (1, 2). The use of rexinoids led to the demonstration that RXRs are active and permissive signaling molecules in heterodimers with the farnesol X receptor (FXR) [or bile acid receptor (BAR)], the liver X receptors (LXRs), and the peroxisome proliferator-activated receptors (PPARs). The simultaneous activation of several permissive heterodimers underlies in part RXRs pleiotropic functions, affecting numerous receptor signaling pathways, and ranges from the control of cell proliferation, differentiation, and apoptosis (3) to the regulation of glucose and lipid metabolism (4). This pivotal role of the various permissive RXR heterodimers is in fact an emerging theme in multiple metabolic pathways. Heterodimers between RXR and PPARγ, PPARα, LXRα, and FXR/BAR respectively influence glucose, triglyceride, cholesterol, and bile acid homeostasis. Dysregulation of these homeostatic control pathways can result in common metabolic disorders such as obesity, type 2 diabetes, and hyperlipidemia, which are often complicated by the development of atherosclerosis.

In view of the potential implication of RXRs in various metabolic pathways implicated in atherosclerosis, we studied whether modulating the activity of these receptors or of some of their heterodimers affects the development of atherosclerosis in apolipoprotein (apo)E-deficient mice. We demonstrate here that the administration of rexinoids significantly attenuates atherosclerosis development, and we show that this effect might be linked to the activation of reverse cholesterol transport through a stimulation of ABC-1-mediated cholesterol efflux by the RXR/LXR heterodimer.

Experimental Procedures

Animals and Analysis of Lipid and Glucose Metabolism. Five groups of 15 apoE −/− mice in a C57BL/6J background (Transgenic Alliance, L'Arbesle, France) were used. All of the mice were fed ad libitum. Blood was obtained after a 12-h fast by retroorbital puncture under barbital-induced anesthesia and serum aliquots were stored at 4°C or −20°C until analysis. After 11 weeks, the animals were killed, organ weights were recorded, and tissues (aorta, adipose tissue, liver, and heart) were collected for further analysis. Serum lipids (cholesterol and triglycerides), FPLC lipoprotein size-exclusion chromatography, levels of apoA-I, apoA-II, apoC-III, and apoB were determined before and at the end of the study (5). Blood glucose was measured by using a Glucotrend 2 (Roche Diagnostics), and serum insulin was determined by radioimmunoassay (Cis-BioInternational, Gif-sur-Yvette, France). mRNA levels were quantified by Northern blotting by using the indicated probes (5).

Cell Culture Studies and Cholesterol Efflux. Cholesterol efflux experiments were performed as described by Takahashi and Smith (6). Thioglycollate-induced mouse peritoneal macrophages and RAW 264.7 cells plated at 1×10^5 cell/ml were loaded with 50 μg/ml [^{3}H] cholesterol-labeled acetylated low-density lipoprotein (LDL) for 24 h in serum-free DMEM containing 0.2% (vol/vol) BSA. Loaded cells were then washed, and efflux was initiated in medium containing 10 μg/ml apoA-I and the designated ligands. Twenty-four hours later, the medium was removed, centrifuged to remove cell debris, and the radioactivity was measured. The cells were dissolved in 0.2 M sodium hydroxide, and the radioactivity was measured. The percent efflux was calculated by dividing the radioactivity of the media by the sum radioactivity of the media and cell lysate. For Northern blot analysis, total RNA was isolated from mouse peritoneal mac-

Abbreviations: RXR, retinoid X receptor; LXR, liver X receptor; ABC-1, ATP-binding cassette protein 1; PPAR, peroxisome proliferator-activated receptor; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

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rophage and RAW 264.7 cells induced with ligand for 24 h. Membranes were probed for ATP-binding cassette protein (ABC)-1 and glyceraldehyde-3-phosphate dehydrogenase as described (5).

Analysis of Atherosclerotic Lesions. Hearts and aortas of animals from the different treatment groups were fixed at the end of the study in formaldehyde, and serial 10-μm sections were cut between the valves and the aortic crosses for quantitative analysis of atherosclerosis. The distance between each section was 100 μm, and the final magnification was 2.5-fold. Sections were stained with Oil red O and counterstained with hematoxylin. The Oil red O-stained areas of each section were quantified by using a computer-assisted video imaging system (Leica Qwin, Rueil Malmaison, France) by calculating the mean lesion area of sections from each mouse.

Statistical Analyses. Results are presented as mean +/− SD. The significance of differences between means was determined by ANOVA comparison of untreated and treated groups (i.e., five groups). Significant differences were then subjected to post hoc analysis by using the unpaired Student's *t* test, and pairwise comparisons were performed between each treated group and the control group.

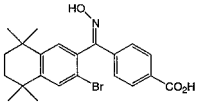
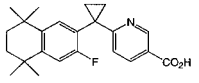
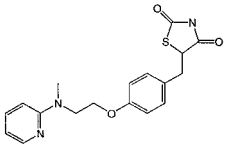
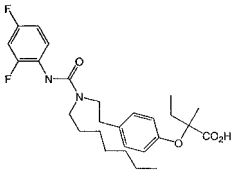
Results and Discussion

ApoE −/− mice are hypercholesterolemic and spontaneously develop severe atherosclerosis (7, 8). These animals not only have fatty streaks, but also develop widespread, fibrous plaque-like lesions at vascular sites typically affected in human atherosclerosis (9, 10). Sixty 8-to-10-week-old female apoE −/− mice were divided into 4 groups of 15 animals each. One group served as control and received normal mouse chow for 11 weeks. The other groups received either a specific RXR agonist (LG100364 at 20 mg/kg/day), a PPARγ agonist (Rosiglitazone or BRL 49653 at 10 mg/kg/day), or a PPARα/γ dual agonist (GW2331 at 5 mg/kg/day) mixed with their chow for 11 weeks. The *in vitro* pharmacological characteristics of the various agonists are shown in Table 1. The potency and efficacy of the doses were estimated for the respective receptors in cell-based transfection assays (Table 1). None of the test compounds affected body weight or food intake (Table 2). Liver weight increased in animals that received the PPARα/γ coagonist and the rexinoid (Table 2), whereas the mass of the different fat pads (perirenal and ovarian) was not different among the groups (data not shown).

To evaluate the extent of atherosclerosis, serial histological sections in the aortic outflow tract were stained with Oil red O, and the lesion area was quantified. Lesion areas were reduced significantly in animals treated with the rexinoid (−50.5%), the PPARγ compound (−17.9%), and the PPARα/γ coagonist (−32.4%; Fig. 1). These results indicate that although PPARγ activation may delay the development of atherosclerosis, its effect is not as robust as that obtained with either the PPARα/γ coagonist or the RXR agonist. When the lesion size was analyzed as a function of the distance from the aortic valves, there were no differences in the distribution of lesions between the groups (Fig. 1*B*).

This improvement in atherosclerotic lesion area was striking, because all treatments increased total serum cholesterol and triglyceride levels (Fig. 2*A*). Control apoE −/− mice have dramatically reduced high-density lipoprotein (HDL) levels, and almost all their cholesterol is contained in lipoproteins corresponding to very LDLs (VLDLs) and lipoprotein remnants (Fig. 2*D*). It was shown that the increase in cholesterol and triglyceride levels on administration of the various receptor agonists was fully accounted for by an increase in these VLDLs and remnants, and not by an increase in "protective" HDLs (Fig. 2*D*). This absence

Table 1. *In vitro* pharmacological characteristics of the receptor agonists

	Cotransfection efficacy, %			Binding K_i, nM	
	RXRα*	PPARγ†	PPAR‡	RXRα	PPARγ
 LG100364	77	46		5.3	
 LG101305	89	38		3.7	
 BRL49653 (Rosiglitazone)	1	100	9		22
 GW2331	2	125	100		

*Efficacy relative to all-*trans* retinoic acid.
†Efficacy relative to BRL49653.
‡Efficacy relative to GW2331.

of an effect of rexinoids on HDL in these apoE −/− animals was opposite the HDL-inducing effects seen in wild-type animals (data not shown) and was substantiated by the reduced serum levels of apoA-I, the most abundant protein associated with HDL (Fig. 2*B*). ApoA-II, which rather predisposes toward atherosclerosis (11), increased after treatment with the PPARα/γ coagonist and the rexinoid, whereas apoC-III and apoB levels were unaffected by treatment (Fig. 2*B*). The steady-state mRNA levels encoding these apos in the liver correlated with the changes in their serum levels (data not shown). Furthermore, fasting serum glucose and insulin concentrations were not different among the groups, suggesting unaltered insulin sensitivity (Fig. 2*C*). In combination, these metabolic data show that the beneficial effects of these compounds are independent of major changes in lipid and glucose metabolism, as evaluated by various steady-state serum parameters.

The most exciting observation of this *in vivo* study is the marked reduction of atherosclerotic lesion size by the rexinoid, immediately raising the question of which RXR heterodimer mediates this effect. PPAR heterodimers constitute potential candidate effectors. Rexinoids activate RXR in PPAR heterodimers, thereby mimicking some effects of PPAR activation. PPARγ, which is almost absent in normal arteries, has been shown to be expressed in atherosclerotic lesions in human and mouse (12–14). Previous *in vitro* studies suggested a key role of PPARγ in inducing macrophage differentiation and foam cell formation (13, 15). In fact, PPARγ promotes monocyte/macrophage differentiation and the uptake of oxidized LDL in these cells through CD36 induction, providing the cells with 9- and 13-hydroxyoctadecadienoic acid, a powerful, natural PPARγ agonist (13, 15). Other studies, however, which demonstrated transcriptional interference with proinflamma-

Table 2. Body and liver weight in animals

	Control	RXR	PPARα/γ	PPARγ
Initial body weight, g	19.85 ± 1.49	19.46 ± 1.23 $P = 0.717$	19.44 ± 0.88 $P = 0.637$	19.11 ± 1.30 $P = 0.204$
Final body weight, g	24.34 ± 1.79	25.30 ± 1.21 $P = 0.248$	24.43 ± 1.22 $P = 0.876$	24.19 ± 0.99 $P = 0.791$
Weight change, g	5.12 ± 1.50	5.57 ± 0.74 $P = 0.339$	4.99 ± 0.60 $P = 0.763$	5.22 ± 0.89 $P = 0.849$
Liver weight, g	1.05 ± 0.18	1.45 ± 0.16 $P < 0.0001$	1.54 ± 0.24 $P < 0.0001$	1.04 ± 0.07 $P = 0.797$

Results are expressed as the mean ± SD. Significance of the difference from the control values by Student's *t* test is indicated.

tory transcription factors NF-κB, activator protein 1, and signal transducers and activators of transcription (16, 17), supported a general antiinflammatory and potential protective effect of PPARγ on macrophage activation (14, 16–18). Consistent with this protective effect on atherosclerosis, it was shown that PPARγ had important inhibitory effects on the

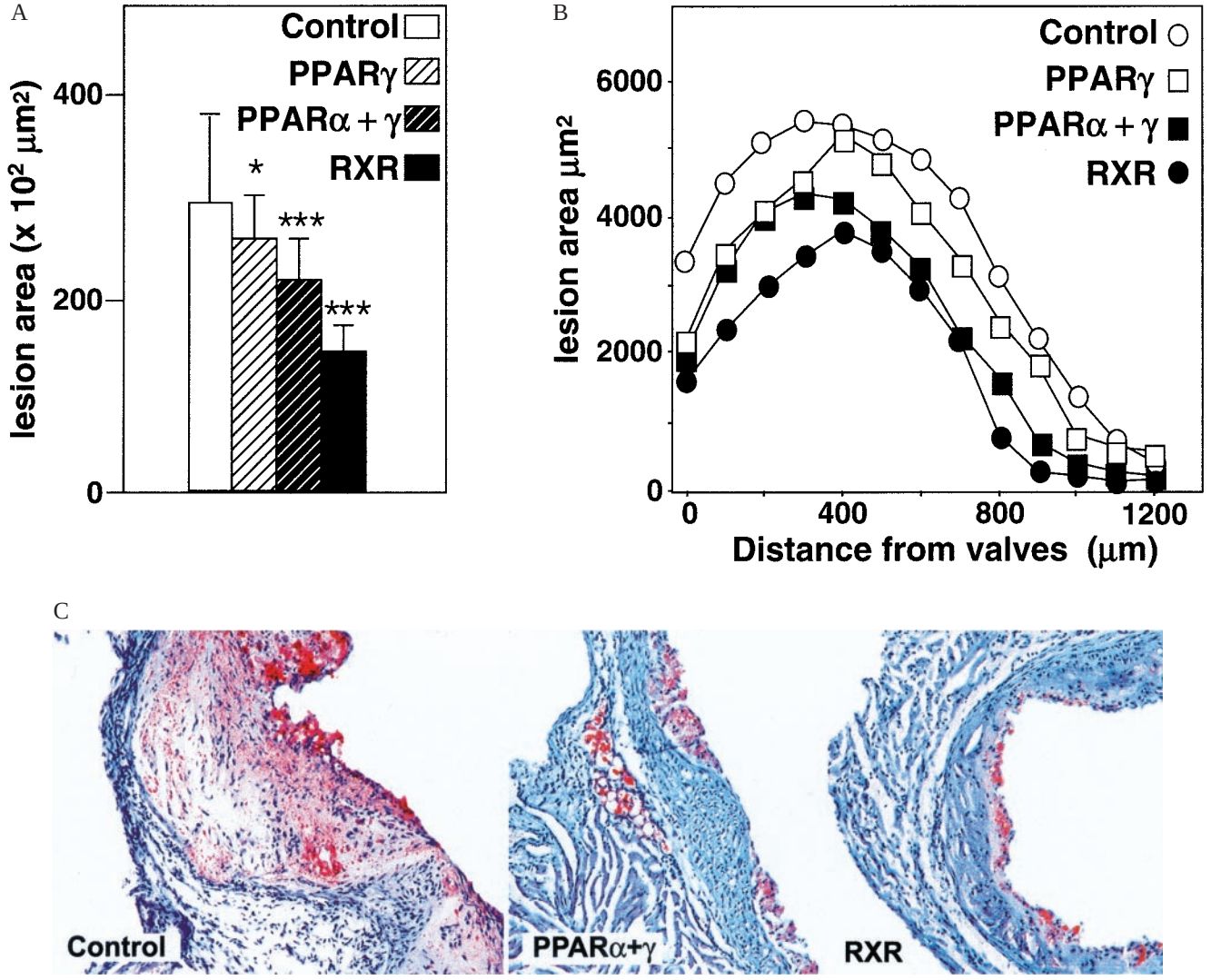


Fig. 1. Area affected by atherosclerotic lesions in the heart outflow tract of apoE −/− mice. (*A*) Different groups of 15 animals each were treated with a PPARγ agonist, a PPARα/γ coagonist, or a rexinoid. Lesion area was determined as specified in *Experimental Procedures*. Results are expressed as the mean ± SD. Significance levels (Student's *t* test) are represented by asterisks (*, $P < 0.05$; *, *, $P < 0.01$; *, *, *, $P < 0.001$). (*B*) Lesion size as a function of distance to the aortic valves in apoE −/− mice treated as specified in *A*. Serial sections were cut from the heart valves to the aortic crosses and stained and analyzed as specified. Each point corresponds to the mean surface of lesions measured for the 15 mice in each group at a given distance from the valves. The corresponding standard deviations are not shown, to improve the clarity of the graphic. (*C*) Representative photographs of atherosclerosis in the aorta of apoE −/− mice, a relevant section from an untreated apoE −/− mouse (control), and an equivalent section of an apoE-deficient mouse after receiving GW2331 (PPARα/γ coagonist) or LG100364 (RXR agonist).

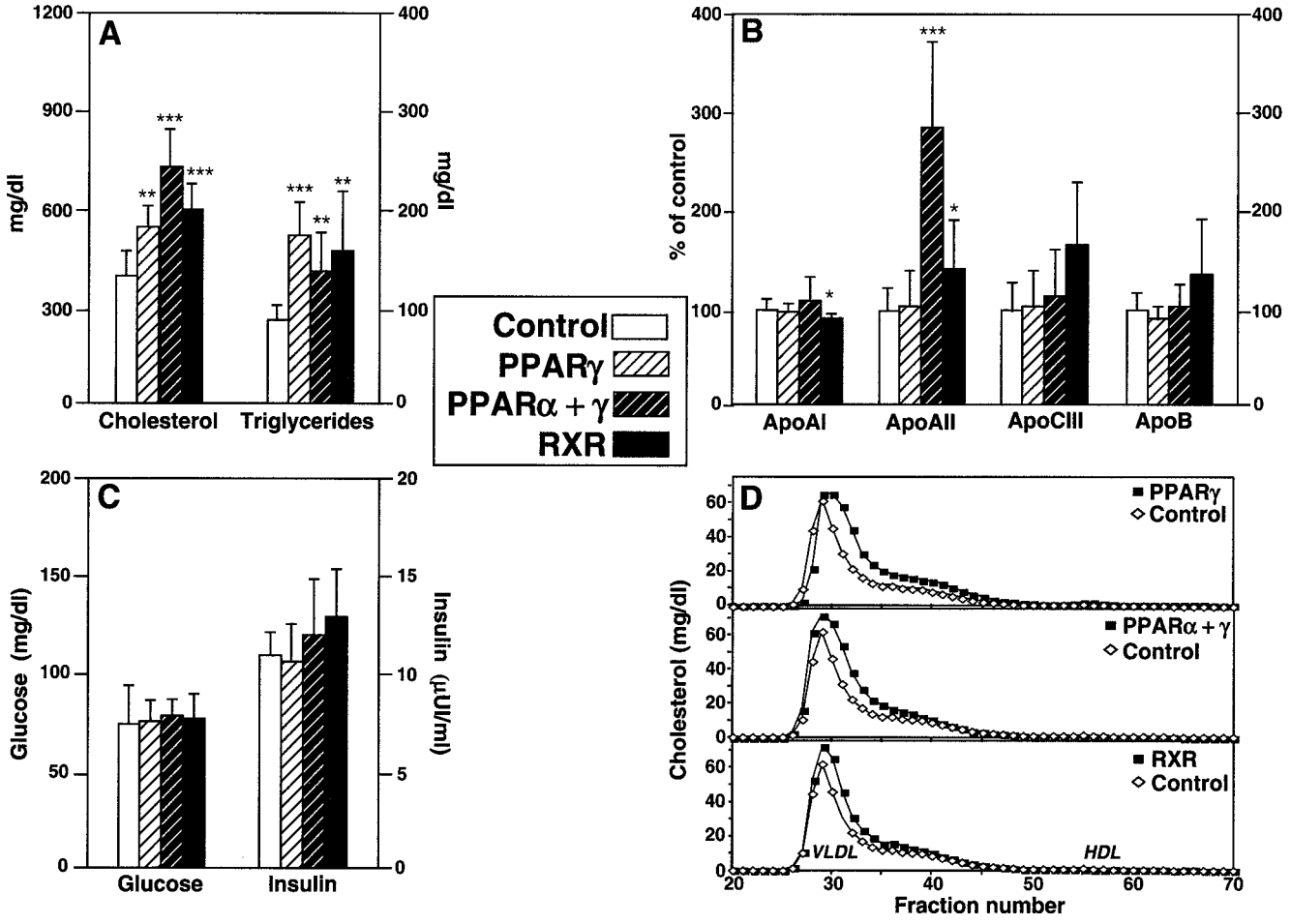


Fig. 2. Changes in lipid (*A*), apolipoprotein (*B*), and glucose (*C*) metabolism by the various treatments in apoE −/− animals. (*A*) Cholesterol and triglyceride levels in apoE −/− animals treated with either a PPARγ agonist, a PPARα/γ coagonist, or a rexinoid. Results are expressed as mean ± SD. Significant differences by Student's *t* test are indicated by asterisks. (*B*) Serum levels of the different apolipoproteins in apoE −/− mice subjected to the different treatments. Values are expressed as a percentage of the value in the control group, and significant differences are indicated. (*C*) Fasting serum glucose and insulin levels in apoE −/− mice. Results are expressed as mean ± SD. No significant differences between the groups existed. (*D*) Representative lipoprotein profiles obtained after size exclusion chromatography of pooled serum of three control apoE −/− animals (open symbols) and of three apoE −/− mice receiving a PPARγ agonist, a PPARα/γ coagonist, or a rexinoid (black closed symbols). Cholesterol was determined in the various fractions. The elution of very LDL and HDL in normal mouse serum is indicated in italics (*Bottom*).

production of matrix metalloproteinases (14), vascular cell adhesion molecule, and intercellular adhesion molecule (19). Furthermore, PPARγ agonists are reported to inhibit migration of vascular smooth muscle cells (20) as well as monocyte/macrophage homing (19) to atherosclerotic plaques. These activities could counterbalance the seemingly proatherogenic effects of PPARγ reported *in vitro* in macrophages (13, 15) and underlay, in part, the antiatherogenic properties reported for some of these compounds in another mouse model of atherosclerosis [i.e., the LDL receptor-deficient mouse (21) and in the clinic (22)]. In addition to the antiatherogenic activities of PPARγ agonists, the PPARα/γ coagonist may affect lesion formation through PPARα signaling pathways. Although no beneficial changes in lipid profiles occurred, PPARα compounds are reported to decrease inflammatory responses (23). We note, however, that PPARα agonists alone (i.e., fenofibrate) have no effect on reducing atherosclerosis in the apoE −/− mice (data not shown).

Although in our study PPAR agonists improve atherosclerosis, rexinoids have additional effects, distinct from PPAR activation, as suggested by the more pronounced improvement in lesion size. A striking example of such distinctive effects was provided recently by the differential tissue-specific regulation of lipoprotein lipase gene expression by RXR (24) and PPARγ (25) agonists in adipose tissue and muscle. These differential regulatory effects can be attributed to the capacity of RXR to form permissive heterodimers with a wide range of nuclear receptors, endowing them with "broad-spectrum" activity, touching numerous physiological processes. Particularly interesting in this context could be the capacity of rexinoids to activate the LXR signaling pathway, which controls several aspects of reverse cholesterol transport (reviewed in ref. 26). LXR stimulates ABC-1- and -8-mediated reverse cholesterol transport from peripheral tissues to the liver (27, 28). In the liver, activation of LXR then enhances cholesterol excretion through the induction of bile acid production (29). Finally, in the gut, LXR inhibits cholesterol (re)absorption, again via an effect on the ABC-1 cholesterol transporter (30). In view of these data, a potential effect of RXR agonists on the ABC-1 transporter was explored. Incubation of the murine monocyte cell line RAW 264.7 with a rexinoid (LG 101305 at 1 μM) or with an LXR ligand [22(R)-hydroxycholesterol at 10 μM] resulted in significant inductions of ABC-1 mRNA levels (Fig. 3*A*). No effects of PPARγ agonists on ABC-1 mRNA levels

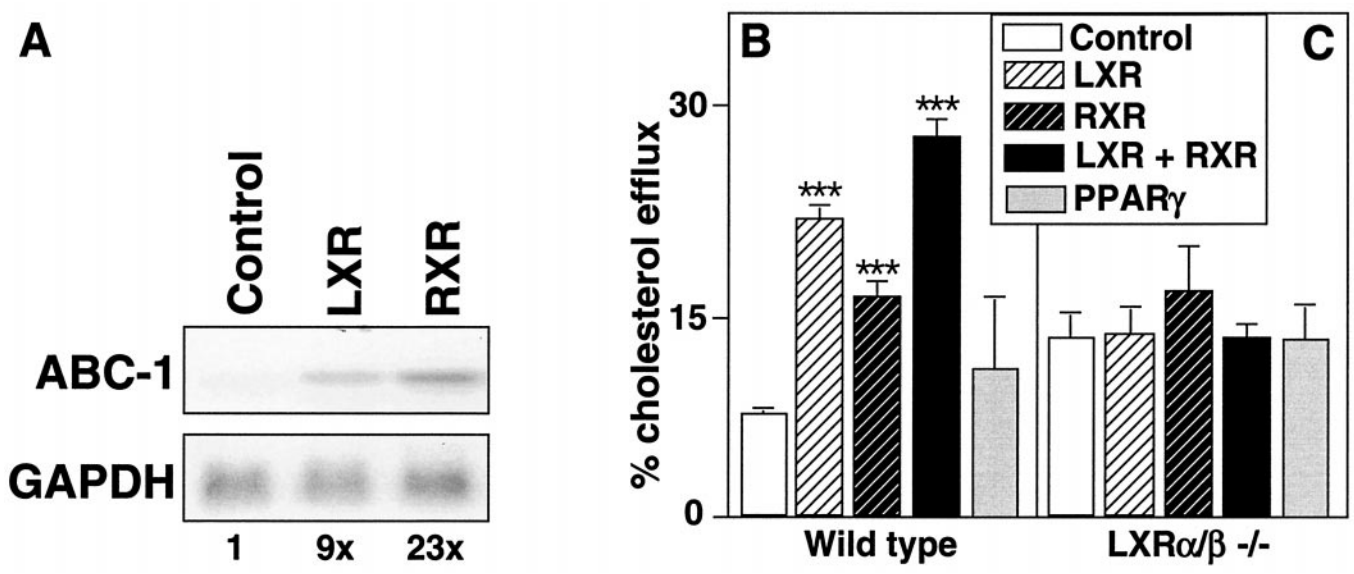


Fig. 3. Ligands for RXR and LXR induce macrophage ABC-1 expression and cholesterol efflux. (*A*) ABC-1 mRNA levels in RAW 264.7 cells treated for 24 h with either a ligand for LXRα [22(R)-OH-Cholesterol; 10 μM] or a rexinoid (LG101305; 1 μM). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control probe. Relative fold induction of ABC-1 mRNA after normalization to the GAPDH expression is indicated under the blots. (*B*) ApoA-I-mediated cholesterol efflux in peritoneal macrophages obtained from control mice. Macrophages were treated with the agonists as specified under *A*. In addition to these treatments, macrophages were also challenged with a PPARγ agonist (BRL 49,653; 10 μM). (*C*) ApoA-I-mediated cholesterol efflux in peritoneal macrophages from LXRα and β double $-/-$ mice.

were observed, an effect attributed to the absence of PPARγ expression under these experimental conditions (data not shown). Similar inductions of ABC-1 mRNA levels were observed after the addition of RXR and LXR agonists to thioglycollate-induced mouse peritoneal macrophages (data not shown). Consistent with changes in ABC-1 expression, addition of RXR or LXR ligands (but not a PPARγ ligand) significantly enhanced cholesterol efflux from mouse peritoneal macrophages, whereas the RXR and LXR compounds together resulted in a slight additional improvement (Fig. 3*B*). Unlike 22(R)-hydroxycholesterol, addition of its stereoisomer 22(S)-hydroxycholesterol, which does not activate LXR, did not induce cholesterol efflux, underscoring the involvement of LXR in this process (data not shown). To prove unequivocally that the rexinoid-mediated effects on cholesterol efflux depended on RXR/LXR heterodimers, we repeated the efflux experiments in peritoneal macrophages from LXRα and β double $-/-$ mice. Unlike in wild-type macrophages, rexinoids and/or 22(R)-hydroxycholesterol were unable to stimulate cholesterol efflux in the LXRα and β $-/-$ mice (Fig. 3*C*). Hence, this absence of cholesterol efflux in this model proved the importance of the LXR/RXR heterodimer in the rexinoid induction of cholesterol efflux from macrophages. Furthermore, these data suggest that ABC-1-mediated cholesterol efflux, and the subsequent stimulation of reverse cholesterol transport, might contribute to the improvement in atherosclerotic lesion size with rexinoids and identify the RXR/LXR heterodimer as the mediator of this effect.

One observation, which might appear at odds with this hypothesis, is the absence of an increase in steady-state HDL cholesterol levels in the rexinoid-treated apoE $-/-$ mice (Fig. 2*D*). An absence of steady-state HDL cholesterol levels does not, however, exclude an increase in reverse cholesterol transport, triggered by ABC-1-mediated cholesterol efflux from peripheral tissues. HDL cholesterol levels are merely a static measure and do not reflect actual cholesterol fluxes. Animals in which scavenger receptor class B type I levels were manipulated provide a nice example of such an apparent paradox. Overexpression of SR-BI in the liver lowered HDL cholesterol levels, but protected against the development of atherosclerosis, indicative of increased reverse cholesterol transport (31, 32). Conversely, SR-BI knockout animals had increased HDL cholesterol levels but suffered from severe atherosclerosis (33).

Finally, additional properties of the rexinoids, independent of their effect on ABC-1, could be invoked to explain some of their antiatherogenic effects. Although no specific data are available for rexinoids, some older studies with various vitamin A derivatives and retinoids hint toward multiple alternative mechanisms. These mechanisms include the modulation of immune and inflammatory responses (34, 35), changes in cytokine-induced responses in macrophages (36) and smooth muscle cells (37), and altered differentiation/proliferation of macrophages (13, 15) and smooth muscle cells (38). The expression of several permissive RXR heterodimers (including PPAR and LXR) in these immune-modulatory cells does not allow us to exclude an eventual contribution of the above mechanisms in the improvement in atherosclerosis in apoE $-/-$ mice.

The most important conclusion from our data is the significant reduction in atherosclerotic lesions in apoE $-/-$ animals treated with rexinoids. Rexinoids stimulate ABC-1 mRNA levels as well as cholesterol efflux from macrophages in an LXR-dependent fashion, suggesting a pivotal role of RXR and some of its permissive heterodimer partners in the protection of the vascular wall against insults. Therefore, further research to explore the mechanisms of their protective activity as well as their effects in other animal atherosclerosis models and in human atherosclerosis is warranted.

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