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Selective Activation of Liver X Receptors by Acanthoic Acid-related Diterpenes.

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Running Title: Activation of the LXR pathway by DTPs

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Non-standard abbreviations: DTP, diterpene; LXR, Liver X Receptor; NOS-2, type-2 inducible nitric oxide synthase; IL-6, interleukin 6.

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Abstract

Terpenoids constitute a large family of natural steroids that are widely distributed in plants and insects. We investigated the effects of a series of diterpenes structurally related to acanthoic acid in macrophage functions. We found that diterpenes with different substitutions at the C4 position in ring A are potent activators of Liver X Receptors (LXRα and LXRβ) in both macrophage cell lines from human and mouse origin and primary murine macrophages. Activation of LXR by these diterpenes was evaluated in transient transfection assays and gene expression analysis of known LXR-target genes, including the cholesterol transporters ABCA1 and ABCG1, the sterol regulatory element-binding protein 1c (SREBP-1c) and the apoptosis inhibitor of macrophages (Spα/AIM). Moreover, active diterpenes greatly stimulated cholesterol efflux from macrophages. Interestingly, these diterpenes antagonize inflammatory gene expression mainly through LXR-dependent mechanisms, indicating that these compounds can activate both LXR activation and repression functions. Stimulation of macrophages with acanthoic acid diterpenes induced LXR-target gene expression and cholesterol efflux to similar levels observed with synthetic agonists GW3965 or T1317. These effects observed in gene expression were deficient in macrophages lacking both LXR isoforms (LXRα,β-/-). These results show the ability of certain acanthoic acid diterpenes to activate efficiently both LXRs and suggest that these compounds can exert beneficial effects from a cardiovascular standpoint through LXR-dependent mechanisms.

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Introduction

Traditional medicine is recently becoming more widely appreciated as therapeutic approach for the treatment of numerous disorders. Interestingly, approximately 60% of market compounds used clinically for the treatment of tumors or infectious diseases are natural products derivatives. Terpenoids are the largest and most widespread class of secondary metabolites, found mainly in plants and lower invertebrates, and a few of them have been used for therapeutic purposes for centuries (de las Heras et al., 2003). Terpenoids exhibit several beneficial effects from a biological perspective, including potent anti-proliferative, antihypercholesterolemic and antidiabetic properties. Based on these favorable biological activities, terpenoids have therefore attracted substantial interest as a source of alternative pharmacological agents (Aggarwal et al., 2004; de las Heras et al., 2003; Molnar et al., 2006).

Nuclear receptors are ligand-activated transcription factors that regulate the expression of genes involved in numerous biological processes such as reproduction, development and metabolism (Mangelsdorf et al., 1995). This superfamily includes the classic hormone receptors activated by steroid hormones, thyroid hormones, and vitamins A and D and several other receptors often called “orphan receptors”, whose endogenous ligands were initially unknown (Mangelsdorf and Evans, 1995). It is now accepted that intermediate products of lipid metabolism activate many of these orphan receptors and, with few exceptions, all orphan receptor ligands are derived from cholesterol, phospholipid and fatty acid metabolism (Chawla et al., 2001; Repa and Mangelsdorf, 2000). For example, endogenous ligands for liver X receptors (LXRs) and peroxisome proliferator-activated receptors (PPARs) are oxidized forms of cholesterol and fatty acids, respectively. Interestingly, active compounds derived from natural products can activate several members of the nuclear receptor family. Indeed, nuclear receptors evolved to regulate efficiently crucial pathways in response to lipophilic molecules derived from diet and the environment (Chawla et al., 2001). In addition, compounds purified from herbal remedies directly activate some members of the nuclear receptor superfamily. For example, the 6,7-dimethylesteculin, isolated from several Chinese herbal teas, has been reported to activate the constitutive androstane receptor (CAR), previously implicated in bilirubin clearance (Huang et al., 2004; Lazar, 2004). Abietic acid, a diterpene

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found in oleoresin synthesized by conifer species, exerts anti-inflammatory activity in part through activation of PPARγ (Takahashi et al., 2003), and pseudolaric acid B, isolated from *Pseudolarix kaempferi* and acanthoic acid act as a PPARα and LXR activators, respectively (Jaradat et al., 2002; Jayasuriya et al., 2005).

A novel pimarane diterpene, acanthoic acid, (–)primara-9(11),15-dien-19-oic acid, was recently isolated from the root bark of *Acanthopanax koreanum* (Nakai), which has been traditionally used as a tonic and sedative as well as in the treatment of rheumatism and diabetes in Korea (Kim et al., 1988). This natural product is biologically attractive because it has been shown to exhibit an excellent suppression of the major proinflammatory cytokines, interleukin-1 (IL-1β) and tumor necrosis factor-α (TNF-α), by human monocytes/macrophages and in the sera of silicosis, fulminant hepatitis and cirrhosis models (Kang et al., 1996). Acanthoic acid has been reported to inhibit COX-2 and NOS-2 activity (Suh et al., 2001). In addition, oral administration of acanthoic acid to mice significantly reduced the granuloma formation and fibrosis in experimental silicosis and cirrhosis (Kang et al., 1996).

Previous work showed that a family of structural isomers of acanthoic acid, represented by compound **1** (see Figure 1), exhibit a very low non-specific cytotoxicity and inhibit the synthesis of TNF-α at low μmolar concentrations (Chao et al., 2005). In this study we evaluated the biological activities of members of this new family that have different substitutions at the carbon 4 position. Our data show that some of these derivatives efficiently activate both LXRα and LXRβ, the two isoforms of the LXR subfamily present in mammals (Peet et al., 1998a). Transient transfection assays showed potent LXR activity induced by DTPs. Active DTPs strongly induced the expression of established LXRs target genes in macrophages and promoted macrophage cholesterol efflux. These data indicate that DTPs derived from acanthoic acid are activators of LXRα and LXRβ and suggest that DTPs are valuable medicinal components to use in the control of cholesterol metabolism and lipid homeostasis.

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Materials and Methods

Reagents and plasmids. Reagents were obtained from Sigma-Aldrich (St. Louis, MO), Roche (Mannheim, Germany), Merck (Darmstadt, Germany), Bio-Rad (Hercules, CA) and Promega (Madison, WI). DTPs were synthesized in the laboratory of E. Theodorakis (UCSD), as described in ref. 16 (*Fig1*). GW3965 and LG268 were kindly provided by Jon Collins and Tim Willson (GlaxoSmithKline). T1317 was from Cayman Chemical (Ann Arbor, MI). Ligands including DTPs were dissolved in ethanol or DMSO before use in cell culture. Antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), Calbiochem (San Diego, CA). Tissue culture serum and media were from BioWhittaker (Walkersville, MD). pCMX expression plasmids for PPARγ, LXRα, LXRβ and RXRα, and minimal promoters containing multiple binding sites for LXR, PPAR and the GAL4 response element (UAS-LUC) have been described previously (Castrillo et al., 2003b; Venkateswaran et al., 2000) and were a gift from Dr P. Tontonoz (Howard Hughes Medical Institute, UCLA).

Animals. LXRαβ$^{+/+}$ (WT) and LXRαβ$^{-/-}$ (DKO) mice (mixed Sv129/C57bl6 background) were maintained on standard chow under pathogen-free conditions. These mice were obtained from a collaboration with Drs D. Mangelsdorf (HHMI, UTSW) and Dr. Peter Tontonoz (first described in (Peet et al., 1998b).

Cell culture and transfections. Cell lines (HEK 293T, THP-1 and RAW264.7) were obtained from ATCC. Murine peritoneal macrophages were obtained from thioglycolate-injected mice as described (Castrillo et al., 2000). Macrophages were cultured in RPMI or DMEM containing 10% fetal bovine serum (FBS, Gibco). For serum-free ligand treatments, cells were cultured in medium supplemented with 0.2% bovine serum albumin and receptor ligands in DMSO vehicle. Transfections were performed in triplicate using JetPeI transfection reagent (PolyPlus Transfection) with a JetPeI:DNA ratio of 3:1. HEK 293T cells were transfected in suspension in DMEM supplemented with 5% lipoprotein-deficient serum. Cells were plated in 48-well plates at a density of 5x10^{4} cells/well. 24 h after transfection, cells were treated with DMSO, the DTPs or LXR agonists and further incubated for 24 hr before harvest. Reporter activities were assayed using the luciferase assay system per manufacturer's instructions (Promega). GFP

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was used as a control for transfection efficiency. WT-LXRα (full length murine LXRα) and LXRα-ΔAF2 (murine LXRα lacking the AF2 activation domain) stable cell lines were generated by retroviral transduction of RAW 264.7 cells using a pBabe-puro based expression vector as described (Venkateswaran et al., 2000).

Isolation and culture of marrow-derived macrophages. Mice were sacrificed by CO_2 chamber euthanasia. Total bone marrow was obtained from mice by flushing pelvises, femurs, and tibiae with DMEM. Bone marrow-derived macrophages were prepared as described (Castrillo et al., 2003a). Briefly, bone marrow mononuclear phagocytic precursor cells were propagated in suspension by culturing in DMEM containing 10% FBS, 0.5 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 nM IL-3 (Peprotech, Rocky Hill, NJ) and 0.22 nM recombinant murine CSF-1 (Peprotech) in tissue culture plates. For the maturation process, IL-3 and the recombinant CSF-1 were replaced with 30% L929-conditioned medium (a source of CSF-1) and the cells were cultured in tissue culture plates. The precursor cells became adherent within 3 days of culturing. For priming of macrophages, the 30% L929-conditioned medium was removed from the macrophage monolayer and replaced with medium without L929-conditioned medium.

Western blot analysis. Cytosolic and nuclear extracts were obtained as described previously (Castrillo et al., 2000). Samples containing equal amounts of protein (30 μg per lane of cytosolic) were boiled in 250 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2% ß-mercaptoethanol, and size-separated in 10% SDS-PAGE. The gels were processed as recommended by the supplier of the antibodies and after blotting onto a polyvinylidine difluoride (PVDF) membrane, proteins were revealed following the enhanced chemoluminescence (ECL) technique (Amersham Pharmacia Biotech). Different exposure times of the films were used to ensure that bands were not saturated. Quantification of the films was performed by laser densitometry (Molecular Dynamics). ABCA1 protein levels were detected using a rabbit ABCA1 antiserum (a gift from Dr M. Fitzgerald, Massachusetts General Hospital, USA). β-actin was used to normalize protein loading.

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RNA analysis and real time quantitative PCR. Total RNA was extracted using Trizol reagent (Life Technologies, Inc). Real-time quantitative PCR (SYBRgreen) analysis was performed with an ABI 7900 sequence detector as described (Laffitte et al., 2001). Briefly, 1 µg of total RNA was reverse transcribed with random hexamers using the Taqman reverse transcription reagent kit (Applied Biosystems) according to the manufacturer's protocol. Each Taqman reaction (20 µl) contained 25 ng of cDNA, 500 nM forward primer, 500 nM reverse primer, and 10 µl of Power SYBR Green PCR Master mix (Applied biosystems). PCR thermocycling parameters were 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. All samples were analyzed for 36B4 expression in parallel. The quantitative expression values were extrapolated from standard curves for 36B4. Each sample was run in duplicate and was normalized to 36B4. The replicates were then averaged and fold induction was determined. Statistical analysis of mRNA expression data was performed by using the two-tailed, homoscedastic *t* test. Primer sequences are available on request.

Cholesterol efflux. Efflux assays were performed essentially as described (Venkateswaran et al., 2000). Briefly, murine peritoneal macrophages were plated at 50% confluence in 24-well plates. On day 2, the cells were washed and incubated for 24 h in medium DMEM containing 2% BSA (fatty acid free, Sigma) supplemented with an ACAT inhibitor (58-035; 2 µg/ml) and [^{3}H]cholesterol (1 µCi/ml) either with DMSO or LXR/RXR ligands (0.5 µM GW3965 and 50 nM LG268) or 10 µM of DTPs. On day 3, the cells were washed twice with PBS and then incubated for 2-4 h in fresh medium, devoid of radiolabeled cholesterol, but containing the indicated DTP or GW/LG ligands. The cells were rewashed before addition of 1 ml medium B (DMEM containing 0.2% BSA) in the absence or presence of apoA-I (15 µg/ml). After 4 h of incubation, the medium was removed and centrifuged at 14,000**g** for 10 min, and the radioactivity was determined by liquid scintillation counting. The cells were dissolved in isopropanol and an aliquot used to determine total cell-associated radioactivity. The apoAI-dependent efflux of radioactive cholesterol from the cells into the medium was determined as a percentage of the total radioactivity in the cells and medium for each condition. Each efflux assay was performed in triplicate.

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Detection of CD36 expression on monocytes. THP-1 human monocytes were incubated with the DTPs (10 μM), T1317 (0.5 μM) and rosiglitazone (2 μM) for 24h at 37°C. The cells were subsequently incubated for 30 min in the dark with a PE-conjugated anti-human CD36 antibody, and the expression of CD36 was detected by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA).

Statistical analysis. Unless otherwise stated, data are expressed as mean ± standard deviation (SD). To compare means between two independent experiments Mann-Whitney rank sum test was used. The results were considered significant at $P<0.05$. Data were analyzed by SPSS for Windows statistical package version 9.0.1.

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Results

Activation of LXR by acanthoic acid-related diterpenes. Since the diterpene structure shows a number of similarities with steroid derivatives, we hypothesized that acanthoic DTPs could function as nuclear receptor activators, including those involved in the control of lipid metabolism such as LXRs and PPARs. To evaluate this possibility, we used a series of DTPs (table 1) structurally related to acanthoic acid in transient transfection assays in HEK 293T. These cells express low levels of LXRs and PPARs endogenously (Fig. 1A). Transfection experiments were performed using minimal promoters containing binding sites for LXR or PPAR fused to a luciferase-reporter, along with pCMX vectors expressing PPARγ, LXRα, LXRβ and RXRα. As Fig 1B shows, none of the DTPs analyzed promoted PPARγ activity. However, a robust LXR-dependent reporter activity was observed in cells transfected with LXRα and LXRβ and stimulated with DTPs. Positive controls for maximal PPAR or LXR activation in this system assays were performed using rosiglitazone and T1317, respectively. DTPs1, 3 and 5 were consistently the most active diterpenes in LXRα and LXRβ assays. We also screened a panel of nuclear receptors in transient transfections using GAL4 fusion expression vectors and a synthetic promoter with four copies of yeast GAL4 response element. As Fig.1C shows, DTP1 (5-10 μM) promoted LXR activity whereas no significant changes were observed in RAR, RXR, PXR and PPAR activity using the same culture conditions. A dose response is shown in Fig. 2. DTPs 1, 3 and 5 induced LXR activity with a Ka in the 0.8-1.2 μM range, irrespectively of the LXR isoform, whereas DTPs 2 and 4 served as low-activity controls since ca. a 2-fold activation was observed when assayed at 10 μM. Analysis on cell viability over a 24 h period of treatment showed no significant effects when DTPs were used up to 20 μM (not shown).

To evaluate the effects of these DTPs on nuclear receptor target genes in macrophages, we measured CD36 expression, a PPARγ target (Tontonoz et al., 1998), and ABCA1 expression as a control for LXR activation (Costet et al., 2000; Repa et al., 2000b; Venkateswaran et al., 2000). PPARγ and LXRs are expressed significantly in THP-1 differentiated macrophages (Fig. 1A). As Fig. 3 shows, DTPs failed to induce CD36 expression, whereas increased significantly ABCA1 expression in naïve THP-1

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cells (DTPs 1 and 3), and in TPA-differentiated THP-1 cells (DTPs 1, 3 and 5, and to a lesser extend DTP2). Furthermore, we confirmed the effect of DTPs on LXR-target genes in primary cells. As Fig. 4A shows, DTPs 1, 3 and 5 promoted the expression of ABCA1 in peritoneal macrophages. Moreover, increased expression of ABCA1 was observed in response to DTPs 1, 3 and 5 in RAW 264.7 macrophages stably expressing LXRα, whereas a significant reduction in ABCA1 inducibility was observed in cells expressing an inactive form of LXRα (LXRα-ΔAF2). It is important to note that RAW cells express considerable levels of LXRβ endogenously, but lack expression of LXRα (Joseph et al., 2004). Collectively, these data indicate that DTPs effectively promote LXRα and LXRβ activity in macrophages.

Induction of activity by DTPs is abolished in macrophages lacking LXRα and LXRβ. In order to evaluate if the effects of these compounds on LXR target gene expression are indeed receptor dependent, bone marrow progenitors were isolated from WT and LXRα/$\beta^{-/-}$ mice and differentiated *in vitro*. As shown in Fig 5A, WT macrophages showed a robust induction of ABCA1 in response to DTPs 1, 3 and 5, whereas LXRα/$\beta^{-/-}$ cells failed to respond to these compounds. Similar data were obtained at the mRNA levels of ABCA1 and SREBP-1c, another established LXR target gene (Repa et al., 2000a) (Fig. 5B).

In addition to bone marrow macrophages, we also evaluated the expression of ABCA1, ABCG1, SPα/AIM and SREBP-1c in thioglycollate-elicited peritoneal macrophages stimulated with DTPs. As shown in Fig. 6A treatment with DTPs increased the expression of these four established LXR target genes in macrophages, in particular with the most active molecules DTP1, 3 and 5. We also evaluated the contribution of full activation of LXR/RXR heterodimers with combination of a specific synthetic ligand for RXR receptors (LG268) in combination with DTPs. A potent synergistic effect was observed when both stimuli were used in combination suggesting that DTPs can efficiently activate LXR/RXR heterodimers (Fig 6).

Next, we tested the ability of DTPs to promote macrophage cholesterol efflux. Given the important role of ABC transporters in the reverse cholesterol efflux pathway and the potent induction of

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ABCA1 mRNA and protein observed by DTPs, it is likely that these compounds induce cholesterol efflux from macrophages. As Fig. 7 shows, DTP1, 3 and 5 promoted significantly the efflux of cholesterol to ApoAI acceptors. A dose-dependent response is shown in the lower panel of the figure. Importantly, the apparent Ka values for efflux activation ranged in the same order of magnitude to those observed for the expression of LXR-target genes, such as ABCA1.

Since activation of LXR by synthetic agonists has also been shown to repress inflammatory gene expression, we evaluated if these diterpenes can also exert anti-inflammatory properties using WT and LXR$\alpha/\beta^{-/-}$ macrophages. We also used GW3965 and Dexamethasone (Dex) as controls of LXR and Glucocorticoid Receptor (GR) activation respectively. As shown in Fig.8, GW, Dex, DTP1 and DTP5 were able to repress the expression of NOS-2, IL-6 and Rantes in WT macrophages (left part of each panel). The effect of GW compound was abolished in LXR$\alpha/\beta^{-/-}$ cells as previously reported and Dex inhibited the expression of inflammatory genes in both cell types. Interestingly, repression of NOS-2, IL-6 and Rantes by DTPs was severely compromised in LXR$\alpha/\beta^{-/-}$ macrophages (70-80% of the effect was abolished) indicating that DTPs exert anti-inflammatory properties mainly through LXR-dependent pathways.

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Discussion

Macrophages play an important role in both lipid metabolism and the innate immune response. Particularly, the contribution of macrophages in processes associated with cholesterol accumulation and efflux, and the activation of inflammatory pathways is likely to impact the course of chronic diseases such as atherosclerosis (Glass and Witztum, 2001; Hansson et al., 2002). The liver X receptors (LXRα and LXRβ) are ligand-activated transcription factors that belong to the nuclear receptor superfamily. LXR form heterodimers with the Retinoid X Receptor (RXR) and are activated by of oxysterols. LXR/RXR heterodimers control the expression of genes involved in the absorption, efflux and excretion of cholesterol and therefore play central roles in the transcriptional control of lipid metabolism. (Castrillo and Tontonoz, 2004; Repa and Mangelsdorf, 2002; Tontonoz and Mangelsdorf, 2003). While LXRβ is more widely expressed, LXRα is preferentially expressed in the liver, fat, intestine and macrophages. Targeted deletion of the *LXRα* gene in mice led to massive cholesteryl ester accumulation in the liver when these animals were supplemented with high cholesterol diet (Peet et al., 1998b). Moreover, atherosclerosis studies in $ApoE^{-/-}$ and $LDLR^{-/-}$ mice demonstrated that LXR expression in the macrophage is beneficial against lesion development (Tangirala et al., 2002). Similarly, synthetic ligands for both receptors increase reverse cholesterol transport and provide atheroprotection in mice (Joseph et al., 2002). Several LXR target genes contribute to these favorable changes in lipid metabolism, including ABC transporters (ABCA1 and ABCG1), apolipoptorein E (ApoE), phospholipid transport protein (PLTP) and the bile acid enzyme Cyp7A1 (reviewed recently (Tontonoz and Mangelsdorf, 2003), and references therein). It is likely that other LXR functions in the macrophage are also involved in atheroprotection, such as reduction in inflammatory mediators (Castrillo et al., 2003a; Fowler et al., 2003; Joseph et al., 2003). Therefore, activation of LXRs presents a number of beneficial effects from a cardiovascular and immune standpoint, and the study of LXR biology is a subject of increasing interest.

Previous studies demonstrated that acanthoic acid derivatives exerted anti-inflammatory actions in various animal models, and can activate nuclear receptors (Chao et al., 2005; Jayasuriya et al., 2005). Using different cells and gene expression strategies we investigated the biological actions of a series of

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acanthoic acid isomers bearing distinct substitutions at the C4. Our data suggest that the rigid tricyclic motif of these compounds is essential to the activation of LXR. In addition, functionalization of the C4 position can further enhance this activation. In particular, the presence of non polar groups at the C4 positions (DTPs 1,3 and 5 respectively) resulted in potent activation of LXR in transient transfection assays and target gene expression. Such activation was less pronounced with compounds 2 and 4 containing more polar functionalities at the C4 position. Our data show that most of the biological properties are related to the C4 modification in the A ring. Regarding the nature of the substitutions, the presence of short ester, amide and alcoholic groups at the C4 positions resulted in potent activation of LXR in transient transfection assays and target gene expression.

Terpenoids are structurally similar to the cholesterol molecule. Furthermore, recent work has identified various modified forms of cholesterol as the physiological activators of LXRs (Janowski et al., 1999; Yang et al., 2006). The discovery of oxysterols as the endogenous LXR ligands and subsequent gene targeting studies in mice provided strong evidence that LXR plays a central role in cholesterol metabolism. Consequently, it has been clearly demonstrated that LXRs play an important role as cholesterol sensors throughout the body.

Previous studies on macrophage inflammatory pathways (i.e. release of TNF-α in LPS-activated monocytes) showed that several DTPs exerted different anti-inflammatory actions (Chao et al., 2005). In the present work we have established a preferential activation of the LXR pathway with potential anti-atherogenic and anti-inflammatory properties for some of these molecules. DTPs 1, 3 and 5 presented potent activation of LXR target gene expression in both primary cells and macrophage cell lines. Gene expression analysis in RAW cells demonstrated that DTPs can activate either LXRα or LXRβ. Particularly, active DTPs induced LXR-dependent gene expression in RAW cells that express only LXRβ and these effects were significantly enhanced when LXRα was ectopically expressed. Moreover, studies in primary macrophages showed that DTPs potently induced the expression of Spα/AIM, an LXRα selective gene and other dual targets including ABCA1 and ABCG1 demonstrating that these compounds can activate both LXR isoforms. We have also provided further evidence that these DTPs exert specific

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LXR-dependent actions with loss of function experiments. The effects on LXR target gene expression observed with active DTPs were completely abolished in macrophages obtained from LXRα,β-/- mice. Finally, DTPs 1, 3 and 5 induced macrophage cholesterol efflux at the same range of concentrations that activated LXR targets genes.

In addition to control lipid metabolic gene expression, LXRs negatively regulate the expression of pro-inflammatory genes induced by external stimuli and in chronic inflammation associated with atherogenesis (Castrillo and Tontonoz, 2004; Joseph et al., 2003). Gene expression analysis in WT and $LXR\alpha/\beta^{-/-}$ macrophages showed that acanthoic acid DTPs can also exert anti-inflammatory properties. DTP1 and DTP5 inhibited the expression NOS-2, IL-6 and Rantes, three classic NFκB pro-inflammatory targets in macrophages. This effect was comparable to the repressive actions observed with LXR and GR synthetic agonists. Interestingly, the anti-inflammatory actions of DTPs are largely LXR-dependent. Given the broad repressive actions of NFκB target genes observed with LXR agonists (Joseph et al., 2003), it is likely that these DTPs also present potent inhibition of several NFκB targets in macrophages through LXR dependent mechanisms.

It is important to note that active DTPs presented in this study exert an unusual efficacy in LXR activation, an effect that is comparable to the well-studied LXR synthetic agonists GW3965 and T1317. Limited studies using higher concentrations of GW or T1317 (up to 10uM) did not modify substantially the similar effects observed with DTPs and synthetic agonists (not shown). This is unexpected since natural ligands that activate nuclear receptors usually present lower efficiency than synthetic high-affinity compounds. In addition to this, preliminary studies testing the viability of cells treated with different concentrations of these compounds (induction of apoptosis and necrosis) showed very low toxic effects, perhaps in part due to the antiapoptotic mechanisms triggered by LXR, such as those mediated by Spα/AIM (Joseph et al., 2004; Valledor et al., 2004).

In conclusion our data show that synthetic diterpenes, structurally related to acanthoic acid are potent activators of LXRs and open new strategies, including dietetic and pharmacological approaches to control cholesterol metabolism in the course of atherogenesis.

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Footnotes

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Legends to Figures.

Figure 1. ***Activation of LXRα and LXRβ by acanthoic acid-related DTPs.*** A; HEK 293T, THP-1 or THP-1 cells pretreated for 24 h with 20 nM TPA to promote differentiation into macrophages were maintained in culture and the mRNA levels of PPARγ, LXRα and β were determined by quantitative RT-PCR. B; HEK 293T cells were co-transfected with the indicated pCMX nuclear receptor expression vectors, including RXR and the corresponding RE-linked to a luciferase reporter gene. After transfection, cells were stimulated for 24 h with 1 μM of rosiglitazone, 1 μM T1317 and 10 μM of the indicated DTPs and the luciferase activity was measured in the cell extracts. C; HEK293T cells were transfected with a synthetic promoter containing four copies of the GAL4 response element and the GAL4 DBD fused to LXRα, RARα, RXRα, PXR or PPARγ and stimulated for 24h with DTP1 (concentrations 5 and 10uM) and the corresponding nuclear receptor agonists (GW3965 1uM, AM580 100nM, LG268 50nM, Rifampicin 5uM and Rosiglitazone 1uM). Results show the mean ± SD of at least 3 experiments. *$*P<0.05$ vs.* the condition with vehicle.

Figure 2. ***Dose-dependent activation of LXRα and LXRβ by acanthoic acid-related DTPs.*** HEK 293T cells were transfected as described in Fig. 1 and incubated for 24 h with the indicated concentrations of DTPs. After homogenization, the luciferase activity was measured. Results show the mean activity ± SD of 3 experiments.

Figure 3. ***Expression of ABCA1 in THP 1 cells treated with acanthoic acid-related DTPs.*** THP 1 monocytes or THP 1 cells differentiated to macrophages after 24 h of treatment with 20 nM TPA were maintained in culture and stimulated with 2 μM rosiglitazone, 1 μM GW3965 and 10 μM of the indicated DTPs and the expression of CD36 and ABCA1, markers of PPARγ and LXR activation, respectively, were determined by flow cytometry (CD36) or by Western blot (ABCA1). Results show the mean ± SD

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of 5 experiments. **P<0.001 vs.* the condition with vehicle, or a representative blot out of four. The content of β-actin is shown to ensure equal load per lane.

Figure 4. ***Activation of LXR in macrophages by acanthoic acid-related DTPs.*** Elicited peritoneal macrophages were maintained in culture and activated for 48 h with 0.5 μM T1317 and 10 μM of the indicated DTPs. The expression of ABCA1 was determined by Western blot (panel A). RAW 264.7 cells stably expressing either vector alone (mock), LXRα or an activation deficient form (LXRαΔAF2) of this nuclear receptor were treated as described for the peritoneal macrophages and the mRNA levels of ABCA1 and 36B4 were determined at 24h by quantitative RT-PCR. Results show a representative blot out of four (A) and the mean ± SD of 3 experiments. **P<0.05 vs.* the condition with vehicle.

Figure 5. ***Expression of LXR-targets in bone marrow-derived macrophages from WT and DKO for LXR after challenge with acanthoic acid-related DTPs.*** Macrophages from WT and LXRα,β-/- mice were maintained in culture and activated for 48 h with 0.5 μM GW3965 and 10 μM of the indicated DTPs. The expression of ABCA1 was determined by Western blot (panel A). The mRNA levels of ABCA1, SREBP-1c and 36B4 were determined at 18 h by quantitative RT-PCR. Results show a representative blot out of four (A) and the mean ± SD of at least 3 experiments. **P<0.01 vs.* the condition with vehicle.

Figure 6. ***Expression of LXR-dependent genes in peritoneal macrophages treated with acanthoic acid-related DTPs.*** Elicited peritoneal macrophages were maintained in culture and activated for 18 h with 100 nM LG268, 1 μM GW3965, 10 μM of the indicated DTPs or the combinations indicated. The expression of ABCA1, ABCG1, SPα/AIM and SREBP-1c was determined by quantitative RT-PCR. Results show the mean ± SD of 4 experiments. **P<0.01;* ^{a}*P<0.05 vs.* the condition with vehicle and without LG268, respectively.

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Figure 7. ***Cholesterol efflux from cholesterol-loaded peritoneal macrophages treated with acanthoic acid-related DTPs.*** Macrophages were loaded with radiolabeled cholesterol followed by treatment with 1 μM GW3965, 50 nM LG268 and 10 μM of the indicated DTPs. The cholesterol efflux was represented as fold induction compared to ApoA-I treated cells and supplemented with vehicle control (upper panel). The dose response of cholesterol efflux after treatment with LXR/RXR ligands and DTPs is shown in the lower panel. Results show the mean $\pm$ SD of four experiments. **P<0.01 vs.* the condition with vehicle.

Figure 8. ***Expression of inflammatory genes in macrophages treated with DTPs.*** Peritoneal macrophages obtained from WT and and LXRα,β-/- mice were cultured with GW3965 (1uM), Dex (1uM) and DTPs (10uM) for 24h and then challenged with LPS (100ng/ml) for another 6h. Espression of NOS-2, IL-6 and Rantes was evaluated by quantitative RT-PCR.

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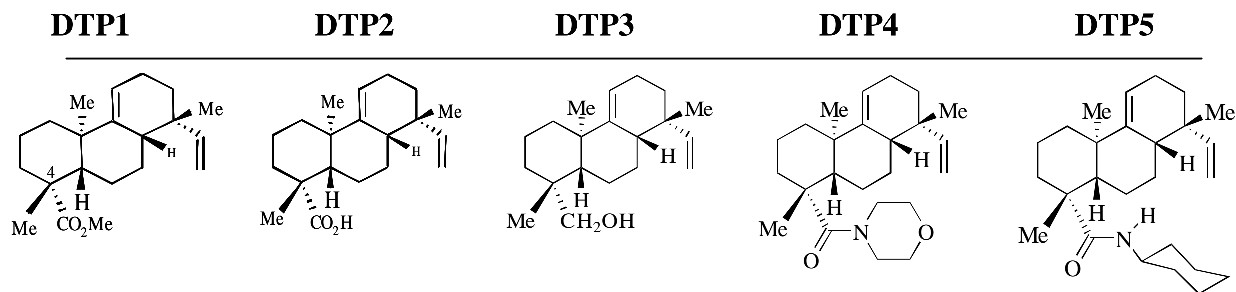
Table 1. ***Chemical structure and proposed generic names of acanthoic acid derived DTPs modified at the C4 position used in this study.***

Diterpenes: DTP1, Diterpene Methyl Ester; **DTP2,** Diterpene Carboxylic Acid; **DTP3**, Diterpene Methyl Alcohol; **DTP4**, Diterpene Carboxyl Morpholine; **DTP5**, Diterpene Carboxyl Piperidine.



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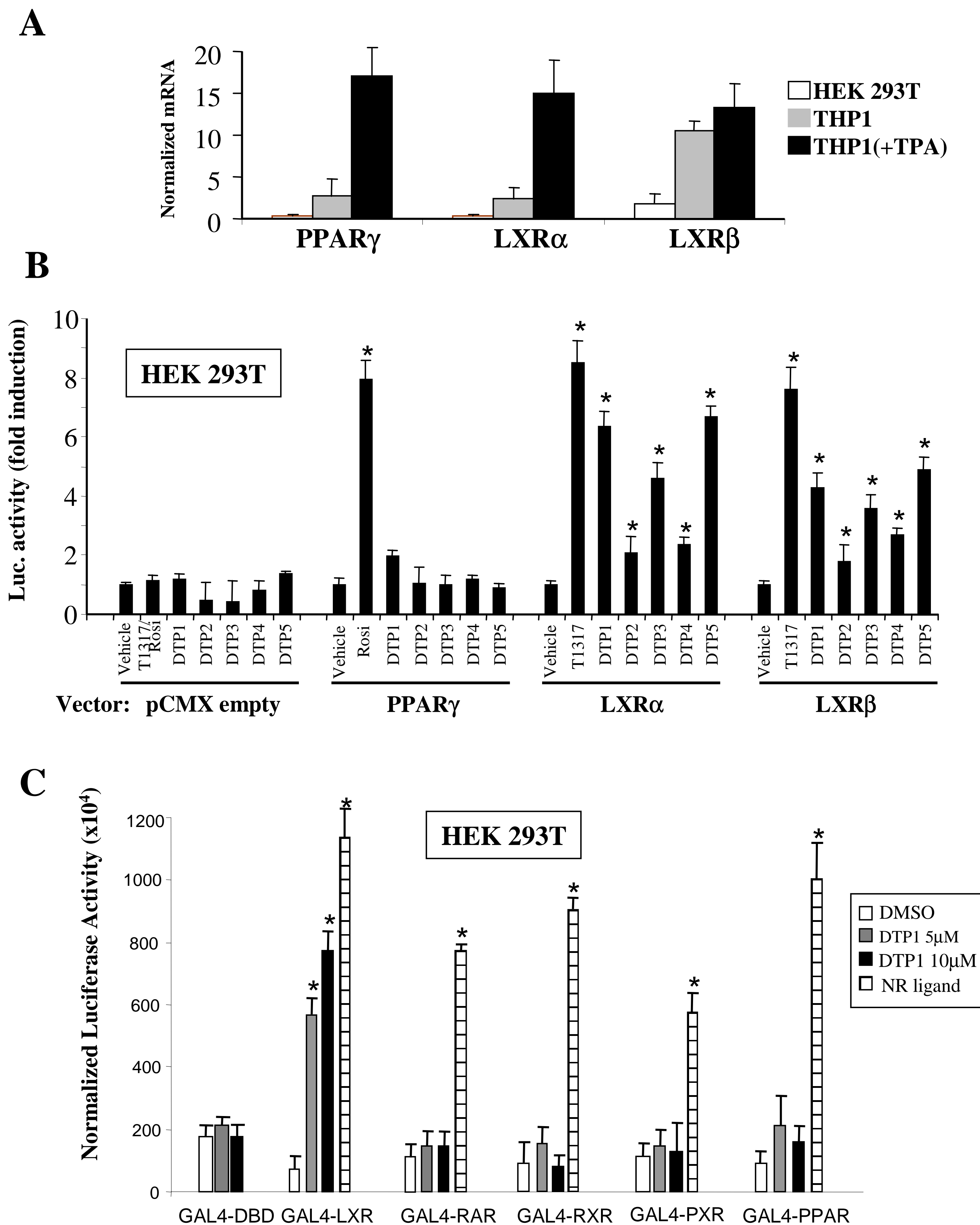

Fig. 1

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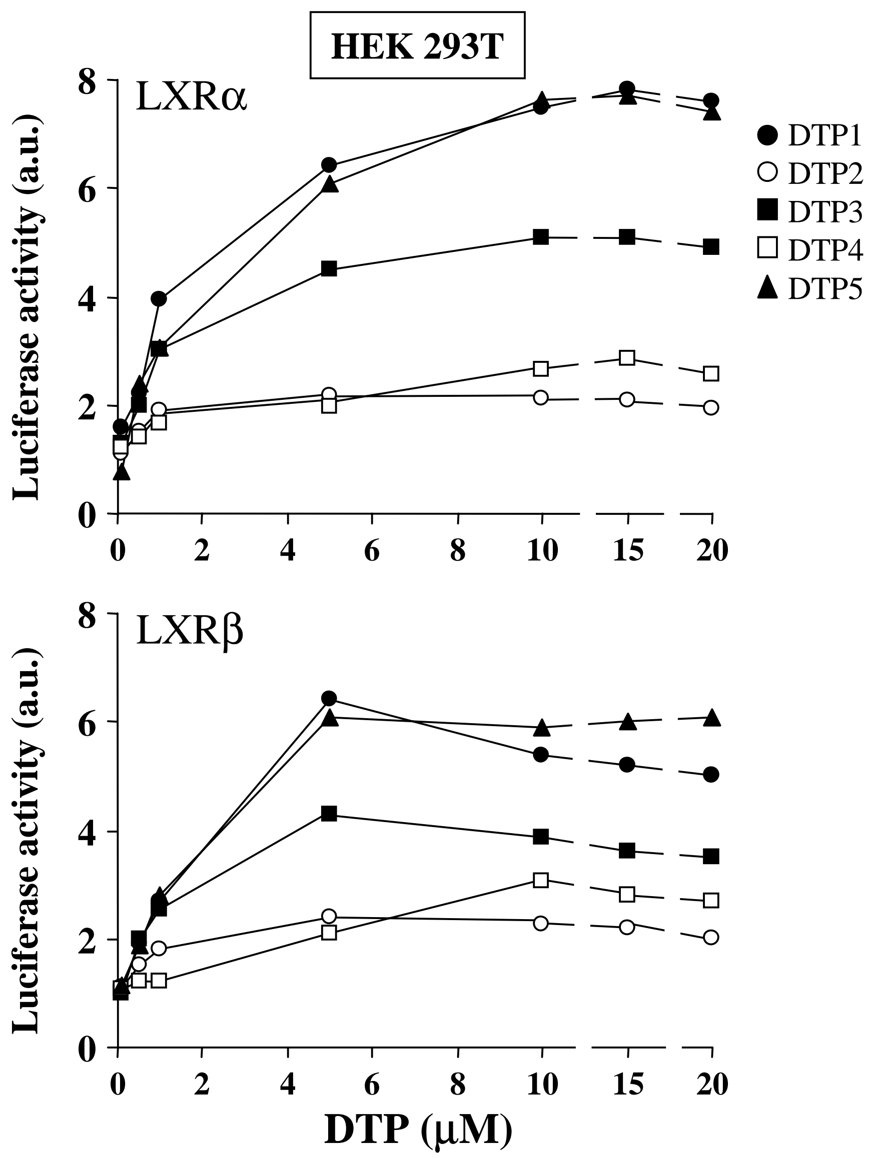

Fig. 2

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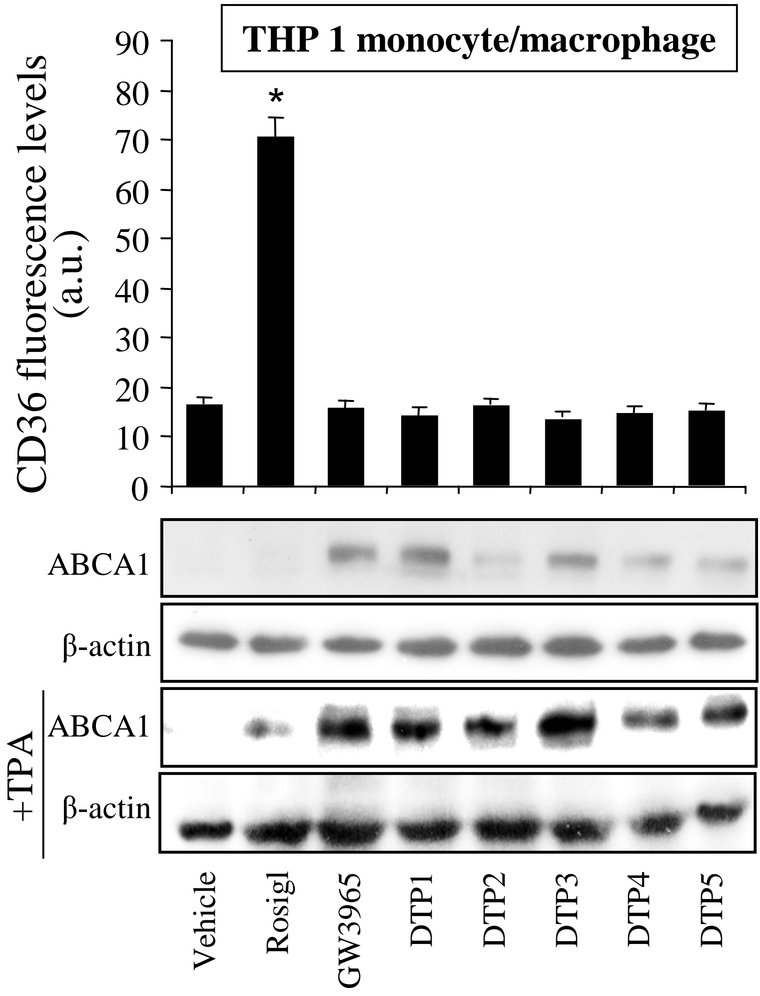

Fig. 3

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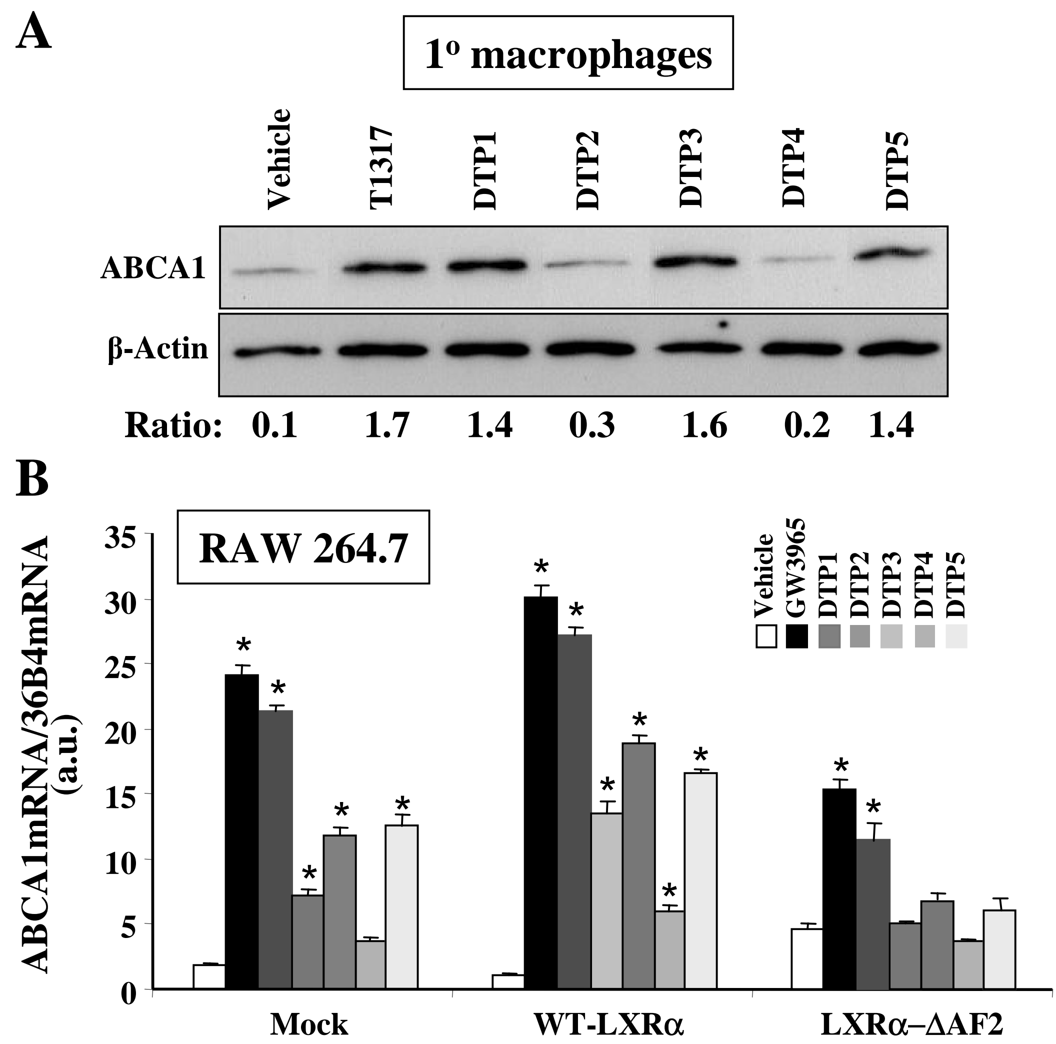

Fig. 4

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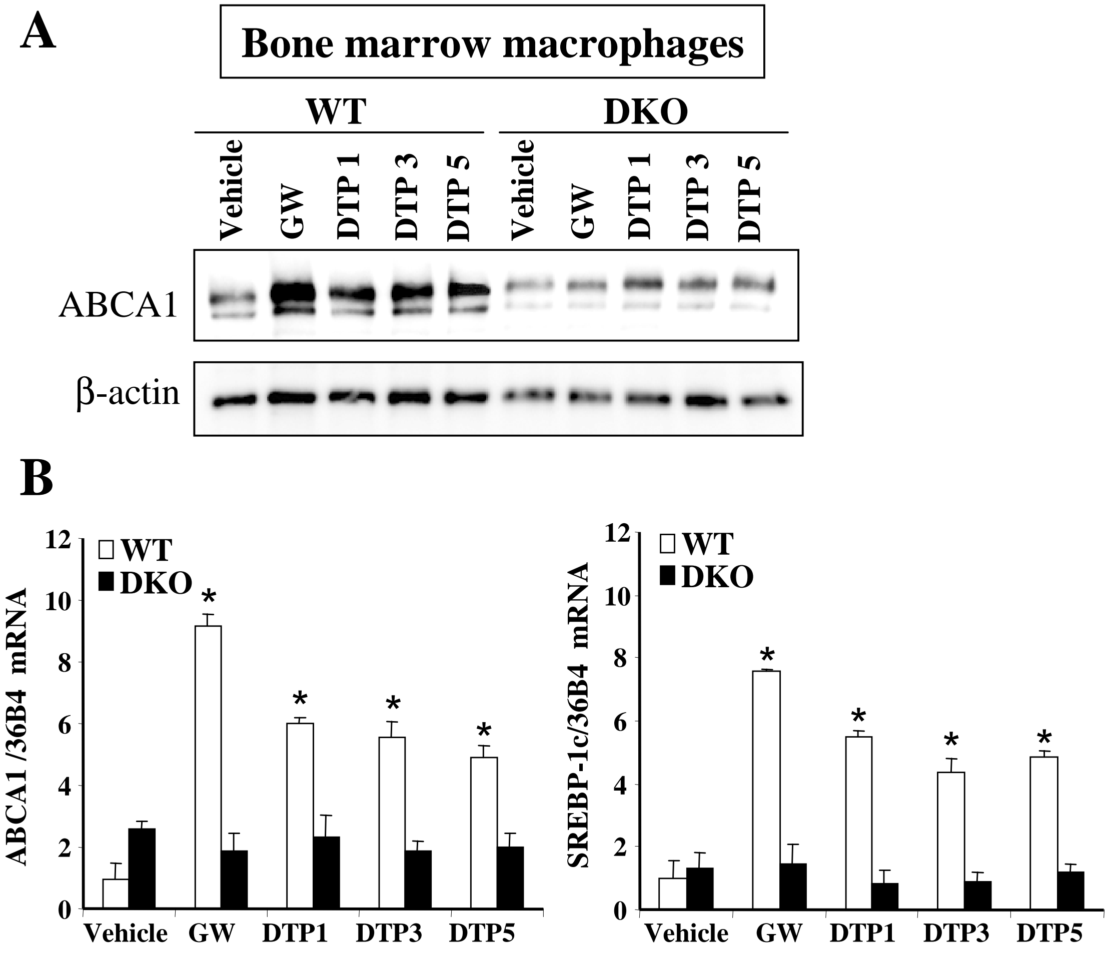

Fig. 5

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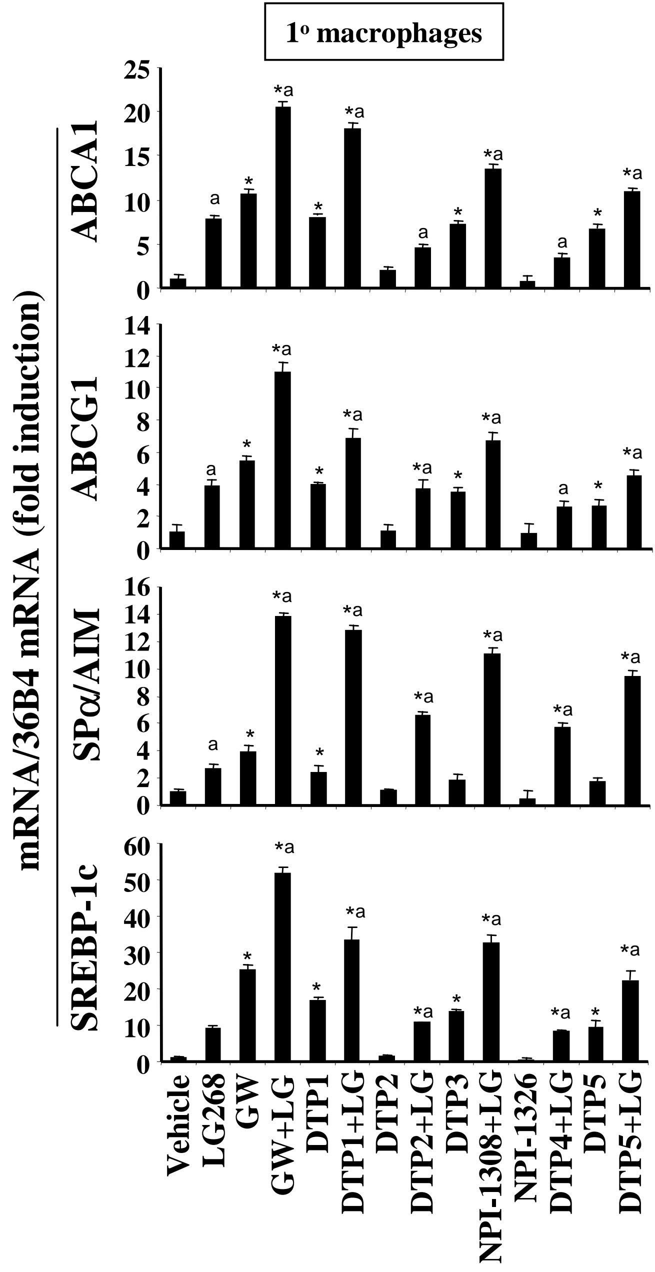

Fig. 6

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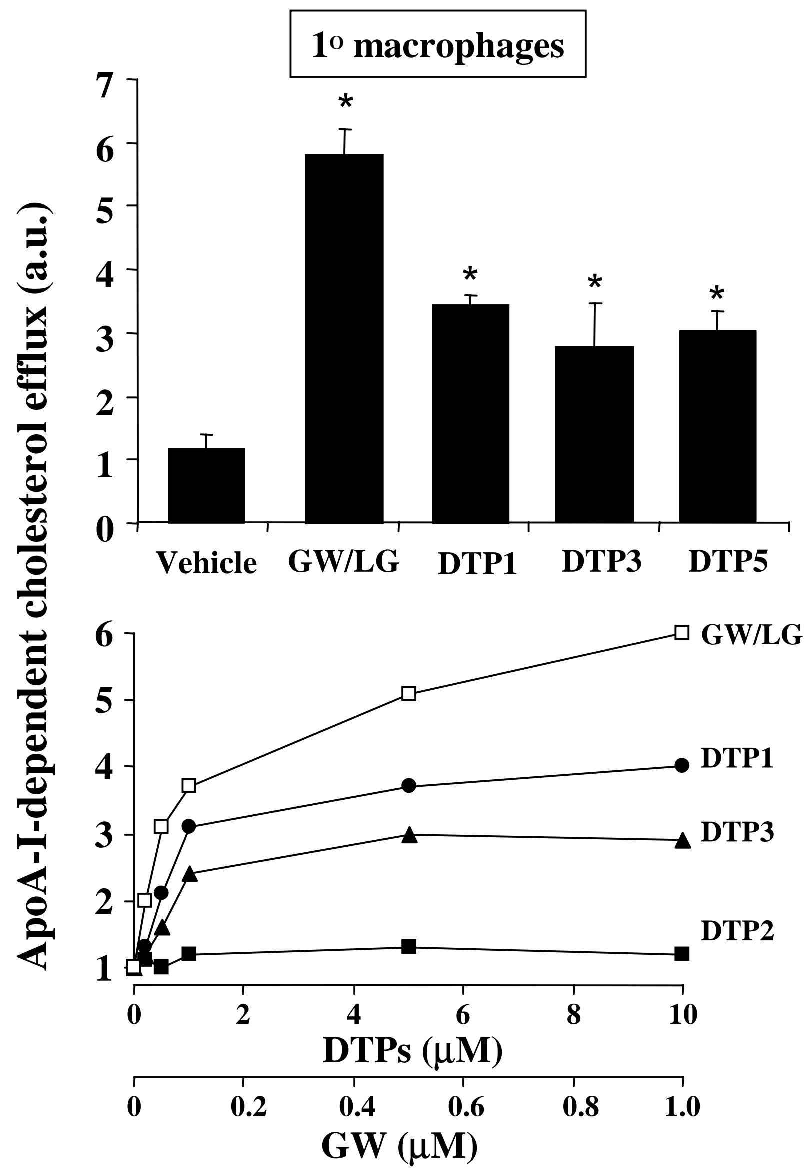

Fig. 7

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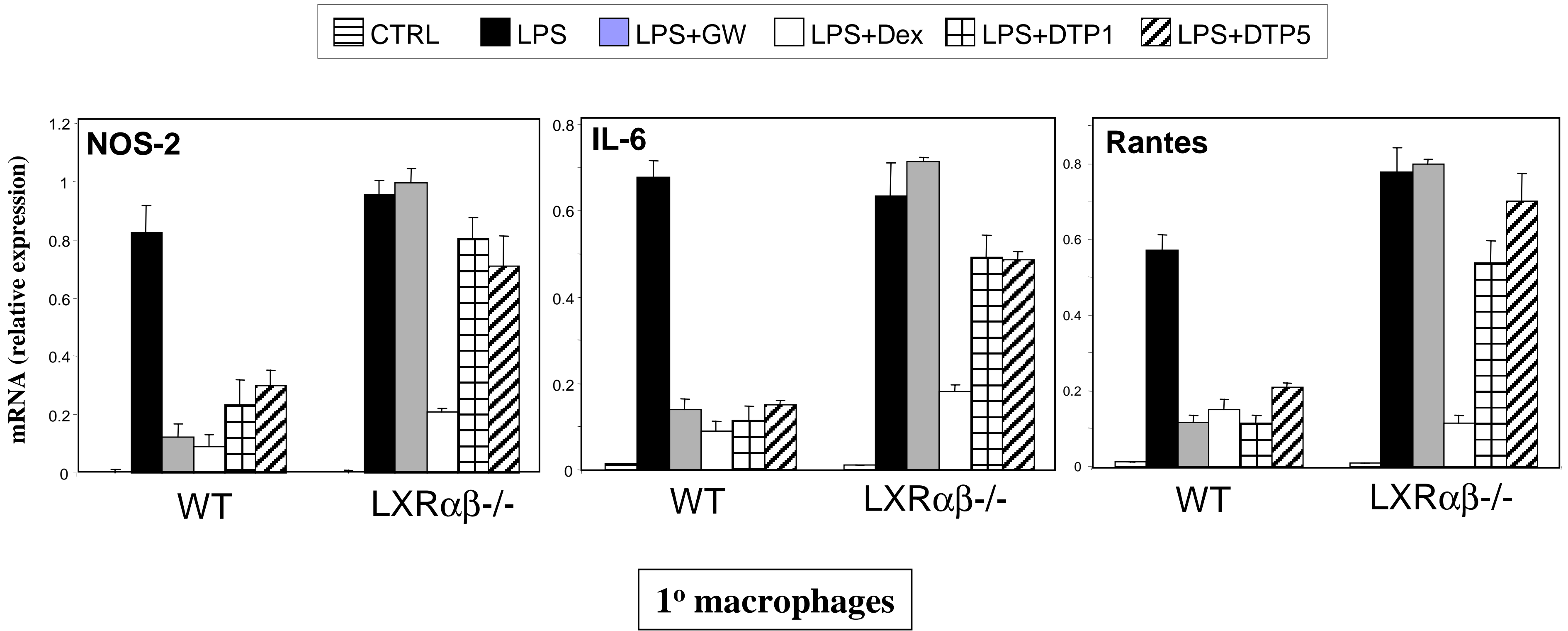

Fig. 8

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