Inhibition of Macrophage CD36 Expression and Cellular Oxidized Low Density Lipoprotein (oxLDL) Accumulation by Tamoxifen

A PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR) γ-DEPENDENT MECHANISM

Miao Yu1,2, Meixiu Jiang3, Yaniu Chen2, Shuang Zhang4, Wenwen Zhang1, Xiaoxiao Yang5, Xiaoj Li6, Yan Li6, Shengzhong Duan*, Jihong Han†‡3, and Yajun Duan‡‡‡4

From the 1College of Life Sciences, 2School of Medicine, and the 3State Key Laboratory of Medicinal Chemical Biology, Collaborative Innovation Center of Biotherapy, Nankai University, Tianjin 300071, the 4Institute of Translational Medicine, Nanchang University, Nanchang 330000, the 5College of Biomedical Engineering, Hefei University of Technology, Hefei 230009, and the 6Institute for Nutritional Sciences, Chinese Academy of Sciences, Shanghai 200031, China

Macrophage CD36 binds and internalizes oxidized low density lipoprotein (oxLDL) to facilitate foam cell formation. CD36 expression is activated by peroxisome proliferator-activated receptor γ (PPARγ). Tamoxifen, an anti-breast cancer medicine, has demonstrated pleiotropic functions including cardioprotection with unfailingly elucidated mechanisms. In this study, we determined that treatment of ApoE-deficient mice with tamoxifen reduced atherosclerosis, which was associated with decreased CD36 and PPARγ expression in lesion areas. At the cellular level, we observed that tamoxifen inhibited CD36 protein expression in human THP-1 monocytes, THP-1/PMA macrophages, and human blood monocyte-derived macrophages. Associated with decreased CD36 protein expression, tamoxifen reduced cellular oxLDL accumulation in a CD36-dependent manner. At the transcriptional level, tamoxifen decreased CD36 mRNA expression, promoter activity, and the binding of the PPARγ response element in CD36 promoter to PPARγ protein. Tamoxifen blocked ligand-induced PPARγ nuclear translocation and CD36 expression, but it increased PPARγ phosphorylation, which was due to that tamoxifen-activated ERK1/2. Furthermore, deficiency of PPARγ expression in macrophages abolished the inhibitory effect of tamoxifen on CD36 expression or cellular oxLDL accumulation both in vitro and in vivo. Taken together, our study demonstrates that tamoxifen inhibits CD36 expression and cellular oxLDL accumulation by inactivating the PPARγ signaling pathway, and the inhibition of macrophage CD36 expression can be attributed to the anti-atherogenic properties of tamoxifen.

Tamoxifen (Nolvadex) and its immediate metabolite, 4-hydroxytamoxifen, belong to the class of drugs called selective estrogen receptor modulators (1). Dependent on target tissue/molecules, tamoxifen can function either as an estrogen receptor (ER) antagonist, such as in the mammary tissue, or as an ER agonist, such as in the endometrium (2). In the breast tissue, inactivation of ER by tamoxifen inhibits the growth of breast cancer cells. Therefore, tamoxifen is the most commonly used anti-hormonal drug for adjuvant treatment of patients with ER-positive breast cancer (2). In addition, tamoxifen has been determined to have a preventive function on breast cancer in the population of women with a high risk of developing this disease (3).

Besides the effect on breast cancer, several lines of evidence have demonstrated the cardioprotective effects of tamoxifen. In animal models, tamoxifen reduces atherosclerosis in wild-type mice and pro-atherogenic animal models, such as ApoE-deficient (ApoE−/−) mice and surgically postmenopausal monkeys (4–6). In humans, tamoxifen lowers the incidence of fatal myocardial infarction, reduces the intima-media thickness of the common carotid artery in the postmenopausal women, and promotes the endothelium-dependent flow-mediated dilation in male patients with advanced atherosclerosis (7–9).

Atherosclerosis is one of the major causes of coronary heart disease and a chronic pathological process with disorders of lipid metabolism and/or inflammation (10, 11). Formation of lipid-laden macrophage/foam cells in the intima is a major hallmark in the early stage of lesion development. Uncontrolled uptake of oxidized low density lipoprotein (oxLDL), excessive cholesterol esterification, and/or impaired cholesterol efflux can result in accumulation of cholesterol esters that are stored...
as lipid droplets and subsequently trigger foam cell formation (12). CD36, a member of type B scavenger receptor family, is an 88-kDa glycosylated transmembrane protein and initially identified as the receptor for oxLDL (13). Cells transfected with CD36 expression vector demonstrate a high capacity for uptake of oxLDL, which is blocked by anti-CD36 antibody (14). Several studies have indicated that macrophage CD36 can facilitate foam cell formation and lesion development (15, 16). However, the controversial effects of genetic deletion of CD36 expression have been reported (17, 18).

Increased CD36 expression has been determined during the monocyte/macrophage differentiation (19). Interestingly, macrophage CD36 expression can be activated by its ligand, oxLDL, which leads to a positive forward loop of CD36 expression/oxLDL accumulation (20, 21). The further studies suggest that activation of macrophage CD36 transcription by oxLDL depends on activation of peroxisome proliferator-activated receptor γ (PPARγ) (19, 21). PPARγ is a ligand-activated transcription factor. Both 9-hydroxyoctadecadienoic acid and 13-hydroxyoctadecadienoic acid contained in oxLDL can serve as endogenous PPARγ ligands (21). The thiazolidinediones class of antidiabetic drugs, such as troglitazone, rosiglitazone, and pioglitazone, are synthetic PPARγ ligands (22). They can also substantially increase macrophage CD36 expression. Despite inducing macrophage CD36 expression, the PPARγ agonist can still reduce atherosclerosis which might be due to the multiple biological functions of PPARγ, such as anti-inflammation and activation of cholesterol efflux/reverse cholesterol transport (23–25).

Binding of ligand to PPARγ protein results in formation of a heterodimer of PPARγ with another nuclear protein, retinoid X receptor α. The complex of PPARγ-retinoid X receptor α can bind to the PPAR response element (PPRE), which contains a conserved sequence of AGGTCAAGGTCA (the 6 nucleotides separated by a nucleotide were repeated, thus, PPRE is also named as the direct repeat 1 (DR1)) in target genes including CD36, and initiate their transcription (21). Besides the ligand binding, the phosphorylation status of PPARγ also influences PPARγ transcriptional activity. The existence of an action site of extracellular signal-regulated kinases 1/2 (ERK1/2) in the N-terminal of PPARγ indicates that PPARγ is a target for ERK1/2 activity. In fact, phosphorylation of PPARγ inhibits, whereas dephosphorylation of PPARγ activates its transcriptional activity including on CD36 expression (22, 26).

Although both clinical and basic studies have demonstrated that tamoxifen has atheroprotective properties, the underlying mechanisms have not been fully elucidated. In the current study, we hypothesized that tamoxifen can inhibit macrophage CD36 expression and cellular oxLDL accumulation, which can be attributed to its anti-atherogenic properties. We also attempted to unveil the involved mechanisms.

Results

Inhibition of Atherosclerosis by Tamoxifen Is Associated with Decreased CD36, FABP4, and PPARγ Expression in Lesion Areas—The inhibitory effects of tamoxifen on atherosclerosis have been reported with different animal models (4–6). At the cellular level, our previous study demonstrates that tamoxifen reduces macrophage adipocyte fatty acid-binding protein (FABP4) expression (27). Expression of CD36 and FABP4 can facilitate macrophage/foam cell formation, and both of them are molecular targets of PPARγ. To determine whether the reduction of atherosclerosis by tamoxifen is related to CD36 and FABP4 expression, we fed ApoE−/− mice a high-fat diet (HFD, 0.5% cholesterol and 21% fat) or HFD containing tamoxifen for 16 weeks. Compared with mice on HFD alone, we determined that tamoxifen reduced en face atherosclerotic lesions (Fig. 1A), which is consistent with other reports.

In contrast to our previous report that demonstrates that tamoxifen reduces total and LDL-cholesterol levels in C57BL/6 wild type mice fed normal chow (28), in the current study, we determined that tamoxifen had little effect on serum lipid profiles of mice fed HFD (Fig. 1B), which suggests that the effect of tamoxifen on the lipid profile might be in an animal model-dependent manner, and the inhibition of lesions in ApoE−/− mice by tamoxifen should be completed by mechanisms other than amelioration of lipid profiles. In fact, when we determined expression of CD36 and FABP4 in lesion areas by immunofluorescence staining the cross-sections of aortic root, we found that both of them were substantially reduced by tamoxifen (Fig. 1C, top and middle panels, and D, left and middle panels). CD36 and FABP4 are two pro-atherogenic molecules that are activated by PPARγ, therefore, we determined PPARγ expression in lesion areas. Similarly, tamoxifen inhibited PPARγ expression (Fig. 1, C, low panel, and D, right panel).

Taken together, the results in Fig. 1 indicate that inhibition of atherosclerosis by tamoxifen is associated with reduction of CD36 and FABP4 expression, which might be completed by action of PPARγ. Since we have reported that tamoxifen regulates macrophage FABP4 expression by the combined PPARγ and glucocorticoid receptor (GR) pathways (27), therefore, in this study, we mainly focused on the regulation of macrophage CD36 expression and the involved mechanisms by tamoxifen.

Tamoxifen and 4-Hydroxytamoxifen Inhibit Human Monocyte/Macrophage CD36 Protein Expression and Cellular oxLDL Accumulation—We initially determined the effect of tamoxifen on CD36 expression in vitro. Both THP-1 monocytes (Fig. 2A) and PMA-derived THP-1 (THP-1/PMA) macrophages (Fig. 2D) were treated with tamoxifen and 4-hydroxytamoxifen at different concentrations for 24 h, respectively, and the CD36 protein expression was determined by Western blot analysis. Normally, two bands of CD36 protein can be determined with cell lines, such as THP-1 monocyte, THP-1/PMA macrophages, and J774/RAW macrophages; and one band with primary macrophages or tissue samples. The results in Fig. 2, A and D, demonstrate that both tamoxifen and 4-hydroxytamoxifen decreased CD36 protein expression in a concentration-dependent manner, in both cell types. We then treated THP-1 monocytes (Fig. 2B) and THP-1/PMA macrophages (Fig. 2E) with 5 μM tamoxifen or 4-hydroxytamoxifen for the indicated times. The results of the time course study indicate that inhibition of monocyte or macrophage CD36 expression by tamoxifen and 4-hydroxytamoxifen occurred quickly (~6 h after treatment) and lasted for 24 h of the treatment.

CD36 is a cellular membrane protein. Inhibition of total CD36 protein levels by tamoxifen can consequently reduce cell...
surface CD36 protein levels. Indeed, the results of the FACS assay demonstrate that tamoxifen and 4-hydroxytamoxifen reduced cellular surface CD36 protein levels in both THP-1 monocytes (Fig. 2C) and THP-1/PMA macrophages (Fig. 2F).

Functionally, the decreased cell surface CD36 protein can result in reduced cellular oxLDL accumulation and foam cell formation. Therefore, we conducted Oil Red O staining and observed that tamoxifen and 4-hydroxytamoxifen substantially decreased oxLDL uptake by macrophages suggesting that the foam cell formation is inhibited (Fig. 2G, top panel). The presence of anti-CD36 antibody attenuated tamoxifen and 4-hydroxytamoxifen-reduced oxLDL accumulation in macrophages (Fig. 2G, bottom panel) confirming that the reduction of cellular oxLDL accumulation/foam cell formation by tamoxifen or 4-hydroxytamoxifen mainly depends on inhibition of CD36 expression.

To determine whether tamoxifen or 4-hydroxytamoxifen can also alter CD36 expression in primary macrophages, human blood monocyte-derived macrophages (BMDMs) were treated with tamoxifen and 4-hydroxytamoxifen followed by determination of CD36 protein expression by both Western blot analysis and FACS assay. The results in Fig. 2H show that tamoxifen and 4-hydroxytamoxifen markedly reduced CD36 protein levels in whole cellular extract and cell surface of BMDMs.

Tamoxifen and 4-Hydroxytamoxifen Inhibit Macrophage CD36 Expression at the Transcriptional Level—To determine whether the inhibition of CD36 expression by tamoxifen and 4-hydroxytamoxifen is at the transcriptional level, we initially determined changes of CD36 mRNA levels in response to tamoxifen and 4-hydroxytamoxifen treatment by real time RT-PCR. The results in Fig. 3A demonstrate that either tamoxifen or 4-hydroxytamoxifen can substantially reduce CD36 mRNA levels. Expression of CD36 mRNA can be induced by PPARγ activation. As shown in Fig. 3B, rosiglitazone, a synthetic PPARγ ligand, induced CD36 mRNA expression. However, the induction was inhibited by tamoxifen or 4-hydroxytamoxifen in a concentration-dependent manner, which implies that tamoxifen may regulate CD36 transcription. To determine it, we constructed a CD36 promoter, which includes the PPRE motif, and treated the promoter with tamoxifen and 4-hydroxytamoxifen. The results in Fig. 3C indicate that both tamoxifen and 4-hydroxytamoxifen inhibited CD36 promoter activity. Furthermore, we determined that high expressing PPARγ activated the CD36 promoter, which was further enhanced by rosiglitazone (Fig. 3D). However, both tamoxifen and 4-hydroxytamoxifen inhibited PPARγ-activated CD36 promoter activity, which further confirms the inhibition of CD36 transcription by tamoxifen and 4-hydroxytamoxifen.

The role of PPRE in tamoxifen-inhibited CD36 transcription was also defined by EMSA. As expected, tamoxifen (lane 2 and 3, Fig. 3E) and 4-hydroxytamoxifen (Fig. 3E, lanes 4 and 5) reduced the binding of PPRE to nuclear protein in a concentration-dependent manner with a greater effect by 4-hydroxytamoxifen. The specific binding of the PPRE to PPARγ protein was confirmed by the competition with addition of...
anti-PPARγ antibody (Fig. 3E, lane 6 versus 1) or unlabeled probe (Fig. 3E, lane 7 versus 1) in the reaction system. Taken together, the results in Fig. 3 indicate that tamoxifen and 4-hydroxytamoxifen inhibited macrophages CD36 expression at the transcriptional level, which is related to the alteration of PPARγ activity.

Tamoxifen Inhibits Mφ CD36 Expression and oxLDL Uptake—To investigate the effect of tamoxifen on PPARγ transcriptional activity, we initially determined if tamoxifen and 4-hydroxytamoxifen can antagonize PPARγ-induced CD36 expression by treating THP-1/PMA macrophages with tamoxifen or 4-hydroxytamoxifen at different concentrations.

FIGURE 2. Tamoxifen and 4-hydroxytamoxifen inhibit human monocyte and macrophage CD36 protein expression and reduce macrophage oxLDL accumulation. THP-1 monocytes (A–C) and THP-1/PMA macrophages (D–F) received the following treatment: A and D, tamoxifen (Tam) and 4-hydroxytamoxifen (4-OH Tam or 4-OH) at the indicated concentrations for 24 h; B and E, 5 μM tamoxifen and 4-hydroxytamoxifen for the indicated times; C and F, 5 μM tamoxifen and 4-hydroxytamoxifen overnight. CD36 protein expression in cellular extract (A, B, D, and E) and cell surface CD36 protein in intact cells (C and F) were determined by Western blot and FACS, respectively; G, THP-1/PMA macrophages were treated with tamoxifen and 4-hydroxytamoxifen at the indicated concentrations overnight. After treatment, cells were preincubated with normal IgG or anti-CD36 antibody for 1 h. After removal of normal IgG or anti-CD36 antibody, cells were incubated with 50 μg/ml of oxLDL for 3 h. Cellular lipid accumulation was determined by Oil Red O staining. H, human BMDMs received the indicated treatment overnight and used for the following determination: top panel, CD36 protein expression in cellular extract; lower panel, cell surface CD36 protein in intact cells. *, p < 0.05 (n = 3).
concentrations overnight followed by extraction of nuclear protein and determination of the binding of PPAR
target genes. Reduced interaction between PPRE and PPAR
expression was induced by troglitazone, another PPAR
b) tamoxifen or 4-hydroxytamoxifen at the indicated concentrations in the presence of rosiglitazone (Rosi; 10 μM) for 24 h. Expression of CD36 mRNA was determined by real time RT-PCR. *, versus control alone; #, versus group received rosiglitazone treatment, p < 0.05 (n = 3). C and D, 293T cells were transfected with DNA for the CD36 promoter, Ppar expression vector plus Renilla luciferase DNA for 6 h followed by the indicated treatment overnight. The cellular lysate was extracted to determine the activity of firefly and Renilla luciferase. *, versus pCD36 alone; #, versus pCD36 + rosiglitazone, p < 0.05 (n = 3). E, THP-1/PMA macrophages were treated with tamoxifen and 4-hydroxytamoxifen at the indicated concentrations overnight followed by extraction of nuclear protein and determination of the binding of PPARγ protein in nuclear extract with PPRE by EMSA. To confirm the specific interaction between the radiolabeled PPRE probe and PPARγ protein, either anti-PPARγ antibody (lane 6) or excess unlabeled PPRE probe (×50, lane 7) was added to the reaction system with nuclear protein extracted from control cells (lane 1). The sequence of the PPRE probe is: tttttcTGACTTacttg. This experiment was repeated three times and a representative image presented.

FIGURE 3. Tamoxifen and 4-hydroxytamoxifen inhibit CD36 expression at the transcriptional level. THP-1/PMA macrophages received the following treatment. A, 5 μM tamoxifen or 4-hydroxytamoxifen for 24 h; B, tamoxifen or 4-hydroxytamoxifen at the indicated concentrations in the presence of rosiglitazone (Rosi; 10 μM) for 24 h. Expression of CD36 mRNA was determined by real time RT-PCR. *, versus control alone; #, versus group received rosiglitazone treatment, p < 0.05 (n = 3); C and D, 293T cells were transfected with DNA for the CD36 promoter, Ppar expression vector plus Renilla luciferase DNA for 6 h followed by the indicated treatment overnight. The cellular lysate was extracted to determine the activity of firefly and Renilla luciferase. *, versus pCD36 alone; #, versus pCD36 + rosiglitazone, p < 0.05 (n = 3). E, THP-1/PMA macrophages were treated with tamoxifen and 4-hydroxytamoxifen at the indicated concentrations overnight followed by extraction of nuclear protein and determination of the binding of PPARγ protein in nuclear extract with PPRE by EMSA. To confirm the specific interaction between the radiolabeled PPRE probe and PPARγ protein, either anti-PPARγ antibody (lane 6) or excess unlabeled PPRE probe (×50, lane 7) was added to the reaction system with nuclear protein extracted from control cells (lane 1). The sequence of the PPRE probe is: tttttcTGACTTacttg. This experiment was repeated three times and a representative image presented.

centrations in the presence of rosiglitazone. CD36 protein expression was induced by rosiglitazone as expected. However, both tamoxifen and 4-hydroxytamoxifen blocked the induction (Fig. 4A). As shown in Fig. 4B, we also determined that tamoxifen and 4-hydroxytamoxifen blocked CD36 expression induced by troglitazone, another PPARγ ligand, in human BMDMs.

Besides ligand activation, expression and phosphorylation of PPARγ can also impact PPARγ activity on expression of its target genes. Reduced interaction between PPRE and PPARγ protein in nuclear extract isolated from tamoxifen-treated macrophages (Fig. 3E) also implies that tamoxifen may inhibit PPARγ expression as well as nuclear translocation. Indeed, the results in Fig. 4C show that PPARγ protein expression was moderately reduced (top panel), whereas phosphorylated PPARγ (pi-PPARγ) was inversely regulated (middle panel) by tamoxifen and 4-hydroxytamoxifen, which suggests that tamoxifen treatment also increases phosphorylation of PPARγ (Fig. 4C, bottom, pi-PPARγ/PPARγ), another mechanism inactivating PPARγ.

Activation of PPARγ by ligands can induce PPARγ nuclear translocation to enhance its transcriptional activity. The results of Western blot analysis (Fig. 4C) and EMSA (Fig. 3E) demonstrate that tamoxifen is able to reduce PPARγ protein expression and nuclear levels. To determine whether tamoxifen can also reduce ligand-induced PPARγ nuclear translocation, we transfected HeLa cells with the PPARγ expression vector and then treated the transfected cells with rosiglitazone or rosiglitazone plus tamoxifen or 4-hydroxytamoxifen. In control cells, PPARγ mainly localized in cytosol (Fig. 4D, 1st column of left panel). Activation of PPARγ by rosiglitazone resulted in PPARγ nuclear translocation (Fig. 4D, 2nd column of left panel). However, such nuclear translocation was substantially blocked by either tamoxifen or 4-hydroxytamoxifen (Fig. 4D, 3rd or 4th column of left panel or right panel). Taken together, the results in Fig. 4 demonstrate that tamoxifen decreases PPARγ activity by reducing PPARγ expression and nuclear translocation while increasing PPARγ phosphorylation, which can lead to inhibition of CD36 expression.

Existence of the ERK1/2 action site in PPARγ protein suggests that PPARγ is a substrate for ERK1/2 activity (26). Several studies have demonstrated that the phosphorylation status of PPARγ can influence its transcriptional activity. In fact, dephosphorylation of PPARγ increases while phosphorylation of
PPAR\textsubscript{γ} decreases its transcriptional activity (26, 29). The increased Pi-PPAR\textsubscript{γ} by tamoxifen or 4-hydroxytamoxifen (Fig. 4C) suggests that tamoxifen can activate ERK1/2. Indeed, both concentration and time course studies show that tamoxifen had no effect on ERK1/2 expression while substantially increasing phosphorylated ERK1/2 (Pi-ERK1/2) (Fig. 5A), the active form of ERK1/2.

We previously reported that MEK1/2 inhibitors (U0126 and PD98059) induce CD36 expression by dephosphorylating PPAR\textsubscript{γ} through inhibition of ERK1/2 (30). The increased Pi-ERK1/2 by tamoxifen (Fig. 5A) indicates that tamoxifen may interact with U0126 to influence CD36 expression. Indeed, we observed that inhibition of CD36 by pre-treatment with tamoxifen was restored by U0126 (Fig. 5B). Inversely, the induction of CD36 expression by pre-treatment with U0126 was reduced by tamoxifen because the reduced Pi-ERK1/2 by U0126 was moderately increased by tamoxifen (Fig. 5C). At the transcriptional level, we determined that MEK1/2 inhibitors increased CD36 promoter activity, which can be blocked by tamoxifen or 4-hydroxytamoxifen. Taken together, the results in Fig. 5 demonstrate the inverse correlation between ERK1/2 activity and CD36 expression mediated by U0126 and/or tamoxifen in which the phosphorylation status of PPAR\textsubscript{γ} is changed (Fig. 4C) (30).

To further confirm that tamoxifen inhibited CD36 expression by inactivating PPAR\textsubscript{γ}, we isolated macrophages from macrophage Ppar\textsubscript{γ}-specific knock-out (MacPpar\textsubscript{γ} KO) mice and the corresponding control (Ppar\textsubscript{γ}\textsuperscript{fl/fl}) mice (Fig. 6A).
treated the cells with tamoxifen and 4-hydroxytamoxifen in vitro. Compared with cells isolated from mice expressing PPARγ, deficiency of PPARγ expression reduced CD36 basal levels in control samples (Fig. 6, B and C), which indicates the importance of PPARγ in CD36 expression. Tamoxifen inhibited CD36 mRNA expression in control macrophages (Pparγfl/fl) but not the cells lacking PPARγ expression (Fig. 6B, MacPparγ KO). Similarly, tamoxifen or 4-hydroxytamoxifen reduced CD36 protein expression only in the cells expressing PPARγ (Fig. 6C). Correspondingly, we determined that deficiency of PPARγ expression substantially reduced cellular oxLDL accumulation in macrophages. Tamoxifen inhibited cellular oxLDL accumulation in macrophages expressing PPARγ in a CD36-dependent manner (Fig. 6D, top left panel), whereas it had little effect on cellular oxLDL accumulation in Pparγ-deficient cells (Fig. 6D, top right panel). The similar changes in cellular cholesteryl ester levels were obtained in response to tamoxifen treatment in either control or Pparγ-deficient macrophages (Fig. 6D, bottom panel).

Although we determined that tamoxifen inhibited CD36 expression and cellular oxLDL accumulation in control cells but not Pparγ-deficient cells (Fig. 6, C and D), the deficiency of PPARγ expression substantially reduced basal levels of CD36 expression and cellular lipid content, which implies it might be difficult to observe the further reduction of both by tamoxifen treatment, which is due to the sensitivity of detections. We previously reported that inhibition of cellular glutathione (GSH) production by inhibitor of glutamate-cysteine ligase (the rate-limiting enzyme for GSH production), L-buthionine-S,R-sulfoximine (BSO), activates macrophage CD36 expression and cellular oxLDL accumulation (31). Interestingly, the activation occurs at the translational level because BSO enhances CD36 translational efficiency (31). To further confirm that tamoxifen inhibited CD36 expression and oxLDL accumulation in control...
cells but not Ppar-deficient cells, we pre-treated all cells with BSO, which can increase basal levels of CD36 expression and cellular lipid content. After removal of BSO, cells were treated with tamoxifen followed by determination of CD36 protein expression and cellular lipid content. BSO increased CD36 expression and cellular lipid content in both control and Ppar-deficient cells. However, we still observed that tamoxifen decreased CD36 protein and oxLDL accumulation in control cells, but not Ppar-deficient cells (Fig. 6, E and F). Taken together, the results in Figs. 4–6 clearly demonstrate that tamoxifen inhibits CD36 expression and cellular oxLDL accumulation mainly by inactivating PPARγ.

Inhibition of Macrophage CD36 Expression by Tamoxifen in Vivo—To define if inhibition of macrophage CD36 expression by tamoxifen is also in a PPARγ-dependent manner in vivo, we initially fed ApoE−/− mice HFD or HFD containing tamoxifen (2 mg/100 g of food) for 2 weeks, and collected peritoneal macrophages to determine CD36 expression. The results in Fig. 7A indicate that administration of tamoxifen reduced macrophage CD36 expression in vivo.

Next, we conducted a similar experiment as above with MacPpar KO and Pparfl/fl (control) mice. Consistently, we determined that lack of Ppar expression decreased basal levels of macrophage CD36 in vivo (Fig. 7B). Treatment of tamoxifen inhibited macrophage CD36 expression in Pparfl/fl mice (Fig. 7B, right), whereas had little effect on macrophage CD36 expression in MacPpar KO mice (Fig. 7B, left). In contrast, tamoxifen reduced adipose tissue CD36 and PPARγ expression in both MacPpar KO mice and Pparfl/fl mice (Fig. 7C), which was due to that expression of PPARγ in adipose tissues is nor-
mal in both mouse types. Therefore, the results of in vivo studies also demonstrate that tamoxifen-inhibited CD36 expression is dependent on inactivation of PPARγ.

**Discussion**

Formation of macrophage/foam cells is the initial and critical step in the development of atherosclerosis. Binding and internalization of oxLDL, which is mainly mediated by CD36 expression, enhance foam cell formation. In addition, the interaction between CD36 and oxLDL in macrophages can trigger a signaling response that is pro-inflammatory and pro-atherogenic (12). Compared with many other feedback inhibitory biological processes, the accumulation of oxLDL in macrophages by CD36 action is the forward positive cycles because 9- and 13-hydroxyoctadecadienoic acid contained in oxLDL can function as PPARγ ligands to induce CD36 expression. Although tamoxifen is used as an anti-breast cancer medicine, it has been observed to have pleiotropic effects including atheroprotection. In the current study, we determined that tamoxifen inhibited CD36 expression in human monocytes and macrophages (Fig. 2, A–F and H). Functionally, inhibition of CD36 expression by tamoxifen reduced macrophage cellular oxLDL accumulation/foam cell formation, which is in a CD36-dependent manner (Fig. 2, D and G). In addition, we determined that tamoxifen inhibited CD36 expression in mouse peritoneal macrophages and adipose tissues, which demonstrates that inhibition of CD36 expression is neither cell-type nor species-dependent (Fig. 7).

Several lines of evidence have demonstrated that tamoxifen has cardioprotective effects by different mechanisms. Both in vitro and in vivo studies indicate a lipid lowering function of tamoxifen, and the function might be executed in an ER/sex independent manner (32, 33). Consistently, tamoxifen inhibits atherosclerosis in male mice and enhances the endothelium-dependent flow-mediated dilation in male patients with advanced atherosclerosis (5, 9). In our study, we determined that tamoxifen inhibited CD36 expression in male mice in vitro (Fig. 6), and administration of tamoxifen to male mice reduced CD36 expression in both peritoneal macrophages and adipose tissues (Fig. 7). These results suggest that inhibition of macrophage CD36 expression by tamoxifen might be completed by mechanisms that are unrelated to ER activity. To further confirm it, we completed the following experiments. We treated J774 and RAW macrophages, two murine macrophage cell lines originally derived from a female and a male mouse, with tamoxifen. We previously determined expression of ER in J774 cells, but not RAW cells, by RT-PCR (data not shown). Although the basal levels of CD36 protein in RAW cells is much higher than J774 cells, tamoxifen inhibited CD36 expression in both cell lines (Fig. 8A, top panel). Similarly, CD36 expression in macrophages isolated from male ApoE−/− mice is higher than cells isolated from female ApoE−/− mice, which suggests that CD36 expression might be in a sex-dependent manner. However, tamoxifen demonstrates similar inhibitory effects on CD36 expression in both cell types (Fig. 8A, lower panel). In addition, we treated cells with ER antagonist (ICI) or 17β-estradiol (E2), and determined that ICI induced CD36 expression in both RAW and J774 cells. E2 had little or moderately inhibitory
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Tamoxifen clearly reduced the binding of PPARγ to the PPRE in CD36 promoter (Fig. 3E), which might be due to that inhibition of PPARγ expression by tamoxifen can lead to reduced nuclear PPARγ protein levels (Fig. 4, C and D). In addition, we determined that tamoxifen enhanced PPARγ phosphorylation by activating ERK1/2, which is consistent with previous reports indicating that tamoxifen induces transforming growth factor β (TGFβ) production, a cytokine inhibiting CD36 expression though phosphorylation of PPARγ (5, 6, 30).

Despite activation of macrophage CD36 expression, PPARγ agonists have been demonstrated to inhibit foam cell formation and atherosclerosis, which is due to that activation of PPARγ enhances macrophage cholesterol efflux and inhibits production of inflammatory cytokines (24, 25). Although tamoxifen inactivated PPARγ by reducing PPARγ expression and activating PPARγ phosphorylation simultaneously, we determined that tamoxifen activated expression of ATP-binding cassette transporter A1 (ABCA1) and ABCG1 (Fig. 8C), two important molecules facilitating cellular free cholesterol efflux to extracellular cholesterol acceptors, apolipoprotein AI (apoAI) and high density lipoprotein (HDL), respectively. The activation of ABCA1 and ABCG1 expression is another action of tamoxifen to inhibit foam cell formation, which is obviously completed by a pathway independent of PPARγ, and needs further investigation. In addition, expression of LDL receptor was also activated by tamoxifen (30). In the current study, we determined that inhibition of CD36 expression by tamoxifen is mainly completed by inactivating PPARγ. Tamoxifen effectively blocked PPARγ ligand-induced CD36 mRNA and protein expression and CD36 promoter activity (Figs. 3, B and D, and 4, A and B). Tamoxifen clearly reduced the binding of PPARγ protein with the PPRE in CD36 promoter (Fig. 3E), which might be due to that inhibition of PPARγ expression by tamoxifen can lead to reduced nuclear PPARγ protein levels (Fig. 4, C and D). In addition, we determined that tamoxifen enhanced PPARγ phosphorylation by activating ERK1/2, which is consistent with previous reports indicating that tamoxifen induces transforming growth factor β (TGFβ) production, a cytokine inhibiting CD36 expression though phosphorylation of PPARγ (5, 6, 30).

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by tamoxifen (Fig. 8C). Thus, the inhibition of foam cell formation by tamoxifen is completed by its multiple biological functions including inhibition of CD36 expression and cellular oxLDL accumulation.

**Experimental Procedures**

**Materials**—Tamoxifen, 4-hydroxytamoxifen, phorbol 12-myristate 13-acetate (PMA), rosiglitazone, troglitazone, 17β-estradiol (E2), and fulvestrant (ICI182780, ICI) and other chemicals were purchased from Sigma. Rabbit anti-CD36 and PPARγ polyclonal antibodies were purchased from Proteintech Group (Chicago, IL). Rabbit anti-PPARγ polyclonal antibody was purchased from Abcam (Cambridge, MA). Rabbit anti-ERK1/2 and pi-ERK1/2 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The reverse transcription kit was purchased from Promega Corporation (Madison, WI). LDL was purchased from Athens Research & Technology, Inc. (Athens, GA) and used to prepare oxLDL as described (20).

**Cell Culture**—THP-1 cells (a human monocytic cell line), 293T cells (a cell line derived from human embryonic kidney), and J774 or RAW cells (murine macrophage cell lines, originally derived from a female or male mouse) were purchased from American Type Culture Collection (Manassas, VA). 293T cells were cultured in complete DMEM, whereas the remaining cells were cultured in complete RPMI 1640 medium containing 10% FBS, 50 μg/ml of penicillin/streptomycin, respectively.

To induce monocyte/macrophage differentiation, THP-1 cells at a density of 2.5 × 10⁵ cells/cm² were added with 100 nM PMA and cultured overnight in complete RPMI 1640 medium. PMA was then removed by aspiration and the cells were washed twice with PBS. The derived THP-1/PMA macrophages were cultured in complete RPMI 1640 medium for another 2 days.

Human BMDMs were obtained as follows: monocytes were isolated from the blood of healthy donors, which was obtained from Tianjin Blood Bank (Tianjin, China), with a standard protocol. The isolated monocytes were then cultured in RPMI 1640 medium containing 20% FBS for 1 week to differentiate into macrophages (BMDMs). All treatments were completed in serum-free medium.

**In Vivo Studies**—The protocols for animal studies were granted by the Ethics Committee of Nankai University and conform to the Guide for the Care and Use of Laboratory Animals published by NIH.

To determine the effect of tamoxifen on atherosclerosis, and expression of CD36, FABP4, and PPARγ in lesion areas, Apoe⁻/⁻ mice (~8 weeks old, male) were randomly divided into two groups (15 mice/group), and fed either HFD or HFD containing tamoxifen (2 mg/100 g of food) for 16 weeks. At the end of the experiment, blood and artery samples were collected followed by determination of serum lipid profiles, en face lesions by Oil Red O staining, and expression of CD36, FABP4, and PPARγ, respectively, by immunofluorescent staining as described (43).

MacPparγ KO mice were generated as described (44), and the Pparγfl/fl mice were used as the corresponding control mice. PPARγ expression was determined by both reverse transcription PCR (RT-PCR) and real time RT-PCR with total RNA extracted from macrophages isolated from MacPparγ KO mice and Pparγfl/fl mice, respectively, and the following primers: forward, 5′-GAGCTGACCCAAATGGTTGCTGATT-3′; and backward, 5′-TGGCCATAGGGAGTTAGAAGGGTT-3′.

The effect of tamoxifen on macrophage CD36 expression in vivo was initially determined with Apoe⁻/⁻ mice (males, ~8 weeks old). The mice were divided into two groups (5 mice/group) and fed HFD or HFD containing tamoxifen (2 mg/100 g of food), respectively, for 2 weeks. The mice were i.p. injected with 4% thioglycollate solution (3 ml/mouse) 5 days before the end of the experiment. After euthanasia, peritoneal macrophages were individually collected for determination of CD36 and PPARγ expression by Western blot analysis (45).

The role of PPARγ in tamoxifen-inhibited macrophage CD36 expression and cellular oxLDL accumulation was determined in MacPparγ KO and Pparγfl/fl mice. In vitro, peritoneal macrophages were collected from MacPparγ KO and Pparγfl/fl mice separately. Cells were then treated with tamoxifen or 4-hydroxytamoxifen followed by determination of CD36 expression by Western blot analysis and cellular oxLDL accumulation by Oil Red O staining and quantitation of cellular cholesteryl ester levels, respectively. In vivo, both MacPparγ KO and Pparγfl/fl mice (both are males) were also divided into two groups (3 mice/group) and fed HFD or HFD containing tamoxifen (2 mg/100 g of food) for 2 weeks. At the end of the experiment, both peritoneal macrophages and adipose tissues were individually collected from each mouse and used to determine CD36 and PPARγ expression, respectively, by Western blot analysis.

**Determination of CD36, ERK1/2, pi-ERK1/2, PPARγ, and pi-PPARγ Protein Expression by Western Blot Analysis**—After treatment, cells were lysed with an ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 50 μg/ml of aprotinin/leupeptin). The cellular lysate was centrifuged with a Microfuge for 15 min at 4 °C, and the supernatant was collected as whole cellular extract. Expression of CD36, ERK1/2, pi-ERK1/2, PPARγ, and pi-PPARγ protein in extract was determined by Western blot analysis, respectively (45).

**Determination of Cell Surface CD36 Protein Levels by FACS Assay**—After treatment, THP-1 monocytes, THP-1/PMA macrophages, and human BMDMs were collected and washed twice with PBS, respectively. Cells (~1 × 10⁶) were initially blocked with 300 μl of PBS containing 1% BSA, 0.1% NaN₃, and 5% rabbit serum for 30 min at room temperature, and then added with 10 μl of anti-human CD36 polyclonal antibody that was pre-conjugated to fluorescein isothiocyanate isomer 1 (FITC) (Chemicon International Inc., Temecula, CA). Cells in the control group were added with the same concentration of FITC-conjugated normal rabbit IgG. After a 1-h incubation at room temperature, cells were washed 3 times with PBS and then conducted fluorescence-activated cell sorting (FACS) assay.

**Isolation of Total RNA and Determination of CD36 mRNA Expression by Real Time RT-PCR**—After treatment, total RNA was isolated from THP-1/PMA macrophages as described (45). The same amount of total RNA (2 μg) from each sample was
used to synthesize cDNA with a reverse transcription kit followed by real time PCR using a SYBR Green PCR master mix (Bio-Rad) with the forward primer: 5’-TCCTGCGGAGATGTTAATTGCA-3’; and the reverse primer: 5’-ACGGTGGATTCATACGAGTATG-3’. Expression of CD36 mRNA was normalized by GAPDH mRNA in the corresponding samples. The GAPDH primers were: forward, 5’-GGTGTTCTCTTGACTCACA-3’ and reverse, 5’-GTGTCGTGATGGCCAAATTCTGTTG-3’.

**Determination of Cellulosomal oxLDL Accumulation by Oil Red O Staining**—After treatment, THP-1/PMA macrophages or peritoneal macrophages isolated from MacPparry KO and Ppary-KO mice in 24-well plates were incubated with rabbit anti-CD36 polyclonal antibody or normal rabbit IgG (0.3 µg/sample) for 1 h. After normal IgG or anti-CD36 antibody removal, cells were added with 50 µg/ml of oxLDL and incubated for another 3 h. Cells were then fixed in 4% paraformaldehyde for 30 min, washed twice with PBS, and stained with Oil Red O solution (0.3% Oil Red O in 60% isopropyl alcohol) to determine cellular oxLDL accumulation as described (31). To determine cellular cholesteryl ester levels, after incubation with oxLDL as above described, cells in 10-cm plates were used to extract total cellular lipid followed by determination of cholesteryl esters with an assay kit (Wako Chemicals) as described (46).

**Preparation of Plasmid DNA and Determination of CD36 Promoter Activity**—The Ppar γ expression vector (pEGFP-C2-PPAR γ) was generated as described (31). The human CD36 promoter (from −1186 to +10) was constructed by PCR with human genomic DNA isolated from THP-1 monocytes and the following primers: forward, 5’-CCGCCTGAGCCCTTCCAGTGACAGC-3’; reverse, 5’-CCCCAGTTGTCACTCCGTCACT-3’ (the underlined letters represent the conserved sites for XhoI and HindIII, respectively). After the sequence was confirmed, the PCR product was digested with XhoI and HindIII followed by ligation with pGL4 luciferase reporter vector and transformed into Escherichia coli to amplify.

To analyze CD36 promoter activity, 293T cells at about 95% confluence in 48-well plates were transfected with DNA for CD36 promoter (pCD36), pEGFP-C2-PPAR γ, and Renilla (for internal normalization) using Lipofectamine™ 2000 (Invitrogen). After 24 h transfection plus treatment, cellular lysate was extracted and used to determine activity of firefly and Renilla luciferase using the Dual Luciferase Reporter Assay System (Promega, Madison, WI).

**Determination of PPARγ Nuclear Translocation by Immunofluorescent Staining**—HeLa cells were plated on coated 20-mm glass bottom dishes and cultured in DMEM containing 10% FBS. At ~90% confluence, cells were transfected with the PPARγ expression vector for 4 h followed by treatment for 24 h. After removal of medium, cells were fixed in 4% paraformaldehyde for 20 min, washed twice with PBS, and stained with DAPI solution (100 ng/ml in PBS) for 5 min. After washing with PBS, cells were observed and photographed to assess localization of the transfected PPARγ with a fluorescence microscopy.

**Extraction of Nuclear Protein and Electrophoretic Mobility Shift Assay (EMSA) of PPARγ-DNA Binding Activity**—After treatment and washing with cold PBS, cells were used to extract nuclear protein followed by determination of the binding activity of PPARγ to the PPRE in CD36 promoter by EMSA as described (45). To confirm the specific interaction between the radiolabeled PPRE probe with PPARγ protein in nuclear extract, we added either anti-PPARγ antibody or excess (×50) unlabeled PPRE probe to the reaction system with nuclear extract of control sample. The sequence of PPRE probe was 5’-TGGCCTcTGACTTACTcctg. The underlined nucleotides in capital letters on each side of the small letter c in the middle is the putative PPRE in human CD36 promoter.

**Data Analysis**—Data were generated from at least three independent experiments. Values are represented as mean ± S.E. All data were initially subjected to a normal distribution analysis with SPSS software (1-sample K-S of non-parametric test), and then analyzed by a parametric statistics, post hoc test of one-way analysis of variance if the data were in normal distribution. A difference was considered to be statistically significant at p < 0.05.

**Author Contributions**—J. H. and Y. D. designed the study; M. Y., M. J., Y. C., S. Z., W. Z., X. Y., X. L., and S. D. performed the experiments; J. H. and Y. D. prepared the manuscript.

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