9HODE Stimulates Cell Proliferation and Extracellular Matrix Synthesis in Human Mesangial Cells via PPARγ

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Plasma oxidized low-density lipoprotein (OX-LDL) levels are elevated in patients with renal diseases, including diabetic nephropathy. We examined effects of OX-LDL on cell proliferation and extracellular matrix (ECM) production by using normal human mesangial cells. Furthermore, we examined possible involvement of peroxisome proliferator-activated receptor gamma (PPARγ). Mesangial cell proliferation with OX-LDL, 9-hydroxy-10,12-octadecadienoic acid (9HODE), and 13-hydroxy-9,11-octadecadienoic acid (13HODE), the major components of OX-LDL, were determined by 5-bromo-2′-deoxyuridine (BrdU) or 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) incorporation. The effect of OX-LDL on mesangial cell proliferation with PD98059 pretreatment was determined by BrdU incorporation. Type IV collagen, fibronectin, and PPARγ expression with OX-LDL or 9HODE or 13HODE was determined by Western blotting. Type IV collagen expression with antisense oligonucleotide against PPARγ pretreatment was also determined by Western blotting. The effect of PD98059 pretreatment on PPARγ expression was determined by Western blotting. In mesangial cells exposed to isolated OX-LDL from human plasma, BrdU incorporation was increased, and this increase was deleted by PD98059. Type IV collagen expression was significantly increased by OX-LDL. 9HODE and 13HODE increased BrdU and MTT incorporation into mesangial cells and also increased expressions of Type IV collagen and fibronection, the major components of ECM. PPARγ expression in mesangial cells was stimulated by 9HODE. The reduction of PPARγ synthesis by pretreatment of antisense oligonucleotide against PPARγ remarkably attenuated Type IV collagen synthesis induced by 9HODE. PPARγ expression induced by 9HODE was also reduced by PD98059 pretreatment. These findings demonstrate that 9HODE, the major component of OX-LDL, stimulates cell proliferation and ECM production of human mesangial cells. In addition, the stimulatory effects are, at least in part, mediated by PPARγ, which may exist in downstream of ERK1/2 pathway. Exp Biol Med 229:1053–1060, 2004.

Key words: 9HODE; oxidized LDL; mesangial cell; PPARγ

Introduction

Glomerulosclerosis is a major morphological change in patients with renal diseases, including diabetic nephropathy. Mesangial cell proliferation and extracellular matrix (ECM) expansion are common features of glomerulosclerosis (1, 2). Increased mesangial cells in the early phases of glomerular disease may influence the synthesis and deposition of ECM proteins, leading eventually to sclerosis and renal failure. The association between proliferation and subsequent ECM deposition has been observed in several experimental glomerulonephritis models (2, 3). Hyperlipidemia is a common feature in patients with chronic renal diseases, including nephrotic syndrome and diabetes (4, 5). It has been reported that low-density lipoprotein (LDL) induces mesangial cell proliferation and causes expansion of the ECM in glomerulosclerosis (6), indicating the possible involvement of lipid metabolism derangement in the development of renal diseases.

Clinical data have shown that serum oxidized LDL (OX-LDL) levels were higher in patients with diabetes than in patients without diabetes (7). Levels of OX-LDL were shown to be higher in patients with diabetic nephropathy than in patients without diabetic nephropathy (7). Increased plasma OX-LDL levels have been shown to correlate with

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the incidence of coronary disease and nephropathy (8, 9). In addition to LDL, OX-LDL has been shown to stimulate a variety of cellular responses: (i) the generation of eicosanoids in mesangial cells (10, 11), (ii) the expansion of adhesion molecules in mouse glomerular endothelial cells (12), (iii) the adhesion of monocytes to human mesangial cells (13), and (iv) the expression of cytokines contributing to the infiltration and differentiation of monocytes in mouse mesangial cells (14). Levels of OX-LDL promote the infiltration of macrophages, modify the growth and the viability of glomerular cells, and modulate secretion of some vasoactive mediators (11). In the early stages of glomerular injury, LDL is deposited in the glomerulus, where it becomes locally oxidized by the action of mesangial cells (11). In addition to the established risk factors for the development of nephropathy in patients with diabetes, notably hyperglycemia and hyperlipidemia, modified LDLs such as oxidized LDL and glycated LDL can cause severe nephropathy and atherogenic lesions.

Both 9-hydroxy-10,12-octadecadienoic acid (9HODE) and 13-hydroxy-9,11-octadecadienoic acid (13HODE) are major components of OX-LDL. These are oxidized metabolites of linoleic acid and are formed during oxidation of LDL (15). Nagy *et al.* (15) have identified these two components of OX-LDL as endogenous activators and ligands of peroxisome proliferator-activated receptor gamma (PPARγ) in macrophages.

Peroxisome proliferator-activated receptor gamma is a member of a nuclear hormone receptor superfamily originally shown to play a critical role in adipocyte differentiation and glucose homeostasis. It is widely distributed in human and animal tissues, including renal mesangial cells (16) and proximal tubular cells (17), and has been implicated as a regulator of cellular proliferation and inflammatory responses in the kidney (18). These findings suggest that PPARγ plays diverse roles in cell growth, differentiation, and ECM accumulation in renal mesangial cells. *In vitro* studies have shown that thiazolidinediones (TZDs), PPARγ ligands, inhibit mesangial cell proliferation (16). However, a previous report suggested that troglitazone significantly enhanced glomerular expression of monocyte chemoattractant protein, a critical cytokine for the development of rat mesangioproliferative glomerulonephritis (19). Therefore, the effects of PPARγ ligands on mesangial cells remain controversial.

In the present study, we examined the effects of 9HODE, the major component of OX-LDL, on cell proliferation and ECM production by using normal human mesangial cells. In addition, we investigated the possible involvement of PPARγ on mesangial cell proliferation and ECM production in mesangial cells.

Methods

Materials. We purchased normal human mesangial cells from Clonetics/Bio Whittaker Inc. (Walkersville, MD), 9HODE and 13HODE from Cayman Chemical Co. (Ann Arbor, MI), PD98059 from Sigma-Aldrich Inc. (St. Louis, MO), and primary antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All other reagents were of chemical grade and were purchased from standard suppliers.

Mesangial Cell Culture. Normal human mesangial cells were cultured in RPMI-1640 medium (GIBCO/Invitrogen Corp, Carlsbad, CA) in the presence of 20% fetal bovine serum (FBS) (GIBCO), 1 %(v/v) insulin transferrin seleninium (ITS) (100 ng/ml insulin, 55 ng/ml transferrin, 50 pg/ml selenous acid; GIBCO) and 1 %(v/v) penicillin-streptomycin (GIBCO). The cells were incubated with this culture medium in a 5% CO_2–95% O_2 incubator at 37°C, and the medium was changed every 2 days. Cells between passages 4 and 9 were used for experiments.

LDL Preparation and Oxidization. Low-density lipoprotein was prepared from normal healthy human plasma by ultracentrifugation and dialyzed at 4°C for 24 hrs against 0.02 *M* phosphate-buffered saline (PBS) as described previously (4). It was filtered through a 0.22-μm filter and stored at 4°C. Low-density lipoprotein (2 mg/ml) was incubated with 10 μ*M* $CuSO_4$ at 37°C for 6 hrs without EDTA. The reaction was stopped by the addition of EDTA (5 m*M*). The OX-LDL was dialyzed against PBS at 4°C for 24 hrs and then filtered through a 0.22-μm filter and stored at 4°C. The degree of LDL modification was determined by measuring the quantity of thiobarbituric acid reactive substances (TBARS), and the OX-LDL possessed TBARS values of an average of 10.6 nmol/mg protein. The OX-LDL was used within 10 days.

5-Bromo-2′-Deoxyuridine (BrdU) Incorporation. Uptake of BrdU into human mesangial cells and its incorporation into DNA were determined by the Cell Proliferation enzyme-linked immunosorbent assay system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Cells were seeded onto 96-well plates and grown under normal conditions. On the third day, the cells were washed with PBS and were serum starved for 24 hrs. After starvation, the cells were preincubated with LDL or OX-LDL (5–100 μg/ml), 9HODE or 13HODE (0.001–1 μg/ml) dissolved in 0.1 % ethanol for 24 hrs. In controls, incubation medium containing 0.1% ethanol was added. The cells were then incubated at 37°C for 2 hrs after the addition of 10 μ*M* BrdU in FBS-free culture medium. Next, the medium was removed, and the cells were incubated for 30 mins at room temperature in fixative solution and then in the blocking reagent included in the assay kit. Peroxidase-labeled anti-BrdU monoclonal antibody solution was added and incubated for 90 mins at room temperature. After the cells were washed three times with PBS for 5 mins, substrate solution was added and the cells were incubated for 30 mins at room temperature. The reaction was stopped by the addition of 1 *M* sulphuric acid, and the optical density of each sample at 450 nm was measured by using a Kinetic Microplate-Reader (Molecular Devices Co., Sunnyvale, CA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) Incorporation. The number of living cells in the human mesangial cell cultures was assayed by an MTT method. The incubation medium was completely aspirated after incubation with 9HODE or 13HODE (0.001–1 μg/ml) or vehicle for 24 hrs. The MTT-formazan product was dissolved in a phosphate-buffer solution. After the addition of RPMI 1640 medium containing 10% MTT, the cells were incubated at 37°C for 4 hrs, the medium was aspirated, and the cells were lysed by the addition of 100 μl dimethylsulfoxide (DMSO). Then, 10 μl was collected from each sample and diluted in 90 μl of fresh DMSO. After the sample was mixed on a mechanical plate mixer, the optical density of each sample at the test and reference wavelengths of 550 and 650 nm was measured with a Kinetic Microplate-Reader (Molecular Devices).

Cell Counting. Cells were seeded onto culture dishes and grown under normal conditions. On the third day, the cells were washed with PBS and were serum starved for 24 hrs. After starvation, the cells were preincubated with 9HODE or 13HODE (0.1–1 μg/ml) for 24 hrs. Controls were vehicle treated. After the medium was removed from the culture dishes, 2 ml trypsin-EDTA (0.25% trypsin, 1m*M* EDTA4Na) was added to each dish for 2 mins. After all cells were removed, the samples were mildly agitated to obtain a homogenous suspension. Trypan blue solution (0.4%) was added to aliquots of cell suspensions in a 1:1 ratio. A hemocytometer was used to count live and dead cells.

Western Blot Analysis. Mesangial cells were lysed in lysis buffer containing leupeptin and phenylmethylsulfonyl fluoride. Protein concentrations were determined with the Lowry protein assay. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was incubated in 10 ml of blocking buffer (Tris-buffered saline [TBS], containing 0.1% Tween 20 and 5% nonfat dry milk) for 1 hr at room temperature followed by the addition of anti–Type IV collagen, anti-fibroncectin, or anti-PPARγ antibody (Santa Cruz Biotechnology) overnight at 4°C. Then, the membrane was given three 5-min washes with TBS/Tween 20 and incubated with anti-goat or anti-rabbit antibody (1:5000) for 2 hrs at room temperature. The membrane was again washed with TBS/Tween 20, and protein was detected by the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham).

Effect of PD98059. Before the experiment, mesangial cells were incubated in serum-free medium and pretreated with PD98059 (50 μ*M*, a extracellular-signal regulated protein kinase 1/2 inhibitor) for 2 hrs before treatment with OX-LDL, 9HODE, or vehicle. After 2 hrs preincubation with PD98059, cells were incubated with 100 μg/ml of OX-LDL or 0.1 μg/ml of 9HODE for 24 hrs in the presence of 50 μ*M* of PD98059. Then, the cells were used for cell proliferation assays and Western blot analysis.

Antisense Transfection. Antisense and sense oligonucleotides were designed by using the PPARγ cDNA nucleotide sequence. The oligonucleotides were synthesized by Invitrogen as follows: PPARγ antisense, 5′-CTCTGTGTCAACCATGGTCAT-3′; PPARγ sense, 5′-ATGACCATGGTTGACACAGAG-3′ (20). The transfection reagent lipofectin (Invitrogen) was used to introduce the oligonucleotide into the mesangial cells. For these studies, 2 $\times 10^4$ cells/well were seeded in 6-well tissue culture plates, and the cells were incubated under normal conditions until 60%–70% confluent. Cells were then transfected with 0.3 μ*M* oligonucleotides and 10 μg of lipofectin according to the manufacturer's instructions (21). Twenty-four hours later, PPARγ protein expression was assessed by Western blotting to confirm the effectiveness of the antisense treatment. After 24-hr treatment with sense or antisense PPARγ oligonucleotides, cells were treated with 0.1 μg/ml of 9HODE or vehicle for 24 hrs. Type IV collagen expression was assessed by Western blotting as described above.

Data Analysis. All data represent mean ± SEM. The statistical analysis of the means was performed by analysis of variance followed by Duncan's multiple range tests for individual comparisons of the means.

Results

The effects of 5–100 μg/ml LDL or OX-LDL on BrdU incorporation into human mesangial cells after 24 hrs of incubation are shown in Figure 1a. Incorporation of BrdU after LDL treatment was significantly increased at 10 ($P < 0.05$), 50, and 100 μg/ml ($P < 0.01$) compared with control. Incorporation of BrdU after OX-LDL treatment was significantly increased at 5, 10, 50, and 100 μg/ml ($P < 0.01$) compared with control. Incorporation of BrdU was stimulated by OX-LDL more effectively than by native LDL at 5, 10 ($P < 0.05$), 50, and 100 μg/ml ($P < 0.01$). To investigate the involvement of mitogen-activated protein kinase (MAPK) in cell proliferation induced by LDL and OX-LDL, we examined the effect of PD98059 on BrdU incorporation (Fig. 1b). Mesangial cells were incubated with 50 μ*M* of PD98059 for 2 hrs before the addition of 100 μg/ml of LDL or OX-LDL. Pretreatment with PD98059 blocked the increase in BrdU incorporation by mesangial cells treated with LDL and OX-LDL for 24 hrs.

The effect of LDL or OX-LDL on the synthesis of ECM protein was assessed by Western blotting and is shown in Figure 1c. Exposure of cultured human mesangial cells to 100 μg/ml of LDL and OX-LDL for 24 hrs significantly ($P < 0.01$) stimulated Type IV collagen production. Type IV collagen synthesis was stimulated more effectively by OX-LDL than by LDL ($P < 0.01$).

Major components of OX-LDL include 9HODE and 13HODE. We examined the effects of these HODEs on cell proliferation and ECM production in mesangial cells for further experiments. Figure 2a and b shows the effects of

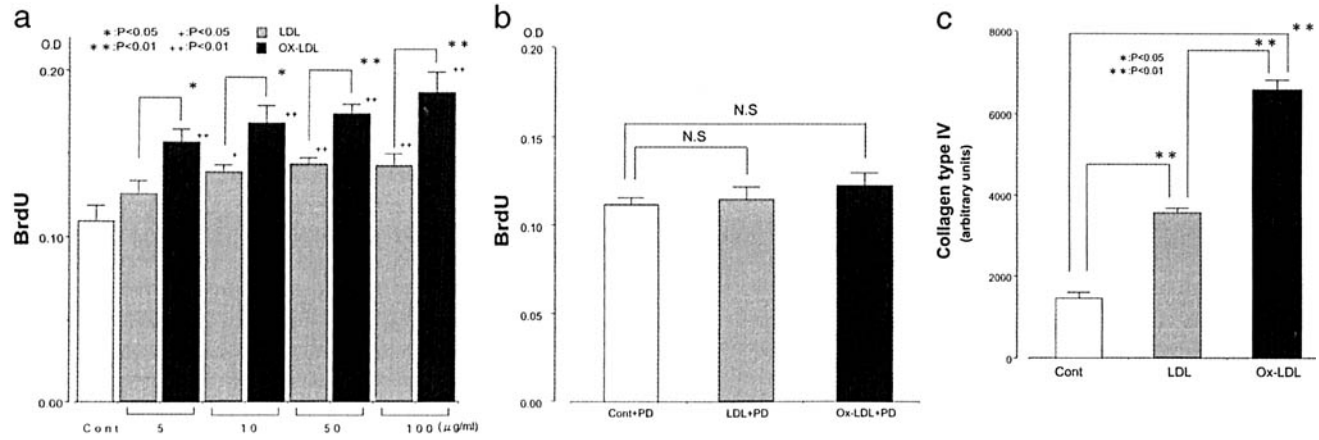

Figure 1. Cell proliferation was inhibited by PD98059. (a) Mesangial cells were cultured in serum-free RPMI medium for 24 hrs and incubated for 24 hrs with 0–100 μg/ml low-density lipoprotein (LDL) or oxidized LDL (OX-LDL). 5-Bromo-2′-deoxyuridine (BrdU) incorporation was assessed. (b) Mesangial cells were preincubated with PD98059 (50 μ*M*, 2 hrs) and incubated for 24 hrs with 100 μg/ml LDL or OX-LDL. Incorporation of BrdU was assessed. (c) Mesangial cells were incubated for 24 hrs with 100 μg/ml of LDL or OX-LDL. Cell proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted with a polyclonal antibody against Type IV collagen (1:200). Bars represent mean ± SE of at least four separate experiments. $+P < 0.05$; $++P < 0.01$ versus control. $*P < 0.05$; $**P < 0.01$ versus LDL.

0.001–1 μg/ml of 9HODE and 13HODE on BrdU incorporation. After a 24-hr incubation with 9HODE and 13HODE, BrdU incorporation was significantly increased at concentrations of 0.01 ($P < 0.05$), 0.1, and 1 μg/ml ($P < 0.01$) in human mesangial cells. To assess the number of living cells, the effects of 9HODE and 13HODE were examined by measuring MTT incorporation. The effects of 0.001–1 μg/ml 9HODE and 13HODE on MTT incorpo-

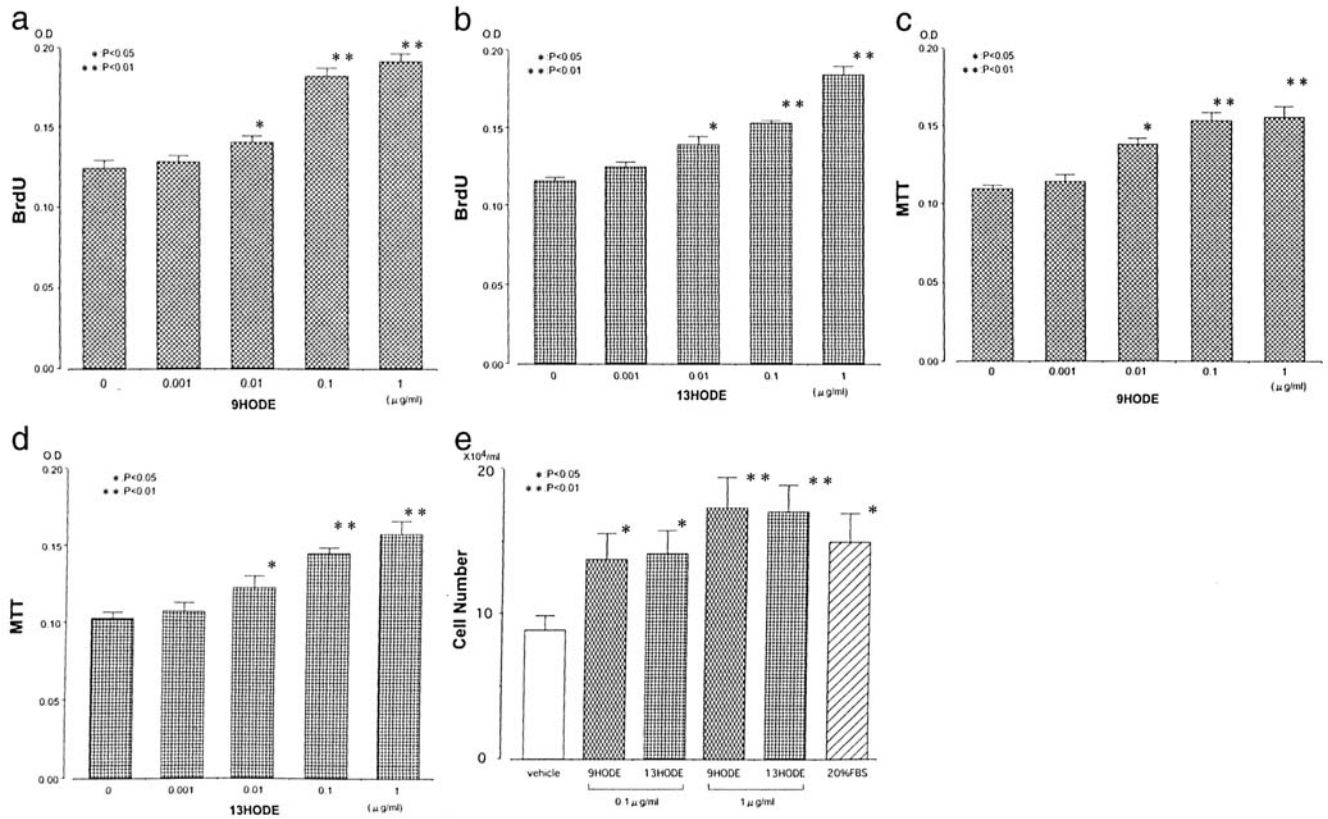

Figure 2. Mesangial cells were cultured in serum-free RPMI medium for 24 hrs and incubated for 24 hrs with 0–1 μg/ml 9-hydroxy-10,12-octadecadienoic acid (9HODE) and 13-hydroxy-9,11-octadecadienoic acid (13HODE). 5-Bromo-2′-deoxyuridine incorporation (a, b) and MTT incorporation (c, d) were assessed. Mesangial cells were cultured in serum-free RPMI medium for 24 hrs and incubated for 24 hrs with 0.1–1 μg/ml 9HODE or 13HODE. Living cell number (e) was counted. Bars represent mean ± SE. $*P < 0.05$; $**P < 0.01$ versus vehicle-treated control.

ration are shown in Figure 2c and d. Incorporation of MTT was significantly increased by 9HODE and 13HODE at 0.01 ($P < 0.05$), 0.1, and 1 μg/ml ($P < 0.01$). These data show that major components of OX-LDL increased mesangial cell proliferation in a concentration-dependent manner. Figure 2e shows the result of cell number by direct counting. Levels of 0.1–1μg/ml of 9HODE and 13HODE significantly increased mesangial cell number compared with vehicle-treated control.

Figure 3a and b shows the effect of 24 hrs of exposure to 9HODE and 13HODE on the expression of Type IV collagen, as assessed by Western blotting. Expression of Type IV collagen was significantly increased by 9HODE and 13HODE at 0.01 ($P < 0.05$), 0.1, and 1 μg/ml ($P < 0.01$). The expression of fibronectin, another component of the ECM, was also assessed by Western blotting. After a 24-hr incubation with 0.001–1 μg/ml 9HODE and 13HODE, fibronectin production by mesangial cells was significantly increased. The density of bands was increased at 0.01 ($P < 0.05$), 0.1, and 1 μg/ml ($P < 0.01$), as shown in Figure 3c and d. The expression of β-actin had no significant differences (data not shown).

To clarify the mechanism by which HODEs stimulate ECM production, we used 9HODE and investigated the expression of PPARγ in cultured human mesangial cells by Western blotting. The mesangial cells were incubated with 0.001–1 μg/ml 9HODE for 24 hrs, and the expression of PPARγ was evaluated by measuring the density of bands. As shown in Figure 4a, the expression of PPARγ was significantly increased by 9HODE at 0.01 ($P < 0.05$), 0.1, and 1 μg/ml ($P < 0.01$). In our preliminary study, 0.33 μ*M* (0.1 μg/ml) of 9HODE showed similar efficacy with 10 μ*M* of troglitazone.

Figure 4b shows the effect of an antisense oligonucleotide against PPARγ. Human mesangial cells pretreated for 24 hrs with a PPARγ antisense oligonucleotide had reduced Type IV collagen expression compared with cells pretreated with a PPARγ sense oligonucleotide, indicating that endogenous PPARγ could be involved in Type IV collagen expression. After 24 hrs of incubation with 0.1 μg/ml 9HODE, the cells with PPARγ sense pretreatment exhibited a significant increase in Type IV collagen expression. In contrast, cells pretreated with PPARγ antisense oligonucleotides did not show any significant increase in Type IV collagen expression.

Figure 4c shows the effect of PD98059 pretreatment on PPARγ expression induced by 9HODE. Expression of PPARγ was enhanced by 0.1 μg/ml of 9HODE in the absence of PD98059. In contrast, the increase in PPARγ expression induced by 9HODE was prevented by the presence of PD98059.

Discussion

In the present study, we first demonstrated that OX-LDL had potent effects on cell proliferation and Type IV collagen production in normal human mesangial cells. Type IV collagen is a major component of the ECM, along with fibronectin and laminin (22–25). Our data demonstrated that OX-LDL was a more potent stimulator of mesangial cell proliferation and ECM protein production than was native LDL. These findings indicate that OX-LDL could be more responsible than native LDL for the induction of glomerulosclerosis in diabetic nephropathy accompanied by lipid disorders.

Other investigators have reported an inhibition of proliferation and induction apoptosis in numerous cell types treated with highly or maximally oxidized LDL and a relatively higher dose of oxidized lipids (26–28). However, other reports indicate that minimally or moderately oxidized LDL has different and potentially very important biological roles in the vascular wall. Minimally oxidized LDL produces endothelial dysfunction through adherence and penetration of monocytes (29–31).

As stated earlier, 9HODE and 13HODE are major components of OX-LDL. In the present study, 9HODE and 13HODE concentration dependently increased mesangial cell proliferation, as evaluated by cell number and DNA synthesis. In addition, 9HODE and 13HODE clearly increased the production of both Type IV collagen and fibronectin, which are major components of the ECM. We confirmed that 9HODE and13HODE had similar effects on mesangial cell proliferation and ECM production. Both 9HODE and 13HODE have been shown to increase vascular epidermal growth-factor production by human monocyte/macrophage cell lines, human acute monocytic leukemia (THP-1) cells, and human coronary artery endothelial cells (32). However, other components of OX-LDL did not have such stimulatory effects in macrophages (15). The present data are compatible with previous observations in nonmesangial cells (15, 32) and indicate that these major components of OX-LDL are responsible for mesangial cell proliferation and ECM production.

The activation of MAPK-signaling pathways has been associated with the stimulatory effects of LDL, relevant to vascular pathology, in cultured cells implicated in macrovascular disease (33–35) and more recently in renal disease (36–38). Whether or not MAPK signals stimulate or inhibit cell proliferation (38) remains controversial. Reports have shown that MAPK signals are associated with the induction of cell apoptosis (39), and another report suggested that MAPK signals stimulated ECM production (37). At least three parallel series of MAPK cascades exist, referred to as P38 MAPK, JNK, and ERK1/2 (40, 41). Native LDL and OX-LDL have been demonstrated to activate ERK 1 and 2, which are early mitogenic signals in mesangial cells (40). In the present study, we showed that the effect of mesangial cell proliferation induced by OX-LDL was blocked by PD98059, a MEK inhibitor. Therefore, OX-LDL may cause mesangial cell proliferation *via* an ERK1/2-dependent pathway. Furthermore, PD98059 pretreatment reduced the expression of PPARγ induced by 9HODE in the present

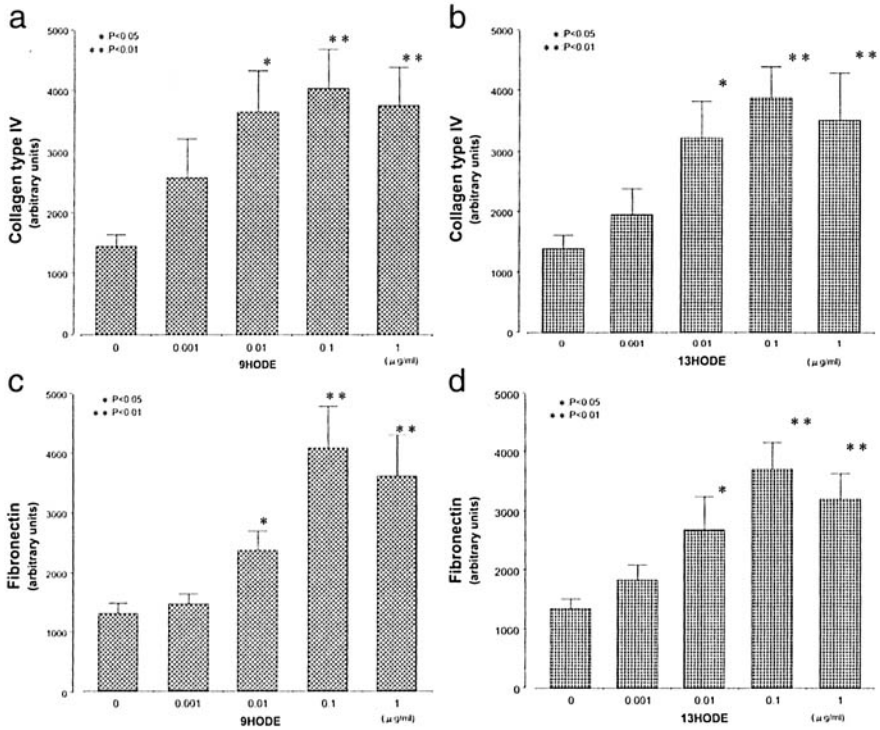

Figure 3. Mesangial cells were cultured in serum-free RPMI medium for 24 hrs and incubated for 24 hrs with 0–1 μg/ml 9-hydroxy-10,12-octadecadienoic acid and 13-hydroxy-9,11-octadecadienoic acid. Cell proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted with a polyclonal antibody against Type IV collagen (1:200) (a, b) or fibronectin (c, d). Bars represent mean ± SE of at least four separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ versus vehicle-treated control.

study. This observation indicates that the MAPK pathway operates upstream of PPARγ and is involved in mesangial cell proliferation and ECM production in mesangial cells induced by 9HODE.

A member of the nuclear hormone receptor superfamily, PPARγ has been implicated as a regulator of cellular proliferation and inflammatory responses in the kidney (42). It is expressed by both monocytes/macrophages and foam cells in atherosclerotic plaques (43). Reports have shown that PPARγ is activated by OX-LDL in monocytes/macrophages (43). Both 9HODE and 13HODE, major components of OX-LDL, were recently recognized as ligands for PPARγ in macrophages (15). We showed that 9HODE increased the expression of PPARγ in mesangial cells. Moreover, pretreatment with antisense PPARγ not only reduced PPARγ expression but also remarkably decreased both basal and 9HODE-induced Type IV collagen expression. The present data indicate that PPARγ could play an

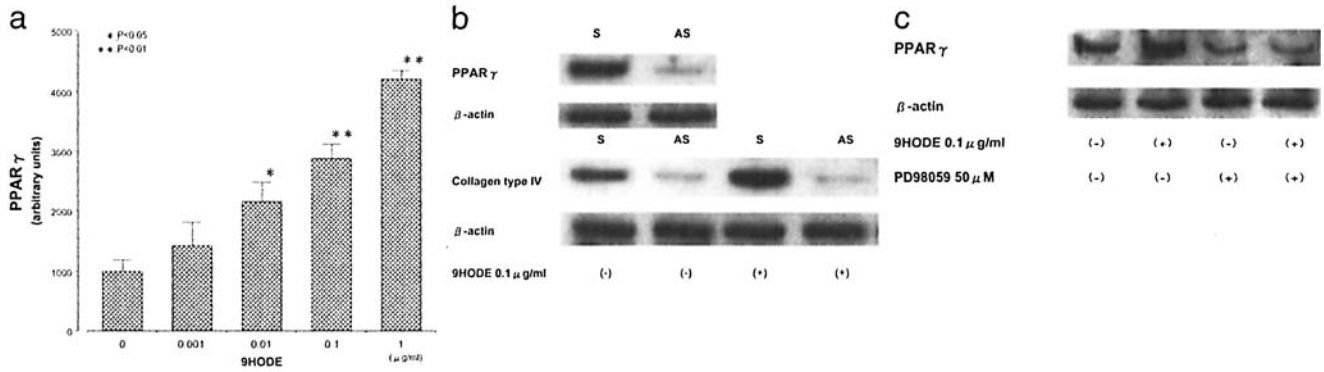

Figure 4. Peroxisome proliferator-activated receptor gamma (PPARγ) stimulation by 9-hydroxy-10,12-octadecadienoic acid (9HODE) was inhibited by PD98059. (a) Mesangial cells were incubated for 24 hrs with 0–1 μg/ml 9HODE. Cell proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with a polyclonal antibody against PPARγ (1:1000). (b) Mesangial cells were preincubated with PPARγ sense or antisense oligonucleotide (0.3 μ*M*) and lipofectin (10 μg/ml) for 24 hrs and incubated with or without 9HODE (0.1 μg/ml). Cell proteins were separated by SDS-PAGE and immunoblotted with polyclonal antibodies against PPARγ (1:1000) and Type IV collagen (1:200). (c) Mesangial cells were preincubated with or without PD98059 (50 μ*M*, 2 hrs) and incubated for 24 hrs with or without 0.1 μg/ml 9HODE. Cell proteins were separated by SDS-PAGE and immunoblotted with a polyclonal antibody against PPARγ (1:1000). Bars represent mean ± SE of at least four separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ versus vehicle-treated control.

important role in 9HODE-stimulated ECM protein production. On the other hand, several reports indicate that TZDs, PPARγ ligands, might reduce microalbuminuria (44, 45). Further studies should be necessary to explain the difference between the present *in vitro* data and the previous clinical data on the role of PPARγ in the diabetic nephropathy.

In our study, 9HODE stimulated PPARγ expression with about 30-fold efficacy compared with troglitazone. Thus, in contrast to other oxidized fatty acids, HODEs are among the most potent agents available as PPARγ ligands in the previous reports (15, 43). As presented in this report, even lower doses of 9HODE were effective to increase PPARγ expression in mesangial cells compared with other cell types such as monocyte/macrophage and THP-1 cells (32). This suggests a significant role for HODEs in mesangial cells and, by consequence, renal diseases.

However, OX-LDL and 7-ketocholesterol, an oxidized lipid in OX-LDL particles, have been reported to stimulate the production of fibronectin through reactive oxygen species and the subsequent activation of SP-1 in rat mesangial cells, whereas 4-hydroxynonenal or 25-hydroxycholesterol have not (4). In addition to HODE, 7-ketocholesterol may contribute to mesangial cell proliferation and ECM synthesis by OX-LDL.

Modified LDLs such as OX-LDL and glycated LDL cause renal failure and other atherogenic lesions in many patients with chronic renal diseases and diabetes. In addition to lipid-lowering drugs, antioxidants may be valuable in preventing renal dysfunction in the setting of chronic renal failure.

In conclusion, we first demonstrated that 9HODE, a major component of oxidized-LDL, stimulates mesangial cell proliferation and ECM protein synthesis. A MAPK-PPARγ-dependent pathway may be involved in the stimulation of ECM production by 9HODE.

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