A redox-sensitive pathway mediates oxidized LDL-induced downregulation of insulin-like growth factor-1 receptor

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Abstract Oxidized low density lipoprotein (OxLDL) has multiple proatherogenic effects, including induction of apoptosis. We have recently shown that OxLDL markedly downregulates insulin-like growth factor-1 receptor (IGF-1R) in human aortic smooth muscle cells, and that IGF-1R overexpression blocks OxLDL-induced apoptosis. We hypothesized that specific OxLDL-triggered signaling events led to IGF-1R downregulation and apoptosis. We examined OxLDL signaling pathways and found that neither IGF-1R downregulation nor the proapoptotic effect was blocked by inhibition of OxLDL-triggered extracellular signal-regulated kinase, p38 mitogen-activated protein kinase (MAPK), or peroxisome proliferator-activated receptor γ (PPARγ) signaling pathways, as assessed using specific inhibitors. However, antioxidants, polyethylene glycol catalase, superoxide dismutase, and Trolox completely blocked OxLDL downregulation of IGF-1R and OxLDL-induced apoptosis. Nordihydroguaiaretic acid, AA-861, and baicalein, which are lipoxygenase inhibitors and also have antioxidant activity, blocked IGF-1R downregulation and apoptosis as well as reactive oxygen species (ROS) production. These results suggest that OxLDL enhances ROS production possibly through lipoxygenase activity, leading to IGF-1R downregulation and apoptosis. Furthermore, anti-CD36 scavenger receptor antibody markedly inhibited OxLDL-induced IGF-1R downregulation and apoptosis as well as ROS production. In conclusion, our data demonstrate that OxLDL downregulates IGF-1R via redox-sensitive pathways that are distinct from OxLDL signaling through MAPK- and PPARγ-involved pathways but may involve a CD36-dependent mechanism.—Higashi, Y., T. Peng, J. Du, S. Sukhanov, Y. Li, H. Itabe, S. Parthasarathy, and P. Delafontaine. **A redox-sensitive pathway mediates oxidized LDL-induced downregulation of insulin-like growth factor-1 receptor. *J. Lipid Res.* 2005.** 46: **1266–1277.**

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Advanced atherosclerotic lesions are characterized by irregular thickening of the arterial intima, inflammatory cell accumulation, extracellular lipid, and fibrous tissue deposition (1–4). Fibrous cap formation arises from the migration and proliferation of vascular smooth muscle cells (SMCs) and from matrix deposition (2). The development of atherosclerotic lesions, especially in their early stage, is thought to be dependent on the oxidation of LDLs, which are taken up by scavenger receptors on macrophages, leading to foam cell formation (5, 6). Although the accumulation of SMCs plays an important role in the development of atherosclerotic plaque, plaques prone to erosion and rupture are rich in lipid-laden macrophages and generally have a thin fibrous cap and a relative reduction in SMC number (1, 3, 4). SMC accumulation in plaque can be considered to result from the balance between cell proliferation and apoptosis. Recently, we reported that in early human atherosclerotic plaques, oxidized low density lipoprotein (OxLDL)-positive SMCs often express the proapoptotic protein BAX (7). In advanced plaques, OxLDL-positive areas of the intima show higher BAX immunoreactivity and terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated deoxyuridine triphosphates (dUTP) nick end-labelling-positive SMCs (8). Taken together, these findings suggest that OxLDL-induced apop-

Abbreviations: ERK, extracellular signal-regulated kinase; HASMC, human aortic smooth muscle cell; 13-HODE, 13-(*S*)-hydroxyoctadecadienoic acid; 13-HPODE, 13-hydroperoxyoctadecadienoic acid; IGF-1, insulin-like growth factor-1; IGF-1R, insulin-like growth factor-1 receptor; MAPK, mitogen-activated protein kinase; NDGA, nordihydroguaiaretic acid; nLDL, native low density lipoprotein; OxLDL, oxidized low density lipoprotein; PEG, polyethylene glycol; PPARγ, peroxisome proliferator-activated receptor γ; ROS, reactive oxygen species; SMC, smooth muscle cell; SOD, superoxide dismutase; SR-A, scavenger receptor class A; TBARS, thiobarbituric acid-reactive substances.

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The online version of this article (available at http://www.jlr.org) contains an additional figure.

tosis is a major cause of cell death in advanced plaques, contributing to the relative reduction in SMC number.

The proapoptotic effect of OxLDL on vascular SMCs is not fully understood. Mechanisms may include changes in the stimulation of various signal transduction pathways, such as the tumor necrosis factor receptor (9), Fas-Fas ligand (9, 10), and mitogen-activated protein kinase (MAPK) and Jun kinase pathways (9, 11), and in the generation of reactive oxygen species (ROS) (12). In addition to the activation of proapoptotic signal pathways, interference in cell survival signaling could be involved in OxLDL effects on SMCs. We have found that OxLDL markedly downregulates insulin-like growth factor-1 (IGF-1) and IGF-1 receptor (IGF-1R) in cultured SMCs (13). IGF-1 is a potent survival factor for vascular SMCs (14, 15). Its antiapoptotic effects are mediated via the IGF-1R, which is tyrosine phosphorylated after IGF-1 binding and then activates a number of downstream mediators, including phosphatidylinositol 3-kinase and the PKB/Akt axis. Recently, we reported that constitutive expression of IGF-1R in human aortic smooth muscle cells (HASMCs) could overcome OxLDL-induced apoptosis mainly through the activation of the phosphatidylinositol 3-kinase-PKB/Akt pathway (16). These findings suggest that IGF-1R downregulation plays a crucial role in OxLDL-induced apoptosis of SMCs.

In this report, we investigate the signaling pathways that mediate the OxLDL-induced downregulation of IGF-1R. We show that OxLDL regulates IGF-1R via redox-sensitive pathways. This redox-sensitive mechanism is dependent on ROS, which is probably generated via lipoxygenase pathways, the activation of which could be mediated by CD36.

MATERIALS AND METHODS

Materials

Reagents were obtained as follows: LY294002, U0126, and PD98059 from Cell Signaling (Beverly, MA); rabbit anti-human IGF-1R (β-chain; C-20) antibody and rabbit anti-scavenger receptor class A (SR-A) antibody (H-190) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-p44/p42 MAPK (E10) monoclonal antibody, rabbit anti-p44/p42 MAPK antibody, rabbit anti-Akt antibody, and anti-phospho-Akt (Ser-473) monoclonal antibody from Cell Signaling; anti-human CD36 monoclonal antibody (clone FA6-152) from Beckman-Coulter (Fullerton, CA); Trolox, prostaglandin F2α, AA-861, and nordihydroguaiaretic acid (NDGA; a lipoxygenase inhibitor) from Biomol Research Laboratories (Plymouth Meeting, PA); apocynin (a NADPH oxidase inhibitor), MK-886, and REV5901 *para*-isomer from Calbiochem (San Diego, CA); baicalein, *N*-acetyl cysteine, and polyethylene glycol (PEG)-catalase (bovine) from Sigma (St. Louis, MO); and superoxide dismutase (SOD; bovine) from MP Biomedicals (Irvine, CA).

Cell culture

Cultured HASMCs (Cambrex Bio Science, Walkersville, MD) were grown in SmBM medium supplemented with 5% fetal calf serum, antibiotics, 0.5 μg/ml human recombinant epidermal growth factor, 5 mg/ml insulin, and 1 μg/ml human recombinant fibroblast growth factor. The cells were used for experiments at passages 4–10.

Preparation of native low density lipoprotein and OxLDL

Native low density lipoprotein (nLDL) was separated from human plasma by sodium bromide stepwise density gradient centrifugation (17), then dialyzed against PBS containing 0.25 mmol/l EDTA to remove sodium bromide. OxLDL was prepared as described previously (18). Briefly, an aliquot of the nLDL fraction was passed through a 10DG desalting column (Bio-Rad) to re-

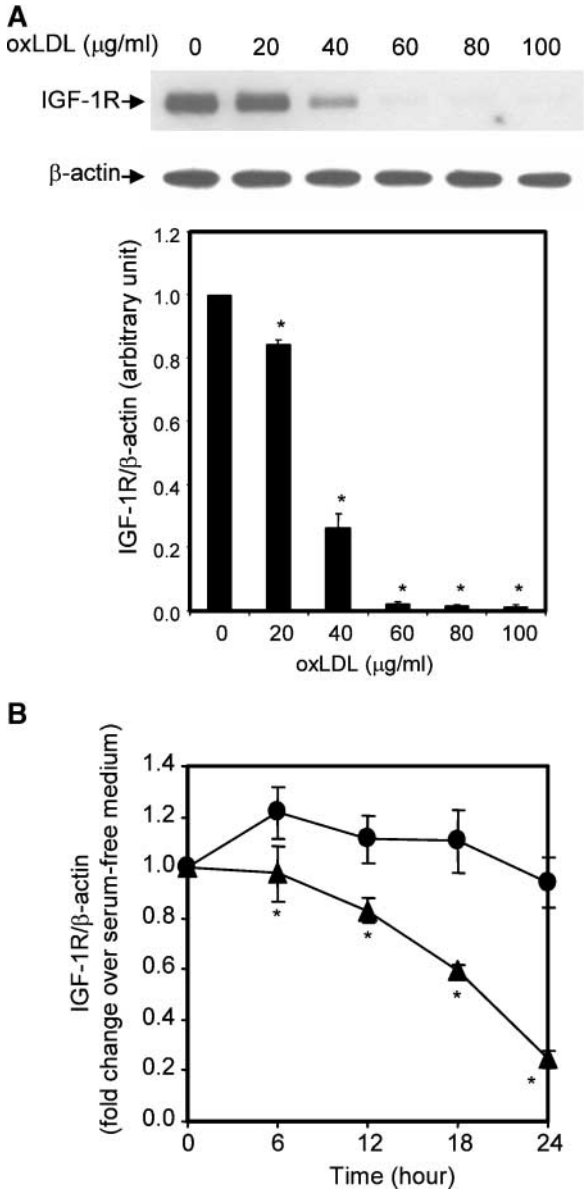

Fig. 1. Oxidized low density lipoprotein (OxLDL) decreased insulin-like growth factor receptor (IGF-1R) expression in human aortic smooth muscle cells (HASMCs). A: HASMCs were incubated with 0–100 μg/ml OxLDL for 24 h, and IGF-1R expression was assessed by Western blot analysis. IGF-1R band intensity after normalization to β-actin (solid bars). Values are expressed as means ± SEM (n = 4). * $P < 0.01$ versus nontreated cells. B: Time-course assessed by immunoblotting in HASMCs exposed to 60 μg/ml OxLDL (triangles) or native low density lipoprotein (nLDL) (circles) for 0–24 h. The graph represents values of IGF-1R band intensity (means ± SEM, n = 4) relative to serum-free medium at each time point after normalization for β-actin. * $P < 0.01$ versus nLDL-treated cells at each time point.

move EDTA, then the nLDL fraction (0.2 mg/ml, diluted in PBS) was incubated with 5 μmol/l $CuSO_4$ at 37°C for 3 h. The reaction was stopped by adding EDTA (final concentration, 0.25 mmol/l). It is likely that such OxLDL has considerable amounts of peroxidized lipids. The OxLDL prepared under these conditions showed an increase in relative mobility on agarose gel electrophoresis, and the value for thiobarbituric acid-reactive substances (TBARS) in OxLDL was 18.6 ± 1.2 nmol malondialdehyde/mg protein. TBARS was not detectable in nLDL.

Western blot analysis

Cells were washed with PBS and lysed in buffer containing 150 mmol/l NaCl, 20 mmol/l Tris-Cl, pH 7.2, 1 mmol/l EDTA, 1% Nonidet P-40, 5 mmol/l dithiothreitol, 0.1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l sodium orthovanadate, 0.1 mol/l okadaic acid, 0.1 μmol/l aprotinin, 10 μg/ml leupeptin, and 10 mmol/l NaF. Lysates were subjected to 10% SDS-PAGE and Western blotting with polyclonal anti-IGF-1R antibody. Immunopositive bands were visualized by ECL (Amersham). Blots were stripped and reprobed with monoclonal anti-β-actin antibody as a control for equal loading.

Apoptosis analysis by flow cytometry

Annexin V binding and propidium iodide staining were carried out using the Annexin V-FITC Apoptosis Detection Kit 1 (BD Pharmingen) and flow cytometry. Briefly, cells exposed with or without 60 μg/ml nLDL or OxLDL were washed twice and resuspended at a concentration of 1×10^6 cells/ml, and 100 μl of cells was mixed with 5 μl each of Annexin V-FITC and propidium iodide for 15 min at room temperature in the dark. After adding 400 μl of binding buffer, cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences).

DNA fragmentation analysis

Fragmented DNA in cytoplasmic fractions was detected and quantified by Cell Death Detection ELISA kits (Roche Molecular Biochemicals) according to the manufacturer's protocol. Briefly, cells were exposed to 60 μg/ml nLDL or OxLDL for 24 h in serum-free medium, then lysed in 100 μl of lysis buffer and centrifuged for 10 min at 200 *g*. The resulting supernatants were placed on streptavidin-coated microtest plates, together with biotinylated anti-histone antibody and peroxidase-conjugated anti-DNA antibody. After incubation and wash, the peroxidase activity retained in the immunocomplex was determined photometrically with 2,2′-azinobis (3-ethylbenzthiazolinesulfonic acid) as substrate (absorbance at 405 nm with a reference wavelength at 490 nm).

Quantification of ROS production

Generation of intracellular superoxide was measured as described by Hsieh et al. (12) using the redox-sensitive dye hydroethidine (Molecular Probes, Eugene, OR). After reaching 80–90% confluence on a 96-well plate (FluoroNunc™ black plate; Nalge Nunc, Rochester, NY), cells were washed once with serum-free medium and then incubated with nLDL or OxLDL in serum- and phenol red-free medium for the periods indicated in the figure legends. During the last 30 min of incubation, 5 μmol/l hydroethidine (final) was added to the culture. The fluorescence intensity was read directly from the culture plate at an emission wavelength of 590 nm and an excitation wavelength of 485 nm (for hydroethidine) with a SynergyHT microplate reader (Bio-Tek Instruments, Winooski, VT). To measure hydoperoxide amount

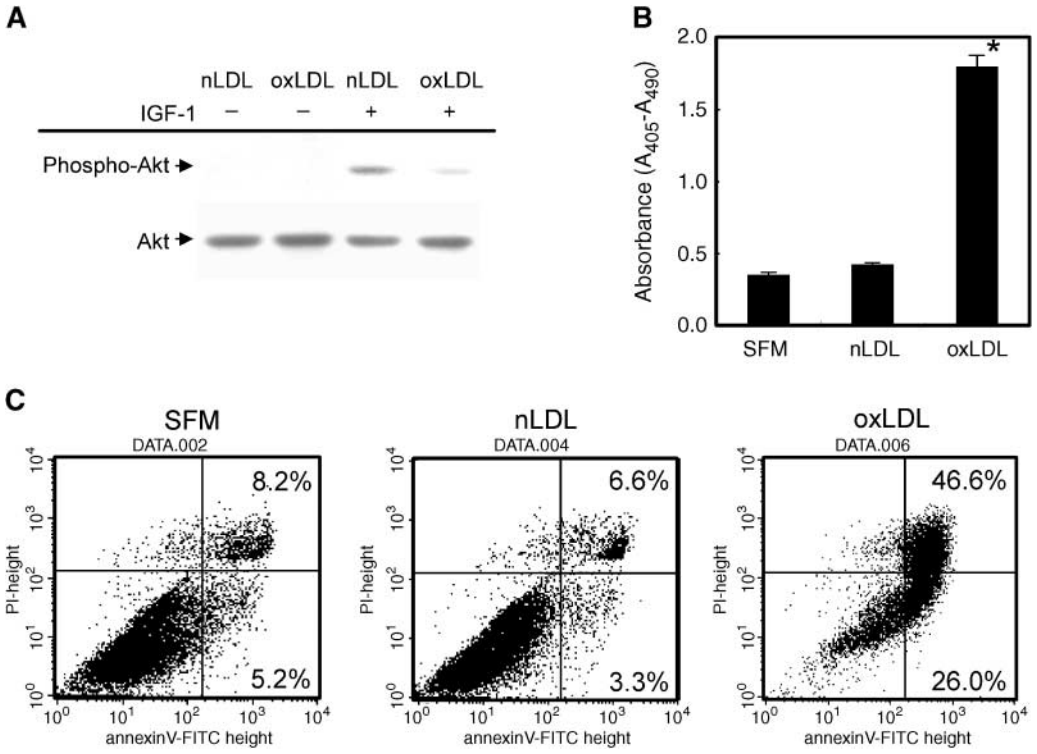

Fig. 2. OxLDL blocked IGF-1-induced Akt phosphorylation and induced apoptosis of HASMCs. A: Native or OxLDL-treated HASMCs were exposed to 10 ng/ml IGF-1 for 10 min, and Western blotting of cell lysates for phospho-Akt and total Akt was performed. B: After incubation with nLDL or OxLDL (60 μg/ml) for 24 h, the cells were stained with annexin V-FITC and propidium iodide and then analyzed by flow cytometry. C: Detection of DNA ladder formation in HASMCs by quantitative cell death detection ELISA. Results are means ± SEM. * $P < 0.001$ versus nLDL. SFM, serum-free media.

in cell culture, we used the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit from Molecular Probes according to the manufacturer's instructions. To examine potential inhibitory effects of apocynin, NDGA, fucoidan, anti-CD36 antibody, SOD, and PEG-catalase, these were added to the culture medium 1 h before the addition of nLDL or OxLDL.

Real-time PCR

RNA was extracted using the RNeasy kit (Qiagen, Inc.), and cDNA was synthesized using the first-strand cDNA synthesis kit with random primers (Amersham Biosciences). Real-time PCR was performed using the iCycler (Bio-Rad Laboratories) with the primers for human CD36 5′-AGGACGCTGAGGACAACAC-3′ and 5′-GCCACAGCCAGATTGAGAAC-3′. We used a two-step amplification protocol with an annealing temperature of 60°C for 40 cycles. Relative expression was calculated from cycle threshold [Ct; relative expression = $2^{-(S°Ct - C°Ct)}$] values using β-actin as an internal control for each sample.

Statistical analysis

Data are presented as means ± SEM. Statistical analysis was performed using one-way ANOVA or Student's *t*-test, with $P < 0.05$ considered significant. All experiments were performed a minimum of three times.

RESULTS

OxLDL downregulates IGF-1R expression in human SMCs

We examined the time- and dose-dependence of OxLDL effects on IGF-1R expression in HASMCs. As shown in **Fig. 1A**, 40 μg/ml OxLDL decreased receptor expression by 70% at 24 h. At doses of 60 μg/ml or higher, OxLDL downregulated IGF-1R expression by more than

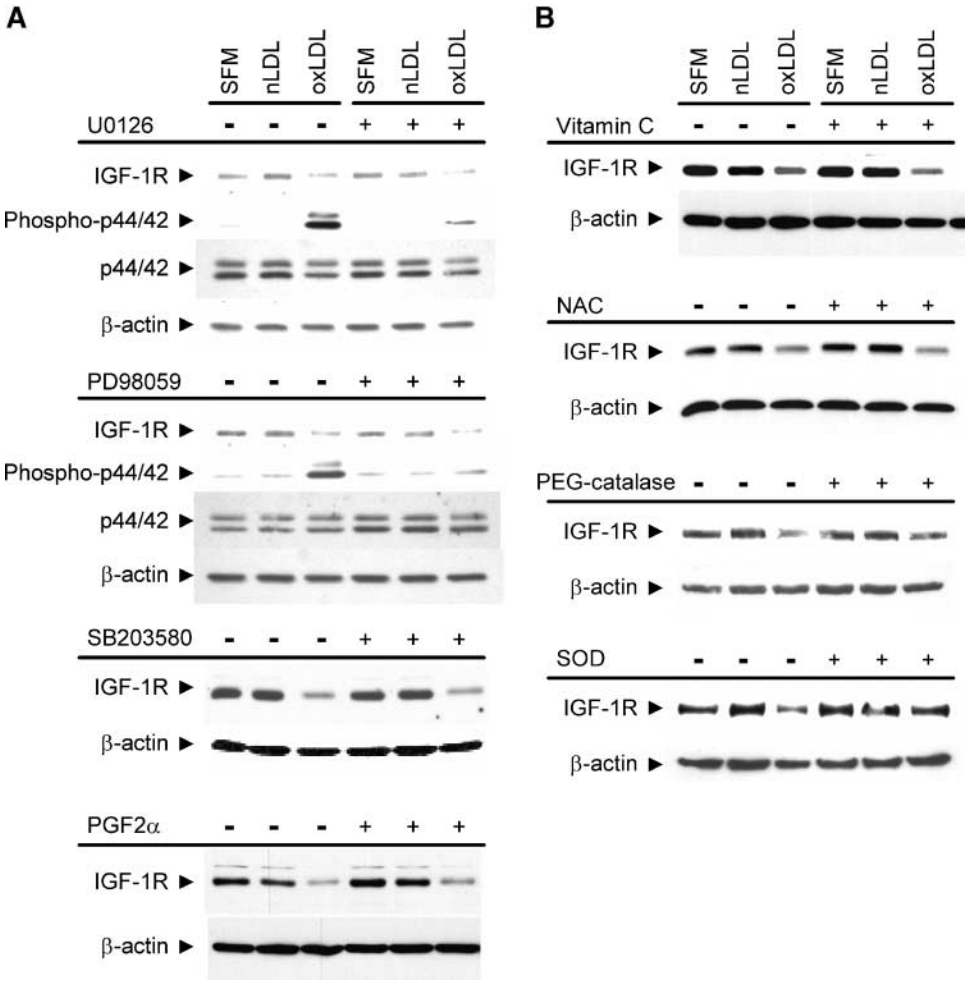

Fig. 3. Polyethylene glycol (PEG)-catalase and superoxide dismutase (SOD) blocked OxLDL downregulation of IGF-1R. A: IGF-1R expression level was determined in HASMCs incubated with 60 μg/ml nLDL or OxLDL in combination with 300 nmol/l U0126 [a mitogen-activated protein kinase (MEK) inhibitor], 10 μmol/l PD98059 (a MEK inhibitor), 10 μmol/l SB203580 [a p38 mitogen-activated protein kinase (MAPK) inhibitor], or 200 nmol/l prostaglandin F2α (PGF2α; a peroxisome proliferator-activated receptor γ antagonist) for 24 h. IGF-1R β-chain, phosphorylated p44/42 MAPK, and β-actin were detected by Western blotting as described in Materials and Methods. B: Vitamin C (50 μmol/l), *N*-acetyl cysteine (NAC; 5 mmol/l), SOD (1,000 U/ml), or PEG-catalase (5,000 U/ml) was added to HASMC 1 h before starting incubation with lipoproteins (60 μg/ml nLDL or OxLDL). At 24 h, IGF-1R expression level was determined by Western blotting. SFM, serum-free media.

80%. Suppression of IGF-1R levels was readily detectable after 12 h of incubation with OxLDL (compared with nLDL; Fig. 1B), and levels decreased progressively in a time-dependent manner. IGF-1R downregulation correlated with reduced IGF-1R signaling (**Fig. 2A**). We examined levels of IGF-1-inducible phosphorylation of Akt after 16 h of nLDL or OxLDL (60 μg/ml) coincubation. We observed Akt phosphorylation after 10 min of exposure to IGF-1, and OxLDL caused a significant decrease in the level of phosphorylation of Akt without affecting the total amount of Akt protein. In previous studies, we showed that OxLDL treatment causes apoptosis of vascular SMCs (13, 16). In the current study, we confirmed that a significant number of the cells were apoptotic after 24 h of OxLDL treatment, as assessed by detection of DNA fragmentation (Fig. 2B) and by annexin V binding (26% of analyzed cells were positive for annexin V binding and negative for propidium iodide staining; Fig. 2C).

Antioxidants blocked OxLDL-enhanced ROS production and IGF-1R downregulation

In various cell types, including SMCs, multiple signaling pathways can be activated by OxLDL, including a p38 MAPK-dependent pathway (11) and an extracellular signal-regulated kinase (ERK)-dependent pathway (19, 20). We examined whether these pathways were involved in IGF-1R downregulation by OxLDL, using well-known inhibitors of those pathways. As shown in **Fig. 3A**, OxLDL treatment caused p44/42 MAPK phosphorylation, indicating that OxLDL can stimulate the ERK pathway, as has been shown previously (19, 20). However, the addition of mitogen-activated protein kinase (MEK) inhibitors [PD98059 (21) or U0126 (22)] did not block IGF-1R downregulation, although p44/42 MAPK phosphorylation was decreased significantly (Fig. 3A). Similarly, the p38 MAPK inhibitor, SB203580 (23)

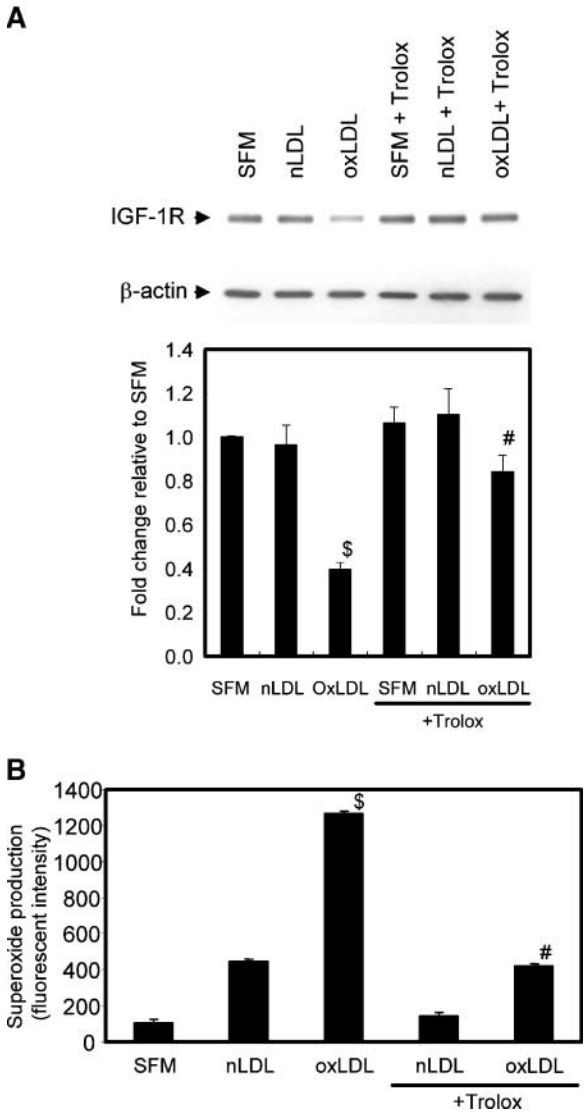

Fig. 4. The antioxidant Trolox prevented OxLDL downregulation of IGF-1R and reactive oxygen species (ROS) production. A: Trolox (250 μmol/l) was added to HASMC 30 min before incubation with lipoproteins (60 μg/ml nLDL or OxLDL, 24 h). IGF-1R expression in each treatment group was assessed by Western blot analysis. The signal intensity of the IGF-1R band was expressed relative to serum-free medium after normalization for β-actin. The graph represents means ± SEM (n = 4). $^{\$}P < 0.001$ versus nLDL; $^{\#}P < 0.001$ versus OxLDL. B: Superoxide production was assessed after the cells were incubated with lipoproteins with or without Trolox for 24 h. Data represent means ± SEM (n = 8). $^{\$}P < 0.001$ versus nLDL; $^{\#}P < 0.001$ versus OxLDL. SFM, serum-free media.

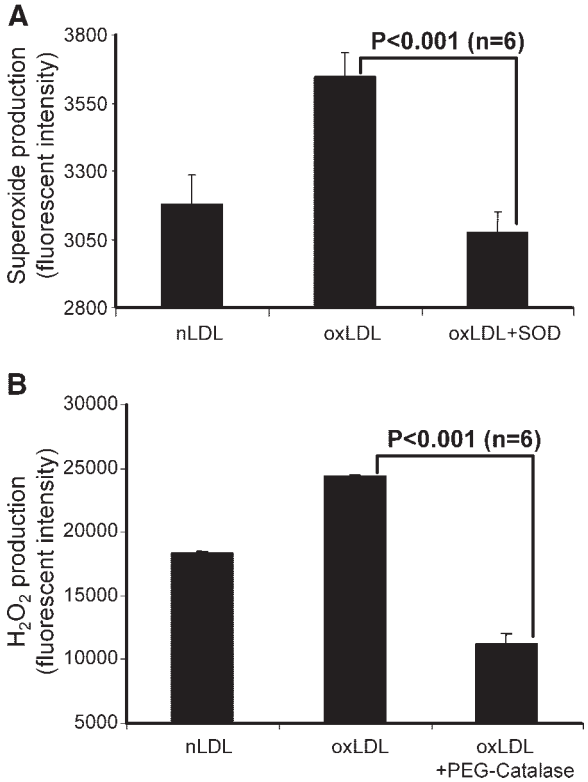

Fig. 5. Effect of SOD and PEG-catalase on OxLDL-generated ROS. A: SOD (1,000 U/ml) was added to HASMCs 1 h before incubation with lipoproteins (60 μg/ml nLDL or OxLDL, 24 h). Superoxide production was assessed as described in Materials and Methods. Data represent means ± SEM (n = 8). B: PEG-catalase (5,000 U/ml) was added to HASMCs 1 h before incubation with lipoproteins (60 μg/ml nLDL or OxLDL, 24 h). Hydroperoxide production was assessed as described in Materials and Methods. Data represent means ± SEM (n = 8).

(10 μmol/l), did not block IGF-1R downregulation (Fig. 3A), although this concentration of inhibitor effectively blocks the kinase activity (data not shown). These data indicate that p38 MAPK and ERK pathways likely are not involved in IGF-1R downregulation by OxLDL. The peroxisome proliferator-activated receptor γ (PPARγ) is also reported to be involved in OxLDL signaling (24, 25). However, prostaglandin F2α, which is known to diminish PPARγ activity (26), could not reverse the effect of OxLDL on IGF-1R expression (Fig. 3A). To examine whether oxidative stress evoked by OxLDL causes IGF-1R downregulation, we used several antioxidants with distinctive modes of action, including *N*-acetyl cysteine, vitamin C, SOD, PEG-catalase (Fig. 3B), and Trolox (**Fig. 4**). Among those, PEG-catalase, SOD (Fig. 3B), and Trolox (Fig. 4A) prevented OxLDL downregulation of IGF-1R. We further confirmed that Trolox, PEG-catalase, and SOD suppress OxLDL-enhanced production of ROS (for Trolox, shown in Fig. 4B; for PEG-catalase and SOD, shown in **Fig. 5**). Further information is provided in the supplementary Figure online.

Possible involvement of lipoxygenase activity in OxLDL-enhanced ROS production

There are multiple potential enzymatic sources for ROS production in the vasculature. These include NADPH oxidase, a major source of ROS in response to vasoactive agonists such as angiotensin II, tumor necrosis factor-α, and thrombin (27, 28), and lipoxygenases, such as 5-lipoxygenase, which is a key enzyme in leukotriene production (29, 30), and 12/15-lipoxygenase, whose biologic functions are unknown (31, 32). We found that NDGA and AA-861, both of which are 5-lipoxygenase inhibitors, markedly reduced OxLDL-induced ROS production. There was 85% less production of superoxide with OxLDL plus 5 μmol/l NDGA compared with OxLDL only-treated cells (**Fig. 6A**). Similarly, 10 μmol/l AA-861 blocked OxLDL-

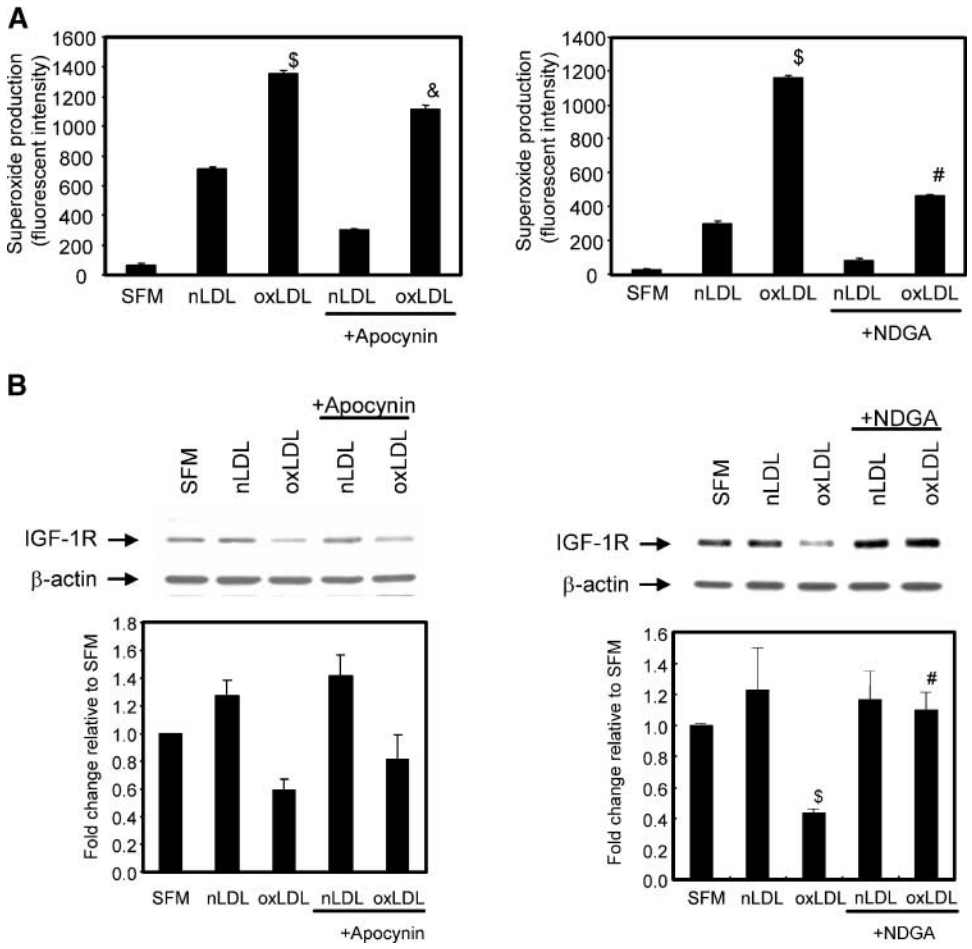

Fig. 6. Role of NADPH oxidase and of lipoxygenase in OxLDL's effect on HASMCs. Apocynin (a NADPH oxidase inhibitor; 50 μmol/l) or nordihydroguaiaretic acid (NDGA; a 5-lipoxygenase inhibitor; 5 μmol/l) was added to HASMCs 1 h before incubation with lipoproteins (60 μg/ml nLDL or OxLDL, 24 h). A: Effect on ROS production. Superoxide was measured using hydroethidine, as described in Materials and Methods. Results are means ± SEM (n = 8). $^{\$}P < 0.001$ versus nLDL; $^{\&}$ not significant versus OxLDL; $^{\#}P < 0.001$ versus OxLDL. B: Effect on IGF-1R. IGF-1R expression level in each treatment group was assessed by Western blot analysis. IGF-1R band signal intensity was expressed relative to serum-free medium after normalization for β-actin. The graph represents means ± SEM (n = 4). $^{\$}P < 0.05$ versus nLDL; $^{\#}P < 0.01$ versus OxLDL. SFM, serum-free media.

induced superoxide production by 81% (n = 8, $P < 0.001$). The same doses of NDGA or AA-861 were effective in attenuating OxLDL downregulation of IGF-1R [5 μmol/l NDGA, 84% reduction in OxLDL downregulation of IGF-1R (Fig. 6B); 10 μmol/l AA-861, 82% reduction in OxLDL downregulation of IGF-1R (n = 4, $P < 0.001$; **Fig. 7A**)]. We studied inhibitors of other well-known enzymes that produce ROS, such as allopurinol [a xanthine oxidase inhibitor (33)] and apocynin [a NADPH oxidase inhibitor (34)]. A total of 50 μmol/l apocynin, which is a potent inhibitor of NADPH oxidase, had no effect on ROS production and IGF-1R downregulation by OxLDL (Fig. 6A, B). A total of 100 μmol/l allopurinol also failed to inhibit OxLDL downregulation of IGF-1R (data not shown). Although NDGA and AA-861 inhibit 5-lipoxygenase, and AA-861 is reported to be a highly specific 5-lipoxygenase inhibitor, these agents can also function as antioxidants. Therefore, we tested the ability of MK-886 and REV5901 *para*-isomer, two inhibitors of 5-lipoxygenase translocation, to block OxLDL downregulation of IGF-1R. Interestingly, neither of these inhibitors blocked IGF-1R downregulation (Fig. 7A, B), strongly suggesting that the inhibitory effects of NDGA and AA-861 were likely related to their effects on free radical formation. Additionally, baicalein, a 12-lipoxygenase inhibitor that also has antioxidant properties, almost completely inhibited OxLDL downregulation of IGF-1R [72% inhibition with 5 μM baicalein (n = 4, $P < 0.01$; Fig. 7C)].

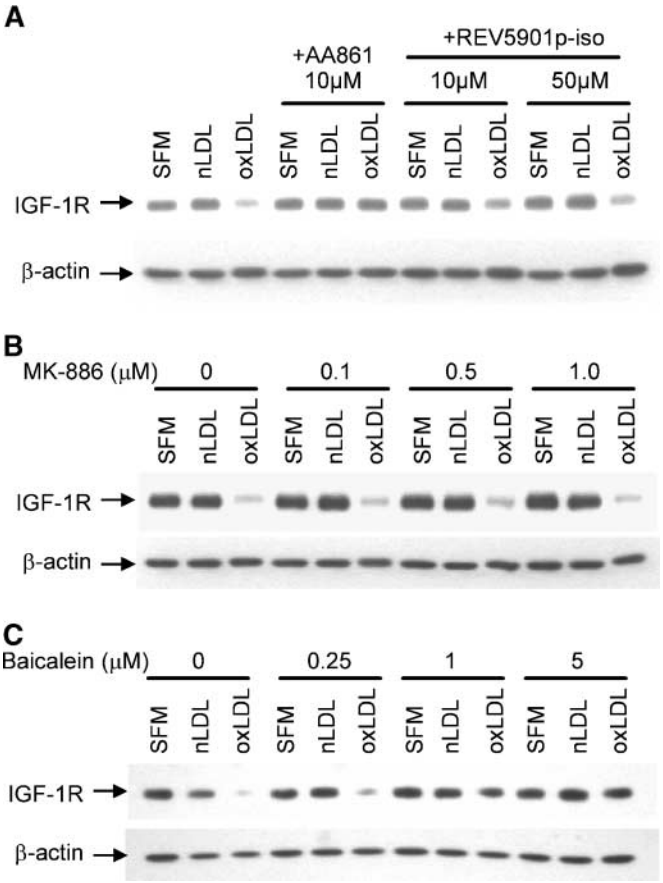

Fig. 7. The lipoxygenase inhibitors AA-861 and baicalein prevented OxLDL downregulation of IGF-1R, but the translocation inhibitors MK-866 and REV5901 *para*-isomer (p-iso) did not. AA-861 and REV5901 *para*-isomer (A), MK-866 (B), or baicalein (C) was added to HASMCs 1 h before incubation with lipoproteins (60 μg/ml nLDL or OxLDL, 24 h). IGF-1R expression in each treatment group was assessed by Western blot analysis. Four independent experiments were performed, and representative results are shown. SFM, serum-free media.

CD36 functions as an OxLDL receptor mediating ROS production and IGF-1R downregulation

Among a number of cell surface receptors for OxLDL, both the macrophage receptor CD36 (35) and the SR-A (36) are reported to be expressed on SMCs. We examined whether these receptors mediated OxLDL downregulation of IGF-1R or ROS production. Preincubation or coincubation with an anti-CD36 blocking antibody significantly reduced OxLDL-induced IGF-1R downregulation and ROS production (**Fig. 8**), whereas fucoidan, which can antagonize OxLDL binding to SR-A (37), was without effect. Anti-SR-A antibody, which blocks acetylated LDL binding to and uptake by human SMCs, failed to show any preventive effect on OxLDL downregulation of IGF-1R (data not shown). These results indicate that CD36 is, at least in part, involved in OxLDL-induced ROS production and IGF-1R downregulation. As reported elsewhere (24, 25), we found that the expression levels of CD36 were regulated by OxLDL. Real-time PCR analysis revealed that OxLDL caused a 2-fold increase in CD36 mRNA content versus nLDL-treated cells at 16 h (**Fig. 9**). Our finding is consistent with the report that OxLDL induced CD36 gene expression in cultured macrophages through PPARγ activation (24, 25). Additionally, we observed that AA-861 coincubation completely prevented OxLDL upregulation of CD36 mRNA (Fig. 9).

Lipoxygenase inhibitors, and also anti-CD36 antibody, prevent apoptotic cell death induced by OxLDL

We have shown previously that downregulation of IGF-1R contributes to the induction of apoptosis by OxLDL (16). Because Trolox, lipoxygenase inhibitors, and anti-CD36 antibody restored IGF-1R expression levels in HASMCs incubated with OxLDL, we assessed the effect of these agents on OxLDL-induced cell death. As shown in **Fig. 10** (top right panel), OxLDL caused an increase in early apoptotic cell number (cells positive for annexin V binding and negative for propidium iodide staining) and in late apoptotic or necrotic cell number (cells positive for annexin V binding and propidium iodide staining). Trolox and NDGA markedly reduced these cell populations [percentages were not significantly different from those of nLDL-treated cells: Trolox (Fig. 10, middle panels); NDGA (Fig. 10, bottom panels)]. Blocking of OxLDL binding to CD36 partially, but substantially, reduced OxLDL-induced cell death [anti-CD36 plus OxLDL, 16.4% apoptosis; normal IgG plus OxLDL, 27.1% apoptosis ($P < 0.05$)]. These results strongly suggest that oxidative stress, primarily in response to lipoxygenase activation, is the main cause of

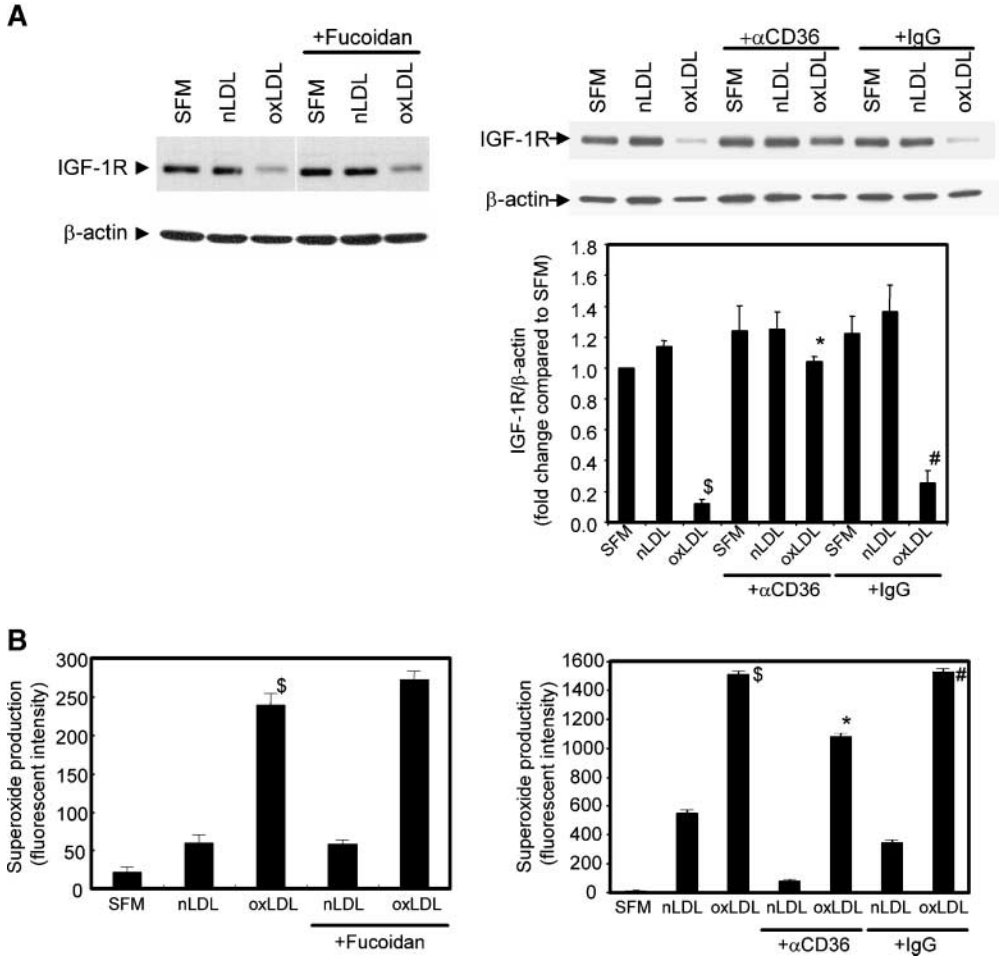

Fig. 8. Blocking CD36, but not scavenger receptor class A, prevented OxLDL-induced downregulation of IGF-1R (A) and ROS production (B). HASMCs were pretreated with fucoidan (40 nmol/l) or anti-CD36 antibody (20 μg/ml) for 30 min before incubation with lipoproteins (60 μg/ml nLDL or OxLDL, 24 h). A: Cell lysates were collected and subjected to Western blot analysis. IGF-1R band signal intensity was expressed relative to serum-free medium after normalization for β-actin. The graph represents means ± SEM (n = 4). $^{\$}P <$ 0.001 versus nLDL; * $P < 0.001$ versus OxLDL; $^{\#}$ not significant versus OxLDL. B: ROS production was measured as described in Materials and Methods. Results represent means ± SEM (n = 8). $^{\$}P < 0.001$ versus nLDL; * $P < 0.005$ versus OxLDL; $^{\#}$ not significant versus OxLDL. SFM, serum-free media.

HASMC death induced by OxLDL and that this effect of OxLDL is at least partially mediated by CD36.

DISCUSSION

In this study, we determined the critical signaling pathway whereby OxLDL downregulates IGF-1R in HASMCs. It has been reported that OxLDL stimulates a variety of signal pathways in several cell types, including the sphingomyelin-ceramide pathway (38), Ca-dependent signaling (39, 40), protein kinase C (41), tyrosine kinase-dependent activation of ERK (19, 20), and a p38 MAPK pathway (11). OxLDL can also activate PPARγ-inducible gene expression, because the oxidized fatty acids contained in OxLDL, 13-hydroperoxyoctadecadienoic acid (13-HPODE) and 13-(*S*)-hydroxyoctadecadienoic acid (13-HODE), are ligands for PPARγ (25). Furthermore, OxLDL stimulates the generation of ROS (12). In our study, we could not demonstrate the involvement of ERK, p38 MAPK, or PPARγ pathways in the OxLDL-induced downregulation of IGF-1R (Fig. 3A); however, we showed that the antioxidants PEG-catalase, SOD, and Trolox can prevent OxLDL downregulation of IGF-1R, indicating that a redox-sensitive signal pathway plays a critical role in the receptor downregulation (Figs. 3B, 4A).

PEG-catalase in a cell culture system scavenges ROS (hydrogen peroxide) after being incorporated into cells. SOD converts superoxide into hydrogen peroxide, thereby reducing cellular oxidative stress. Trolox is a water-soluble derivative of vitamin E (42), whose primary function is as an antioxidant. We found that PEG-catalase, SOD, and Trolox almost completely blocked ROS generation enhanced by OxLDL (Fig. 4B). Thus, there was clearly an inverse relationship between ROS production and IGF-1R expression. Interestingly, the other antioxidants tested (*N*-acetyl cysteine and vitamin C) had no effect on IGF-1R downregulation in response to OxLDL (Fig. 3B). One

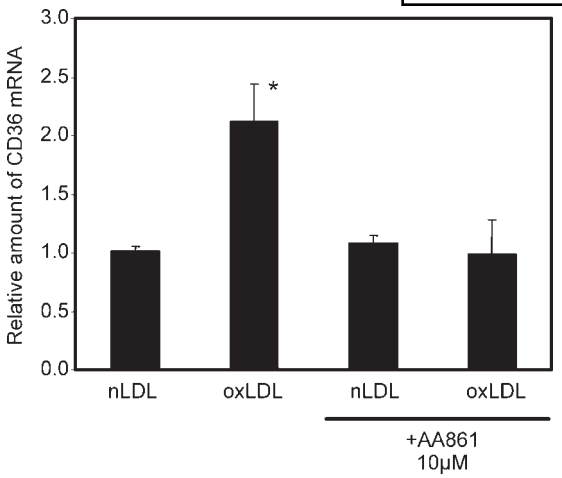

Fig. 9. OxLDL-induced upregulation of CD36 mRNA level, which was blocked by AA-861. AA-861 (10 μmol/l) was added to HASMCs 1 h before incubation with lipoproteins (60 μg/ml nLDL or OxLDL, 16 h). Total RNA was isolated and subjected to reverse-transcription reaction, followed by real-time PCR analysis for CD36 mRNA expression, as described in Materials and Methods. A representative result from three separate experiments is shown. * $P <$ 0.01 versus nLDL-treated cells.

possible explanation for these results could be attributable to the cell permeability and/or distribution of each compound to the subcellular site of the OxLDL effect. Indeed, it is notable that Trolox is active in both lipid and aqueous phases of the subcellular compartment (42), whereas *N*-acetyl cysteine and vitamin C distribute to the aqueous phase. It is also notable that vitamin C failed to significantly reduce ROS production by OxLDL (S. Sukhanov, unpublished data), consistent with a model wherein the OxLDL effect is via hydrophobic radical formation. We hypothesize that the reduced accessibility of *N*-acetyl cysteine and vitamin C to the site of OxLDL ROS production limited their ability to suppress the OxLDL-induced ROS production and downregulation of IGF-1R.

Because we hypothesized that Trolox, SOD, and PEG-catalase inhibition of IGF-1R downregulation was attributable to their antioxidant effects, we investigated the potential sources of oxidative stress in response to OxLDL. There are a variety of nonenzymatic and enzymatic sources for ROS production reported in vivo (43). Thus, there is accumulating evidence that the membrane NADPH oxidase is the predominant source of ROS production in vascular tissue, including SMCs (27, 28, 44). Lipoxygenases, such as 5-lipoxygenase, which is a key enzyme in leukotriene production, and 12/15-lipoxygenase, which is expressed in smooth muscle (45, 46), are a family of lipid-peroxidizing enzymes that are another source of ROS (31, 32). To our surprise, we failed to demonstrate the involvement of NADPH oxidase in OxLDL-induced ROS generation and in IGF-1R downregulation, based on experiments using apocynin, which is a potent NADPH oxidase inhibitor (34) (Fig. 6). Meanwhile, we found that NDGA (Fig. 6) and AA-861 (Fig. 7A), which are 5-lipoxygenase inhibitors, blocked the effect of OxLDL on ROS production and on IGF-1R expression, supporting a model in which OxLDL-generated ROS are critical mediators of IGF-1R downregulation. Because two structurally distinct 5-lipoxygenase inhibitors could block OxLDL effects on ROS production and on IGF-1R expression, and the blockade was almost complete, we first considered that 5-lipoxygenase activity was the primary source of ROS production, leading to IGF-1R downregulation. However, inhibition of 5-lipoxygenase translocation with MK-886 or REV5901 *para*-isomer did not block IGF-1R downregulation, suggesting that the effects of NDGA and AA-861 are unlikely caused by an inhibition of 5-lipoxygenase but rather are the result of their antioxidant properties. However, the possibility that 5-lipoxygenase is already in a translocated form in these cells cannot be excluded. Previous reports indicate that 5-lipoxygenase is expressed predominantly in macrophages in atherosclerotic lesions (47), whereas 12/15-lipoxygenase is expressed in SMCs in addition to macrophages (48). In preliminary experiments, we have detected immunoreactive bands corresponding to 5-lipoxygenase and 12/15-lipoxygenase by Western blot analysis of human aortic SMC lysates (Y. Higashi, unpublished data).

CD36, a class B scavenger receptor, is reported to be expressed on human SMCs (35). We recently showed, using DNA and protein microarrays, that CD36 mRNA and protein expression are upregulated by OxLDL in HASMCs (49). Prevention of OxLDL binding to CD36 by anti-CD36 antibody significantly blocked OxLDL-enhanced ROS production and the decrease in IGF-1R expression (Fig. 8). This result suggests that the OxLDL-stimulated ROS production is, at least in part, mediated through OxLDL binding to CD36. It has been reported that CD36 stimulates ROS production by initiating a signal cascade involving Src kinase family members in microglia cells (50), and c-Src has been reported to regulate NADPH oxidase-dependent superoxide production in vascular SMCs (44). Our study suggests that OxLDL binding to CD36 results in enhanced ROS generation by lipoxygenase(s), consistent with a novel functional relationship between CD36 and lipoxygenase to generate ROS in SMCs. Alternatively, CD36 may function to keep OxLDL on the cell surface or even to internalize OxLDL into cells. Because OxLDL contains a lot of peroxidized fatty acids, such as 13-HPODE (25), having high concentrations of OxLDL at the proximity of the cell or in intracellular vesicle structures (i.e., endosome and lysosome) can facilitate those peroxidized fatty acids to be incorporated into the cell; nevertheless such incorporation is reported to be scarce (51). It is possible that the small amount of peroxidized fatty acid, which is incorporated from OxLDL, initially provides the "peroxide tone" evoked for the lipoxygenase reaction, resulting in more peroxidized fatty acid generation. This is consistent with our finding of the lipoxygenase inhibitors' effect on ROS production. Interestingly, we observed that OxLDL incubation induced the upregulation of CD36 mRNA, which is consistent with enhanced CD36 gene expression by OxLDL in macrophages (24, 25) (Fig. 9). Moreover, AA-861 blocked this upregulation (Fig. 9). In macrophages, it is proposed that OxLDL can induce CD36 expression,

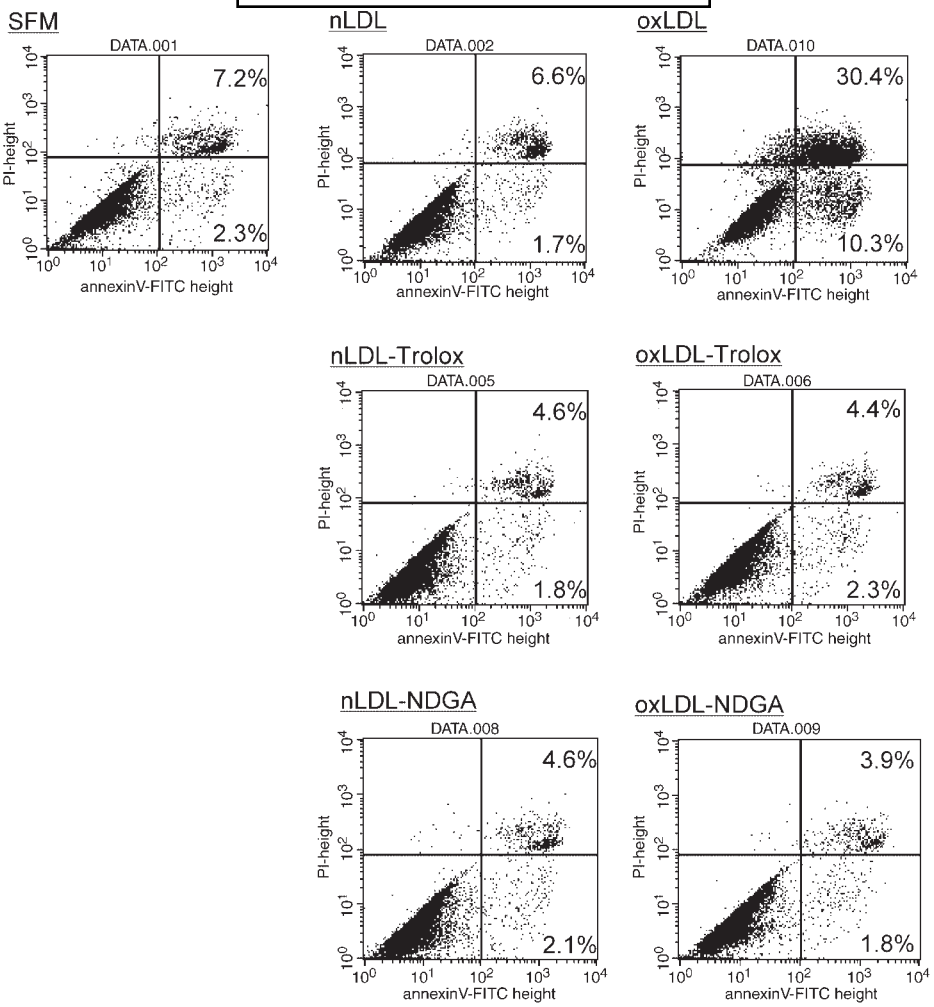

Fig. 10. OxLDL-induced apoptosis was blocked by the antioxidant Trolox and by NDGA. Trolox (250 μmol/l) or NDGA (5 μmol/l) was added to HASMCs 30 min or 1 h before incubation with lipoproteins (60 μg/ml nLDL or OxLDL, 24 h), respectively. Analysis for annexin V binding and propidium iodide staining was conducted as described in Materials and Methods. SFM, serum-free media.

possibly through PPARγ activation by ligands such as 13-HPODE and 13-HODE contained in OxLDL (24, 25).

We found that Trolox suppressed not only OxLDL-induced IGF-1R downregulation but also HASMC apoptosis induced by OxLDL (Fig. 10). A similar effect was observed with lipoxygenase inhibitors (Fig. 10) and anti-CD36 antibody (i.e., blunting of IGF-1R downregulation was accompanied by reduced apoptosis in response to OxLDL). Thus, there is an obvious inverse relationship between IGF-1R expression level and the induction of cell death. Because IGF-1 is a potential survival factor for SMCs (16), our data suggest that the decrease or restoration of IGF-1R expression directly contributes to the rate of HASMC apoptosis. Indeed, this observation supports our previous reports, in which we found that IGF-1 and IGF-1R expression are reduced in areas of atherosclerotic plaque positive for OxLDL (8) and that these areas have increased SMC apoptotic rates (7). This hypothesis is supported by our recent study showing that adenovirally mediated IGF-1R expression in HASMC can prevent the cells from OxLDL-induced apoptosis (16).

In summary, our findings indicate that OxLDL downregulates IGF-1R through redox-sensitive pathways, which involves the CD36 scavenger receptor and is ERK, p38 MAPK, and PPARγ independent. Our observation of cell death suppression by anti-CD36 antibody or by antioxidants indicates that the CD36-dependent/redox-sensitive pathway is also critical for OxLDL-induced apoptosis. In light of our recent finding that adenovirally mediated overexpression of IGF-1R rescues HASMC from OxLDL-induced cell death (16), we propose that IGF-1R downregulation via a lipoxygenase-dependent, CD36/redox-sensitive pathway plays a significant role in HASMC apoptosis induced by OxLDL. This mechanism would contribute to the progressive depletion of SMCs in atherosclerotic plaque and to plaque rupture. Our data suggest that in addition to its role in atherosclerotic plaque development (46–48, 52, 53), lipoxygenase may play a role in plaque destabilization.

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