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OxLDL induces endothelial dysfunction and death via TRAF3IP2. Inhibition by HDL3 and AMPK activators

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Abstract

Oxidized low-density lipoprotein (OxLDL) induces endothelial cell death through the activation of NF- κ B and AP-1 pathways. TRAF3IP2 is a redox-sensitive cytoplasmic adapter protein and an upstream regulator of IKK/NF- κ B and JNK/AP-1. Here we show that OxLDL-induced death in human primary coronary artery endothelial cells (EC) was markedly attenuated by the knockdown of TRAF3IP2 or the lectin-like OxLDL receptor 1 (LOX-1). Further, OxLDL induced Nox2/superoxide-dependent TRAF3IP2 expression, IKK/p65 and JNK/c-Jun activation and LOX-1 upregulation, suggesting a reinforcing mechanism. Similarly, the lysolipids present in oxLDL (16:0-LPC and 18:0-LPC) and minimally modified LDL also upregulated TRAF3IP2 expression. Notably, while native HDL3 reversed OxLDL-induced TRAF3IP2 expression and cell death, 15-lipoxygenase-modified HDL3 potentiated its pro-apoptotic effects. The activators of the AMPK/Akt pathway, adiponectin, AICAR and metformin attenuated superoxide generation, TRAF3IP2 expression, and OxLDL/TRAF3IP2-mediated EC death. Further, both HDL3 and adiponectin reversed OxLDL/TRAF3IP2-dependent monocyte adhesion to endothelial cells *in vitro*. Importantly, *TRAF3IP2* gene deletion and the AMPK activators reversed OxLDL-induced impaired vasorelaxation *ex-vivo*. These results indicate that OxLDL-induced endothelial cell death and dysfunction are mediated via TRAF3IP2, and that native HDL3 and the AMPK activators inhibit this response. Targeting TRAF3IP2 could potentially inhibit progression of atherosclerotic vascular diseases.

Keywords

Atherosclerosis; Oxidative stress; NADPH oxidases; Endothelial dysfunction

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Introduction

Atherosclerosis is characterized by chronic oxidative stress and inflammatory changes in the vascular tissue. Increased local and systemic levels of oxidized low-density lipoprotein (OxLDL) induce endothelial cell activation, dysfunction, apoptosis and impaired vasorelaxation, and contribute causally to atherosclerosis development and progression [1–3]. In endothelial cells, OxLDL signals mainly via the lectin-like oxidized low density lipoprotein receptor 1 (LOX-1, encoded by *OLR1*) [4, 5], and activates diverse cellular second messengers, including NF- κ B and AP-1, two oxidative stress-responsive transcription factors important in the regulation of cytokines, chemokines, and adhesion molecules. Some of the induced cytokines also activate NF- κ B and AP-1, and can reinforce the inflammatory signaling cascade. Of note, *LOX-1* is an NF- κ B and AP-1-responsive gene [6, 7], indicating that in addition to signaling via LOX-1, OxLDL can also upregulate its expression, resulting in positive amplification in its pro-inflammatory and pro-apoptotic signaling. Furthermore, while OxLDL is a product of chronic oxidative stress, it can also exert pro-oxidant effects by inducing ROS generation by the NADPH oxidases (Nox) [8].

The cytoplasmic adapter protein TRAF3IP2 (a.k.a. CIKS or Act1) is an upstream regulator of IKK/NF- κ B and JNK/AP-1 [9, 10], and a mediator of various autoimmune and inflammatory diseases. We have recently demonstrated that the induction TRAF3IP2 is redox-sensitive. In cardiomyocytes, induction of TRAF3IP2 expression by angiotensin II was dependent on Nox2-dependent superoxide generation [11]. Further, advanced oxidation protein products (AOPPs), formed during chronic oxidative stress, also induced TRAF3IP2 expression via Nox2/superoxide [12]. Of note, AOPPs induce endothelial dysfunction via NF- κ B [13, 14] and are implicated in atherosclerosis. In endothelial cells, high glucose-induced endothelial dysfunction is TRAF3IP2 dependent [14]. However, whether TRAF3IP2 plays a role in OxLDL-induced endothelial dysfunction and death has not been reported.

In contrast to OxLDL, native high-density lipoprotein (HDL) is considered to be anti-atherogenic [15]. In addition to its well-known function of facilitating cholesterol efflux from peripheral tissues (reverse cholesterol transport), HDL also exhibits anti-oxidant and anti-inflammatory properties [15]. By removing oxidized phospholipids from LDL, and from cells of the arterial wall, including endothelial cells, HDL prevents LDL oxidation. However, under conditions of chronic oxidative stress and inflammation [16], HDL can undergo oxidation itself, to become proinflammatory. For instance the enzyme 15-lipoxygenase (15LO), expressed at high levels in the artery wall during the early stages of atherosclerosis [17, 18], has been shown to modify HDL *in vitro* [19]. The 15LO-modified HDL (15LO-HDL) subsequently loses its ability to promote cholesterol-efflux, and signals via LOX-1 to activate pro-inflammatory pathways similar to those of OxLDL [19]. However, whether native HDL3 and 15LO-HDL3 differentially modulate Ox-LDL-induced TRAF3IP2 expression, and endothelial dysfunction and death is not known.

Adiponectin is an adipocyte-derived cytokine that exerts potent anti-inflammatory and anti-atherogenic effects. It suppresses the accumulation of modified lipoproteins in the vascular

wall, and inhibits adhesion molecule expression and monocyte-endothelial adhesion [20]. We previously reported that adiponectin blocks IL-18-induced apoptosis of endothelial cells via the AMP-activated protein kinase (AMPK)-dependent activation of Akt [21]. Interestingly, the anti-diabetic drug metformin, that exerts both anti-oxidant and anti-inflammatory effects independently of its effect on metabolism, also activates AMPK/Akt [22]. Thus the AMPK/Akt pathway may be an important mechanism in their anti-inflammatory response.

Here we have investigated the role of TRAF3IP2 in mediating the pro-inflammatory and pro-apoptotic responses of endothelial cells to OxLDL. Our results show for the first time that OxLDL induces endothelial cell dysfunction, cell death and impaired vasorelaxation in part via TRAF3IP2. Further, OxLDL induces TRAF3IP2 in a LOX-1/Nox2-dependent manner, and while native HDL antagonizes the effect of OxLDL, 15LO-modified HDL3 potentiates both TRAF3IP2 induction and endothelial cell death. Further, the AMPK activators adiponectin, AICAR and metformin block OxLDL-induced TRAF3IP2 expression and EC apoptosis through AMPK-dependent Akt activation. These results indicate that TRAF3IP2 is an important mediator of OxLDL-induced pathological effects in the vasculature, and that targeting TRAF3IP2 could potentially inhibit the progression of atherosclerotic vascular diseases.

Materials and methods

Materials

human medium OxLDL (#770202), nLDL (#770200), and HDL3 (#770300) were purchased from KALEN Biomedical, LLC. (Montgomery Village, MD). Metformin hydrochloride (#2864; Tocris Bioscience), recombinant human adiponectin (1065-AP), anti-LOX-1 antibodies (#AF1798; used in immunoblotting and for blocking OxLDL/LOX-1 interaction), and normal goat IgG (#AB-108-C) were purchased from R & D Systems (Minneapolis, MN). Compound C (a cell-permeable pyrazolopyrimidine compound that acts as a potent, selective, reversible, and ATP-competitive inhibitor of AMPK), DPI (#300260; 10 μ M in DMSO for 30 min) and the solvent control DMSO were purchased from EMD Biosciences (Gibbstown, NJ). Tiron (#D7389) was purchased from Sigma-Aldrich. AICAR (catalog # EI-330) was from Biomol (Plymouth Meeting, PA). 16:0 Lyso PC (16:0-LPC; 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; #855675) and 18:0 Lyso PC (18:0-LPC; 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; #855775) were supplied by Avanti Polar Lipids, Inc. (Alabaster, AL). Minimally modified LDL (mmLDL) was generously provided by Yury I. Miller of the University of California at San Diego, and is characterized as previously described [23, 24]. All tissue culture supplies, Vybrant Cell Adhesion Assay kit (V-13181), TRIzol and Alexa Fluor® 488 F(ab')₂ fragment of goat anti-rabbit IgG (H+L; #A11070) were purchased from Invitrogen (Chicago, IL). Anti-TRAF3IP2 (#IMG-563), and peroxidase-conjugated affinity purified goat anti-rabbit IgG (#111-035-046), and goat anti-mouse IgG were purchased from Imgenex (San Diego, CA) and Jackson Immuno Research (Baltimore, PA) respectively. Antibodies against JNK1 (#sc-1648), phospho-IKK α / β (Ser¹⁸⁰/Ser¹⁸¹; sc-23470-R), phospho-JNK (Thr¹⁸³/Tyr¹⁸⁵; sc-6254), ICAM-1 (6.5B5; #sc-18853), and VCAM-1 (H-276; #sc-8304) were purchased from Santa Cruz

Biotechnology, Inc (Santa Cruz, CA). AMPK and phospho-AMPK (#9957), Akt (#9272), pAkt (Thr308, #9275), phospho-p65 (Ser536; #3031), p65 (#3034), α -tubulin (# 2144), and Lamin A/C (# 2032) antibodies were from Cell Signaling Technology, Inc. (Beverly, MA). Affinity-purified rabbit polyclonal antibodies that detect the active (cleaved) p17/19 form of caspase-3, but not the p35 precursor form (AB3623) were purchased from Millipore (Billerica, MA, USA). Quantitect cDNA synthesis kit (# 205310) and EndoFree® Plasmid Maxi kit (#12362) were from Qiagen (Valencia, CA). Cell Death Detection ELISA^{PLUS} kit was from Roche Applied Science. SuperSignal West Pico Chemiluminescent substrate (#34080) and NE-PER Nuclear Extraction kit were purchased from Thermo Fisher Scientific Inc. (Houston, TX).

Cell Culture

Clonetics® human aortic endothelial cells (EC, #CC-2535; Lonza) were previously described [14], and cultured at 37°C in endothelial basal medium-2 (EBM-2, #CC-3156) supplemented with EGM-2 SingleQuots. THP-1 cells (human acute monocytic leukemia cell line) and the mouse monocytic cell line RAW264.7 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). THP-1 cells were cultured as previously described in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum and 0.05 mM 2-mercaptoethanol [14]. RAW264.7 cells were cultured in DMEM+10% FCS, and used below passage number 10. EC were used between passages 4 to 8. At 60%–70% confluency the media was changed to EBM-2 (without supplements), and incubated with indicated agonists and antagonists.

Adenoviral vectors and lentiviral shRNA particles

Ad.siNox2, Ad.siGFP, Ad.GFP, Ad.dnAMPK and Ad.kdAMPK were previously described [11, 12]. At 70–80% confluency, EC were infected at ambient temperature with adenovirus in PBS at the indicated multiplicity of infection (MOI). After 1 h, the adenovirus was replaced with culture medium. Assays were carried out 24 or 48 h later. At 50% confluency, EC were infected with lentiviral particles in complete medium for 48 hrs. Lentiviral particles expressing shRNA against human TRAF3IP2 IKK β , p65, JNK1, c-Jun, and GFP were purchased from Santa Cruz Biotechnology Inc., and were previously described [14]. Lentiviral particles expressing LOX-1 shRNA (*OLR1*; TRCN0000060518) were from Sigma-Aldrich. To increase lentivirus infection efficiency, cells were co-treated with the cationic polymer Polybrene® (5 μ g/ml in water; sc-134220). Neither shRNA nor Polybrene® affected cell viability. The siRNA and shRNA had no off-target effects, and at the indicated moi and duration of infection, did not affect EC adherence, shape and viability (trypan blue-dye exclusion; data not shown).

Endothelial-monocyte adhesion assay

Adhesion of monocytic cells to endothelial cells was analyzed by the Vybrant Cell Adhesion assay kit as previously described [14]. In brief, EC were plated in a 24-well plate. When at 70 to 80% confluency, the complete medium was replaced with EBM-2 containing OxLDL for 2 hours. THP-1 cells were labeled with 5 μ M calcein AM in serum-free RPMI 1640 for 30 minutes at 37°C, washed twice with pre-warmed RPMI 1640, and resuspended in the

same medium. The labeled cells (5×10^4 cells) were then added to EC and incubated for 1 hr at 37° C. The non-adherent cells were removed by gently washing three times with ice-cold PBS. Finally 200 μ l of PBS were added to each well. Endothelial-monocyte adhesion was quantitated by measuring fluorescence at excitation and emission wavelengths of 485 and 535nm, respectively, using BioTek Fluorimeter. Wells containing EC without THP-1 cells served as blanks.

RT-qPCR

Total RNA was isolated using the TRIzol method. One microgram of RNA was reverse transcribed using the Quantitect cDNA synthesis kit. TRAF3IP2, cytokine, chemokine and adhesion molecule expression was analyzed by RT-qPCR using TaqMan® probes and Eppendorf Realplex⁴ system [11, 12, 14]. Data were shown as fold change (2^{-C_t}).

Nuclear protein extraction

The nuclear and cytoplasmic fractions were separated using the NE-PER nuclear extraction kit according to the manufacturer's instructions and has been previously described [14]. Protein levels were quantified using the Bradford method. Lamin B served as an invariant control.

Immunoblotting and ELISA

EC were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4 , 1 mM PMSF, protease inhibitor cocktail, and 1% NP-40) for 30 min at 4°C and lysates were collected after centrifugation at 14,000g for 20 min at 4°C. Protein concentrations in clear cell lysates were determined using Bradford's reagent and BSA as a standard. For western blots, equal amounts of total protein (lysates were boiled in 6x Laemmli buffer containing SDS) were separated by SDS-PAGE [11, 12, 14]. Separated proteins were transferred to PVDF membrane and blocked in PBST (phosphate-buffered saline + 0.2% Tween-20) containing 5% non-fat dry milk powder. The membrane was then incubated with primary antibody overnight at 4°C. The primary antibodies were diluted in PBST solution containing 2.5% nonfat dry milk powder. The membranes were washed in PBST (3x5 minutes) and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The washed blot was then developed using enhanced chemiluminescence reagent and exposed to X-ray film. Images of the bands were scanned by reflectance scanning densitometry, and the intensity of the bands was quantified using Scion Image software.

Immunoprecipitation (IP) and immunoblotting (IB)

IP/IB experiments were performed as previously described [25, 26]. For IP, equal amounts of whole cell extracts were incubated overnight with anti-TRAF3IP2 antibodies attached to agarose beads at 4 °C under slow rotation. After washing 3 times in a buffer containing 50 mM Tris-Cl, 150 mM NaCl, 0.1% Nonidet P-40, the bound proteins were eluted from the beads by boiling in SDS sample buffer for subsequent SDS-PAGE and IB. The antibodies against LOX1 and AMPK are described above.

Detection of superoxide

Superoxide (O_2^-) generation was quantified as previously described [26] using the lucigenin-enhanced chemiluminescence assay. In brief, EC were rinsed twice in PBS and resuspended in Krebs/HEPES buffer (in mM: 115 NaCl, 20 HEPES, 1.17 K_2HPO_4 , 1.17 $MgSO_4$, 4.3 KCl, 1.3 $CaCl_2$, 25 $NaHCO_3$, and 11.7 glucose pH 7.4) that contained no NADPH. After incubation at 37 °C for 30 min, the tubes were placed in a Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany). Dark-adapted lucigenin (5 μ M) and either OxLDL alone or OxLDL combined with DPI or Tiron were added. Luminescence was measured for 10 s with a delay of 5 s. After background luminescence was subtracted, results were expressed as relative light units (RLU) per second per 5×10^4 cells. Studies were also performed using EC infected with Ad.siNox2 (moi 100 for 48 h) prior to the assay. Ad.siGFP served as a control.

Cell death detection

Following 48 h incubation in ECM plus 0.5% bovine serum albumin, EC were treated with the indicated concentration of OxLDL for 24 h. Cell death was analyzed by a photometric enzyme immunoassay (cell death detection ELISA^{PLUS} kit, catalog No. 11920685001, Roche Applied Science) [14]. The assay is based on the quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. After removal of the unbound antibodies, the amount of peroxidase retained in the immunocomplex was determined photometrically using ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) as the substrate. Cell viability was also assessed by a microplate-based MTT cell viability assay, and viability was expressed as percentage of MTT absorbance at 562/650 nm in the absence of OxLDL. In addition, cell death was analyzed by DNA laddering using the Apoptotic DNA Ladder Kit (11 835 246 001; Roche Applied Science) and by determining the levels of cleaved caspase-3.

Animals

The investigation conformed to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health, and all protocols were approved by the Institutional Animal Care and Use Committees at University of Texas Health Sciences Center in San Antonio, TX and Tulane University in New Orleans, LA. All animals were housed in a temperature- (22°C) and light-controlled (12 h light and 12 h dark) environment with *ad libitum* access to water and food. TRAF3IP2-null mice on a C57Bl/6 background are previously described [27]. These mice exhibit no basal phenotypic abnormalities. Breeding, litter size and the sex ratio, growth, body and heart weights were comparable between TRAF3IP2-null and wild type C57Bl/6 control mice [27]. All studies were carried out on male mice 3 months of age.

Monocyte adhesion to endothelium *ex vivo*

The assay describing *ex vivo* adhesion of monocytes to endothelium was previously reported [28]. Thoracic aortas were isolated from WT and TRAF3IP2-null mice, opened longitudinally, cut into pieces, layered and pinned onto agarose gels, washed, treated with

OxLDL (50 µg/ml for 3 h), and then incubated with RAW264.7 mouse monocytes loaded with calcein AM [14]. After 15 min the vessels were washed in DMEM+0.5% BSA to remove unbound cells, and the cells firmly adhered to the vessels were photographed. The number of adhered monocytes was counted in four fields using fluorescent microscopy, and results from 6 animals summarized.

Collection of thoracic aorta and contraction studies

Thoracic aortas were collected, placed in gassed (95% O₂+5%CO₂) Krebs-Hensleit solution composed of 119 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO₄, 1.8 mmol/L CaCl₂, 1.2 mmol/L KH₂PO₄, 24.9 mmol/L NaHCO₃ and 11.1 mmol/L glucose, and then cut into 3 mm length rings. Aortic rings were attached to holders and then placed in an organ bath filled with the buffer solution. Following 1 h equilibration, aortic rings were incubated with nLDL or OxLDL (100 µg/ml) for 1 h. Tissues were washed three times, and vasorelaxant responses were measured as previously described [29]. Rings were contracted to submaximal tone with 1 µM phenylephrine (PE) and subsequently relaxed with cumulative concentrations of the endothelium-dependent vasodilator acetylcholine (ACh). The percentage was calculated by considering contractions obtained immediately prior to the addition of ACh as 100%.

Statistical analysis

Comparisons between controls and various treatments were performed by ANOVA with post hoc Dunnett's *t*-tests. All assays were performed at least three times, and the error bars in the figures indicate the S.E. Though a representative immunoblot is shown in the main figures, changes in protein/phosphorylation levels from three independent experiments were semi-quantified by densitometry, and were shown as ratios and fold changes from untreated or respective controls at the bottom of the panels whenever the results are less clear.

Results

OxLDL induces cell death in primary human EC via TRAF3IP2

Previously, we demonstrated that TRAF3IP2, an upstream regulator of NF-κB and AP-1 pathways, plays a critical role in AOPPs-induced cardiomyocyte injury [12]. Here we have investigated its role in OxLDL-induced EC death. Consistent with earlier reports [30, 31], incubation of EC with OxLDL induced cell death in a concentration dependent manner, with a peak effect observed at 50 µg/ml (Supplementary Fig. S1-A). These results were confirmed by the detection of increased levels of the active (cleaved) form of caspase-3 (Supplementary Fig. S1-A, inset) and by an apoptotic genomic DNA fragmentation assay (Supplementary Fig. S1-B). Furthermore, knockdown of LOX-1, the cell surface receptor mainly responsible for OxLDL signaling in EC [4, 5], or blocking the interaction between OxLDL and LOX-1 with antibodies, each markedly attenuated OxLDL-induced EC death (Fig. 1A). An increase in the levels of the cleaved fragment of caspase-3 (Fig. 1B) supported these results. Notably, knockdown of TRAF3IP2 blunted OxLDL-induced EC death (Fig. 1C, cleaved caspase-3 levels are shown in Fig 1D). Thus OxLDL induces EC death via a LOX-1/TRAF3IP2 pathway.

OxLDL induces endothelial cell death via TRAF3IP2-mediated IKK/NF- κ B and JNK/AP-1 activation

TRAF3IP2 is an upstream regulator of NF- κ B and AP-1, and both are reported to contribute to cell death in a stimulus- and cell type-specific manner. We and others have previously reported that TRAF3IP2 physically interacts with IKK and JNK, and mediates activation of both NF- κ B and AP-1. Here, we investigated whether OxLDL-induced EC death is mediated by IKK/NF- κ B and JNK/AP-1. Indeed, OxLDL induced activation of both IKK (Fig. 2A) and JNK (Fig. 2B) in EC in part via TRAF3IP2, and while knockdown of IKK β attenuated OxLDL-induced p65 activation (Fig. 2C), knockdown of JNK1 inhibited c-Jun phosphorylation (Fig. 2D). Furthermore, knockdown of TRAF3IP2 attenuated activation of both p65 and c-Jun, as evidenced by the reduced levels of nuclear p-p65 and p-c-Jun levels (Fig. 1E). Importantly, knockdown of p65 and JNK1 each inhibited OxLDL-induced caspase-3 activation (Fig. 1F, inset) and EC death (Fig. 1F). These results indicate that OxLDL induces EC death in part via TRAF3IP2-dependent IKK/NF- κ B and JNK/AP-1 activation.

To date, TRAF3IP2 has been shown to play a critical role in IL-17 signaling. TRAF3IP2 physically associates with IL-17R via a SEFIR-SEFIR interaction, and activates both IKK and JNK. Since OxLDL induces TRAF3IP2 expression via LOX-1, and activates IKK/NF- κ B and JNK/AP-1 in part via TRAF3IP2, we next investigated whether OxLDL-induced NF- κ B and AP-1 activation is mediated via LOX-1/TRAF3IP2 physical interaction. Amino acid analysis of LOX-1 coding region revealed neither a SEFIR nor a TIR domain (data not shown), suggesting that TRAF3IP2 may not bind LOX-1. Further, immunoblotting of TRAF3IP2 immunoprecipitates with anti-LOX-1 antibodies revealed no significant binding between TRAF3IP2 and LOX-1 either at basal conditions or following OxLDL treatment (Fig. 2G, left hand panel). However, confirming our earlier published report, TRAF3IP2 was found associated with IKK β , and OxLDL enhanced their binding (Fig. 2G, right hand panel). These results indicate that OxLDL-mediated TRAF3IP2-dependent NF- κ B and AP-1 activations are not mediated by LOX-1/TRAF3IP2 physical association.

OxLDL induces proinflammatory gene expression and monocyte adhesion to EC via TRAF3IP2

OxLDL induces NF- κ B and AP-1 activation, through which it regulates both *LOX1* and other proinflammatory genes [4, 5]. We next investigated the role of TRAF3IP2 in these pathways. Addition of OxLDL markedly elevated both LOX-1 mRNA and protein expression in the EC, and this response was attenuated by TRAF3IP2 knockdown (Fig. 3A). Further, OxLDL induced the expression of IL-18 and other proinflammatory genes important in the induction of EC dysfunction and death, and these responses were similarly attenuated by TRAF3IP2 knockdown (Fig. 3B and Table 1).

Since OxLDL induced ICAM-1 and VCAM-1 expression in TRAF3IP2-dependent manner (Table 1), and monocyte adhesion to EC is a critical initial step in atherogenesis, we next investigated whether OxLDL-induced endothelial-monocyte adhesion *in vitro* and *ex vivo* is TRAF3IP2-dependent. EC transduced with lentiviral TRAF3IP2 shRNA were incubated with OxLDL, and then layered with calcein AM-loaded THP-1 monocytes. While a

significant increase in monocyte-endothelial cell adhesion was detected in the control GFP shRNA treated cells, THP-1 cell adhesion was markedly reduced following TRAF3IP2 knockdown (Fig. 3C). Importantly, in supporting *ex vivo* studies, segments of thoracic aorta from both wild type and TRAF3IP2-null mice were preincubated with OxLDL and then layered with calcein AM-loaded RAW264.7 cells. There was a marked increase in RAW264.7 adherence to WT vessels as compared to vessels isolated from TRAF3IP2-null mice (Fig. 3D). However, very few monocytes were found adhered to the endothelium of vessels isolated from either WT or TRAF3IP2-null mice treated with nLDL. These results indicate that TRAF3IP2 plays a critical role in several of the OxLDL-induced inflammatory changes that underlie the initiation and progression of atherosclerosis.

OxLDL induces TRAF3IP2 expression via Nox2-dependent superoxide generation

Having determined that TRAF3IP2 mediates OxLDL induction of its receptor LOX-1 (Fig. 2), as well as other proinflammatory genes (Fig. 2B and Table 1), we next investigated whether TRAF3IP2 itself is regulated by OxLDL. Addition of OxLDL significantly increased the expression of TRAF3IP2 mRNA and protein in the EC, a response that was inhibited by either LOX-1 knockdown or the addition of LOX-1 blocking antibodies (Fig. 4A). Furthermore, OxLDL enhanced the generation of superoxide in EC, and this enhancement could be partially inhibited by the superoxide scavenger Tiron, by the NADPH oxidase inhibitor DPI, or by the knockdown of Nox2 (Fig. 4B). Similarly, Tiron, DPI and Nox2 knockdown each inhibited TRAF3IP2 induction (Fig. 4C). Thus OxLDL-induces TRAF3IP2 expression in part via Nox2/superoxide-dependent signaling and suggests a positive reinforcing mechanism in the Ox-LDL proinflammatory pathway.

Lysophosphatidylcholine (LPC) constitutes up to 40% of the total lipid content of OxLDL and is formed during the oxidation of LDL. Since LPCs induce LOX-1 [32] and exert proinflammatory effects [33, 34], we next investigated whether 16:0-LPC (Palmitoyl-LPC) and 18:0-LPC (Stearoyl-LPC) induce TRAF3IP2 expression. In addition, we also investigated whether minimally modified LDL (mmLDL), which exerts both proinflammatory and pro-atherogenic effects [35, 36], also induce TRAF3IP2 expression in EC. Our results indicate that pretreatment with 16:0-LPC and 18:0-LPC each induced TRAF3IP2 expression in EC (Fig. 4D). Similarly, mmLDL upregulated TRAF3IP2 expression (Fig. 4D). However, OxLDL appears to be a more effective inducer of TRAF3IP2 in EC (Fig. 4D). These results indicate that OxLDL and its lipid constituents, as well as mmLDL, can all upregulate TRAF3IP2 expression in EC, and strongly suggest that targeting TRAF3IP2 may blunt endothelial cell dysfunction and possibly death.

HDL3, but not modified HDL3, reverses OxLDL-induced endothelial cell death and dysfunction

While OxLDL promotes oxidative stress, inflammation and injury, native HDL exerts potent anti-oxidative, anti-inflammatory and pro-survival effects [15]. Based on their composition, HDLs differ in their anti-atherogenic potential. While HDL2 is large and lipid-rich, HDL3 is small and protein-rich, and is considered to be more effective in accepting cellular cholesterol and promoting its efflux [37, 38]. To investigate the role of HDL on the OxLDL/LOX-1/TRAF3IP2 pathway, we confined our studies to HDL3. HDL3 can undergo

oxidative modification under chronic oxidative and inflammatory conditions, and lose its anti-atherogenic properties, and become proinflammatory [16]. For example, 15-lipoxygenase (15LO), an enzyme present at undetectable levels in normal vessels, is expressed at high levels in atherosclerotic vessels, and can oxidatively modify HDL3 (15LO-HDL3) [17–19]. Therefore, we investigated whether native HDL3 and 15LO-HDL3 differentially affect OxLDL-induced TRAF3IP2 expression. Native HDL3 markedly attenuated OxLDL-induced TRAF3IP2 expression, whereas in contrast, 15LO-HDL3 enhanced its expression (Fig. 5A; results from three independent experiments are summarized on the right). Further, native HDL3 inhibited OxLDL-induced EC death, whereas 15LO-HDL3 potentiated its pro-apoptotic effects (Fig. 5B; cleaved caspase-3 levels confirmed these results, Fig. 5B, inset). Similarly, native HDL3 inhibited OxLDL-induced ICAM-1 and VCAM-1 expression (Fig. 5C; left and right panels respectively) and endothelial-monocyte adhesion (Fig. 5D), whereas the effects of 15LO-HDL3 on the OxLDL-induced changes were additive. Importantly, incubation with 15LO-HDL3 alone induced TRAF3IP2 expression, and LOX-1 knockdown attenuated this effect (Fig. 5E). These results indicate that while native HDL3 reverses OxLDL-induced TRAF3IP2-dependent endothelial dysfunction and death, the effects of 15LO-HDL3 are reinforcing.

Adiponectin reverses OxLDL-induced TRAF3IP2 expression and endothelial cell death

The adipocyte-derived cytokine adiponectin is known to exert anti-inflammatory and anti-apoptotic effects [39]. In patients with atherosclerotic cardiovascular disease, circulating levels of adiponectin correlate inversely with those of the proinflammatory and proapoptotic cytokines. Adiponectin exerts anti-inflammatory and anti-apoptotic effects via AMPK phosphorylation and Akt activation [21]. Therefore we examined whether adiponectin blocks OxLDL-induced TRAF3IP2 expression and endothelial cell death. Initially, we determined whether adiponectin activates Akt in EC via AMPK. Our results show adiponectin induced AMPK phosphorylation (Fig. 6A), a response that was markedly attenuated by the forced expression of mutant AMPK by adenoviral transduction (Fig. 6A), or by Compound C, a pharmacological AMPK inhibitor (Supplementary Fig. S2-A). Further, forced expression of mutant AMPK inhibited adiponectin-induced Akt activation (Fig. 6B). Importantly, in addition to attenuating OxLDL-induced TRAF3IP2 expression (Fig. 6C), adiponectin reversed OxLDL-induced Akt suppression (Fig. 6D). Adiponectin also blunted OxLDL-induced EC death, an effect reversed by mutant AMPK (Fig. 6E). The AMPK inhibitor Compound C similarly reversed the pro-survival effects of adiponectin on OxLDL-induced EC death (Supplementary Fig. S2-B), as did mutant Akt (Supplementary Fig. S3). These results indicate that adiponectin blocks OxLDL-induced EC death via AMPK-dependent Akt activation.

AICAR and metformin reverse OxLDL-induced TRAF3IP2 expression and endothelial cell death

AICAR, a pharmacological activator of AMPK, exerts anti-inflammatory and anti-apoptotic effects [21]. Metformin, an anti-diabetic insulin-sensitizing drug, also has an anti-inflammatory effect. AMPK activation is considered as one of the mechanisms by which metformin exerts its protective effects [22]. Since adiponectin reversed OxLDL-induced TRAF3IP2 expression and EC death, we hypothesized that AICAR and metformin would

antagonize the pro-apoptotic effects of OxLDL via AMPK activation and TRAF3IP2 suppression. Similar to adiponectin, both AICAR and metformin induced AMPK phosphorylation (Supplementary Fig. S4-A), and AMPK-dependent Akt activation (Supplementary Fig. S4-B and C). Moreover, both reversed OxLDL-induced suppression of Akt activation (Supplementary Fig. S4-D). Importantly, AICAR and metformin inhibited OxLDL-induced TRAF3IP2 expression in a dose-dependent manner (Fig. 7A and 7B) and reversed its pro-apoptotic effects (Fig. 7C). These results indicate that the AMPK activators AICAR and metformin block the pro-apoptotic effects of OxLDL via AMPK-dependent Akt activation.

So far we have demonstrated that HDL3 (Fig. 5A), adiponectin (Fig. 6C), AICAR (Fig. 7A) and metformin (Fig. 7B) all attenuate OxLDL-induced TRAF3IP2 expression in EC. Since TRAF3IP2 is a redox-sensitive adapter molecule, and AMPK activation is known to block oxidative stress, we next investigated whether HDL3, adiponectin, AICAR and metformin block superoxide generation in EC via AMPK. Therefore, EC transduced or not with a dominant negative mutant form of AMPK (dnAMPK) were treated with HDL3, adiponectin, AICAR or metformin prior to OxLDL addition. Superoxide (O_2^-) production was quantified using the lucigenin-enhanced chemiluminescence assay. Confirming the results in Fig. 4B, OxLDL induced a robust increase in superoxide, and this effect was markedly attenuated by HDL3, adiponectin, AICAR or metformin pretreatment (Fig. 7D). However, forced expression of dnAMPK partially reversed this inhibitory effect (Fig. 7D). The superoxide scavenger Tiron served as a positive control, and significantly attenuated OxLDL-induced superoxide generation. Together, these results indicate that reduction in oxidative stress by the AMPK activators HDL3, adiponectin, AICAR or metformin might have contributed to TRAF3IP2 inhibition. We further investigated whether a physical association between AMPK and TRAF3IP2 might have contributed to attenuated downstream signaling. To this end, TRAF3IP2 was immunoprecipitated from EC treated or not with OxLDL, and then blotted with anti-AMPK antibodies. Using this technique, no binding between AMPK and TRAF3IP2 was evident, either at basal conditions or after OxLDL treatment (Fig. 7D). These results suggest that the AMPK activators may attenuate TRAF3IP2 expression by inhibiting ROS generation.

OxLDL impairs acetylcholine (ACh)-mediated vasorelaxation in part via TRAF3IP2, and this response is reversed by the AMPK activators

We have demonstrated that TRAF3IP2 plays a role in OxLDL-induced endothelial dysfunction (Fig. 2) and death (Fig. 1C) *in vitro*. We next investigated whether TRAF3IP2 contributes to OxLDL-induced impaired vasorelaxation *ex-vivo*. Our results show that while OxLDL impairs ACh-mediated, endothelium-dependent vasorelaxation *ex-vivo*, *TRAF3IP2* gene deletion reverses this effect (Fig. 8A). Further, all three AMPK activators, adiponectin, AICAR and metformin reversed OxLDL-induced impaired vasorelaxation (Fig. 8B). These results indicate that TRAF3IP2 mediates OxLDL-induced impaired vasorelaxation *ex vivo*, and that the AMPK activators antagonize OxLDL-induced responses.

Discussion

Here we describe studies that demonstrate for the first time the critical role of the cytoplasmic adapter protein TRAF3IP2 in OxLDL-induced endothelial dysfunction and injury *in vitro*, and impaired vasorelaxation *ex vivo*. Further, while native HDL3 inhibits, 15LO-modified HDL3 (15LO-HDL3) potentiates TRAF3IP2 induction. The AMPK activators adiponectin and AICAR inhibit TRAF3IP2 expression and antagonize OxLDL-induced cell injury and death. The anti-diabetic drug metformin inhibits OxLDL-induced TRAF3IP2 expression and endothelial cell death in part via AMPK-dependent Akt activation. These results indicate that targeting TRAF3IP2 can potentially inhibit progression of atherosclerotic vascular diseases (Fig. 8C).

In addition to being a product of the ROS generated in chronic oxidative stress, OxLDL is also a potent inducer of oxidative stress. In endothelial cells, NADPH oxidases are the predominant ROS-generating enzymes. Among the known seven Nox isoforms, endothelial cells express Nox1, 2, 4 and 5. We have previously shown that Nox1, Nox2 and Nox4 all contribute to TRAF3IP2 induction in a cell- and stimulus-specific manner [11, 12, 14, 25, 26]. While Nox1 and 2 generate superoxide, the usual measurable product of Nox4 activity is hydrogen peroxide [40]. In fact, we recently reported that both superoxide and hydrogen peroxide induce TRAF3IP2 expression [12]. Here we show that OxLDL induces TRAF3IP2 induction in human endothelial cells in a dose- and time-dependent manner, and pretreatment with the Nox inhibitor DPI or Nox2 knockdown markedly attenuates TRAF3IP2 induction, confirming that the regulation of TRAF3IP2 is redox-sensitive. In fact, the *TRAF3IP2* promoter region contains several oxidative stress-responsive transcription factors, including C/EBP, IRF-1 and AP-1 [14, 41]. Our unpublished data show that OxLDL induces TRAF3IP2 expression via increased transcription, and not mRNA stability.

Both NF- κ B and AP-1 play critical roles in OxLDL-induced endothelial dysfunction and death. TRAF3IP2 is an upstream regulator of IKK and JNK [9, 10]. In fact TRAF3IP2 has been shown to physically associate with IKK [10]. Using reciprocal immunoprecipitation and immunoblotting, we recently reported that TRAF3IP2 binds IKK γ , and that IKK γ binds JNK [11], suggesting that TRAF3IP2 may serve as a scaffold for JNK/IKK γ interactions, and their interactions may play a role in NF- κ B and AP-1 activation. Here we report that the activation in EC of NF- κ B via IKK, and AP-1 via JNK by OxLDL is inhibited by the knockdown of TRAF3IP2, and that TRAF3IP2 knockdown significantly reduces OxLDL-induced endothelial cell death.

OxLDL is a product of inflammation and is also a potent activator of inflammatory signaling. Here we show that OxLDL, via LOX-1, induces the expression of various proinflammatory cytokines, chemokines and adhesion molecules in part via TRAF3IP2. Interestingly, *OLR1*, the gene encoding LOX-1 is also NF- κ B and AP-1 responsive, implying amplification in OxLDL/LOX-1 signaling. However, this amplification does not appear to involve a physical association between LOX-1 and TRAF3IP2. Using IP/IB, we could demonstrate no binding between LOX-1 and TRAF3IP2 in EC, either at basal conditions or after OxLDL treatment. In addition, analysis of LOX-1 amino acid sequence,

revealed neither a SEFIR nor a TIR domain that are known to play a role in IL-17R/ TRAF3IP2 interaction, making it highly unlikely that TRAF3IP2 may physically associate with LOX-1 in inducing NF- κ B or AP-1 activation.

Of note, several of the inflammatory molecules induced by OxLDL are NF- κ B and AP-1 responsive, as well as potent activators of these transcription factors, suggesting that upon induction by OxLDL, they may further amplify inflammatory signaling in the vessel wall, and contribute to endothelial dysfunction and injury. Several of these cytokines also induce TRAF3IP2 transcription [25, 41], indicating a critical role for TRAF3IP2 in vascular inflammation and injury. In fact, TRAF3IP2 knockdown blunted OxLDL-induced adhesion molecule expression and endothelial-monocyte adhesion *in vitro*, and TRAF3IP2 gene deletion inhibited monocyte adhesion to arterial wall *ex vivo*. Further, TRAF3IP2 gene deletion reversed OxLDL-induced, acetylcholine-mediated impaired vasorelaxation, indicating that TRAF3IP2 plays a critical role in three of the OxLDL-induced pathological phenomena that contribute to atherogenesis: endothelial dysfunction, injury and impaired vasorelaxation.

An important observation in the present study is that HDL3 blocks OxLDL-induced endothelial cell death. Independent of its well-described effects on promoting cholesterol efflux via scavenger receptor class B type I and ATP-binding cassette transporters A1 and G1, HDL3 also exerts antioxidant, anti-inflammatory and anti-apoptotic effects. Here we show that HDL3 inhibits TRAF3IP2 induction. However, HDL3 has been shown to undergo modifications during chronic oxidative and inflammatory conditions, and 15-lipoxygenase, an enzyme abundantly present in atherosclerotic, but not normal vessels [17–19], induces one such modification. Oxidatively modified HDL3 loses its vasculoprotective effects, and become proinflammatory. Interestingly, while native HDL3 inhibits LOX-1 expression, 15LO-HDL3 signals in part via LOX-1. Further, we also show that, like OxLDL, 15LO-HDL3 induces TRAF3IP2 expression. Moreover, its effects on TRAF3IP2 are additive when combined with OxLDL suggesting additional unique signaling pathways. It also potentiates OxLDL-induced endothelial cell death. This synergistic interaction between OxLDL and the modified HDL3 may further potentiate oxidative stress and inflammatory signaling, resulting in endothelial cell injury and dysfunction, and progression of atherosclerosis.

In addition to HDL3, we also show that adiponectin reversed OxLDL-induced TRAF3IP2 expression. Further, adiponectin restored Akt levels via AMPK, and inhibited OxLDL-induced endothelial cell death. Its pro-survival effects were abrogated by Compound C, a pharmacological inhibitor of AMPK, and forced expression of activation-deficient mutant AMPK. AICAR, an AMP mimic and AMPK activator, similarly inhibited OxLDL-induced endothelial cell death. Further, our results also show that the anti-diabetic drug metformin antagonized the pro-apoptotic effects of OxLDL via AMPK activation. Interestingly, HDL3 has also been shown to activate AMPK, indicating that AMPK activators exert potent pro-survival effects in endothelial cells by reversing OxLDL-induced TRAF3IP2 expression and Akt suppression. Of note, AMPK activation has been shown to inhibit oxidative stress, and oxidative stress plays a critical role in TRAF3IP2 induction, implying that HDL3, adiponectin, AICAR and metformin might have attenuated TRAF3IP2 upregulation by blocking ROS generation in EC. In fact, here we show that all four attenuated superoxide

generation in AMPK-dependent manner. However, this inhibition did not involve a physical interaction between AMPK and TRAF3IP2.

In conclusion, our results indicate that TRAF3IP2 plays a critical role in OxLDL-induced endothelial dysfunction, injury and impaired vasorelaxation. HDL3 and the AMPK activators inhibit TRAF3IP2 expression and reverse the pro-apoptotic effects of OxLDL. Targeting TRAF3IP2 could potentially inhibit progression of atherosclerotic vascular diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACh	acetylcholine chloride
Act1	NF- κ B activator 1
AP-1	activator protein-1
AICAR	5-Aminoimidazole-4-carboxamide-1-(R)-d-ribonucleoside
AMPK	5'-Adenosine monophosphate-activated protein kinase
CIKS	Connection to IKK and SAPK/JNK
dn	dominant negative
DPI	diphenylene iodonium
EBM	endothelial cell basal medium
EC	endothelial cell
EDR	endothelium-dependent relaxation
EGM	endothelial cell growth medium
GFP	green fluorescent protein
ICAM-1	intercellular adhesion molecule-1
IKK	inhibitory κ B kinase
IP/IB	immunoprecipitation/immunoblotting
JNK	c-Jun N-terminal kinase
LDL	low-density lipoprotein

LOX-1	lectin-like oxidized low-density lipoprotein receptor 1
LPC	Lysophosphatidylcholine
mmLDL	minimally modified low-density lipoprotein
OxLDL	oxidized LDL
moi	multiplicity of infection
OLR1	Oxidized Low Density Lipoprotein (Lectin-Like) Receptor
NF-κB	nuclear factor kappa B
Nox	NADPH oxidase
NADPH	nicotinamide adenine dinucleotide phosphate
PE	L-phenylephrine hydrochloride
ROS	reactive oxygen species
SAPK	stress-activated protein kinase/Jun kinase
shRNA	small hairpin RNA
SR-A	scavenger receptor type A
TNF-α	tumor necrosis factor- α
TRAF	TNFR-associated factor
TNF	tumor necrosis factor
TRAF3IP2	TRAF3-interacting protein 2
VCAM-1	vascular cell adhesion molecule-1
WT	wild-type

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Highlights

- OxLDL induces endothelial injury and impaired vasorelaxation via TRAF3IP2.
- Native HDL3 inhibits, but oxidatively-modified HDL3 potentiates OxLDL-induced TRAF3IP2 expression.
- AMPK activators adiponectin, AICAR and metformin blunt OxLDL-induced TRAF3IP2 induction and signaling.
- TRAF3IP2 is a potential therapeutic target in atherosclerotic vascular diseases.

Fig. 1. Oxidized low-density lipoprotein (OxLDL) induces human coronary artery endothelial cell (EC) death via TRAF3IP2

A, OxLDL induces EC death via LOX-1. EC treated with OxLDL (50 μ g/ml) for 24 h were analyzed for mono and oligonucleosomal fragmented DNA in cytoplasmic extracts using a cell death ELISA. Normal LDL (nLDL) served as the control. Experiments were also performed after LOX-1 knockdown by lentiviral shRNA (moi 0.5 for 48 h), or treatment with a blocking antibody (10 μ g/ml for 1 h). Knockdown of LOX-1 was confirmed by immunoblotting (right hand). Akt served as an off-target. * P < 0.001 versus nLDL, † P < 0.01 versus OxLDL (n=8). **B**, The active form of caspase-3 (p17/19) at 8 h was analyzed by immunoblotting using cleared whole cell lysates (inset; n=3). **C**, OxLDL induces EC death via TRAF3IP2. EC transduced with lentiviral TRAF3IP2 shRNA (moi 0.5 for 48 h) were treated with OxLDL (50 μ g/ml) for 24 h. Cell death was analyzed as in **A**. Knockdown of TRAF3IP2 was confirmed by immunoblotting. Akt served as an off-target (n=3). * P < 0.001 versus nLDL, † P < 0.01 versus OxLDL (n=8). **D**, The active form of caspase-3 (p17) was also analyzed by immunoblotting as in **A** (n=3).

Fig. 2. OxLDL induces endothelial cell death via IKK/NF- κ B and JNK/AP-1-dependent signaling

A, B, OxLDL induces IKK β and JNK activation via TRAF3IP2. EC transduced with lentiviral TRAF3IP2 shRNA (moi 0.5 for 48 h) were incubated with OxLDL (50 μ g/ml for 1 h). Phospho-IKK β (A) and phospho-JNK (B) levels were analyzed by immunoblotting using cleared whole cell homogenates and activation-specific antibodies (n=3). C, D, OxLDL induces p65 and c-Jun activation via IKK β and JNK respectively. EC transduced with lentiviral IKK β (C) or JNK1 (D) shRNA (moi 0.5 for 48 h) were treated with OxLDL as in A, and analyzed for phospho-p65 (C) or phospho-c-Jun (D) levels by immunoblotting (n=3).

Knockdown of IKK β and JNK1 was confirmed by immunoblotting and are shown on the respective right hand panels. E, OxLDL activates p65 and cJun via TRAF3IP2. EC transduced with lentiviral TRAF3IP2 shRNA and then treated with OxLDL as in A were analyzed for phospho-p65 and phospho-c-Jun levels by immunoblotting using nuclear protein extracts and activation-specific antibodies. Lamin and GAPDH served as loading and purity controls (n=3). F, OxLDL induces EC death via NF- κ B and JNK. EC transduced with lentiviral p65 or JNK1 shRNA (moi 0.5 for 48h) were incubated with OxLDL (50 μ g/ml for 24 h). Cell death was analyzed by ELISA. The active form of caspase-3 (p17/19) at 8 h was determined by immunoblotting (inset; n=3). * P < 0.001 vs. nLDL, † P < 0.01 vs. nLDL $\pm$ GFP (n=8). G, TRAF3IP2 fails to physically associate with LOX-1.

EC were treated with or without OxLDL (50 μ g/ml for 15 min), and TRAF3IP2 and LOX-1 binding was analyzed by immunoprecipitation/immunoblot (IP/IB) using whole cell lysates (n=3). IKK β served as a positive control (right hand panels).

Fig. 3. TRAF3IP2 mediates OxLDL-induced LOX-1 and IL-18 expression, and monocyte adhesion to endothelium
 A, B, OxLDL induces LOX-1 (A) and IL-18 (B) expression in part via TRAF3IP2. EC transduced with lentiviral TRAF3IP2 shRNA (moi 0.5 for 48 h) were treated with OxLDL, and then analyzed for LOX-1 (6 h; A) and IL-18 (2 h; B). mRNA expression was analyzed by RT-qPCR using TaqMan® probes. LOX-1 and mature IL-18 levels were analyzed by immunoblotting using cleared whole cell lysates (insets; n=3). A, B, * $P < 0.01$ versus nLDL, † $P < 0.5$ versus OxLDL (n=3). C, OxLDL induces endothelial-monocyte adhesion via TRAF3IP2. EC at 50% confluency were transduced with lentiviral TRAF3IP2 shRNA (moi 0.5 for 48 h), treated with OxLDL (50 μ g/ml) for 2 h, and then incubated for 1 h with calcein AM-loaded THP-1 cells. Monocyte adhesion to endothelial cells was quantified by measuring fluorescence at excitation and emission wavelengths of 485 and 535 nm. Wells containing EC without THP-1 cells served as blanks. * $P < 0.001$ versus nLDL, † $P < 0.01$ versus OxLDL+GFP shRNA (n=12). D, TRAF3IP2 gene deletion blunts monocyte adhesion to endothelium *ex vivo*. Thoracic aortas from WT and TRAF3IP2-null mice, were opened longitudinally, cut into pieces, washed, treated with OxLDL (50 μ g/ml for 3 h), and then incubated with RAW264.7 mouse monocytic cells loaded with calcein AM. After 15 min the vessels were washed in DMEM+0.5% BSA, layered on agarose gel, and photographed. A representative photograph is shown in D. The number of adhered monocytes was counted in four fields using fluorescent microscopy, and results from 6 animals are summarized on the right. * $P < 0.05$ versus nLDL, † $P < 0.05$ versus respective OxLDL-treatment (n=6).

Fig. 4. OxLDL, its lipid constituents, and mmLDL all induce TRAF3IP2 expression

A, OxLDL induces TRAF3IP2 expression via LOX-1. EC treated as in Fig. 1A were analyzed for TRAF3IP2 expression. mRNA expression at 1 h was analyzed by RT-qPCR. Protein levels at 2 h were quantified by immunoblotting (inset, $n=3$). $*P < 0.001$ versus nLDL, $†P < 0.01$ versus OxLDL ($n=5$). B, OxLDL induces superoxide generation via Nox2. EC loaded with dark-adapted lucigenin (5 μ M) were incubated with OxLDL (50 μ g/ml) alone or following DPI (10 μ M in DMSO for 30 min) or Tiron (5 mM for 1 h) pre-treatment. Studies were also performed using EC transduced with Ad. siNox2 (moi 100 for 48 h) prior to OxLDL addition. Superoxide ($O_2^{\cdot-}$) production was quantified using the lucigenin-enhanced chemiluminescence assay. Luminescence was measured for 10 s with a delay of 5 s. After subtracting background luminescence, results (RLU/second/ 5×10^4 cells) are expressed as fold increase from untreated controls. The knockdown of Nox2 was confirmed by immunoblotting, and is shown in the inset. Akt served as an off-target. $*P < 0.001$ versus nLDL, $†P < 0.01$ versus OxLDL $\pm$ respective controls ($n=12$). C, OxLDL induces TRAF3IP2 expression via Nox2/superoxide. EC treated as in A, but for 2 h, were analyzed for TRAF3IP2 expression by immunoblotting ($n=3$). D, Lysolipids in OxLDL and mmLDL induce TRAF3IP2 expression. EC were treated with 16:0-LPC (Palmitoyl-LPC), 18:0-LPC (Stearoyl-LPC) or mmLDL, each at 50 mg/ml for 2 h, and analyzed for TRAF3IP2 expression by immunoblotting ($n=3$).

Fig. 5. HDL3, but not 15-lipoxygenase-modified HDL3 (15LO-HDL3), reverses OxLDL-induced endothelial dysfunction and death
 A, HDL3 inhibits, but 15LO-HDL3 potentiates, OxLDL-induced TRAF3IP2 expression. EC were incubated with HDL3 or 15LO-HDL3 (100 µg/ml for 6 h) prior to OxLDL addition (50 µg/ml for 2 h). TRAF3IP2 expression was analyzed by immunoblotting. Densitometric results from 3 independent experiments are summarized on the right. * $P < 0.05$, ** $P < 0.01$ versus nLDL; † $P < 0.05$ versus OxLDL; § $P < 0.05$ versus OxLDL (n=3). B, HDL3 inhibits, but 15LO-HDL3 potentiates, OxLDL-induced EC death. EC treated as in A, but for 24 h with OxLDL were analyzed for cell death by ELISA. The active form of caspase-3 at 8 h was determined by immunoblotting (inset; n=3). * $P < 0.001$ versus nLDL, ** $P < 0.05$ versus OxLDL, § $P < 0.05$ versus OxLDL † $P < 0.01$ versus OxLDL ± HDL3 (n=8). C, HDL3 inhibits, but 15LO-HDL3 potentiates, OxLDL-

induced ICAM-1 and VCAM-1 expression. EC treated as in A were analyzed for ICAM-1 and VCAM-1 expressions by immunoblotting (n=3). D, HDL3 inhibits, but 15LO-HDL3 potentiates, OxLDL-induced endothelial-monocyte adhesion. EC at 70% confluency were treated with HDL3 or 15LO-HDL3 (100 µg/ml for 6 h) prior to OxLDL (50 µg/ml for 2h), and then incubated with calcein AM-loaded THP-1 cells. Endothelial-monocyte adhesion was quantified after 1 h as detailed under Fig.

2C. * $P < 0.01$ versus nLDL, † $P < 0.01$ versus OxLDL, § $P < 0.05$ versus OxLDL (n=8). E, 15LO-HDL3 induces TRAF3IP2 expression via LOX-1. EC transduced with lentiviral LOX-1 shRNA (moi 0.5 for 48 h) were treated with 15LO-HDL3 (100 µg/ml for 2 h). TRAF3IP2 expression was analyzed as in A (n=3).

Fig. 6. Adiponectin inhibits OxLDL-induced TRAF3IP2 expression and endothelial cell death

A, Adiponectin induces AMPK activation. EC transduced with Ad.dnAMPK or Ad.kdAMPK (moi 100 for 24 h) were incubated with adiponectin (30 μ g/ml) for 1 h, and then analyzed for total and phospho-AMPK levels by immunoblotting (n=3). **B**, Adiponectin induces Akt activation via AMPK. EC treated as in **A** were analyzed for total and phospho-Akt levels by immunoblotting (n=3). **C**, Adiponectin attenuates OxLDL-induced TRAF3IP2 expression. EC were pre-treated with adiponectin (30 μ g/ml for 1 h) prior to OxLDL addition (50 μ g/ml for 2 h). TRAF3IP2 expression was analyzed by immunoblotting (n=3). **D**, Adiponectin reverses OxLDL-induced Akt suppression. EC treated as in **C**, but for 1 h, were analyzed for total and phospho-Akt levels by immunoblotting. Results from three independent experiments are summarized in the lower panel. * $P < 0.05$ versus nLDL, † $P < 0.05$ versus OxLDL (n=3). **E**, Adiponectin attenuates OxLDL-induced EC death via AMPK. EC transduced with Ad.dnAMPK or Ad.kdAMPK (moi 100 for 24 h) were incubated with adiponectin (30 μ g/ml for 1 h) followed by OxLDL addition (50 μ g/ml for 24 h). Cell death was analyzed by ELISA. The active form of caspase-3 at 8 h was determined by immunoblotting (inset; n=3). * $P < 0.001$ versus nLDL, † $P < 0.01$ versus OxLDL, § $P < 0.05$ versus adiponectin (n= 8/group).

Fig. 7. The AMPK activators AICAR and metformin inhibit OxLDL-induced TRAF3IP2 expression and endothelial cell death
A, B, AICAR and metformin inhibit TRAF3IP2 expression. EC incubated with indicated concentrations of AICAR (**A**) or metformin (**B**) for 1 h prior to the addition of OxLDL (50 μg/ml for 2 h) were analyzed for TRAF3IP2 expression by immunoblotting (n=3). **C,** AICAR and metformin inhibit OxLDL-induced EC death. EC incubated with AICAR (30 μM for 1 h) or metformin (100 μM for 1 h) prior to OxLDL (50 μg/ml for 24 h) were analyzed for cell death by ELISA. The active form of caspase-3 at 8 h was determined by immunoblotting (inset, n=3). **P* < 0.001 versus nLDL, †*P* < 0.01 versus OxLDL (n= 8/group). **D,** HDL3, adiponectin, AICAR and metformin each attenuates OxLDL-induced superoxide generation in AMPK-dependent manner. EC transduced with Ad.dnAMPK (moi 100 for 24 h) were incubated with HDL3, adiponectin, AICAR or

metformin at 50 mg/ml. Superoxide generation was analyzed as in 4B. $*P < 0.001$ versus nLDL, $\dagger P < \text{at least } 0.01$ versus OxLDL, $\S *P < 0.01$ versus OxLDL+Ad.GFP (n=12). E, TRAF3IP2 does not physically associate with AMPK. EC treated with or without OxLDL (50 $\mu\text{g/ml}$) for 15 min were analyzed for TRAF3IP2 and AMPK physical association by IP/IB (n=3).

Fig. 8. OxLDL-induced impaired vasorelaxation is TRAF3IP2-dependent, and is reversed by the AMPK activators

A, *TRAF3IP2* gene deletion reverses OxLDL-induced impairment of acetylcholine (ACh)-mediated vasorelaxation *ex-vivo*. Aortic rings from thoracic aorta (3 mm) of *TRAF3IP2*-null and WT mice were equilibrated for 60 minutes under 0.5g tension, incubated with OxLDL (100 µg/ml) for 1 h, washed, pre-contracted with phenylephrine (PE; 1 µM) and then relaxed with cumulative concentrations of ACh for measurement of endothelium-dependent relaxation. **P* < 0.01 versus WT-OxLDL (n=6/at each ACh concentration). **B**, The AMPK activators reverse OxLDL-induced impaired vasorelaxation. Following equilibration, aortic segments from thoracic aorta of WT mice were incubated with adiponectin (30 µg/ml), AICAR (30 µM) or metformin (100 µM) for 3 h followed by OxLDL (100 µg/ml for 1 h). Vasorelaxation was measured as in **A**. **P* < 0.01 versus nLDL, † *P* < 0.05 versus OxLDL (n=6/at each ACh concentration). **C**, Schema showing inhibition of OxLDL-induced TRAF3IP2 expression and endothelial cell death by HDL3 and the AMPK activators adiponectin, AICAR and metformin.

Table 1

TRAF3IP2 knockdown inhibits OxLDL-induced proinflammatory cytokine, chemokine and adhesion molecule expression.

Gene	OxLDL			
	versus nLDL	GFP siRNA	TRAF3IP2 siRNA	Percent reduction TRAF3IP2 versus GFP siRNA
	Fold change			
IL-6	4.70±0.31 *	4.50±0.24	2.78±0.21 †	38
TNF-α	1.84±0.21 *	1.76±0.19	1.23±0.20 †	30
MCP-1	2.89±0.34 *	2.78±0.20	1.72±0.21 †	38
MIP2	3.38±0.20 *	3.42±0.23	2.06±0.22 †	40
KC	2.78±0.14 *	2.89±0.24	1.81±0.14 †	37
ICAM-1	6.21±0.31 *	6.01±0.30	3.38±0.24 †	44
VCAM-1	4.62±0.28 *	4.23±0.27	2.64±0.25 †	38

EC transduced with lentiviral TRAF3IP2 or GFP shRNA (moi 0.5 for 48 h) were treated with OxLDL (50 µg/ml for 2 h), and then analyzed for cytokine, chemokine, and adhesion molecule expression. mRNA expression was analyzed by RT-qPCR using TaqMan® probes.

* $P < 0.01$ versus nLDL.

† $P < 0.05$ versus OxLDL+GFP siRNA (n=3).