Triglyceride-Rich Lipoproteins Prime Aortic Endothelium for an Enhanced Inflammatory Response to Tumor Necrosis Factor-α

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Abstract—High levels of triglyceride-rich lipoproteins (TGRLs) in blood are linked to development of atherosclerosis, yet the mechanisms by which these particles initiate inflammation of endothelium are unknown. TGRL isolated from human plasma during the postprandial state was examined for its capacity to bind to cultured human aortic endothelial cells (HAECs) and alter the acute inflammatory response to tumor necrosis factor-α. HAECs were repetitively incubated with dietary levels of freshly isolated TGRL for 2 hours per day for 1 to 3 days to mimic postprandial lipidemia. TGRL induced membrane upregulation of the low-density lipoprotein family receptors LRP and LR11, which was inhibited by the low-density lipoprotein receptor–associated protein-1. TGRLs alone did not elicit inflammation in HAECs but enhanced the inflammatory response via a 10-fold increase in sensitivity to cytokine stimulation. This was reflected by increased mitogen-activated protein kinase activation, nuclear translocation of NF-κB, amplified expression of endothelial selectin and VCAM-1, and a subsequent increase in monocyte-specific recruitment under shear flow as quantified in a microfabricated vascular mimetic device. (***Circ Res*. 2007;100:381-390.)**

Key Words: triglyceride-rich lipoprotein ■ endothelium ■ monocyte ■ NFκB ■ vascular adhesion molecule-1 ■ atherosclerosis

Atherosclerosis is initiated by an inflammatory response resulting in monocyte recruitment to arterial intima and the gradual development of lipid-rich plaques. Whether because of diet or genetic predisposition, elevated triglyceride-rich lipoproteins (TGRL) levels have been associated with an increased risk of atherogenesis.[1–3] Although published data implicate lipid oxidation as a critical link between vascular inflammation and atherosclerosis, it has not been established that lipoprotein oxidation is the primary mediator early in disease progression.

Oxidized lipoproteins are taken up by scavenger receptors and oxidized low-density lipoprotein receptor-1 (LOX-1)[4] and, on internalization, trigger endothelial inflammation. In the absence of oxidation, low-density lipoprotein (LDL), and TGRL remnant particles infiltrate the vascular endothelium and accumulate in the intima. Chiefly composed of cholesterol-rich very-low-density lipoproteins (VLDLs) and chylomicron particles, TGRL is taken up by LDL family receptors such as VLDL receptor and LDL receptor–related protein (LRP) that bind to apolipoproteins (ie, apoE and apoB-100).[5,6] Uptake of TGRL through this pathway is vital for clearance as LDL-receptor–deficient mice accumulate postprandial TGRLs after a high fat meal.[1,7,8] Although epidemiological evidence has shown a clear link between chronic hyperlipidemia and atherogenesis, there is a paucity of data on how repetitive exposure to elevated TGRL exacerbates vascular inflammation. We focus on the mechanisms by which unmodified TGRL alters the inflammatory response of human aortic endothelial cells (HAECs) to cytokine.

Atherosclerosis develops within discrete vascular lesions, associated with focal expression of acute inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1.[9,10] This in turn triggers activation of mitogen-activated protein kinases (MAPKs) such as Akt, extracellular signal-regulated kinase 1/2 (Erk1/2), and p38.[11,12] The transcription NF-κB translocates from the cytosol to the nucleus where it promotes transcription of VAMs (ie, endothelial [E]-selectin, ICAM-1, and VCAM-1) and chemokines, which guide the recruitment of leukocytes.[9,13,14] Costimulation of aortic endothelium by lipoproteins and cytokines results in the preferential recruitment of monocytes and T cells from the circulation by upregulation of E-selectin and VCAM-1.[9,10] This is central to atherogenesis as apoE knockout mice have been shown to exhibit decreased vascular lesion formation when these adhesion molecules are genetically deleted as compared with wild-type animals.[15,16]

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Here we explore the link between dietary hyperlipidemia and inflammatory atherogenesis. We mimicked a high-fat diet by repetitively exposing HAECs to unmodified postprandial TGRLs, freshly-isolated from human blood, for 2 hours over a period of 1 to 3 days. Inflammatory responses of HAECs were then assessed by acute stimulation (ie, 4 hours) with TNF-α. Membrane binding of TGRL receptor was monitored along with its effect on intracellular signaling pathways, leading to VAM expression and monocyte recruitment on inflamed HAECs in a custom-designed microfluidic flow chamber. We report that although repetitive treatment with TGRL did not itself elicit an inflammatory response, it enhanced the acute response to cytokine stimulation in a manner dependent on LDL family receptor binding and activation of the p38 MAPK and NF-κB pathways. These data reveal how chronic exposure to TGRL primes aortic endothelium for enhanced monocyte recruitment to incipient sites of atherosclerosis.

Methods and Materials

HAEC Culture and Treatment With TGRLs

HAECs were purchased at passage 4 (Cascade Biologics), ≈13 cell divisions following primary isolation, expanded in T75 flasks to 80% to 90% confluence, and then seeded onto 6-well tissue culture plates (BD) between passages 5 and 6 (16 to 19 total cell divisions). HAECs were conditioned in growth media for 2 h/d for up to 3 days with freshly isolated TGRLs (at 2.5 mg/mL unless otherwise indicated) or a control lipid emulsion, Intralipid (at 3 mg/mL). Following incubation, unbound lipoprotein was removed by washing with Hanks' balanced salt solution (without Ca^{2+} or Mg^{2+}). Acute inflammation was stimulated by incubating HAECs with TNF-α for 4 hours and then washing. Where indicated, LDL receptor–associated protein-1 (RAP-1) (100 μg/mL) was added before each TGRL treatment; it remained throughout the incubation and was used to block VLDL receptor as previously described.[1,17]

Triglyceride-Rich Lipoprotein Purification

Blood from healthy human volunteers, as approved by the Human Subjects Institutional Review board of the University of California, Davis, was drawn and prepared at the Clinical Nutrition Research Unit. For the TGRL studies, volunteers consumed controlled meals typical of a Western diet containing 30% total fat (≈20% saturated fat), 15% protein, and 55% carbohydrate. Postprandial blood was drawn 3.5 hours following meal ingestion, a time point previously shown to correlate with elevated circulating TGRL.[18] Blood was collected in 10-mL Vacutainer tubes containing Na^+ EDTA from BD (San Jose, Calif). TGRL, isolated as previously described,[18] contained ≤0.5 endotoxin unit/mL according to a Pyrochrome kit from Associates of Cape Cod (Falmouth, Mass). Samples were stored over nitrogen gas in sealed tubes at 4°C. VLDL and chylomicrons were isolated at an average concentration of 75 and 32 mg/dL of whole blood, respectively.

Quantitative Real-Time RT-PCR

Gene transcripts for E-selectin, ICAM-1, VCAM-1, p50, and p65 were quantified using PCR as described in the online data supplement, available at http://circres.ahajournals.org. Briefly, HAECs were lysed, RNA isolated and quantified, and first-strand cDNA synthesized. The cDNA was then amplified for 40 cycles and results quantified by the $\Delta\Delta C_T$ method.

Protein Isolation and Western Immunoblot Analysis

Protein levels for total and phosphorylated Akt, Erk1/2, p38, IκB, IκB kinase (IKK), p50, and p65 were quantified using Western immunoblot and densitometry of the bands as described in the online data supplement. Briefly, HAECs were lysed, and protein was isolated, run on SDS-PAGE gel, and transferred to nitrocellulose.

Statistical Analysis

Analysis of data were performed using GraphPad Prism version 4.0 software (GraphPad Software Inc, San Diego, Calif). All data are reported as mean±SEM, as indicated. Data were analyzed by ANOVA and secondary analysis for significance with Newman–Keuls post tests. Group comparisons were deemed significant for 2-tailed $P<0.05$.

Results

TGRL Binding and Internalization by HAECs Induces Expression of LDL Receptor

Endothelial response to postprandial lipidemia was modeled by repetitive exposure of cultured HAECs to TGRL, freshly isolated from postprandial blood. TGRL binding to endothelium was directly imaged by phase contrast and fluorescence microscopy (Figure 1). A pericellular distribution of fluorescent TGRL was observed on the majority of HAECs (Figure 1a). Membrane-bound TGRL was endocytosed within 30 minutes of incubation, and confocal sections at ≈1 μm beneath the plasma membrane clearly revealed fluorescent endosomes (Figure 1b). The specificity of binding was assessed by coincubating HAECs with TGRL and RAP-1, a high-affinity LRP antagonist previously reported to block VLDL receptor binding.[17,19] RAP-1 virtually abrogated TGRL membrane fluorescence to the level of IgG2a control (Figure 1c and 1d).

LDL family receptors are reported to be upregulated within atheromatous lesions of apoE knockout mice.[17,20] To elucidate the mechanism of TGRL binding to HAECs, we assessed expression of 2 members, LRP and LR11, by immunofluorescence. Both were expressed on untreated HAECs at levels significantly greater than nonspecific IgG2a (Figure 1e and 1f). Repetitive exposure to TGRL was observed to upregulate LRP by 7-fold and LR11 nearly more than 10-fold more than baseline. Coincubation with RAP-1 significantly reduced LRP and LR11 upregulation as indicated by the shift in mean fluorescence intensity (MFI) toward that of untreated HAECs (MFI of TGRL versus untreated: LRP, 135 versus 59; LR11, 203 versus 84; $P<0.05$). Taken together, these data reveal a positive-feedback mechanism, by which TGRL binds LDL family receptors, is subsequently internalized, and then signals their upregulation on HAECs.

TGRL Primes HAECs for Enhanced Upregulation of Adhesion Molecules

We next determined whether binding of TGRL alone, or in combination with acute TNF-α stimulation, alters transcription and membrane expression of adhesion molecules on HAECs (Figure 2). A 2-hour incubation with TGRL was chosen for priming studies, as elevated TGRL has been detected at this time point in postprandial plasma. Furthermore, 4-hour exposures did not further augment priming of inflammation (data not shown). Incubation with TGRL, whether as a single exposure or repetitively over 3 days, failed to significantly upregulate VAM mRNA or expression. In contrast, acute stimulation for 4 hours with TNF-α (0.3

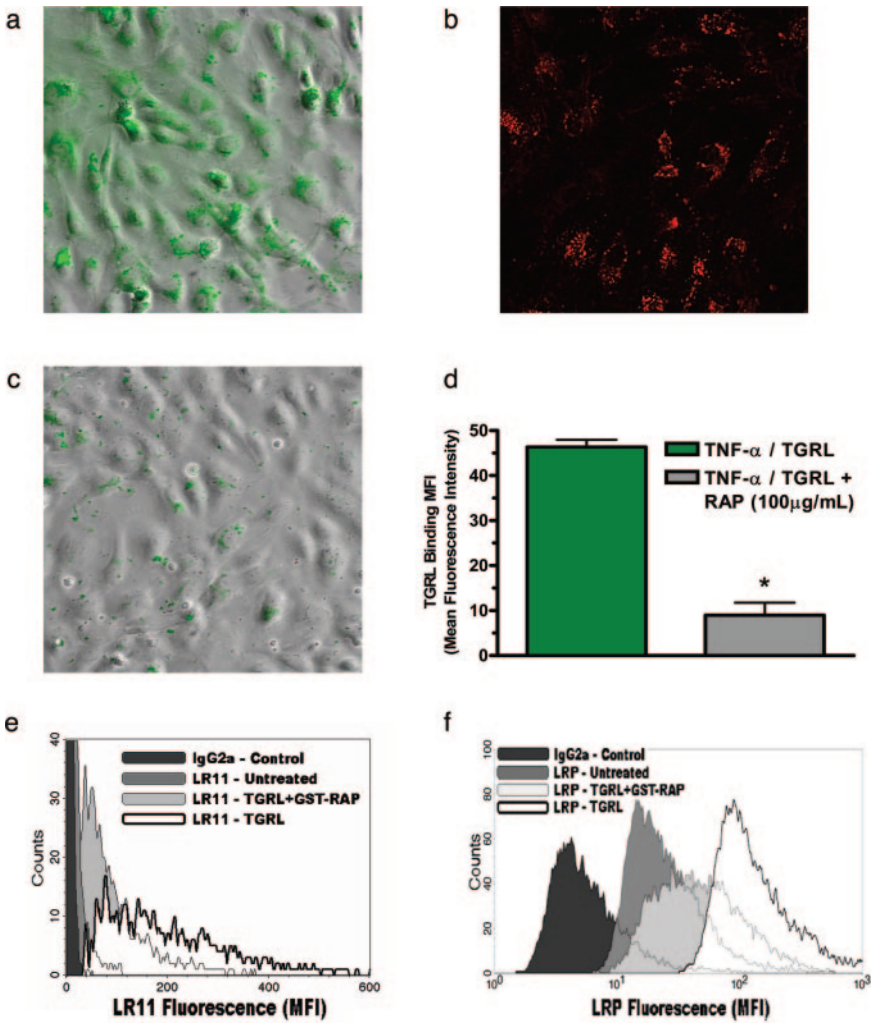

Figure 1. TGRL binds endothelium and upregulates LDL family receptors that are reduced in presence of RAP-1. TGRL conjugated to ATTO-fluorophore was incubated with HAECs and imaged by phase-contrast fluorescence and confocal microscopy. A recombinant RAP-1 peptide was incubated along with fluorescently conjugated TGRL in select wells. a, Representative image of TGRL-ATTO bound to HAECs treated for 2 hours. b, Confocal image of TGRL-ATTO incubated with HAECs for 30 min. c, Representative image of HAECs incubated with TGRL-ATTO in presence of RAP-1 (100 μg/mL). d, Image digitization of TGRL-ATTO and RAP-1 treatment quantified as MFIs. *Significant decrease in TGRL fluorescence ($P<0.05$; n=3±SEM). Representative flow cytometry histograms depict LR11 (e) and LRP (f) expression on untreated HAECs greater than the nonspecific binding of IgG2a antibody control. TGRL upregulated both LRP and LR11, which were significantly reduced in presence of RAP-1 ($P<0.05$; n=3±SEM).

ng/mL) upregulated both VAM transcription and expression. A single 2-hour exposure to TGRL followed a day later by TNF-α stimulation primed a 30% increase in E-selectin expression over TNF-α alone. Expression was not further enhanced over 2 additional days of TGRL exposure, but correlated with a doubling of E-selectin mRNA. TNF-α stimulation upregulated ICAM-1 expression by 6-fold more than unstimulated control, however, priming with TGRL even over 3 days did not enhance mRNA or protein expression over TNF-α alone. In contrast, TGRL priming over 3 days increased VCAM-1 mRNA and expression by ≈70% more than that stimulated by TNF-α alone. Taken together, these data suggest that TGRL alone is not inflammatory to HAECs but differentially amplifies VAM expression elicited by cytokine.

We then examined the sensitivity of HAECs to priming with TGRL by measuring its capacity to augment VAM upregulation elicited over a 1000-fold dose range in TNF-α (Figure 3). VAM expression was compared between culture media and that supplemented with TGRL or Intralipid, a control lipid emulsion lacking apolipoproteins associated with LDL receptor binding. A low concentration of TNF-α (0.003 ng/mL) did not elicit expression of E-selectin or VCAM-1 above unstimulated control. Significantly, priming HAECs with TGRL, but not Intralipid, lowered the threshold dose of TNF-α that stimulated upregulation (0.003 versus 0.03 ng/mL). In this case, TNF-α at 0.03 ng/mL effectively doubled membrane expression of E-selectin and VCAM-1. Though ICAM-1 was expressed on unstimulated HAECs, and was upregulated at 0.003 ng/mL, TGRL did not enhance its expression over the entire dose range of TNF-α. In contrast, VCAM-1 exhibited the greatest sensitivity to TGRL priming, increasing expression relative to Intralipid by ≈100% over the entire dose range of TNF-α. We conclude that TGRL primes HAECs for enhanced inflammation via lowering by 10-fold the threshold dose of TNF-α necessary to elicit upregulation of VCAM-1 and E-selectin.

TGRL Primes HAECs for Enhanced Monocyte Recruitment Under Shear Flow

An early event in atherogenesis is monocyte recruitment to sites of arterial inflammation, whereas neutrophils are typically not observed in developing plaques. We examined the role of TGRL in priming TNF-α–inflamed endothelium for enhanced monocyte recruitment using a custom microfluidic shear flow chamber under a stress of 2 dynes/cm^2 (Figure 4). Recruitment of monocytes and neutrophils onto HAECs were imaged by phase contrast microscopy and categorized as

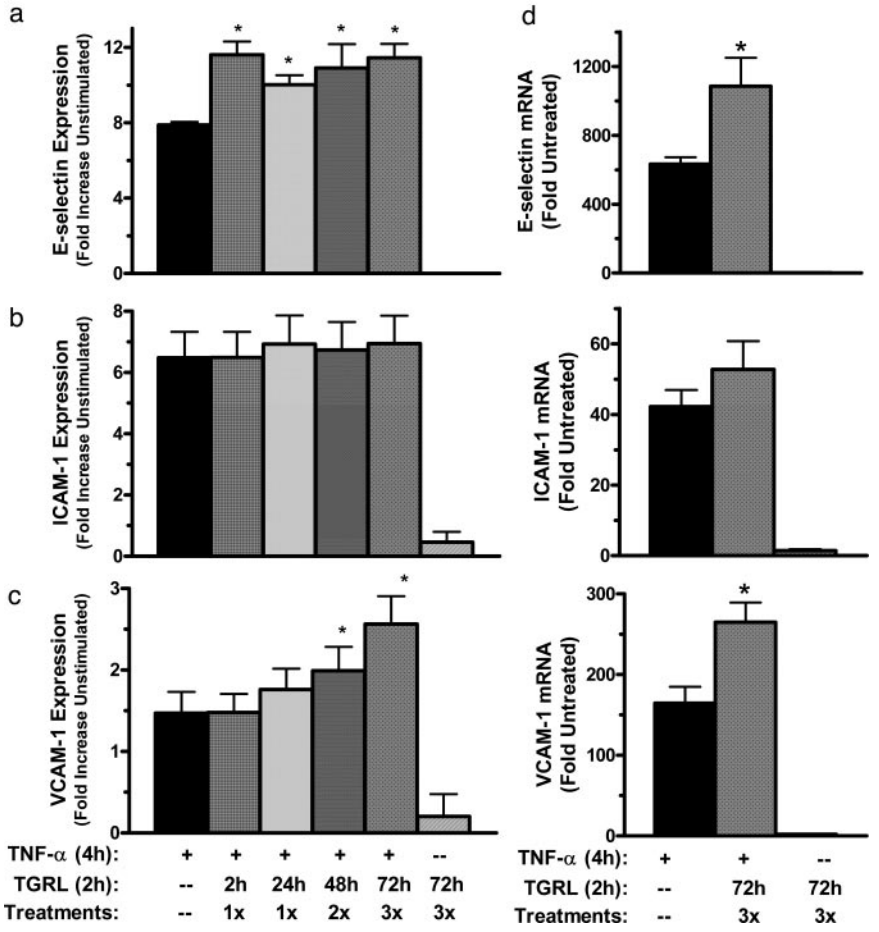

Figure 2. mRNA transcription and expression of VAMs are upregulated in HAECs primed with TGRL and stimulated with TNF-α. HAECs were repeatedly incubated for 2 hours with TGRL (2.5 mg/mL) over the periods denoted on the abscissa and where indicated (+) acutely stimulated with TNF-α (0.3 ng/mL) for 2 hours for quantitative PCR analysis or 4 hours for flow cytometry. Receptor expression of E-selectin (a), ICAM-1 (b), and VCAM-1 (c). Flow cytometry data expressed as fold increase of MFI$\pm$SEM over that measured in untreated HAECs. d, VAM transcript levels assessed by quantitative PCR and expressed as fold increase over untreated HAECs. *Significant upregulation as compared with TNF-α alone ($P<0.05$; n$\geq$3).

rolling, arrested, and transmigrated across the endothelial monolayer. Over a dose range of TNF-α stimulation, neutrophils transitioned from rolling to arrest almost twice as efficiently as monocytes (Figure 4a). Priming HAECs with TGRL doubled monocyte recruitment efficiency at each concentration of TNF-α, whereas neutrophil recruitment did not increase. Consistent with VAM upregulation, TGRL priming did not directly stimulate monocyte recruitment even following repetitive conditioning over 3 days at concentrations up to 5.0 mg/mL (data not shown). However, increasing the concentration of TGRL from 2.5 to 5.0 mg/mL doubled the number of monocytes recruited and transmigrating across TNF-α–inflamed HAECs (Figure I in the online data supplement).

We next examined the VAMs supporting increased monocyte recruitment. In the presence of TNF-α stimulation alone, monocyte arrest and transmigration was supported by $\alpha_4\beta_1$ and β_2 integrins, as blocking each with antibody added up to the control level of 6 monocytes per field (ie, $\alpha_4\beta_1$, 4/field; β_2, 2/field) (Figure 4b). ICAM-1 and VCAM-1 supported firm adhesion; however, capture and rolling required expression of E-selectin, as determined by antibody inhibition (data not shown). Priming HAECs with TGRL doubled monocyte recruitment efficiency, which remained dependent on both β_1 and β_2 integrins (Figure 4c). However, inhibition of β_2 integrins blocked proportionally less monocyte arrest on TGRL-primed HAECs versus TNF-α alone, suggesting that β_1 integrin is predominant. Interestingly, greater cooperativity between β_1 and β_2 integrins was apparent for the TGRL-primed HAECs, as the total number of monocytes arrested was $\approx$30% greater than the sum of monocytes recruited when blocking integrins individually (ie, 12/field versus 9/field, $P<0.05$). These data reveal that priming with TGRL increases monocyte recruitment to inflamed HAECs in a β_1- and β_2-integrin–dependent manner.

VLDL Primes for Enhanced Inflammation Through LDL Family Receptors

We further analyzed TGRL to determine which components were associated with the priming effect by isolating VLDL and chylomicrons separately and incubating HAECs with each before assaying TNF-α–stimulated monocyte arrest (Figure 5a). Neither VLDL nor chylomicron fractions at concentrations commensurate with that applied during TGRL incubation ($\approx$2.5 mg/mL) stimulated monocyte arrest in the absence of TNF-α. Repetitive exposure with VLDL elicited $\approx$70% of the increase in monocyte arrest observed for priming with TGRL. In contrast, chylomicron did not significantly increase monocyte arrest above that stimulated by TNF-α alone. To confirm that TGRL priming was not attributable to oxidized lipoprotein, we treated HAECs with native and oxidized VLDL in the absence of TNF-α and assayed VCAM-1 expression (Figure 5b). Oxidized VLDL significantly upregulated VCAM-1 to levels observed with

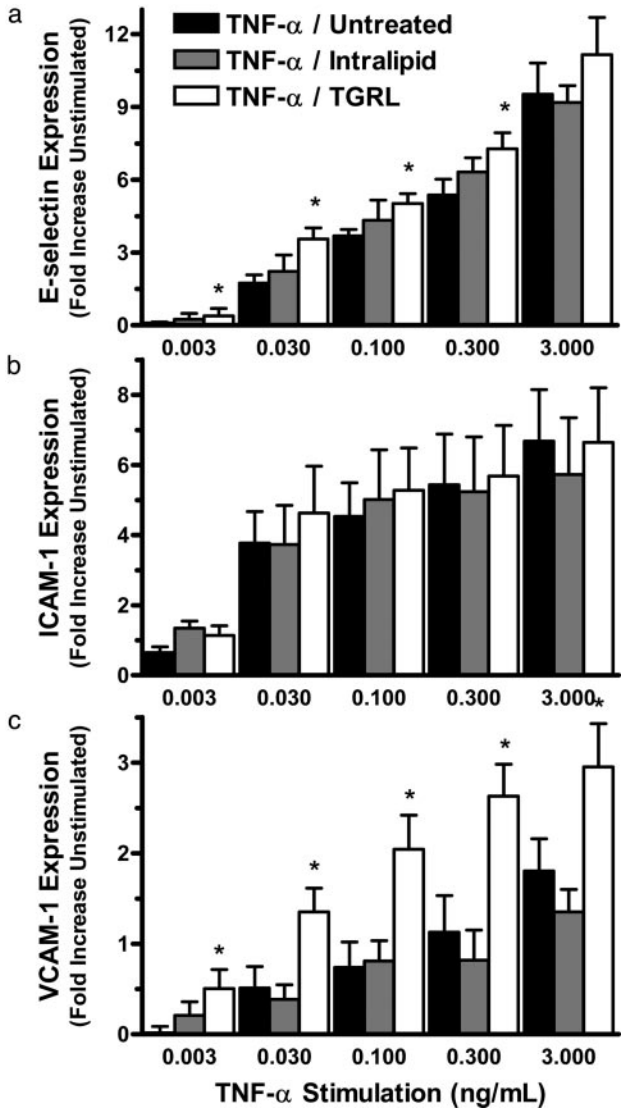

Figure 3. TNF-α–stimulated VAM expression in HAECs primed with TGRL is dose dependent. HAEC monolayers were incubated with culture media (Untreated), TGRL, or Intralipid for 2 h/d over 3 days and then stimulated 4 hours with TNF-α at the indicated doses. Flow cytometry data of (a) E-selectin, (b) ICAM-1, and (c) VCAM-1 expressed as fold increase of MFI over basal expression on untreated HAECs. *Significant upregulation compared with TNF-α–stimulated control ($P<0.05$; n≥3±SEM).

TGRL and TNF-α. This effect was inhibited by treatment of oxidized VLDL with the antioxidant ascorbic acid, whereas VLDL alone or treated with antioxidant did not significantly alter VCAM-1 expression. Together, these data reveal that the inflammatory priming effect of TGRL is largely mediated by VLDL and not associated with oxidized lipoprotein.

To confirm that TGRL binding and internalization are required for the priming response, we assayed VCAM-1 upregulation and monocyte recruitment in the presence of the LRP antagonist RAP-1 (Figure 6). VCAM-1 expression nearly doubled in response to TNF-α, and priming with TGRL augmented expression by 100% (Figure 6a). RAP-1 blocked this augmented VCAM-1 expression down to the level induced by TNF-α alone and likewise reduced monocyte arrest elicited on TGRL-primed HAECs to the level stimulated by TNF-α alone (Figure 6b). These data confirmed that blocking membrane binding and internalization of TGRL by coincubation with RAP-1 abrogates its capacity to augment inflammation, but does not inhibit the response to TNF-α.

TGRL Primes for Inflammation Through Enhanced Activation of p38 MAPK and NFκB

TNF-α–induced inflammatory responses are partially mediated via activation of MAPKs, which in turn trigger the transcription factor NF-κB, known to play an important role in atherogenesis. We focused on this pathway to reveal how repetitive exposure to TGRL itself elicits, or amplifies signaling through TNF-α. Consistent with its lack of direct inflammatory activity, TGRL alone failed to increase MAPK activation above that of untreated HAECs. However, TNF-α significantly activated MAPKs, detected by an increase in phosphorylated Akt, Erk1/2, and p38 (Figure 7a through 7c). Priming with TGRL had the greatest influence in amplifying activation of p38, with an increase of 3-fold more than TNF-α alone. We assessed the role of p38 activation by application of the pharmacological p38 inhibitor SB202190 during TGRL priming. SB202190, in a dose-dependent manner, reduced expression of E-selectin and VCAM-1 in both TNF-α–treated and TGRL-primed HAECs (Figure 7d). Significantly, blocking p38 activity effectively reduced VCAM-1 upregulation in TGRL-primed HAECs to the level observed with TNF-α alone, whereas upregulation of ICAM-1 was unaffected (data not shown). This result is consistent with previous reports on the role of p38 in modulating expression of VAM in response to inflammatory stimulation.[21,22]

TGRL alone did not elicit phosphorylation of IKK but on stimulation with TNF-α increased activation by ≈4-fold, whereas IKK synthesis did not increase (Figure 8a). A concomitant downregulation in the inhibitory protein IκB was observed in HAECs stimulated with TNF-α, which was reduced to undetectable levels in TGRL-primed HAECs (Figure 8b). Notably, priming with TGRL alone resulted in a 2.5-fold increase in mRNA for NF-κB subunit p65 (Figure 8c). Despite the increase in p65 at the transcript level, an increase in p65 and p50 protein was not detectable by Western blot (data not shown). Likewise, TGRL alone did not increase the amount of activated p65 detected in nuclear fractions over untreated HAECs (Figure 8d). In contrast, within 30 minutes of TNF-α stimulation, there was a dramatic increase in nuclear translocation of active phospho-p65. Moreover, TGRL-primed HAECs exhibited a ≈30% increase in NF-κB activation over TNF-α alone. This correlated with amplification in expression of inflammatory gene products and augmented monocyte recruitment. We confirmed that TGRL primed for enhanced inflammatory response through NF-κB by pretreating HAECs with consensus decoy oligonucleotides that prevented its binding to DNA promoter regions, as previously reported for cytokine mediated inflammation.[23] Transfection of HAECs with decoy oligonucleotides, but not a nonspecific scrambled DNA sequence, abrogated upregulation of all 3 adhesion molecules (supplemental Figure II). Taken together, these data reveal that TGRL

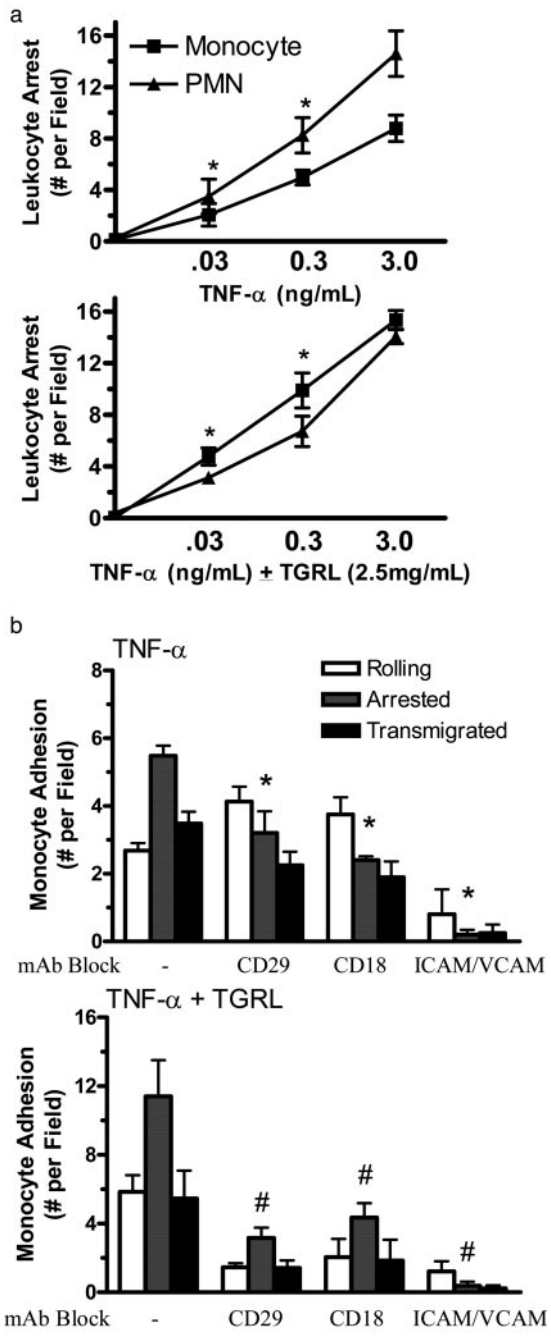

Figure 4. TGRL primes HAECs for monocyte-specific recruitment in an integrin-dependent manner on stimulation with TNF-α. Leukocyte adhesive interactions imaged in microfluidic flow channels assembled over HAEC monolayers and sheared at 2 dyne/cm^2. a, Monocyte and neutrophil arrest on HAECs acutely stimulated over a dose range of TNF-α. Top, TNF-α alone; bottom, HAECs primed with TGRL for 2 h/d over 3 days. *Significant difference in monocyte adhesion over polymorphonuclear leukocyte (PMN) ($P<0.05$; n$\geq$3$\pm$SEM). Monocyte adhesive interactions on HAECs stimulated with TNF-α (0.3 ng/mL) alone (c) or pretreated with TGRL (d). Antibodies were added to block specific adhesion receptors as denoted. Blocking with nonspecific IgG control monoclonal antibodies (denoted as $-$ on the abscissa). *Significant decrease in monocyte arrest and transmigration compared with IgG control ($P<0.05$); #significant decrease in all adhesive interactions ($P<0.05$). Data are number of adherent monocytes$\pm$SEM averaged over 5 separate fields for 3 to 5 blood donors.

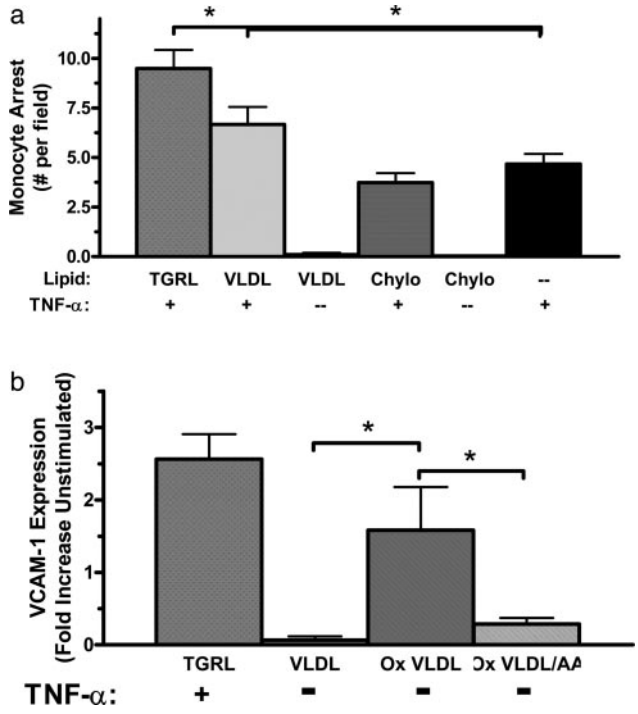

Figure 5. Upregulation of inflammation by TGRL is primarily mediated by nonoxidized VLDL. a, HAECs were incubated with culture media, TGRL (2.5 mg/mL), or isolated TGRL components (VLDL 1.75 mg/mL or chylomicrons 0.75 mg/mL) and then acutely stimulated for 4 hours with TNF-α (0.3 ng/mL), as denoted on the abscissa. *Significant increase in monocyte arrest over TNF-α alone ($P<0.05$). Data are monocyte arrest$\pm$SEM averaged over 5 separate fields for 3 to 5 blood donors. b, HAECs were incubated with unmodified TGRL, VLDL, or oxidized VLDL (OxVLDL) in the presence or absence of ascorbic acid antioxidant (OxVLDL/AA). VCAM-1 expression was detected by flow cytometry and normalized as fold increase over basal media control. *Oxidized VLDL significantly upregulated expression over VLDL alone, and this was significantly reduced in the presence of ascorbic acid ($P<0.05$; n$\geq$3$\pm$SEM)

primes upregulation of p65 along with its inhibitory partner IκB, thereby providing a readily accessible reservoir of cytoplasmic NF-κB for translocation to the nucleus in response to acute inflammatory stimulus.

Discussion

We addressed the hypothesis that repetitive exposure of human aortic endothelium to TGRL exacerbates the inflammatory response to cytokine activation. TGRL levels found to prime for enhanced inflammation in this study were commensurate with circulating levels in normolipidemic individuals (189$\pm$20 mg/dL) hours after consumption of a typical Western diet ($\approx$30% fat, $\approx$20% saturated fat). By comparison, TGRL in metabolic syndrome patients can reach 800 mg/dL and remain elevated for 4 hours following a high-fat meal.[2] Conventional wisdom is that progression of atherosclerosis involves oxidized lipoprotein (OxLDL) binding to scavenger receptors, thereby triggering inflammation. In contrast, we show here that although native TGRL does not directly signal inflammation without first being oxidized, it lowers the threshold dose at which a cytokine elicits VAM expression and increases the efficiency of monocyte recruitment in shear flow.

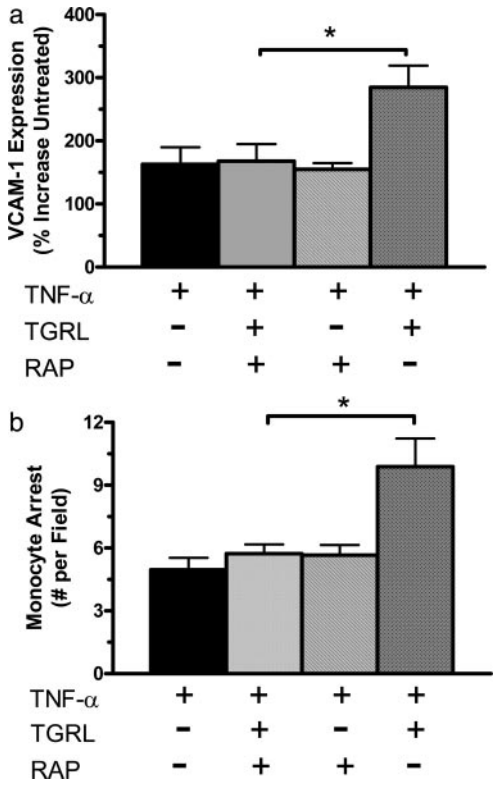

Figure 6. TGRL-primed inflammation is inhibited in the presence of RAP-1. a, VCAM-1 expression on HAEC monolayers primed by TGRL pretreatment for 2 h/d over 3 days and acutely stimulated by TNF-α (0.3 ng/mL) in presence or absence of RAP-1 as denoted on abscissa. *RAP-1 significantly decreased VCAM-1 upregulation ($P<0.05$; n=3). b, Monocyte arrest on HAECs primed with TGRL was significantly downregulated by RAP-1 to the baseline stimulated with TNF-α alone ($P<0.05$; n=3).

We provide the first evidence that TGRL binds aortic endothelium and elicits upregulation of LRP and LR11, known to recognize apoE-laden lipoproteins.[17,19] LRP has been reported to function in VLDL and chylomicron remnant clearance by endocytosis and can also bind tissue factor, resulting in transmembrane signaling involving tyrosine phosphorylation.[5,24,25] LR11 is expressed on smooth muscle cells and its upregulation shown to correlate with cell proliferation and intimal thickening of atherosclerotic arteries.[19,20] Upregulation of lipoprotein receptors suggests that TGRL can elicit a positive-feedback mechanism for increased uptake of apolipoproteins. The finding that RAP-1 blocked TGRL from binding to HAECs may be explained by its capacity to directly antagonize ligand binding to LRP alone, or in a complex with LR11 and other VLDL receptors.[5,17] Experiments to determine whether LR11 and LRP directly colocalize with TGRL and the dependence on caveolae-rich membrane domains during endocytosis of TGRL are underway.[26]

Atherosclerosis exhibits many elements common to chronic inflammatory diseases including upregulation of VAMs and extensive monocyte recruitment.[9,13,14,27] Targeted deletion of E-selectin, ICAM-1, or VCAM-1 in apoE knockout mice revealed that these adhesion molecules are critical for monocyte extravasation into arterial intima.[15,16] A novel vascular mimetic flow chamber was used here to image the multistep process of monocyte recruitment to inflamed HAECs. Monocyte rolling transitioned to arrest and transmigration within a few cell diameters of capture, as also observed by intravital microscopy of mouse models of atherosclerosis.[28] Priming HAECs with TGRL followed by acute inflammation resulted in a doubling of VCAM-1 expression and a 2-fold boost in monocyte arrest and transmigration. Under these conditions, the fraction of monocytes transitioning to arrest and transmigration exceeded that of neutrophils. This may be attributed to an increase in production of the chemokine monocyte chemoattractant protein-1 (data not shown) and activation of β_1 and β_2 integrins on monocytes to bind VCAM-1 and ICAM-1 with high affinity.[29] We hypothesize that repetitive exposure of aortic endothelium to TGRL may provide cues that amplify the efficiency of monocyte recruitment by sensitizing HAECs to inflammatory stimuli and increasing the efficiency of integrin mediated adhesion in shear flow.

The mechanism for this TGRL-mediated effect on endothelium is currently unknown. A recent report by Norata et al has shown that TGRL can modulate the activity of the p38 MAPK pathway, degradation of IκBα and expression of VCAM-1, E-selectin, and monocyte chemoattractant protein-1.[30] Similarly, Dichtl et al has reported that VLDL from fasting, hypertriglyceridemic patients can activate NFκB in vitro and when injected into rats can signal aortic expression of VCAM-1.[31] Although these findings are consistent with our work, there are key points of divergence. Foremost, they found that TGRL alone activated acute inflammation and upregulated ICAM-1, whereas we found no evidence for direct activation in the absence of TNF-α. Moreover, Norata et al reported transient activation of MAPKs including p38 and ERK1/2 within 10 minutes, fading to baseline by 30 minutes, whereas we found a more sustained priming activity of TGRL in that repetitive exposure of HAECs enhanced an acute inflammatory response to cytokine over several hours. Another key difference was that Norata et al and Dichtl et al studied TGRL isolated from hyperlipidemic fasting subjects, as compared with postprandial normolipidemic TGRL applied in our study. TGRL obtained from fasting subjects are metabolically different from postprandial TGRL in that they typically contain smaller particles, which may more readily infiltrate vascular endothelium.[1]

Priming HAECs with TGRL elicited an enhanced inflammatory response over a 1000-fold dose range in TNF-α stimulation. The signaling pathway involved activation of p38 MAPK, as confirmed by pharmacological inhibition of p38 activity with SB202190. In fact, the highest dose of SB202190 effectively abrogated the TGRL priming response, although to a lesser extent blocking TNF-α–stimulated upregulation of E-selectin and VCAM-1. This result might be expected because TNF-α alone potently increased the activity of the Akt and Erk1/2 signaling pathways, whereas enhanced activity of p38 MAPK required costimulation with TGRL. We also assessed the importance of NFκB in the signaling pathway by transfecting HAECs with decoy oligonucleotides that inhibited NF-κB transcriptional activity. The decoy, but not a scrambled peptide, abrogated VAM upregulation in TGRL-primed as well as TNF-α–stimulated HAECs. This confirmed a central role for NF-κB in both

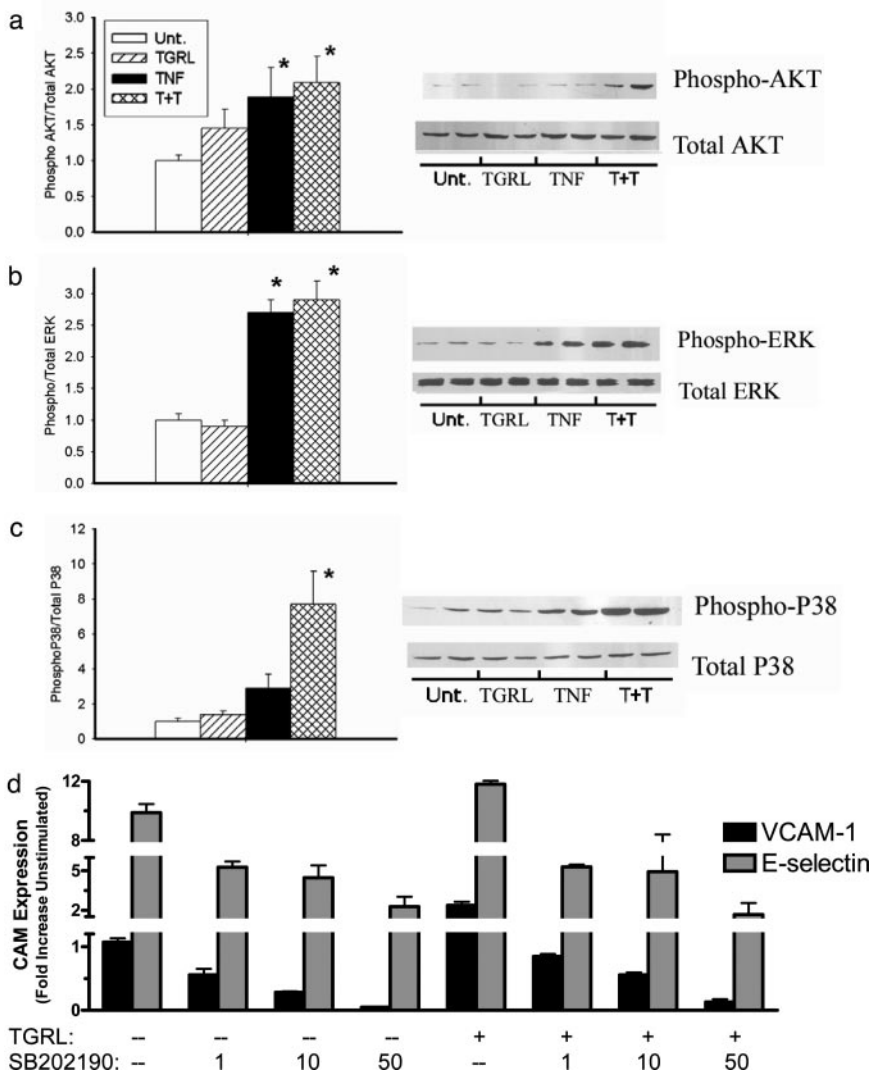

Figure 7. TGRL enhances activation of MAPK over that of TNF-α stimulation. HAEC monolayers incubated with culture media (Untreated) or TGRL with or without TNF-α (0.3 ng/mL) for 15 minutes before cell lysis (a, b, and c) or for 4 hours before flow cytometric analysis (d). TGRL incubations alone did not increase the levels of activated MAPK over untreated control. *, Costimulation with TNF-α significantly upregulated ($P<0.05$) activated p38 over that of TNF-α alone. Data are expressed as fold of untreated based on densitometric analysis of Western blots ($n\geq8\pm$SEM). d, Pretreatment of HAECs with p38 inhibitor SB202190 at concentrations denoted (in μmol/L) before incubations with TGRL and TNF-α significantly inhibited expression of VCAM-1 and E-selectin in a dose-dependent fashion ($P<0.05$). Data are expressed as fold increase of MFI over untreated HAEC controls ($n=3\pm$SEM).

cytokine and TGRL priming of enhanced inflammation. The increase in phosphorylated IKKα (associated with activation of the kinase complex) and p65 mRNA, and concomitant decrease in IκBα inhibitory molecule on costimulation with cytokine and TGRL are consistent with the observed increase in transactivation of NF-κB. We hypothesize that TGRL signals for enhanced p38 MAPK activity that, on cytokine stimulation, leads to more rapid and robust nuclear translocation and transcriptional activity of NF-κB.

Priming of the NF-κB pathway has been observed in vivo in relation to focal susceptibility to atherosclerosis. Hajra et al demonstrated nuclear translocation of NF-κB and induction of proinflammatory genes in susceptible regions of the aortas of hypercholesterolemic LDLR$^{-/-}$ mice.[32] In normal mice, although p65 and IκBs were elevated in such regions, NF-κB activation remained low, suggesting that the pathway was primed but not activated. In this regard, Passerini et al examined the effect of hemodynamics on the steady-state gene expression profiles in the arteries of swine fed a normal diet. In atherosusceptible regions of the vessel, they detected enhanced expression of several proinflammatory cytokines, chemokines, and receptors, as well as elevated transcript levels for components of NF-κB and IκB.[33] They concluded that these regions are primed for inflammation, but activation is kept in check through a dynamic balance of antiinflammatory gene expression. Thus, metabolic risk factors such as elevated TGRL levels may tip the balance toward inflammatory-mediated atherogenesis in susceptible regions of the artery. Modeling repetitive injury of HAECs with native TGRL in our vascular mimetic flow system provides a "laboratory-on-a-chip" approach for gauging its inflammatory potential under defined hemodynamics and dissecting its mechanism(s) of action.

In summary, we demonstrate that repetitive exposure to native TGRL upregulated lipoprotein receptors and primed HAECs for an amplified inflammatory response to TNF-α. Primed HAECs were 10-fold more sensitive to TNF-α–stimulated upregulation of VCAM-1 and E-selectin. Monocytes were preferentially recruited over neutrophils on inflamed HAECs in shear flow as a function of the extent of TGRL priming. This vascular mimetic model of repetitive injury reveals how unmodified lipoproteins contribute to the inflammatory axis of atherogenesis.

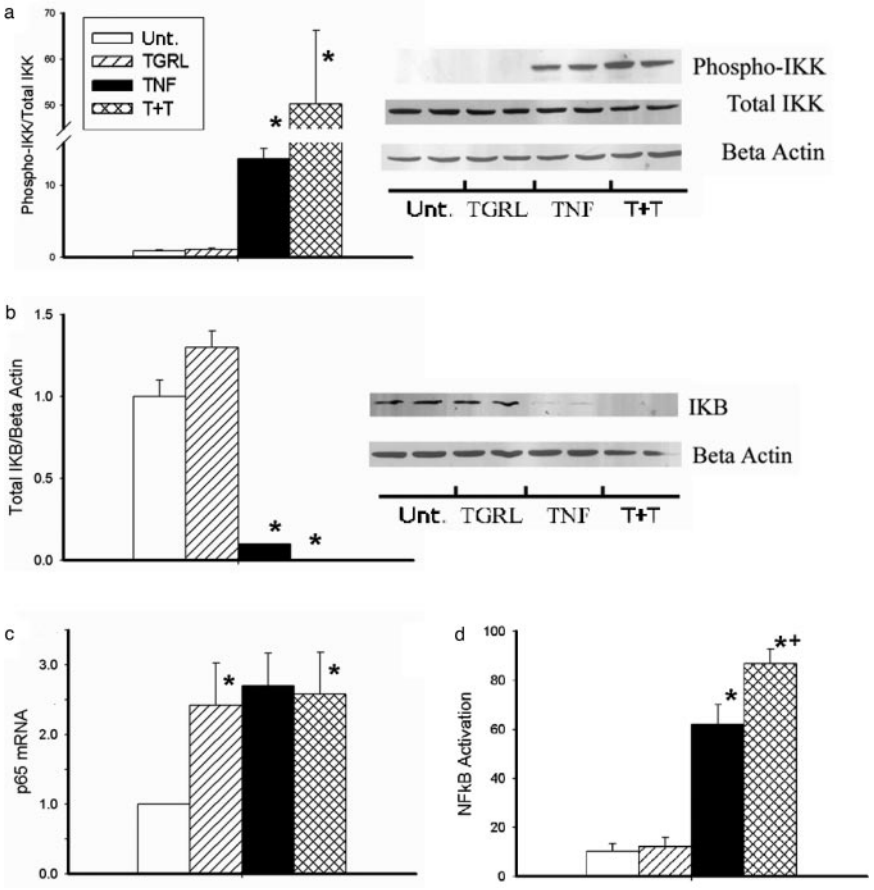

Figure 8. TGRL primes the NF-κB pathway for an enhanced response to TNF-α. HAEC monolayers incubated with culture media (Untreated) or TGRL were incubated in media with or without TNF-α (0.3 ng/mL) for 15 minutes (a and b) or 2 hours (c) immediately before cell lysis. a, TNF-α increased levels of phosphorylated IKK, and TGRL pretreatment significantly upregulated this activation ($P<0.05$). Total IKK levels were elevated and not significantly different between treatments. b, IκB levels were reduced on TNF-α treatment, and TGRL priming abrogated IκB signal ($P<0.05$). TGRL priming alone increased levels of IκB. Data expressed as fold of untreated as quantified by densitometric analysis of Western blots ($n \geq 8 \pm$SEM). c, TGRL priming alone increased p65 mRNA above untreated, expressed as fold increase ($P<0.05$). d, Nuclear translocation of phosphorylated p65 subunit was not different between untreated and TGRL but was increased above TNF-α alone in TGRL-primed HAECs ($P<0.05$). Data expressed as relative luminescence units±SEM of anti–phospho-tyrosine p65 bound to DNA ($n=3$).

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Disclosures

None.

References

1. Karpe F. Postprandial lipoprotein metabolism and atherosclerosis. *J Intern Med*. 1999;246:341–355.
2. Cohn JS, Marcoux C, Davignon J. Detection, quantification, and characterization of potentially atherogenic triglyceride-rich remnant lipoproteins. *Arterioscler Thromb Vasc Biol*. 1999;19:2474–2486.
3. Hodis HN. Triglyceride-rich lipoprotein remnant particles and risk of atherosclerosis. *Circulation*. 1999;99:2852–2854.
4. Chen M, Narumiya S, Masaki T, Sawamura T. Conserved C-terminal residues within the lectin-like domain of LOX-1 are essential for oxidized low-density-lipoprotein binding. *Biochem J*. 2001;355:289–296.
5. Herz J, Hui DY. Lipoprotein receptors in the vascular wall. *Curr Opin Lipidol*. 2004;15:175–181.
6. Martins IJ, Hone E, Chi C, Seydel U, Martins RN, Redgrave TG. Relative roles of LDLr and LRP in the metabolism of chylomicron remnants in genetically manipulated mice. *J Lipid Res*. 2000;41:205–213.
7. Kruth HS. Lipoprotein cholesterol and atherosclerosis. *Curr Mol Med*. 2001;1:633–653.
8. Goudriaan JR, Espirito Santo SM, Voshol PJ, Teusink B, van Dijk KW, van Vlijmen BJ, Romijn JA, Havekes LM, Rensen PC. The VLDL receptor plays a major role in chylomicron metabolism by enhancing LPL-mediated triglyceride hydrolysis. *J Lipid Res*. 2004;45:1475–1481.
9. Libby P. Inflammation in atherosclerosis. *Nature*. 2002;420:868–874.
10. Lusis AJ. Atherosclerosis. *Nature*. 2000;407:233–241.
11. Yuan ZQ, Feldman RI, Sun M, Olashaw NE, Coppola D, Sussman GE, Shelley SA, Nicosia SV, Cheng JQ. Inhibition of JNK by cellular stress- and tumor necrosis factor alpha-induced AKT2 through activation of the NF kappa B pathway in human epithelial Cells. *J Biol Chem*. 2002;277:29973–29982.
12. Puddicombe SM, Davies DE. The role of MAP kinases in intracellular signal transduction in bronchial epithelium. *Clin Exp Allergy*. 2000;30:7–11.
13. O'Brien KD, McDonald TO, Chait A, Allen MD, Alpers CE. Neovascular expression of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in human atherosclerosis and their relation to intimal leukocyte content. *Circulation*. 1996;93:672–682.
14. Ley K, Huo Y. VCAM-1 is critical in atherosclerosis. *J Clin Invest*. 2001;107:1209–1210.
15. Iiyama K, Hajra L, Iiyama M, Li H, DiChiara M, Medoff BD, Cybulsky MI. Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation. *Circ Res*. 1999;85:199–207.
16. Bourdillon MC, Poston RN, Covacho C, Chignier E, Bricca G, McGregor JL. ICAM-1 deficiency reduces atherosclerotic lesions in double-knockout mice (ApoE(-/-)/ICAM-1(-/-)) fed a fat or a chow diet. *Arterioscler Thromb Vasc Biol*. 2000;20:2630–2635.
17. Williams SE, Ashcom JD, Argraves WS, Strickland DK. A novel mechanism for controlling the activity of alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein. Multiple regulatory sites for 39-kDa receptor-associated protein. *J Biol Chem*. 1992;267:9035–9040.
18. Chan JW, Motton D, Rutledge JC, Keim NL, Huser T. Raman spectroscopic analysis of biochemical changes in individual triglyceride-rich lipoproteins in the pre- and postprandial state. *Anal Chem*. 2005;77:5870–5876.

19. Taira K, Bujo H, Hirayama S, Yamazaki H, Kanaki T, Takahashi K, Ishii I, Miida T, Schneider WJ, Saito Y. LR11, a mosaic LDL receptor family member, mediates the uptake of ApoE-rich lipoproteins in vitro. *Arterioscler Thromb Vasc Biol*. 2001;21:1501–1506.
20. Zhu Y, Bujo H, Yamazaki H, Ohwaki K, Jiang M, Hirayama S, Kanaki T, Shibasaki M, Takahashi K, Schneider WJ, Saito Y. LR11, an LDL receptor gene family member, is a novel regulator of smooth muscle cell migration. *Circ Res*. 2004;94:752–758.
21. Read MA, Whitley MZ, Gupta S, Pierce JW, Best J, Davis RJ, Collins T. Tumor necrosis factor alpha-induced E-selectin expression is activated by the nuclear factor-kappaB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways. *J Biol Chem*. 1997;272:2753–2761.
22. Pietersma A, Tilly BC, Gaestel M, de Jong N, Lee JC, Koster JF, Sluiter W. p38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. *Biochem Biophys Res Commun*. 1997;230:44–48.
23. Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. *J Clin Invest*. 2001;107:7–11.
24. Hussain MM, Maxfield FR, Mas-Oliva J, Tabas I, Ji ZS, Innerarity TL, Mahley RW. Clearance of chylomicron remnants by the low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *J Biol Chem*. 1991;266:13936–13940.
25. Webb DJ, Nguyen DH, Sankovic M, Gonias SL. The very low density lipoprotein receptor regulates urokinase receptor catabolism and breast cancer cell motility in vitro. *J Biol Chem*. 1999;274:7412–7420.
26. Yu KC, Chen W, Cooper AD. LDL receptor-related protein mediates cell-surface clustering and hepatic sequestration of chylomicron remnants in LDLR-deficient mice. *J Clin Invest*. 2001;107:1387–1394.
27. Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation*. 2001;104:365–372.
28. van Zandvoort M, Engels W, Douma K, Beckers L, Oude Egbrink M, Daemen M, Slaaf DW. Two-photon microscopy for imaging of the (atherosclerotic) vascular wall: a proof of concept study. *J Vasc Res*. 2004;41:54–63.
29. Wang G, Woo CW, Sung FL, Siow YL, O K. Increased monocyte adhesion to aortic endothelium in rats with hyperhomocysteinemia: role of chemokine and adhesion molecules. *Arterioscler Thromb Vasc Biol*. 2002;22:1777–1783.
30. Norata GD, Grigore L, Raselli S, Seccomandi PM, Hamsten A, Maggi FM, Eriksson P, Catapano AL. Triglyceride-rich lipoproteins from hypertriglyceridemic subjects induce a pro-inflammatory response in the endothelium: molecular mechanisms and gene expression studies. *J Mol Cell Cardiol*. 2006;40:484–494.
31. Dichtl W, Nilsson L, Goncalves I, Ares MP, Banfi C, Calara F, Hamsten A, Eriksson P, Nilsson J. Very low-density lipoprotein activates nuclear factor-kappaB in endothelial cells. *Circ Res*. 1999;84:1085–1094.
32. Hajra L, Evans AI, Chen M, Hyduk SJ, Collins T, Cybulsky MI. The NF-kappa B signal transduction pathway in aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation. *Proc Natl Acad Sci U S A*. 2000;97:9052–9057.
33. Passerini AG, Polacek DC, Shi C, Francesco NM, Manduchi E, Grant GR, Pritchard WF, Powell S, Chang GY, Stoeckert CJ Jr, Davies PF. Coexisting proinflammatory and antioxidative endothelial transcription profiles in a disturbed flow region of the adult porcine aorta. *Proc Natl Acad Sci U S A*. 2004;101:2482–2487.