Lipolysis products from triglyceride-rich lipoproteins increase endothelial permeability, perturb zonula occludens-1 and F-actin, and induce apoptosis

Larissa Eiselein,1 Dennis W. Wilson,2 Michael W. Lamé,3 and John C. Rutledge1
1Division of Endocrinology, Clinical Nutrition, and Vascular Medicine, University of California, Davis, and 2Department of Veterinary Medicine: Pathology, Microbiology and Immunology and 3Department of Veterinary Medicine: Molecular Biosciences, University of California, Davis, California

Submitted 28 June 2006; accepted in final form 22 January 2007

Eiselein L, Wilson DW, Lamé MW, Rutledge JC. Lipolysis products from triglyceride-rich lipoproteins increase endothelial permeability, perturb zonula occludens-1 and F-actin, and induce apoptosis. Am J Physiol Heart Circ Physiol 292: H2745–H2753, 2007. First published January 26, 2007; doi:10.1152/ajpheart.00686.2006.—Products generated from lipoprotein lipase-mediated hydrolysis of triglyceride-rich lipoproteins (TGRL) are reported to increase endothelial layer permeability. We hypothesize that these increases in permeability result from the active rearrangement and dissolution of the junctional barrier in human aortic endothelial cells, as well as induction of the apoptotic cascade. Human aortic endothelial cells were treated with TGRL lipolysis products generated from co-culture of human TGRL plus lipoprotein lipase. Measurement of transendothelial electrical resistance demonstrated a time-dependent decrease in endothelial barrier function in response to TGRL lipolysis products. Immunofluorescent localization of zonula occludens-1 (ZO-1) showed radial rearrangement along cell borders after 1.5 h of treatment with lipolysis products. A concurrent redistribution of F-actin from the cell body to the cell margins was observed via rhodamine phalloidin staining. Immunofluorescent imaging for occludin and vascular endothelial cadherin showed that these proteins relocalize as well, although these changes are less prominent than for ZO-1. Western analysis of cells exposed to lipolysis products for 3 h revealed the fragmentation of ZO-1, a reduction in occludin, and no change of vascular endothelial cadherin. Lipolysis products also increased caspase-3 activity and induced nuclear fragmentation. Treatments did not cause oncosis in cells at any point during the incubation. These results demonstrate that TGRL lipolysis products play an important role in the regulation of endothelial permeability, the organization of the actin cytoskeleton, the localization and expression of junctional proteins, especially ZO-1, and the induction of apoptosis.

endothelium; lipoprotein lipase

DESPITE SIGNIFICANT MEDICAL advances, the complications of atherosclerosis remain the leading cause of death in Western society (2, 8). In this disorder, injury or dysfunction of the endothelium is one of the earliest changes in the artery wall. One parameter observed in endothelial dysfunction is an increase in endothelial layer permeability, which is thought to allow for augmented accumulation of lipoproteins in the subendothelial space (26, 34).

Control over vascular permeability largely depends on the presence of specialized junctions between adjacent cells (4, 18, 19). In endothelial cells, two classical complexes of junctional proteins exist: tight and adherens junctions. The tight junction is the more apical of these intercellular linking junctions and consists of an assembly of membrane-associated and membrane-spanning proteins. Zonula occludens-1 (ZO-1) is a tight junction-associated protein located in the submembranous region of the junction (18, 19). ZO-1 possesses multiple binding domains, allowing it to interact with other cellular proteins. The proline-rich COOH terminus of ZO-1, for instance, mediates binding to the actin cytoskeleton (15, 45), whereas the guanulate kinase domain and acidic domain allow binding to the transmembrane protein occludin (15, 16). It is this unique positioning of ZO-1, coupling the structural and dynamic properties of the cytoskeletal network to the paracellular barrier, and its ability to interact with transcription factors and signaling molecules (4) that make it a key protein in the control of endothelial layer permeability and the assembly and organization of the tight junction (19, 42). Although increased expression of the membrane-spanning tight junction protein occludin has also been shown to correlate with enhanced barrier function (12, 19, 20, 43), occludin knock-out studies have shown that this protein, unlike ZO-1, is not required to form structurally intact tight junctions (35, 36).

In the adherens junction, a family of Ca2+−dependent proteins called cadherins are the major transmembrane components and are responsible for the mechanical cell-cell contacts. Endothelial cells express a specific cadherin called vascular endothelial cadherin (VE-cadherin), which has also been shown to participate in the dynamic regulation of paracellular permeability (41, 44).

Besides their key role in regulating barrier function, junctional proteins may also affect cell survival by signaling cell-cell attachment. Close cell-to-cell contacts are thought to be important in anchorage-dependent cells, such as endothelial cells, acting as survival signals. It has been well documented that cleavage of proteins found in the adherens junction, such as β-catenin or plakoglobin, can interrupt extracellular survival signals required for normal cell maintenance, activating the apoptotic pathway (23). Furthermore, studies showed that VE-cadherin−/− cells are more prone to undergo programmed cell death and that administration of VE-cadherin-blocking antibodies inhibited the protective effect of VEGF on apoptosis (10). Recent in vitro studies have revealed that ZO-1 location and integrity may be compromised in cells undergoing programmed cell death (apoptosis), suggesting that ZO-1 could also be a key modulator of endothelial apoptosis (9, 38). An increasing number of in vivo and in vitro studies have linked apoptosis to the initiation and progression of inflammatory responses.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
AJP-Heart Circ Physiol • VOL 292 • JUNE 2007 • www.ajpheart.org

LIPOLYSIS PRODUCTS INDUCE REARRANGEMENT AND APOPTOSIS

H2746

In recent years, high plasma concentrations of lipoproteins rich in triglyceride content have gained attention as strong and independent risk factors for the development of atherosclerosis (24). This association has largely been related to lipoprotein particle size with smaller particles, such as very low density lipoproteins (VLDL) remnants, being primarily implicated in disease development (31). This work suggests that these smaller particles can enter the artery wall and initiate atherogenesis. Recent work, however, revealed that triglyceride-rich lipoproteins (TGRL), and especially TGRL lipolysis products, can cause vascular injury by other mechanisms. Whereas unmodified TGRL, such as chylomicrons and VLDL, seem to cause vascular injury by other mechanisms. Whereas unmodified TGRL, such as chylomicrons and VLDL, seem to have little effect on endothelial permeability, lipolysis products generated from lipoprotein lipase (LPL)-mediated hydrolysis of TGRL have been shown to decrease barrier function significantly (21, 34).

The goal of this study was to determine whether TGRL lipolysis products could cause an increase in paracellular permeability in human aortic endothelial cells (HAEC). We also asked whether TGRL lipolysis products could modify key junctional proteins, the actin cytoskeleton, and/or activate the apoptotic cascade.

MATERIALS AND METHODS

Cell culture. HAEC were purchased from Cascade Biologics (Portland, OR) and were maintained at 37°C under 5% CO2 in medium 200 supplemented with 2% FBS, 1 µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, and 10 µg/ml heparin (Cascade Biologics). All experiments were performed on cells at passage 5.

Treatments. Treatment groups included serum-free medium, TGRL alone, LPL alone, and TGRL plus LPL. TGRL were obtained from the plasma of young, healthy volunteers by ultracentrifugation in a SW-41 rotor (Beckman Coulter, Fullerton, CA) at 40,000 rpm at 14°C for 18 h, using 1.0063 mg/dl density gradient solution. The protocol for blood collection was approved by a UC Davis School of Medicine Institutional Review Board (no. 20031166-19). After overnight dialysis, the triglyceride concentration was determined with an enzymatic assay kit (Sigma, St. Louis, MO). TGRL were diluted to a final concentration of 150 mg/dl of triglycerides with cell culture medium. The enzyme LPL (Sigma) was added at a concentration of 2 U/ml. All treatments were prepared and then preincubated at 37°C for 30 min before they were added to cell cultures.

Transendothelial electrical resistance measurements. HAEC were plated on fibronectin-coated Transwell cell culture membranes (pore size 1.0 µm) (BD Falcon, Pittsburgh, PA) and grown to confluence in 12-well cell culture plates (BD Falcon). Transendothelial electrical resistance (TER) was measured using an EndOhm chamber (World Precision Instruments, Sarasota, FL). To verify monolayer confluency, experiments were performed 1 day after resistance measurements no longer changed. Baseline TER was determined, and measurements were made every 30 min. All measurements were carried out in a Faraday cage to prevent electrical interference. Ten replicate TEER experiments were performed for each treatment group, and data were analyzed by ANOVA for multiple comparison groups followed by a Bonferroni multiple comparison test. Statistical significance was assigned at P < 0.05.

Immunofluorescence and actin stain. HAEC were grown to confluence on fibronectin-coated 12-mm round coverslips in 24-well medical-grade polystyrene plates (BD Falcon) and were treated as described for 1.5 h. After treatment, cells were fixed with 1% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized and blocked with Superblock (Pierce, Rockford, IL) with 0.05% saponin for 30 min. Fixed cells were incubated with mouse monoclonal anti-ZO-1 (Zymed, South San Francisco, CA) (1:250), rabbit polyclonal anti-occludin (Zymed) (1:250), or mouse monoclonal anti-VE-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA) (1:250) for 1 h. Cells were then treated with goat anti-mouse antibody conjugated to Alexa fluor 568 (Molecular Probes, Eugene, OR) (1:650) or goat anti-rabbit antibody conjugated to Alexa fluor 488 for 30 min. For filamentous actin staining, cells were permeabilized with 0.1% Triton-X 100, blocked with Superblock (Pierce), and incubated with rhodamine-phallolidin (Molecular Probes) for 20 min at room temperature. After cells were mounted, cells were evaluated and photographed with an Olympus Provis system fluorescence microscope. The images shown are representative of at least three separate experiments.

Western blotting. HAEC were grown to confluence in T-75 cell culture flasks (BD Falcon) and then treated as described for 3 h. Cells were scraped from the bottom of the flask and lysed (Pierce NE-PER lysis kit). Protein concentration was determined with the bichinchoninic acid assay (Pierce), and equal amounts of proteins were separated by SDS-PAGE. The 11% total acrylamide, 2.75% crosslinker running gel was overlaid with a 4% total acrylamide, 2.75% crosslinker stacking gel and run at 4°C on a Hoefer SE-600 vertical tank chamber (Hoefer Scientific Instruments, San Francisco, CA) at 10 mA for an average of 5.5 h. Proteins were then transferred onto 0.2-µm polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA), blocked with 5% nonfat milk (Bio-Rad) for 1 h and then probed with ZO-1 monoclonal mouse antibody (Zymed) (1/1,000), occludin polyclonal rabbit antibody (Zymed) (1/1,000), VE-cadherin monoclonal mouse antibody (Santa Cruz) (1/200), or caspase-3 monoclonal mouse antibody (Imgenex, San Diego, CA) (1/500) at 4°C overnight. Membranes were then incubated with horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibody (1/10,000) (Amersham, Piscataway, NJ) for 1 h. Blots were developed with the enhanced chemiluminescence detection system according to manufacturer’s instructions (Amersham). Blots shown are representative of three separate experiments. To ensure loading of equal amounts of protein, all blots were also stripped with Restore Buffer (Pierce), washed, blocked, and probed with mouse monoclonal anti-b-actin (Sigma) (1/5,000) as described.

Caspase-3 activity assay. A caspase-3 cellular activity assay kit was purchased from EMD Biosciences (San Diego, CA), and cell extracts were prepared from endothelial cells grown to confluence in T-75 cell culture flasks according to kit instructions. Protein concentrations of cell extracts were determined by the Bradford assay (Pierce), and equal amounts of proteins were separated by SDS-PAGE. The 11% total acrylamide, 2.75% crosslinker running gel was overlaid with a 4% total acrylamide, 2.75% crosslinker stacking gel and run at 4°C on a Hoefer SE-600 vertical tank chamber (Hoefer Scientific Instruments, San Francisco, CA) at 10 mA for an average of 5.5 h. Proteins were then transferred onto 0.2-µm polyvinylidene difluoride membranes (Bio-Rad) and then treated as described for 3 h. Cells were scraped from the bottom of the flask and lysed (Pierce NE-PER lysis kit). Protein concentration was determined with the bichinchoninic acid assay (Pierce), and equal amounts of proteins were separated by SDS-PAGE. The 11% total acrylamide, 2.75% crosslinker running gel was overlaid with a 4% total acrylamide, 2.75% crosslinker stacking gel and run at 4°C on a Hoefer SE-600 vertical tank chamber (Hoefer Scientific Instruments, San Francisco, CA) at 10 mA for an average of 5.5 h. Proteins were then transferred onto 0.2-µm polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA), blocked with 5% nonfat milk (Bio-Rad) for 1 h and then probed with ZO-1 monoclonal mouse antibody (Zymed) (1/1,000), occludin polyclonal rabbit antibody (Zymed) (1/1,000), VE-cadherin monoclonal mouse antibody (Santa Cruz) (1/200), or caspase-3 monoclonal mouse antibody (Imgenex, San Diego, CA) (1/500) at 4°C overnight. Membranes were then incubated with horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibody (1/10,000) (Amersham, Piscataway, NJ) for 1 h. Blots were developed with the enhanced chemiluminescence detection system according to manufacturer’s instructions (Amersham). Blots shown are representative of three separate experiments. To ensure loading of equal amounts of protein, all blots were also stripped with Restore Buffer (Pierce), washed, blocked, and probed with mouse monoclonal anti-b-actin (Sigma) (1/5,000) as described.

Morphological assessment of apoptosis and/or oncosis. HAEC were grown to confluence on fibronectin-coated 12-mm round coverslips and were treated as indicated. Cells were differentially stained with nucleophilic fluorescent dyes as previously described (40). Briefly, cells were incubated with the membrane-impermeant nucleic acid stain ethidium homodimer-1 (8 µM) (Molecular Probes) for 15 min at 37°C in the dark, followed by three washes in PBS and fixation in 1% paraformaldehyde for 5 min. Next, cells were permeabilized, and nuclei were counterstained by incubation with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (1 µg/ml in PBS with 0.1%

Disease, such as atherosclerosis, but our understanding of the underlying mechanisms is rudimentary (5, 33).

Susceptibility was assigned at P < 0.05.

AJP-Heart Circ Physiol • VOL 292 • JUNE 2007 • www.ajpheart.org

Downloaded from http://ajpheart.physiology.org/ by 10.20.33.6 on April 3, 2017
Triton X-100) for 5 min at room temperature. Cells were then washed in 1% paraformaldehyde and mounted with anti-fade medium. Slides were photographed with an Olympus Provis system fluorescence microscope with a broad-range UV filter. Cells exhibiting round, DAPI-stained (blue) nuclei were categorized as normal, cells with ethidium homodimer-1-stained nuclei (red) were labeled as undergoing oncotic necrosis, and cells displaying DAPI-stained (blue) condensed and fragmented nuclei were called apoptotic. Images shown are representative of three separate experiments.

RESULTS

**TGRL lipolysis products increase endothelial cell layer permeability.** To study the effect of lipolysis products on endothelial cell permeability, we measured TEER across endothelial cell monolayers. Measured TEER values ranged from 10 to 35 Ω. Exposure to TGRL lipolysis products (150 mg/dl TGRL + 2 U/ml LPL) resulted in a time-dependent decrease in resistance (increase in monolayer permeability) compared with control cells (Fig. 1). TEER began to significantly decrease after 90 min of treatment with TGRL lipolysis products and fell to almost 40% of baseline resistance after 330 min. We observed initial increases in TEER at the beginning of treatment for the control (medium, TGRL, and LPL) and TGRL lipolysis product groups (TGRL + LPL). TEER for the medium and TGRL controls returned to ~100% baseline after 330 min, whereas the TEER for cells treated with LPL alone continued to be slightly but not significantly increased.

**TGRL lipolysis products induce junctional protein rearrangement.** To analyze the effect of lipolysis products on the distribution of the tight junction proteins ZO-1 and occludin and the adherens junctional protein VE-cadherin, we performed immunofluorescent staining.

For ZO-1, control cells exposed to cell culture medium, TGRL alone, or LPL alone demonstrated smooth lines of continuous staining along cell-cell contacts (Fig. 2). Cells exposed to TGRL lipolysis products for 1.5 h had discontinuity and irregularity of ZO-1 distribution at the intercellular junctions. In these cells, ZO-1 rearranged from an uninterrupted band along cell borders into radially oriented sporadic aggregates.

A similar trend of rearrangement could be observed for VE-cadherin, although the effect seemed to be more pronounced for ZO-1. As for ZO-1, cells stained with anti-VE-cadherin showed more discontinuous borders for monolayers treated with TGRL lipolysis products compared with the controls. Gaps between adjacent cells were visible.

In monolayers probed with anti-occludin antibody, we were unable to show localization of the junctional protein to the cell borders exclusively. Rather, we observed occludin distributed uniformly across cells and along the cell margin. For monolayers treated with TGRL lipolysis products, occludin became centralized to the cell interior as cells retracted from each other.

**TGRL lipolysis products cause disruption of the F-actin network.** To examine the effects of lipolysis products on the cytoskeletal F-actin network, we stained HAEC monolayers with rhodamine phalloidin. In cells treated with medium, TGRL alone, or LPL alone, actin fibers were mostly organized in peripheral dense bands and in actin stress fibers spanning throughout the cell body (Fig. 3, A–C). After 1.5 h of treatment with lipolysis products, the actin cytoskeleton was disrupted and stress fibers disappeared completely from the central region of the cell, where instead fragmented, short-length filaments were present (Fig. 3D). A moderate number of peripheral dense fibers along cell boarders were still evident in lipolysis product-treated cells. With the disappearance of the actin fibers from the cell interior, there was a prominent marginalization and clustering of microfilaments along cell borders. In addition, there were gaps between adjacent endothelial cells with a moderate amount of F-actin fibers traversing those spaces.

**TGRL lipolysis products cause ZO-1 degradation.** To assess the effect of lipolysis products on the expression of junctional proteins, we performed Western blots probing for ZO-1, occludin, and VE-cadherin.

As shown in Fig. 4A, major bands staining with anti-ZO-1 antibody spanned from ~240 to 160 kDa for extracts from cells treated with cell culture medium, TGRL alone, or LPL alone. When cells were treated with TGRL lipolysis products for 1.5 h, those originally high molecular mass bands were still present. Treatment of cells for 3 h with TGRL lipolysis products significantly reduced the presence of original bands, whereas smaller bands in the 160- to 55-kDa range appeared. When top bands were analyzed for optical density (Fig. 4B), a decrease of ZO-1 protein to 26% of the medium control was observed for cells treated with TGRL lipolysis products for 3 h. At 1.5 h, where ZO-1 rearrangement along cell borders had been observed, no significant ZO-1 fragmentation was detected. Although Fig. 4B also shows that treatment with TGRL alone and LPL alone slightly reduced the density of the top ZO-1 bands, this reduction was not significant (85% and 88% of the medium control, respectively).

Bands staining for the protein occludin were observed at ~65 kDa (Fig. 4A). Treatment with TGRL alone, LPL alone, or TGRL + LPL for 1.5 h did not significantly alter band density compared with the medium control (Fig. 4B). When cells were treated with TGRL lipolysis products for 3 h,
however, occludin protein content was significantly reduced to 78% of medium control. In contrast to ZO-1, no band fragments of smaller molecular mass were detected for occludin.

Analysis of the adherens junctional protein VE-cadherin (bands observed at 130–90 kDa) by Western blotting showed no significant or detectable change in top band density for any of the applied treatments (Fig. 4).

To exclude the possibility of immuoreactivity of the antibody to lipolysis products directly, a control group was included in which TGRL/H11001LPL was incubated in flasks without cells and then processed and treated similarly to other controls. As shown in Fig. 4A, no immunoreactivity of lipolysis products was detected in any of the tested antibodies.

*TGRL lipolysis products increase caspase-3 activity.* To determine whether TGRL lipolysis products could cause programmed endothelial cell death (apoptosis), we analyzed the cellular activity levels of caspase-3, an enzyme activated in apoptotic cells. Caspase-3 activity was measured by fluorometric assay and Western blotting.

Analysis of cell extracts with a caspase-3 activity assay kit showed increased activation of the enzyme for cells treated with TGRL lipolysis products compared with cells treated with cell culture medium (Fig. 5A). Activity started to increase slightly after 1.5 h of treatment. After 3 h of treatment with TGRL lipolysis products, activity was found to be maximal, having increased more than 10 times compared with control cells. The drop in activity after 4 h of treatment with lipolysis products was attributed to the beginning of cell death and loss in function of the enzyme. Cell extracts from cells treated with TGRL alone or LPL alone showed no significant increase in caspase-3 activity compared with control cells.

Caspase-3, like all caspases, requires cleavage of the pro-enzyme to become activated. Activation can be determined by analyzing the cleavage of procaspase-3 into a 17- and 12-kDa larger and smaller catalytic subunit. We were able to monitor those cleavage events via Western blotting (Fig. 5B). Whereas only the pro (inactive) form of caspase-3 (at 32 kDa) was detected for cells treated with medium, TGRL alone, or LPL alone, both the pro and active (cleaved) forms were present in cells treated with TGRL lipolysis products for 3 h (see arrowhead for catalytic subunits in Fig. 5). Unlike with the fluorometric caspase-3 assay kit, we were unable to detect caspase-3 cleavage (activation) for cells treated with TGRL lipolysis products for 1.5 h. No immunoreactivity to the antibody was detected with lipolysis products directly loaded onto the gel without cell lysate.

*TGRL lipolysis products induce apoptosis but not oncosis.* To determine whether treatment with lipolysis products would result in apoptotic or oncocytic cell death, we performed studies that examined nuclear morphology and membrane permeability. Cells were identified as apoptotic when showing condensed or blebbed blue (DAPI-stained) nuclei, whereas cells were characterized as oncocytic when there was indication of permeable cell membranes (evidenced by red nuclei, stained with...
membrane-impermeant ethidium homodimer-1). Staurosporine-treated cells (0.1 μM) were used as positive controls for oncosis and oncosis with apoptosis in these studies (Fig. 6D, right and left insets, respectively).

Cells treated with cell culture medium, TGRL alone, or LPL alone showed no signs of apoptosis or oncosis and exhibited brightly fluorescing round, blue-stained nuclei (Fig. 6, A–C). After 2 h of treatment with TGRL lipolysis products, there were increasing numbers of cells with condensed and fragmented nuclei, an indication of apoptosis (Fig. 6D). There were no signs of cell oncosis in cells treated with lipolysis products for up to 3 h.

**DISCUSSION**

It is well established that oxidized low-density lipoproteins play a role in atherogenesis (11) by inducing endothelial cell apoptosis (28) and increased endothelial permeability (14). There is still a paucity of research, however, on the effects of lipolysis products generated from LPL-mediated hydrolysis of TGRL on vascular cells. LPL is an enzyme central to lipid metabolism and is found bound to the vascular endothelium. There, LPL is responsible for the hydrolysis of TGRL, such as chylomicrons and VLDL, into smaller remnant lipoproteins, free fatty acids, phospholipids, monoglycerides, and diglycerides. Although a few studies have shown that remnant lipoproteins and some free fatty acids cause increases in permeability and apoptosis (3, 22, 25, 37), additional results have indicated that other free fatty acids do not affect or even reduce permeability (22, 25). Our study was designed to supplement the limited information that exists with regard to the effects of lipolysis products from TGRL on endothelial barrier function. To do so, we incubated physiological concentrations of freshly isolated human TGRL with LPL to generate a mix of lipolysis products similar to what the vascular endothelium would encounter after a meal.

Our results show that products generated from LPL-mediated hydrolysis of TGRL can promote a proatherogenic environment in HAEC by increasing paracellular permeability. Specifically, we show that the observed rise in monolayer permeability occurs concurrently with a rearrangement of the tight junction proteins ZO-1 and occludin, adherens junctional VE-cadherin, and cytoskeletal F-actin. Furthermore, we demonstrate that this disturbance of the tight and adherens junctions precedes the activation of the apoptotic cascade and ZO-1 proteolysis. Together, these findings indicate that TGRL lipolysis products can open paracellular junctions and thus may promote the accumulation of lipoproteins in the subendothelial space.

Multiple studies employing cytokines, hormones, and growth factors have demonstrated that ZO-1 localization at the tight junction is important in the regulation of paracellular permeability and that delocalization of ZO-1 from the junction results in decreased barrier function (1, 19). A recent study showed that oxidized phospholipids cause changes in ZO-1 localization along cell-cell contacts and concomitantly increase the diffusional permeability in bovine aortic endothelial cells (13). Our study demonstrates that treatment with lipolysis products results in a similar increase in monolayer permeability in cells of human origin (Fig. 1) and that this rise in permeability occurs concurrently with the rearrangement of ZO-1 along cell borders (Fig. 2). Similar to ZO-1, adherens junctional VE-cadherin was also reorganized and was found to be disrupted from cell borders after treatment with lipolysis products. Pellegrino et al. (30) reported a comparable rearrangement of VE-cadherin with a concurrent rise of transendothelial permeability after treatment of coronary artery endothelial cells.
with lipoprotein (a), a well-known risk factor for the development of atherosclerosis. Although our observed occludin immunoreactivity along HAEC borders was markedly more discontinuous in control cells than in those with the ZO-1 and VE-cadherin stains, we were still able to show centralization of occludin away from cell borders after treatment with lipolysis products, another indication for tight junction disassembly.

Previous studies have also emphasized the importance of the association of the tight junction complex with cytoskeletal elements, particularly F-actin, to form strong paracellular barriers (6, 17, 27, 32). Not only does actin actively participate in the regulation of paracellular permeability by contraction and pulling on junctional components, but loss of anchorage to actin is also thought to destabilize the junction and lead to decreased barrier function. Palladini et al. (29) showed that oxidized cholesterol can induce the dissolution of stress fibers and clustering of F-actin filaments along cell borders and that these changes precede the activation of the apoptotic cascade in bovine endothelial cells. Here, we demonstrate that TGRL lipolysis products can induce similar changes in HAEC and show the disappearance of stress fibers from the cell body as well as marginalization of F-actin in response to treatment with lipolysis products (Fig. 3).

Our results also indicate that the described destabilization of the tight and adherens junctions via VE-cadherin, occludin, and especially ZO-1 and F-actin rearrangement (first detected after 1.5 h of treatment) occurs before activation of the apoptotic cascade (significant activation after 2 h of treatment). This observation might place new emphasis on the importance of the tight and adherens junctions and their cytoskeletal anchoring partners for maintenance of close cell-to-cell contacts and survival pathways, which prevent activation of the default death program.

In our study, the induction of endothelial cell apoptosis was shown by increased caspase-3 activity (Fig. 4A) and cleavage of the procaspase form into the smaller active subunits (Fig. 4B). Our findings support the notion for a proatherogenic role of TGRL lipolysis products in the initiation of atherosclerotic cardiovascular disease because, although apoptosis is an essential component of normal vascular growth and regeneration, abnormally regulated or excessive apoptosis might contribute to atherogenesis by disturbing the integrity of the endothelial cell monolayer. The exact mechanisms and consequences of apoptosis in the development and progression of the disease remain to be elucidated.

We speculate from our findings that the observed induction of apoptosis hinges on changes in localization of ZO-1, occludin, VE-cadherin, and F-actin and destabilization of the tight junction. This might lead to a reduced ability for close cell-cell contact formation and activation of the default death program. Once initiated, the activated apoptotic caspases could further degrade the junction by protein catalysis. This theory is sup-

Fig. 4. A: Western blots of membranes probed with antibody to ZO-1, occludin, VE-cadherin, and β-actin. Endothelial cells treated with TGRL + LPL for 3 h (TL3) show significantly reduced expression of the complete ZO-1 protein and have smaller molecular mass fragments than control cells. Occludin expression was also diminished after 3 h of treatment with lipolysis products (TL3), whereas VE-cadherin expression was largely unchanged. B: densitometric analysis of Western blot. Top bands are compared with medium control. Both ZO-1 and occludin bands were significantly reduced after 3 h of treatment with lipolysis products (TL3) (to 26 and 78% of medium control, respectively). VE-cadherin band density was not significantly altered. M, medium; T, 150 mg/dl TGRL; L, 2 U/ml LPL; TL1.5 and TL3, 150 mg/dl TGRL + 2 U/ml LPL for 1.5 or 3 h, respectively; TL-, 150 mg/dl TGRL + 2 U/ml LPL alone (no cell lysate). *P < 0.05.
ported by the recent finding that junctional proteins can be targeted by enzymes of the apoptotic cascade in epithelial cells. Bojarski et al. (7) have found that ZO-1, among other tight junction proteins, became fragmented during staurosporine-induced apoptosis and that this catalysis of ZO-1 was caspase-3 specific. Here, we show a similar fragmentation of ZO-1 at the height of caspase-3 activity during lipolysis product-induced apoptosis (Fig. 5). Previous studies in our laboratory (39) have shown a similar catalysis of ZO-1 after treatment with thrombin. The proteolysis and reduced expression of key modulators

![Image](http://ajpheart.physiology.org/)

**Fig. 5.** A: caspase-3 activity assay. Extracts from cells treated with 150 mg/dl TGRL + 2 U/ml of LPL show significantly increased caspase-3 activity after 1.5 h (TL1.5) compared with extracts obtained from cells treated with medium (M), TGRL alone (T), or LPL alone (L). Activity is maximal after 3 h of treatment with lipolysis products (TL3). *P < 0.05. B: Western blot of membrane probed with antibody to pro- and active (cleaved) caspase-3. Lysates from cells treated with 150 mg/dl TGRL + 2 U/ml of LPL for 3 h (TL3) show emergence of the cleaved smaller molecular mass active subunits (see arrowhead). Only the larger pro-caspase-3 form is present in control groups treated with medium, TGRL alone, LPL alone, or TGRL + LPL for 1.5 h (TL1.5). No immunoreactivity to the antibody was observed for lanes containing lipolysis products alone (no cell lysate).

![Image](http://ajpheart.physiology.org/)

**Fig. 6.** Ethidium homodimer-1 and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) stain after 3-h treatment. Cells treated with 150 mg/dl TGRL + 2 U/ml LPL exhibit DAPI-stained, clumped, and fragmented nuclei in the absence of ethidium homodimer staining, suggesting apoptosis with intact cell membranes (D). Control cells treated with medium (A), TGRL alone (B), or LPL alone (C) show no signs of apoptosis or necrosis. Insets: examples of oncosis (right) and apoptosis with oncosis (left) from staurosporine-treated cells.
of barrier function, such as ZO-1, have been shown to increase paracellular permeability (19). Interestingly, although a slight reduction in occludin content after 3 h of treatment with lipolysis products was also observed, VE-cadherin protein content did not seem to be affected (at least up to 3 h of treatment with lipolysis products).

Regulated disruptions of the tight and adherens junction and the F-actin cytoskeleton appear to represent crucial steps during lipolysis product-induced apoptosis. During this process, an increase in endothelial permeability may contribute to augmented diffusion of lipids into the subendothelial space via the paracellular route. The accumulation of lipids, such as low-density lipoprotein, beneath the endothelium then is thought to trigger inflammatory events in the arterial wall that lead to plaque development. In this context, our results suggest that increased levels of blood triglycerides and the associated rise in lipolysis products around the vascular endothelium represent important causative factors for the development of the disease. Our study shows a potential pathogenic mechanism by which lipolysis products can injure endothelial cells. Specifically, we have shown that TGL lipolysis products increase monolayer permeability and disrupt key modulators of barrier function, ZO-1, occludin, VE-cadherin, and F-actin. We have also demonstrated that lipolysis products can subsequently induce apoptosis but not necrosis in HAEC.

REFERENCES


