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Research Article

Sphingosine 1-phosphate signal survival and mitogenesis are mediated by lipid-stereospecific binding of triacylglycerol-rich lipoproteins

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Abstract. Proof for the role of triacylglycerol-rich lipoproteins (TRLs) in the development of cardiovascular events is accumulating. We recently reported that postprandial TRLs bind to and internalize into human aortic vascular smooth muscle cells (HA-VSMCs) by a lipid-dependent mechanism. We now show that postprandial TRLs triggered hydrolysis of sphingomyelin and stimulation of the sphingosine kinase producing sphingosine 1-phosphate (S1P). In addition, postprandial TRLs exhibited survival and mitogenic effects. Interestingly, the signals were modulated by the nature of the fatty acids located at the sn-2 position in the triacylglycerol molecules of TRL. This lipid-stereospecific regulation of S1P cellular levels in HA-VSMCs provides a novel insight into the intrinsic role of dietary fatty acids and the mechanism mediated by triacylglycerol-containing postprandial lipoproteins in the pathogenesis of atherosclerosis.

Key words. Sphingolipid; VSMC; triacylglycerol-rich lipoprotein; postprandial metabolism.

Oleic acid is one of the most abundant fatty acids in membrane phospholipids, triacylglycerols, and cholesterol esters and plays a major role in membrane fluidity, adiposity, and lipoprotein metabolism. Stearoyl-CoA desaturase (SCD) is the microsomal enzyme required for the biosynthesis of oleic acid. SCD gene expression is known to be highly regulated [1–3]. Yet oleic acid is one of the most abundant fatty acids in the diet.

Dietary intake of oils rich in oleic acid has been associated with several beneficial effects in healthy subjects and in patients with cardiovascular risk factors [4–7]. Emerging evidence supports the importance of oleic acid in modulating the postprandial response of triacylglycerol-rich lipoproteins (TRLs) [8, 9], which predicts asymptomatic and symptomatic atherosclerosis [10–13]. Histological and cell culture studies indicate that postprandial TRLs can enter into the vascular wall and display a high degree of plasticity in smooth muscle cells (SMCs) [14, 15]. Furthermore, we recently demonstrated a novel lipid-dependent mechanism of clearance of postprandial TRLs [16, 17] that was corroborated by in vitro analysis of TRL interaction with SMCs [18]. Of major interest, postprandial TRLs can stimulate cell proliferation via the activation of mitogen-activated protein kinases (MAPKs) ERK1 and ERK2 ($p44^{MAPK}$ and $p42^{MAPK}$, respectively) in pathways initiated by G protein-coupled receptors [19], suggesting a potential molecular link to their atherogenesis-promoting ability. Similar cellular effects, preceded by an increase in neutral sphingomyelinase activity, sphingomyelin turnover to ceramide, sphingosine, and sphingosine 1-phosphate (S1P), have been shown for oxidized low density lipoproteins [20, 21]. Sphingolipids

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were originally though to play a predominantly structural role as components of lipid bilayers, but are now also known to be required for survival of yeast [22], *Drosophila* [23], and mammals [24, 25]. A defect in serine palmitoyl transferase, the enzyme that catalyzes the first step in the biosynthesis of sphingolipids, impairs cell viability unless cells are supplemented with long-chain sphingoid bases. Moreover, sphingolipid metabolites, including S1P, apparently, play essential roles in cell survival and growth [26, 27], motility [28], and tissue remodeling [29].

Our studies therefore focused on the ability of postprandial TRLs to influence the sphingolipid metabolism of human aortic vascular SMCs (HA-VSMCs). The composition of triacylglycerols in postprandial TRLs is partially dependent on the nature of dietary lipids, and these triacylglycerols represent the primary bulk of the lipid carried by TRLs. Accordingly, we designed two types of TRL with a similar content of oleic acid (monounsaturated fatty acid, MUFA) but differently distributed into the glycerol backbone of the triacylglycerol molecule. They were obtained from healthy volunteers. MUFAs were preferentially localized at the sn-2 position in TRLs (TRL-sn2^{MUFA}) after olive oil intake. High-oleic sunflower oil produced TRLs with saturated fatty acids (SFAs) preferentially localized at the sn-2 position (TRL-sn2^{SFA}) and oleic acid at the sn-1,3 positions [17]. We also explored the lipid-stereospecific effect of postprandial TRLs in inducing survival and/or growth in HA-VSMCs. The data reported in this study suggest that S1P, in a lipid-stereospecific fashion, can be a crucial bioactive component to modulating HA-VSMC responses for the cardiovascular risk factor of postprandial hypertriglyceridemia.

Material and methods

Chemicals

M-199 medium, penicillin/streptomycin, L-glutamine, and fetal calf serum (FCS) were purchased from Life Technologies (Barcelona, Spain). Monoclonal anti-MAPK activated (diphosphorylated ERK 1 and 2) from mouse, monoclonal anti-MAPK (non-phosphorylated ERKs) from mouse, anti-mouse IgG (whole molecule)-alkaline phosphatase from goat, doxorubicin, platelet-derived growth-factor (PDGF), polyvinylidine difluoride (PVDF) membranes, pancreatic lipase (EC 3.1.1.3) from pig, D-erythro-sphingosine, pertussis toxin from *Bordetella pertussis*, and o-phthalaldehyde (OPA) were from Sigma (St. Louis, Mo.). C_{17}-sphingosine 1-phosphate (C_{17} S1P) was from Avanti Polar Lipids (Alabaster, Ala.). Alkaline phosphatase color development reagents (BCIP and NBT) were from BioRad Laboratoires (Madrid, Spain). Hoescht 33258 was from Molecular Probes (Eugene, Ore.). Radiochemicals {[^{3}H]thymidine (22 Ci/mmol), [methyl-^{3}H]choline chloride (83 Ci/mmol), [γ^{32}P]ATP (10 mCi/ml) and [^{32}P]orthophosphate (10 mCi/ml)} were purchased from Amersham Biosciences (Amersham, UK), except for [^{32}P]S1P which was provided by Dr. Spiegel.

Cell culture

HA-VSMCs were obtained from the ATCC (Rockville, Md.). HA-VSMCs were grown in Medium 199 (Gibco-BRL, Life Technologies, Barcelona, Spain) supplemented with FCS (10% v/v), penicillin-streptomycin (50 U/ml), and L-glutamine (2 mM). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Isolation of TRLs

TRLs (Sf > 400, density>0.93 kg/l) were isolated from the plasma of healthy volunteers according to a protocol described previously [16, 17]. The design conformed to the guidelines of the World Medical Assembly (Declaration of Helsinki). Briefly, the test meal consisted of one slice of brown bread (28 g), 100 g of plain pasta (cooked with 200 ml of water), 130 g of tomato sauce, and one skimmed yogurt, providing 1936 kJ of energy. The source of fat (virgin olive oil or high-oleic sunflower oil, 70 g) was supplied mixed with the tomato sauce. The fatty acid composition of the virgin olive oil was as follows: oleic acid (18:1n-9, 72%), palmitic acid (16:0, 12.8%), linoleic acid (18:2n-6, 5.6%), palmitoleic acid (16:1n-7, 3.7%), stearic acid (2.7%), and others (3.2%). The fatty acid composition of high-oleic sunflower oil was: oleic acid (74.3%), linoleic acid (14.2%), palmitic acid (4.1%), stearic acid (4%), palmitoleic acid (3%), and others (0.4%). Blood samples were drawn 2 h after the meal, a time point that corresponded exactly with the rise of total triacylglycerols in plasma under the indicated meal design. The apolipoprotein profile of postprandial TRLs was examined by SDS-polyacrylamide (7.5%) gel electrophoresis separation. Briefly, 10 ml of ethanol:diethyl ether (3:1, v/v) at –20°C was added to 500 µg of TRL protein in 1 ml of 0.15 M NaCl, pH 7.4. The sample was mixed overnight at –20°C, and then centrifuged for 20 min at 720 g. The organic phase was removed, the pellet was washed once with ethanol:diethyl ether (3:1, v/v) and twice with diethyl ether anhydride at –20°C. Delipidated TRLs were dryed under a nitrogen stream and then dissolved with 200 µl of Laemli Sample Buffer. A portion was loaded on a denaturing gel and electrophoresis was carried out at room temperature and 30 mA for 2 h. The gel was stained with Coomassie Brilliant Blue R250 and destained with a solution of methanol:acetic acid:water (5:7:88, v/v/v). Image analysis of Coomassie Brilliant Blue-stained gels indicated that TRL-sn2^{MUFA} (from olive oil) and TRL-sn2^{SFA} (from high-oleic sunflower oil) had

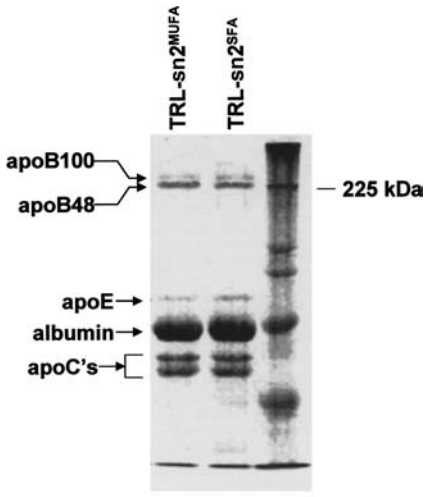

Figure 1. Human postprandial TRL-sn2^{MUFA} and TRL-sn2^{SFA} have an identical apolipoprotein profile. Lane 1, TRL-sn2^{MUFA}; lane *2*, TRL-sn2^{SFA}; lane *3*, perfect marker proteins. Lipoproteins were prepared as described elsewhere [16, 17]; delipidation and the electrophoresis procedure were carried out as described in Materials and methods. In each lane, 50 µg of protein was applied. The arrow on the right marks the position of the 225-kDa marker protein.

the same apolipoprotein profile and concentration (fig. 1). In addition, lipid oxidizability of postprandial TRLs was checked (TBARS level) during isolation and storage, but oxidation of lipids was not detected.

Determination of fatty acids at the sn-2 position of triacylglycerols in TRLs

Triacylglycerols from postprandial TRLs were extracted, partially hydrolyzed by pancreatic lipase from pig (EC 3.1.1.3), and fatty acids analyzed as previously described [16, 17]. In brief, hydrolysis products were separated by thin-layer chromatography using Silica Gel G60 plates in a solvent system containing diethyl ether: hexane: acetic acid (90: 10:1, v/v/v). The band corresponding to 2-monoacylglycerols was scraped off, eluted with hexane, and treated for analysis of fatty acid methyl esters. Total lipid extracts of postprandial TRL-sn2^{MUFA} and TRL-sn2^{SFA} were also analyzed for fatty acid composition.

Determination of S1P in TRLs

Samples of 80 µl postprandial TRLs were mixed with 250 µl of methanol containing 0.6 µl concentrated HCl and 30 pmol C_{17}-S1P, as internal standard [30]. After sonication for 5 min in ice-cold water, lipids were extracted by addition of 500 µl chloroform: NaCl (1 M) (1:1, v/v) and 25 µl NaOH (3 N). The alkaline aqueous phase containing sphingoid base 1-phosphates was collected by centrifugation. Sphingoid base 1-phosphates were efficiently dephosphorylated by ATPase treatment, and the resulting sphingoid bases were then extracted with chloroform, transferred to a fresh tube, and dried under a nitrogen stream. The dried lipid residue was preincubated in 120 µl ethanol at 67 °C for 25 min before derivatization with OPA reagent [50 mg OPA, 1 ml ethanol, 100 µl 2-mercaptoethanol and 50 ml 3% (w/v) boric acid solution adjusted to pH 10.5]. Elution profiles of OPA derivatives of S1P and C_{17}-S1P were obtained by high-performance liquid chromatography analysis using a Beckman System Gold 126 Solvent Module, a 508 Autosampler System and a prepacked C_{18} reversed-phase column. The isocratic eluent was acetonitrile: deionized distilled water (90:10, v/v) and the flow rate was 1 ml/min. The OPA derivatives were detected using a Merck Hitachi FL Detector, with an excitation wavelength of 340 nm and an emission wavelength of 455 nm.

Metabolic labeling of cellular lipids

HA-VSMCs were seeded at a density of 7000 cells/ml in six-multiwell Nunc culture plates and grown to subconfluence in medium containing 10% FCS. After 48 h of culture, medium was replaced by fresh medium containing 1% FCS and [methyl-^{3}H]choline (1 µCi/ml) to label sphingomyelin as described previously [31]. After 48 h incubation, the radioactive medium was removed and cells were chased for 2 h in 1% FCS-enriched medium. Then, cells were treated with TRLs at different concentrations and times of incubation, and the reactions were stopped on ice. Cells were washed three times in cold PBS, harvested with a rubber policeman in 0.2 ml of cold PBS, and sedimented by slow-speed centrifugation. Cell pellets were immediately frozen at –20ºC. For the estimation of cellular S1P concentration, culture medium of subconfluent cells was replaced by phosphate-free DMEM medium containing [^{32}P]orthophosphate (40 µCi/ml) and cells were then incubated for 48 h following the procedure described by Olivera et al. [32]. After the radioactive medium had been removed, the cells were chased for 2 h in phosphate-free medium and then incubated with postprandial TRLs. Cell pellets were obtained as above.

Extraction and analysis of cellular sphingomyelin

Cell pellets were disrupted by sonication and extracted with chloroform: methanol (2:1, v/v). The lipid extract was further washed to remove any trace of free radioactive choline and concentrated under nitrogen. The lipid residue was subjected to mild alkaline hydrolysis to liberate [methyl-^{3}H]choline from phosphatidylcholine (PC), which was quantified by scintillation counting [31]. This technique yields maximal (95%) hydrolysis of PC, without (less than 2%) sphingomyelin hydrolysis. After several washes, the radioactivity in the organic phase containing labeled-sphingomyelin was also quantified. In addition, we determined the amount of choline released by cells after agonist treatment. Data are expressed as

dpm/µg of cell protein. Protein concentrations were determined using the BioRad protein assay.

Measurement of cellular sphingosine kinase activity

Sphingosine kinase activity was determined as described previously [32, 33] with minor modifications. A sample of cell pellets (up to 40 µg of cell protein) and 10 µl of 1 mM D-erythro-sphingosine (dissolved in 5% Triton X-100) were mixed with buffer A (20 mM Tris pH 7.4, 10% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 mM β-glycerophosphate, 15 mM NaF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxypyridoxine) in a total volume of 190 µl, and the reaction was started by addition of 10 µl of [γ^{32}P]ATP (10 µCi, 20 mM) containing 200 mM $MgCl_2$ and incubated for 20 min at 37 °C. Reactions were terminated by addition of 20 µl of 1 N HCl followed by 0.8 ml of chloroform : methanol : HCl (100:200:1 v/v/v). After vigorous vortexing, 240 µl of chloroform and 240 µl of 2 M KCl were added and the phases were separated by centrifugation. The labeled lipids in the organic phase were resolved by thin layer chromatography on silica gel G60 with 1-butanol : ethanol : acetic acid : water (80:20:1:2, v/v/v/v), and radioactive spots corresponding to S1P were then identified and quantified with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Sphingosine kinase activity was expressed as units (pmol of S1P formed per minute)/mg of cell protein.

Measurement of cellular S1P

Lipids from cell pellets were extracted after mild alkaline hydrolysis as previously described [34]. [^{32}P]S1P was separated by thin-layer chromatography using 1-butanol : ethanol : acetic acid : water (80:20:1:2, v/v/v/v) as developing solvent, and then visualized by autoradiography as described above.

Staining of apoptotic nuclei

HA-VSMCs were incubated with postprandial TRLs in medium containing 1% FCS for 72 h in the absence or presence of 6 µg/ml of doxorubicin (lower tested concentration inducing at least 50% of apoptosis in non-treated TRL cells). They were washed in PBS, and fixed in a solution of 4% paraformaldehyde containing 4% sucrose for 20 min. After washing with PBS, fixed cells were incubated with bis-benzimide trihydrochloride (Hoechst 33258; 24 µg/ml of 30% glycerol/PBS) for 10 min. Stained cells were examined with a Photoscope II fluorescent microscope (Thornwood, N. Y.). Apoptotic cells were distinguished by condensed, fragmented nuclear regions. A minimum of 300 cells per treatment were scored.

[^{3}H]Thymidine incorporation assays

After trypsinization, HA-VSMCs were seeded at a density of 7000 cells/ml in 24-multiwell Nunc culture plates, in medium containing 10% FCS for 48 h. The medium was removed, and cells were washed once with medium containing 1% FCS and grown for an additional 48 h to reach quiescence. Cells were then incubated with the indicated concentrations of postprandial TRLs for 48 h and labeled for the last 24 h with [^{3}H]thymidine (0.2 µCi/ml). Cells were washed three times with PBS, precipitated by methanol, and dissolved in 0.1 N NaOH for 2 h. The NaOH (0.5 ml/well) was transferred to a scintillation vial and 5 ml of Ecoscint A was added for liquid scintillation counting. [^{3}H]thymidine radioactivity was expressed as a percent of that measured in control cells grown in medium containing only 1% FCS.

Western blotting of MAPKs

HA-VSMCs were solubilized in 100 µl of lysis buffer (20 mM HEPES pH 7.4, 50 mM NaCl, 30 mM sodium pyrophosphate, 1 mM EGTA, 5 mM β-glycerophosphate, 1% Triton X-100, 0.1 mM sodium orthovanadate, 1 mM PMSF, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin). Cell lysates were subjected to SDS-polyacrylamide (10%) gel electrophoresis (169 V, 140 min), and proteins were then transferred onto nitrocellulose membranes (130 mA, 120 min) using the Mini Protean II Electrophoresis System from BioRad. Prior to the immunoblotting, non-specific protein interactions were blocked using 5% of non-fat milk in TBS-Tween 20 (0.1%) at 37 °C for 1 h. Monoclonal anti-phosphorylated MAPKs (1:1000), monoclonal anti-total MAPKs (1:1000), and secondary anti-IgG antibody (1:6000) were incubated at 37 ºC for 1 h each, with 10-min washes in triplicate between them. Antibodies were detected by reaction between alkaline phosphatase enzyme attached to the secondary antibody and alkaline-phosphatase Color Development Reagents (BCIP and NBT). Color intensity was quantified by the Kodak Digital Science 1D software (Kodak Scientific Imaging Systems; Eastman Kodak, Newhaven, Conn.). To determine if G proteins were involved in MAPK activation, cells were pretreated with 200 ng/ml of pertussis toxin (PTX) for 2 h.

Statistics

Results are expressed as means ± SD. The significance of differences was examined using Student's t test.

Results

Distribution of fatty acids in the triacylglycerols of TRLs

In addition to the apolipoprotein profiles of TRL-sn2^{MUFA} and TRL-sn2^{SFA} that were found to be identical (fig. 1),

Table 1. Composition of major fatty acids is similar between TRL-sn2^{MUFA} and TRL-sn2^{SFA}.

Fatty acid	All positions of the glycerol backbone	
	TRL-sn2^{MUFA} (%)	TRL-sn2^{SFA} (%)
16:0	14.1 ± 0.3	6.6 ± 0.9*
18:0	3.6 ± 0.2	4.6 ± 0.5
18:1n-9	66.0 ± 1.8	70.7 ± 4.0
18:1n-7	3.6 ± 0.4	3.1 ± 0.1
18:2n-6	7.8 ± 0.8	11.6 ± 1.6*
18:3n-3	0.8 ± 0.1	0.2 ± 0.0*
Others	4.1 ± 0.2	3.2 ± 0.4
SFA	18.7	11.9*
MUFA	71.6	75.3
PUFA	9.7	12.8*

Fatty acid methyl esters at all positions of triacylglycerols from postprandial lipoproteins were analyzed by gas chromatography [16, 17]. Values means ± SD from eight separate experiments. * $p < 0.05$ TRL-sn2^{SFA} vs TRL-sn2^{MUFA}.

Table 2. The composition of fatty acids at the sn-2 position of triacylglycerols is different between TRL-sn2^{MUFA} and TRL-sn2^{SFA}.

Fatty acid	The sn-2 position of the glycerol backbone	
	TRL-sn2^{MUFA} (%)	TRL-sn2^{SFA} (%)
16:0	21.82 ± 2.34	31.87 ± 3.12*
18:0	12.23 ± 1.06	27.63 ± 4.01*
18:1n-9	59.69 ± 3.01	35.56 ± 3.36*
18:2n-6	6.26 ± 1.15	4.96 ± 1.01*
SFA	34.04	59.51*
MUFA	59.69	35.56*
PUFA	6.27	4.93*

The hydrolysis products from lipase-treated postprandial lipoproteins were separated by thin layer chromatography, and fatty acid methyl esters of 2-monoacylglycerols were analyzed by gas chromatography [16, 17]. Values means ± SD from eight separate experiments. * $p < 0.05$ TRL-sn2^{SFA} vs TRL-sn2^{MUFA}.

we determined their total fatty acid composition and fatty acids localized at the sn-2 position of the glycerol backbone in the triacylglycerol molecule. Table 1 shows a similar composition of total fatty acids in both postprandial TRLs. As expected, oleic acid (18:1n-9) was the major fatty acid (more than 65% of total fatty acids) followed by palmitic (16:0) and linoleic (18:2n-6) acids. However, there were marked differences between the fatty acids at the sn-2 position of triacylglycerols (table 2). In TRLs from virgin olive oil (TRL-sn2^{MUFA}), MUFAs (mainly oleic acid) represented 60% of total fatty acids at the sn-2 position. In contrast, this stereospecific position was occupied by SFAs (mainly palmitic and stearic, 18:0, acids; almost 60% of total fatty acids) in TRLs from high-oleic sunflower oil (TRL-sn2^{SFA}).

The distribution of lipids (triacylglycerols, phospholipids, and cholesterol esters) and the electrophoretic mobilities on agarose were exactly the same for both postprandial TRLs (data not shown). Additionally, S1P was not detected in the postprandial TRLs.

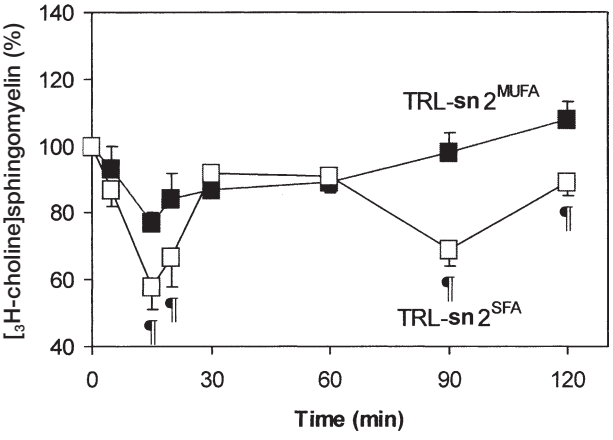

Figure 2. Sphingomyelin turnover is activated by TRL-sn2^{MUFA} and TRL-sn2^{SFA} in HA-VSMCs. Cells were labeled with [methyl-^{3}H]choline (2 µCi/ml) for 48 h and incubated for increasing periods of time with a fixed concentration (100 µg of triacylglycerols/ml) of postprandially obtained TRL-sn2^{MUFA} (filled symbols) or TRL-sn2^{SFA} (open symbols). Radiolabeled-sphingomyelin content is expressed as percent of the values at zero time (283 ± 16 dpm/µg of protein; 16 ± 2 µg of protein/well). Values are means ± SD of at least eight separate experiments. All of them were statistically different to untreated cells. ¶ indicates a statistical significance ($p < 0.05$) compared with TRL-sn2^{MUFA}.

Effect of TRLs on hydrolysis of sphingomyelin in HA-VSMCs

Exposure of bovine aortic [20] and rabbit femoral [21] SMCs to oxidized low density lipoprotein has been shown to activate the sphingomyelin-ceramide pathway. However, there are no studies on the effect of postprandial TRLs, suggested to be proatherogenic lipoproteins, in the sphingomyelin turnover of HA-VSMCs. We investigated this issue and found that acute treatment of HA-VSMCs with TRLs (100 µg of triacylglycerols/ml) induced the hydrolysis of [methyl-^{3}H]choline-labeled sphingomyelin (fig. 2). There was a time-dependent decrease in sphingomyelin, with maximal hydrolysis at 15 min after TRL treatment. Interestingly, the nature of the fatty acids at the sn-2 position of triacylglycerols in postprandial TRLs determined the effect on the transiently activated sphingomyelinase. Thus, TRL-sn2^{MUFA} caused a monophasic response by reducing cellular sphingomyelin content to 80%, but a more pronounced and biphasic response was observed for TRL-sn2^{SFA} (55% of sphingomyelin content at 15 min and a second pulse with 65% at 90 min), indicating that activation of sphingomyelin turnover in HA-VSMCs by postprandial (non-oxidized) TRLs involved a fatty acid-stereospecific mechanism.

Effect of TRLs on activation of the sphingosine kinase pathway and production of S1P in HA-VSMCs

To determine whether postprandial TRLs activate sphingosine kinase, the phosphorylation of D-erythro-sphingosine was monitored in HA-VSMCs by an in vitro assay that we have described previously [32]. Compared with non-treated cells, TRL-sn2^{MUFA} and TRL-sn2^{SFA} induced a similar and rapid increase in the activity of sphingosine kinase by approximately threefold, peaking at 10 min with no return to basal levels (fig. 3). Additionally, TRL-sn2^{SFA}, but not TRL-sn2^{MUFA}, induced a further significant increase in cytosolic sphingosine kinase activity when cell homogenates were assayed after 120 min incubation in the presence of the lipoprotein. The parallel and transient accumulation of S1P by an in vivo assay (table 3) agrees with the proposition that the endogenous conversion of ceramide to S1P is mediated by a fatty acid-stereospecific response of the sphingomyelin-ceramide cycle to postprandial TRLs.

Protective effect of TRLs against doxorubicin-mediated apoptosis in HA-VSMCs

S1P has been proven to cause pleiotropic biological responses, one of which is the prevention of apoptosis [35]. We investigated whether S1P generated after postprandial TRL-treatment of HA-VSMCs could provide protection from apoptosis induced by doxorubicin, an anthracycline drug that causes cell death through different pathways, including the accumulation of intracellular ceramide(s) [36], and the activation of JNK and the tumor suppressor p53 [37]. Apoptosis was assessed by morphological changes in the nucleus characterized by condensed and fragmented regions. As shown in figure 4 and according to the postprandial TRL-induced S1P formation, TRL-sn2^{SFA} provided more efficient protection from doxorubicin-induced apoptosis than did TRL-sn2^{MUFA}, suggesting a role for downstream metabolites of ceramides in the survival response of HA-VSMCs.

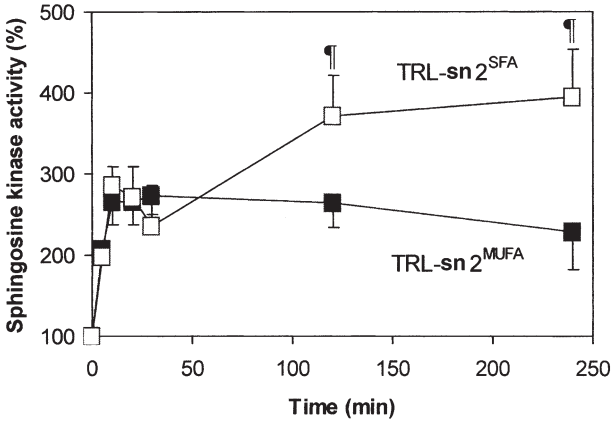

Figure 3. In vitro activation of sphingosine kinase by TRL-sn2^{MUFA} and TRL-sn2^{SFA} in HA-VSMCs. Cells were treated with TRL-sn2^{MUFA} (filled symbols) or TRL-sn2^{SFA} (open symbols) (100 µg of triacylglycerols/ml) for the indicated times. Sphingosine kinase activity was determined in cell extracts using exogenous D-erythro-sphingosine as substrate and [γ^{32}P]ATP as described in Materials and methods. Data are expressed as percent of the control (i.e. untreated cells, 31.42 pmol/min per milligram of protein). Values are means ± SD of at least eight separate experiments. All of them were statistically different to untreated cells. ¶ indicates statistical significance ($p < 0.05$) compared with TRL-sn2^{MUFA}.

Table 3. Early induction by TRL-sn2^{MUFA} and TRL-sn2^{SFA} of sphingosine 1-phosphate in HA-VSMCs.

Treatment	Time (min)	S1P (%)
Control	0	100
PDGF	10	417 ± 9
TRL-sn2^{MUFA}	10	137 ± 5*
	60	79 ± 3*
TRL-sn2^{SFA}	10	336 ± 8*,¶
	60	103 ± 2¶

Cells were grown for 48 h in a phosphate-free medium containing [^{32}P]orthophosphate. After a 2-h chase, they were incubated with TRL-sn2^{MUFA}, TRL-sn2^{SFA} (100 µg of triacylglycerols/ml) or PDGF (20 ng/ml) as a positive control for various times. Cellular [^{32}P]S1P was quantified as described previously [34]. Data are expressed as percent of the values in untreated cells. Values are means ± SD from eight separate experiments. * $p < 0.05$ TRL vs control, ¶ $p < 0.05$ TRL-sn2^{SFA} vs TRL-sn2^{MUFA}.

Mitogenic effect of TRLs in HA-VSMCs

The incorporation of [^{3}H]thymidine into DNA of HA-VSMCs was next tested in the presence of postprandial TRLs. TRL-sn2^{MUFA} and TRL-sn2^{SFA} enhanced (up to two-fold of the control) the thymidine labeling after 48 h incubation in a concentration-dependent manner (fig. 5).

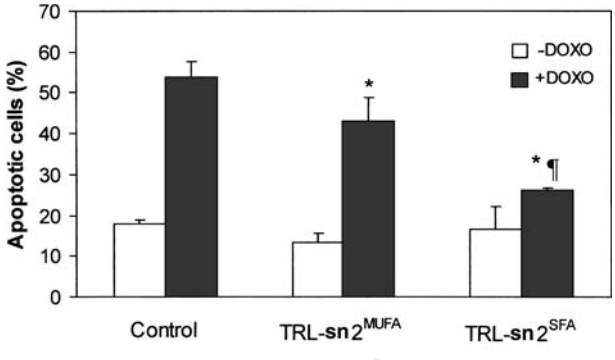

Figure 4. TRL-sn2^{MUFA} and TRL-sn2^{SFA} protect HA-VSMCs against apoptosis induced by doxorubicin. Cells were grown in 12-well plates at low confluence. They were then treated with TRL-sn2^{MUFA} or TRL-sn2^{SFA} (100 µg of triacylglycerols/ml) for 72 h in the absence (open bars) or presence (filled bars) of doxorubicin (6 µg/ml). Apoptotic cells were assessed by staining cells using Hoescht 33258 as described in Materials and methods. The number of apoptotic cells is expressed as percent of the total cells (average of three groups of 100 cells for each experiment). Values are means ± SD of at least eight separate experiments. * and ¶ indicate statistical significance ($p < 0.05$) compared with untreated cells or TRL-sn2^{MUFA}, respectively.

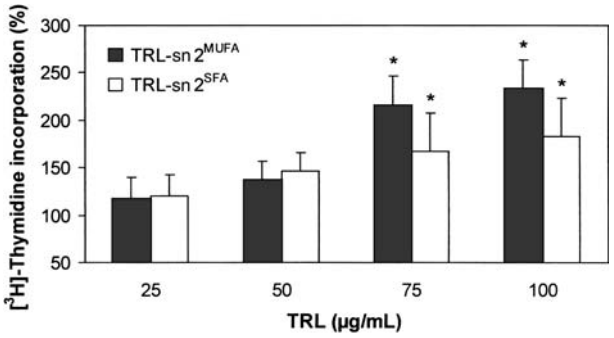

Figure 5. TRL-sn2^{MUFA} and TRL-sn2^{SFA} are mitogenic for HA-VSMCs. Cells were incubated for 48 h with TRL-sn2^{MUFA} (filled bars) or TRL-sn2^{SFA} (open bars) (100 µg of triacylglycerols/ml). [^{3}H]thymidine incorporation was evaluated during the last 24 h. Data are expressed as percent of the radioactivity measured in cells grown in medium containing 1% FCS. The positive control (i.e. cells grown in 10% FCS) displayed a value of 484%. Values are means ± SD of at least eight separate experiments. * Indicates statistical significance ($p < 0.05$) compared with untreated cells.

There was a saturating effect at a concentration of 75–100 µg of triacylglycerols/ml and TRL-sn2^{MUFA} was apparently more mitogenic than TRL-sn2^{SFA}, which agrees with recent reported studies on the proliferation of rat aortic SMCs induced by postprandial TRLs using the MTT assay [19].

Effect of TRLs on the activation of pertussis toxin-sensitive G protein-coupled receptor in HA-VSMCs

The atherogenic cholesterol ester-rich β-migrating very low density lipoprotein and oxidized low density lipoprotein have been shown to stimulate the proliferation of rabbit [38] and bovine [20] aortic SMCs via the activation of MAPKs, a well known downstream target in growth signaling. Therefore, the next objective was to establish the capability of TRL-sn2^{MUFA} and/or TRL-sn2^{SFA} to activate the MAPK pathway in HA-VSMCs. As shown in figure 6B, MAPK isoforms ERK1 and ERK2 (p44^{MAPK} and p42^{MAPK}, respectively) were strong and biphasically phosphorylated by TRL-sn2^{SFA}, but monophasically by TRL-sn2^{MUFA}. The maximal response was noted after 5–10 min in the presence of postprandial TRLs (100 µg of triacylglycerols/ml). TRL-sn2^{SFA} showed a second pulse peaking at 60 min. Total levels of ERK1/ERK2 were similar in control and TRL-treated samples (fig. 6A). Pretreatment of HA-VSMCs with PTX (200 ng/ml) for 2 h partially inactivated MAPKs, which suggests a PTX-sensitive G protein-coupled receptor(s) involved in postprandial TRL-induced activation of ERK1/ERK2.

Discussion

A growing number of studies relating TRLs and vascular lesions have appear since Zilversmit [39] proposed that postprandial lipemia plays a major role in atherosclerosis [10–13]. During a vascular injury, SMCs show a high degree of plasticity, undergoing rapid and reversible phenotypic changes [14, 15]. The postreceptor response of SMCs to TRL interaction has recently been shown to be lipid dependent [18, 19]. However, intracellular mediators in SMCs to TRL extracellular stimuli are largely unknown. The aim of this study was to explore lipid-dependent signaling pathways regulated by postprandial TRLs in HA-VSMCs.

Interestingly we found that subphysiological concentrations of postprandial TRLs may trigger sphingomyelin breakdown in HA-VSMCs. In addition, there was a monophasic activation of sphingomyelinase when the agonist carried MUFAs at the sn-2 position of the glycerol backbone in the triacylglycerol molecule (TRL-sn2^{MUFA}). However, we found a biphasic effect with an agonist carrying SFAs at the same position (TRL-sn2^{SFA}). We cannot rule out the possibility that a minor fatty acid, linoleic acid, could also play a role in these processes. This novel lipid-stereospecific effect for TRL has not been described elsewhere for other lipoproteins. Hydrolysis of sphingomyelin is related to the ability of SMCs and other cell types to synthesize DNA [40]. Accordingly, our findings indicate that TRL-induced sphingomyelinase activation was followed by HA-VSMC mitogenesis. Single activation of sphingomyelinase was enough for cell proliferation. These observations suggest that the second pulse of sphingomyelin hydrolysis is not necessarily involved in the mitogenic response of human SMCs, but its involvement in other cell responses cannot be excluded.

Sphingomyelin turnover is known to generate ceramide which has proven to be an apoptotic factor for cells [41]. Ceramide can be hydrolyzed by ceramidases to liberate the sphingoid base backbone (sphingosine), which can be reacylated to ceramide or phosphorylated to S1P by sphingosine kinase [26]. This bioactive lipid, S1P, acts both intracellularly and extracellularly to cause pleiotropic biological responses: survival and mitogenesis [26, 27, 42], migration [28, 43], and differentiation [44]. To follow up the sphingomyelin turnover in HA-VSMCs mediated by postprandial TRLs and the mediators in intracellular signaling, sphingosine kinase activity both in vitro and in vivo was assayed. According to the in vitro assay, there was a rapid production of S1P in SMCs incubated with TRL-sn2^{MUFA}, however, the increase in S1P in response to TRL-sn2^{SFA} was higher (about two-fold over TRL-sn2^{MUFA}). TRL-sn2^{SFA} further provoked a second peak of S1P. We could not find any biphasic response of S1P in the in vivo assay, suggesting that S1P can be quickly metabolized by HA-VSMCs.

The balance between the intracellular levels of ceramide and S1P and their regulatory effects on different family members of MAPKs determine cell fate involving life and growth [40]. In particular, S1P has been shown to be-

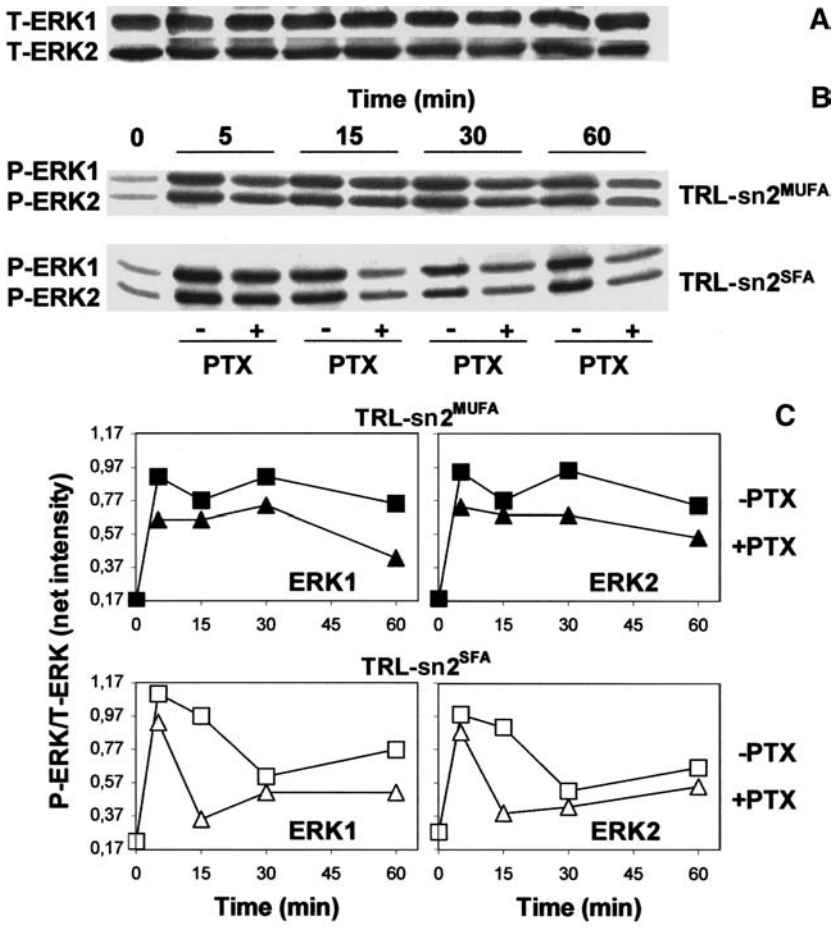

Figure 6. TRL-sn2^{MUFA} and TRL-sn2^{SFA} induce the phosphorylation of ERKs which is mediated by G protein activation in HA-VSMCs. To block G proteins, cells were incubated with PTX (200 ng/ml) for 2 h. TRL-sn2^{MUFA} or TRL-sn2^{SFA} (100 μg of triacylglycerols/ml) were then added for 5, 15, 30, and 60 min. Medium containing 1% FCS was used for 60 min as negative control (similar results were obtained at shorter times). Western blot analysis was made for detecting total (*A*) and phosphorylated (*B*) ERK as described in Materials and methods. The ratios of band-densities from *A* and *B* are also depicted (*C*). Only one panel of lanes for total ERK (1 and 2) is exhibited, as similar findings were found after TRL-sn2^{MUFA} or TRL-sn2^{SFA} incubation. These figures are representative of at least eight separate experiments.

have as a survival mediator, protecting vascular cells from apoptosis induced by both, the withdrawal of growth factors [45] and tumor necrosis factor-α [46]. Therefore, another major question was whether S1P generated by postprandial TRL stimulation could influence HA-VSMC survival, which plays a critical role in plaque stabilization and progression of the vascular lesions [47]. We observed that postprandial TRLs protected HA-VSMCs against doxorubicin-induced apoptosis. This protective effect was enhanced with TRL-sn2^{SFA}, as expected due to its capacity to produce more S1P. Moreover, we found that postprandial TRLs were able to stimulate the MAPK pathway through phosphorylation of ERK1/ERK2. This effect was sensitive to PTX, in agreement with recent studies on the TRL-SMC interaction in a rat model indicating a role for the O-linked sugar domain of the very low density lipoprotein receptor coupled to G proteins [18]. Similar effects have been demonstrated for β-migrating very low density lipoprotein [38] and other pro-atherogenic non-rich-triglyceride lipoproteins [20, 48, 49], including low-density lipoprotein and oxidized low-density lipoprotein.

The importance of sphingolipids in cardiovascular signaling has been illustrated by recent observations which implicate them in physiological processes such as vasculogenesis as well as in frequent pathological conditions, including atherosclerosis and its complications [40]. Our study is the first demonstration that postprandial TRLs (non-oxidized) trigger the sphingomyelin signaling cascade, leading to the production of S1P, survival, and mitogenesis in HA-VSMCs. This provides a mechanistic explanation for the recent findings relating postprandial TRLs with atherosclerosis and underscores the potentially important role of the nature of lipids in TRLs for switching on the signals associated with S1P. The regulation of this or other enzymes controlling S1P levels could be a possible tool to control SMC survival and proliferation in TRL-induced cardiovascular diseases.

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