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Influence of postprandial triglyceride-rich lipoproteins on lipid-mediated gene expression in smooth muscle cells of the human coronary artery

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Aims Postprandial triglyceride-rich lipoproteins (TRL) have a direct effect on vascular smooth muscle cells (SMC) and they increase the risk of atherogenesis. Here, we have tested the hypothesis that the different fatty acid composition of TRL is capable of differentially modifying gene expression in human coronary artery SMC (CASMC). In addition, the effect of TRL on cell proliferation and transcription factor activation was also evaluated.

Methods and results TRL were prepared from plasma of healthy volunteers after the ingestion of meals enriched in refined olive oil (ROO), butter or a mixture of vegetable and fish oils (VEFO). We use cDNA microarrays to determine the genes differentially expressed in TRL-treated CASMC. Correspondence cluster analysis demonstrated that TRL-butter, -ROO and -VEFO provoked different transcriptional profiles in CASMC. Sixty-six genes were regulated by TRL-butter, 55 by –ROO, and 47 by -VEFO. The data revealed that TRL-butter predominantly activated genes involved in the regulation of cell proliferation and inflammation. Likewise, TRL-VEFO induced the expression of genes implicated in inflammation, while TRL-ROO promoted a less atherogenic gene profile.

Conclusion The pathophysiological contribution of TRL to the development of atherosclerosis and the stability of atherosclerotic plaques may depend on the fatty acid composition of TRL. Our findings suggest a role for macrophage-inhibiting cytokine-1 (MIC-1) in coronary artery cardiovascular events.

1. Introduction

Postprandial lipemia is characterized by a rise of triglyceride-rich lipoproteins (TRL) after a rich fat meal and elevated levels of remnant TRL in fasting plasma, and it is associated with an increased risk of coronary artery disease (CAD).[1] TRL can penetrate the artery wall[2] and may have a direct effect on atherosclerosis. While the effect of TRL on macrophages and endothelial cells has been well characterized, little is known about the effect of these lipoproteins on the smooth muscle cells (SMC) of coronary vessels. TRL induce proliferation,[3] enhance coronary vasospastic activity through up-regulation of Rho-kinase[4] and they have been suggested to participate in immune responses by inducing monocyte chemoattractant protein-1 expression in SMC.[5] The role of SMC in the stability of plaques in response to postprandial triglyceridemia is therefore of key importance to understand the progression of CAD. In recent years, we have shown that the fatty acid composition of TRL modulates the pathogenesis of postprandial triglyceridemia,[6] and that it may regulate certain thrombogenic and fibrinolytic markers during the postprandial state in healthy subjects.[7] Hence, we tested the hypothesis that the fatty acid composition of postprandial TRL influences how they modify coronary artery SMC (CASMC) gene expression, which was analyzed using cDNA microarrays. In this way, we have been able to develop a new hypothesis concerning the cellular responses to TRL. Identifying the genes activated in CASMC by TRL with different lipid compositions helps to understand how plaque stability is established, and may lead to the development of effective nutrient-based preventive and therapeutic strategies to combat diet-related diseases.

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2. Methods

2.1 Study design and postprandial lipoprotein isolation

Healthy males participated in this study with a BMI (in kg/m^2) of 23.9 ± 1.9 (mean $\pm$ SD) and a mean age of 27 ± 7. The details of the subjects and the study design have been reported previously.[7] For TRL isolation, the subjects were administered a fat-rich meal containing, refined olive oil (ROO) (Monteolivo), butter (Puleva), or a mixture of vegetable and fish oils (VEFO) (Puleva). Blood samples were drawn 3 h after the ingestion of the meals, and the TRL were immediately isolated from plasma by ultracentrifugation and identified as described.[6] The TRL from the individuals in each group were mixed and the pools used for cell culture studies. The endotoxin concentration in the TRL fractions was <5 pg/mL when determined by the Limulus Amebocyte Lysate assay (Hycult Biotechnology, The Netherlands), far less than that which might induce the gene pattern observed here. Triglycerides (TG) and fatty acid composition in the TRL were determined as described.[8] The study was carried out in accordance with the principles outlined in the Helsinki Declaration.

2.2 Cell culture

The human CASMC (Clonetics, Walkersville, ML) were used up to the eighth passage. CASMC were grown in SMC basal medium (SmBM®) supplemented with 0.5 μg/mL hEGF, 5 mg/mL insulin, 1 μg/mL bFGF, 50 mg/mL gentamicin and 5% FBS. Cells were arrested when subconfluent by maintaining them in serum-free medium for 48 h (quiescent), before they were stimulated with TRL at different concentrations and for different times.

2.3 Microarray analysis: sample preparation and hybridization

A human cDNA microarray was used to study the gene expression profile of CASMC in response to the different TRL. All cDNA spots were present in duplicate and each slide contained the polymerase chain reaction (PCR)-products from the cDNAs of 4376 known genes. Cells were stimulated for 24 h with 100 μg TG/mL of TRL-ROO, TRL-butter, or TRL-VEFO, and quiescent cells were used as a control. Total RNA was extracted from the cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified using the Nanodrop system (Nucliber, Wilmington, USA). Fluorescently labelled cDNA-samples were prepared from 10 μg total RNA in the presence of either Cy3- or Cy5- labelled dCTP (Amersham Bioscience, Freiburg, Germany) and using 600 U of Superscript II reverse transcriptase (Invitrogen, Carlsbad, USA). Each labelling reaction was performed twice. Hybridization was carried out in SlideHyb™ hybridization buffer 1 (Ambion, Huntingdon, UK) under a coverslip in a humidified hybridization chamber[9] (Hauser Prazionstechnik, Vohringen, Germany).

2.4 Microarray analysis: detection and data analysis

Fluorescence signals were detected using a ScanArray5000 confocal laser scanner (PackardBioChip Technologies, Billerica, USA). At least eight data points were accumulated per gene and individual experimental condition (duplicate spots on each array, four hybridizations per sample, inclusive dye-swaps). The signal intensities were quantified with GenePix Pro 4.1 analysis software (Axon Instruments, Union City, USA). Data quality assessment, normalization, and correspondence cluster analysis were performed by procedures that meet or exceed the MIAME-criteria of microarray analysis[10] and using data warehouse software M-CHiPS (http://www.mchips.org).

Significance of signal variations was assessed by the highly stringent 'min-max separation' criterion,[11] which is calculated by taking the minimum distance between all data points of two conditions. For further analysis, only genes that exhibited significant changes between TRL samples and the control were selected. Cluster analyses were performed using the Correspondence Analysis clustering and projection method, which plots the genes and hybridization conditions in the same space. In the resulting plot, the χ^2-distance displayed is a measure of association between genes and hybridizations. Profiles are similar to the average profile plot near the centroid of the map; while greater the distance to the centroid, the greater is the dissimilarity to the average profile. Thus, the more similar two profiles were, the smaller the distance of the data points in the plot. Points with anti-correlated profiles are located on opposite sides of the centroid.[12]

2.5 Quantitative real-time polymerase chain reaction

The mRNA levels for specific genes were determined by real-time PCR in a MX3000P system (Stratagene, La Jolla, USA). Reverse transcription was performed using 3 μg RNA and RevertAid™ M-MuLV RT (Fermentas, Ontario, Canada) according to the manufacturer's instructions. The cDNA template was added to Brilliant SYBR green QPCR Master mix (Stratagene) containing the primer pairs for the genes (*Table 1*). Reactions were performed in triplicate and the change in mRNA expression was calculated using a standard curve. All data were normalized to the endogenous reference P0 and GAPDH genes, and expressed as the change with respect to the controls.

2.6 Proliferation assay

Cell proliferation was analyzed in cells seeded at a density of ~40% with a colorimetric bromodeoxyuridine (BrdU) ELISA kit (Roche, Mannheim, Germany) according to manufactur's instructions. Quiescent cells were stimulated with 50, 100 and 200 μg/mL of TG–TRL for 48 h and the cells were incubated with 10 μM BrdU 15 h prior to analysis.

2.7 Flow cytometric analysis

Quiescent cells were incubated with 100 μg/mL TG–TRL for 12 h. Cell cycle distribution was evaluated by flow cytometry (FACSCANTO II flow cytometer, Beckton Dickinson, Franklin Lakes, USA) using propidium iodide (Sigma, Cedex, France) to label the DNA. Cells were treated and analyzed for cell cycle distribution with FACS DIVA Software (Beckton Dickinson) as described previously.[8]

2.8 Immunoblotting

Quiescent CASMC were stimulated with 100 μg/mL TG–TRL for different times (6, 12, 18, 24, 36, and 42 h) and the protein extracts were analyzed by immunoblotting as described previously[13] with an antibody against cyclin D1 (A-12) (SantaCruz Biotechnology, Santa Cruz, USA), mouse anti-PCNA (proliferating cell nuclear antigen) (SantaCruz) and Rb (Becton Dickinson, Heidelberg, Germany). Specific antigen-antibody complexes were detected with the ECL

Table 1 Gene-specific oligonucleotides used for quantitative reverse transcription polymerase chain reaction (RT–PCR) for seven selected genes

Gene	Forward primer	Reverse primer
HSPA1A	AAGATCTGCGTCTGCTTGGT	CGACCTGAACAAGAGCATCA
MIC-1	AGAGATACGCAGGTGCAGGT	CTCCAGATTCCGAGAGTTGC
IL-8	ATTGCATCTGGCAACCCTAC	CTGCGCCAACACAGAAATTA
SOCS-5	CCCACAGTATCCTGCAACCT	ACCCAGAGTTCATTGGATGC
MCP-1	TGGAATCCTGAACCCACTTC	CCCCAGTCACCTGCTGTTAT
TIMP-1	TGCAGTTTTCCAGCAATGAG	AATTCCGACCTCGTCATCAG
POU2F-1	GGAGTGGAGGTGGTCTGTGT	CAGTGCAGCAACTACCCTCA

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detection kit (Supersignal West Pico Chemiluminescent Substrate, Pierce, USA).

2.9 Electrophoretic mobility shift assay

Quiescent cells were incubated with 100 μg/mL of TG–TRL for 2 h. Nuclear proteins extracts and EMSA (electrophoretic mobility shift assay) assays were performed as described previously.[14] In brief, 10 μg of nuclear protein extracts were incubated with 0.5 ng of oligonucleotide containing the NF-κB or AP-1 binding sequences in a reaction buffer that contained 1 μg of Poly(dI-dC) as a non-specific competitor. Samples were analyzed using a PhosphoImager (Thyphoon 9400, Amersham) (ImageQuant TI 2003.03). Competition experiments were performed by including a 50-fold excess of the unlabelled oligonucleotides (specific competitor) and mutant binding oligonucleotides. The sequences of the double stranded oligonucleotides probes (labelled with T4 kinase and [γ-^{32}P]ATP) were: NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and AP-1 (5′-CGCTTGATGACTCAGCCGGAA-3′) consensus oligonucleotides, and the mutant oligonucleotides were: NF-κB (5′-AGTTGAGGCG ACTTTCCCAGGC-3′) and AP-1 (5′-CGCTTGATGACTTGGCCGGAA-3′) (SantaCruz).

2.10 Biochemical assessment of lipid peroxidation

Quiescent cells were stimulated with 100 μg/mL of TG–TRL. Then, the TRL-containing medium was collected at time 0 and 24 h and the amount of lipid peroxidation was assessed by the analysis of 2-thiobarbituric acid reactive substances (TBARS) as described.[15] Human low-density lipoprotein (LDL) (100 μg/mL protein) was used as a control.

2.11 Statistics

The results were expressed as the mean ± SD. Comparisons of the differences between these mean values were performed using the Student's *t*-test. The data were analyzed with STATVIEW v.5 for WINDOWS (SAS Institute, Cary, USA). The designated level of significance was $P < 0.05$. Microarray data analyzes are shown before.

3. Results

3.1 Fatty acid profile of postprandial triglyceride-rich lipoproteins

The butter meal provided saturated fatty acids (SFA), resulting in the enrichment of TRL-butter with palmitic and stearic acids (*Table 2*). The ROO meal provided monounsaturated fatty acids (MUFA), and TRL-ROO had high amounts of oleic acid (OA). The percentage of MUFA in TRL-VEFO was also high; in addition it contained moderate amounts of the n-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. Moreover, TRL-VEFO had a higher linoleic acid (LA) content than TRL-ROO and TRL-butter. Fatty acids were not detectable in the TRL fraction after the control (non-fat) meal.

Table 2 Fatty acid composition of lipid fractions in postprandial triglyceride-rich lipoproteins (TRL) derived from butter, refined olive oil (ROO), and a mixture of vegetable and fish oils (VEFO)

Fatty acid	% by weight of total fatty acid		
	TRL-butter	TRL-ROO	TRL-VEFO
14:0 (MA)	3.7 ± 0.7		
16:0 (PA)	27.7 ± 2.3[a]	14.2 ± 1.2[b]	10.6 ± 0.7[c]
18:0 (SA)	12.6 ± 1.1[a]	5.4 ± 0.2[b]	6.2 ± 0.6[c]
18:1n-9 (OA)	35.5 ± 5.2[a]	64.5 ± 2.6[b]	59.7 ± 2.8[c]
18:2n-6 (LA)	12.1 ± 1.0[a]	9.6 ± 0.5[b]	15.0 ± 1.6[c]
20:5n-3 (EPA)	0.6 ± 0.4[a]	0.6 ± 0.2[a]	1.6 ± 0.3[b]
22:6n-3 (DHA)	0.9 ± 0.3[a]	0.8 ± 0.3[a]	2.3 ± 0.4[b]
Other	6.9 ± 1.8	4.9 ± 1.4	4.6 ± 1.1
SFA	45.5 ± 2.1[a]	20.8 ± 1.1[b]	17.8 ± 0.7[c]
MUFA	39.9 ± 3.4[a]	67.4 ± 2.4[b]	62.4 ± 3.2[c]
PUFA	14.7 ± 0.6[a]	11.8 ± 0.6[a]	19.8 ± 1.5[b]

MA, miristic acid; PA, palmitic acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid, EPA; eicosapentaenoic acid; DHA, docosahexaenoic acid.

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[a,b,c]Values in arrow with different letter are significantly different, $P < 0.05$. Values are means ± SD, $n = 3$.

3.2 Correspondence cluster analysis of gene expression profile of TRL-stimulated CASMC

A human cDNA expression array containing 4376 known genes that represent different functional classes was used to identify the genes involved in TRL-mediated CASMC activation. Housekeeping genes were included in the array as internal positive controls. Transcriptional profiling was performed with samples obtained from controls (serum-starved cells) and after incubation with TRL-ROO, TRL-butter or TRL-VEFO for 24 h. The samples labelled with either Cy3 or Cy5 were analyzed in competitive hybridizations, and after scanning and quantifying the spot intensities, the data were normalized and filtered. In total, 189 PCR fragments contained a sequence that was differentially transcribed in the samples analyzed according to the highly stringent 'min-max separation' criterion.[11] The results were subjected to correspondence cluster analysis,[12] which is an explorative computational method to investigate the associations between variables, such as genes and hybridizations, in a multi-dimensional space. This analysis simultaneously displays data for two (or more) variables in a low-dimensional projection, thereby revealing associations between them. The results of clustering the profiles obtained after incubation with the different TRL are shown as squares in *Figure 1*, in which the black dots represent differentially transcribed genes. Genes located close to the centroid of the guidelines are simultaneously co-expressed in all the different TRL while genes located close to one of the guidelines and far from the centroid are key regulated genes for each TRL. The co-localization of genes and conditions in the blot is indicative of a strong association between these elements. When classified by correspondence analysis, three distinct clusters were recognized corresponding to the differential gene expression profile produced by TRL-ROO, TRL-butter, and TRL-VEFO. Different TRL regimes produced statistically different transcriptional profiles in CASMC and the distances between TRL-butter and TRL-VEFO indicated particularly large differences in the expression of certain genes.

3.3 Differential expression of specific functional groups of genes in triglyceride-rich lipoproteins-stimulated coronary artery smooth muscle cells

In each of the three clusters, genes were considered statistically differentially expressed if they fulfilled the min-max separation criterion (see Methods) and the change in

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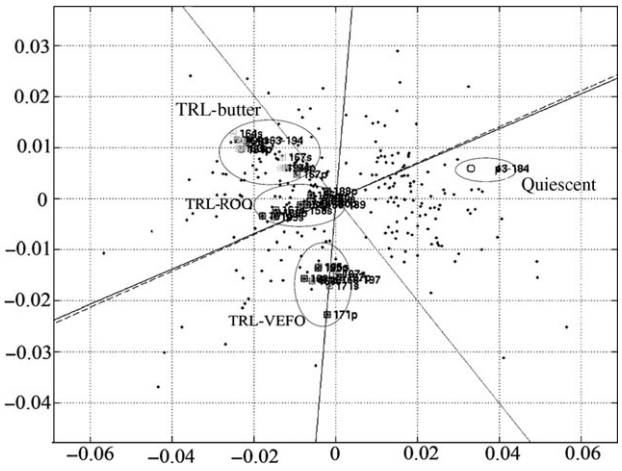

Figure 1 Biplot representation of the results of the correspondence analysis. Transcriptional profiling data were generated from serum-starved cells (Quiescent) cells treated with TRL (triglyceride-rich lipoproteins)-butter, TRL-ROO (refined olive oil), and TRL-VEFO (vegetable and fish oils). In the plot, each hybridization of the RNA following exposure to a specific TRL is depicted as a square, where four hybridizations have been performed for each experimental condition. Each gene differentially transcribed is shown as a black dot. As a consequence of the normalization process, only the median of all the control hybridizations is shown in the diagram as a single square (Quiescent) instead of the individual hybridization. The samples from the different TRL and from the control form distinct clusters showing that the effects of the TRL are clearly different from each other. Each differentially transcribed gene is shown as a black dot and the closer the co-localization of two spots (both genes and TRL); the higher is the degree of association between them. The guidelines displayed in the diagram correspond to the position of genes whose transcription profiles would exhibit a signal in one condition only. The closer a gene lies to one of these guidelines and the further its distance to the centroid, the better its expression is described by the related ideal profile. All genes that are not differentially transcribed are located close to the centroid of the lines but are not shown for clarity.

expression was at least two-fold. As a result 71 differentially expressed genes were identified that exhibited moderate (two- to four-fold) to strong (greater than four-fold) differential expression (*Table 3*). Of these genes, 59 were up-regulated by TRL-butter, 51 by TRL-ROO and 45 by TRL-VEFO. Seven genes were down-regulated by TRL-butter, 4 by TRL-ROO and 2 by TRL-VEFO in three different experiments. The functional classification of these genes revealed that many of them encoded for proteins that were directly or indirectly responsible for the regulation of cell proliferation, migration and inflammatory responses. The strongest differentially expressed genes after exposure to TRL-butter were associated with cell cycle regulation and motility. These included genes that encode for the cell cycle proteins (cyclin D1, cyclin E1), the TGFBR-2 and BMPR-2 receptors, intracellular signalling proteins (MAP3K1, DYRK1A, MKP-3, PLK-3), transcription factors and binding proteins (AP-1, GTF2H1, SP-1, NF-κB), cytoskeletal proteins and those implicated in motility (RAF-1, ANXA2, VIM). Furthermore, INSIG-1 and ODC-1 were among the positive regulators of cell proliferation strongly up-regulated by these TRL. Some genes implicated in migration and/or inflammation were also highly regulated by TRL-butter, such as MIC-1 and DDR-1. TRL-VEFO produced a strong response in genes mostly implicated in inflammation (MIC-1, IL-8, IL1B, MCP-1) and stress-responses (HSPA1L, SAPK-3, SAPK2A). By contrast, TRL-ROO induced changes in gene expression in genes that belong to the functional classes of cell cycle regulators, intracellular signalling, cytoskeleton, transcription factors, receptors and immune response. However, the induction of these genes was moderate when compared with the effects of TRL-butter and TRL-VEFO. All TRL strongly induced the expression of HSP1A, a molecular chaperone that is also involved in stress and apoptosis.

3.4 Confirmation of the microarray data by RT-PCR

Seven of the genes that were considered to be differentially expressed were further analyzed by RT-PCR. The relative quantitative expression of the PCR products was consistent with their differential expression profile in the cDNA arrays: HSPA1A (RT 9.1-fold vs. microarray seven-fold), MIC-1 (6.7 vs. 4.5), IL-8 (4.8 vs. 3.1), SOCS-5 (5.1 vs. 3.8), MCP-1 (4.0 vs. 2.9), TIMP-1 (-3.0 vs. -2.3) and POU2F-1 (-3.2 vs. -2.3).

3.5 Effects of triglyceride-rich lipoproteins on proliferation, entry into S-phase and protein expression

The influence of TRL on the proliferation of CASMC was examined by assessing the effect of TRL on the incorporation of BrdU as a measure of DNA synthesis. Furthermore, the ability of TRL to stimulate quiescent cells to enter the cell cycle was analyzed by FACS analysis.

The incorporation of BrdU into CASMC was significantly increased 48 h after exposure to the TRL in a dose-dependent manner. ELISA assay was carried out in quiescent cells following incubation with physiological concentrations of 50, 100 or 200 μg/mL of TG–TRL. Accordingly, 50 μg/mL of TG was sufficient to promote cell proliferation while the maximal response was obtained at 100 μg/TG–TRL (*Figure 2A*), the concentration used in the following experiments. Proliferation was also dependent on the nature of dietary fats (*Figure 2B*) and TRL-butter induced significantly higher BrdU incorporation into CASMC than TRL-ROO and TRL-VEFO.

The ability of TRL to stimulate the entry of CASMC into the cell cycle was analyzed by FACS. We found that TRL released cells from growth arrest on a lipid dependent-manner (*Figure 3A*). When CASMC were rendered quiescent $92.0 \pm 2.1\%$ of cells were in the G_0/G_1-phase, $1.9 \pm 0.6\%$ were in the S-phase, and $6.1 \pm 1.7\%$ were in the G_2/M-phase (*Figure 3B*). After stimulation with TRL-butter for 12 h, the proportion of cells in G_0/G_1-phase decreased to $74.2 \pm 2.0\%$ while the proportion in S-phase increased to $17.0 \pm 1.9\%$. In cells incubated with TRL-ROO, the proportion of cells in G_0/G_1-phase decreased to $81.4 \pm 2.8\%$ and the proportion in the S-phase increased to $9.4 \pm 1.1\%$. TRL-VEFO did not produce a significant increase in the cells in the S-phase ($6.6 \pm 0.9\%$) when compared with TRL-ROO.

Cyclin D1 is necessary for entry and progression through the G_1/S-phase of the cell cycle. Significantly, TRL produced the transient induction of cyclin D1 within as little as 6 h (*Figure 4A*). The maximal effect was observed for TRL-butter at 12 h and while TRL-ROO induced significantly less cyclin D1 expression, TRL-VEFO provoked the lowest levels of expression (*Figure 4A*). DNA synthesis in the S-phase requires the DNA polymerase cofactor PCNA. PCNA protein levels were significantly higher at 12 and 18 h after TRL-butter stimulation than those found for

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Table 3 Genes regulated by triglyceride-rich lipoproteins (TRL) in coronary artery smooth muscle cells (CASMC) after 24 h of incubation

Biological function gene name	Gene bank	TRL fold change		
		Butter	ROO	VEFO
Molecular chaperones				
Heat-Shock 70 KDa-protein 1A; HSPA1A	NM_005345	+7.25	+6.69	+7.75
Homo sapiens heat shock 70kDa protein 1-like; HSPA1L	NM_005527	+2.71	_	+3.78
Cell cycle/proliferation/apoptosis				
Cyclin D1; CCND1	NM_001759	+5.88	+3.02	+2.44
Cyclin E1; CCNE1	NM_001238	+4.78	+2.65	+2.16
Retinoblastoma; RB1	NM_000321	+3.07	+2.54	_
Retinoblastoma-like2 (p130); RBL2	NM_005611	+2.99	+2.01	_
Proliferating cell nuclear antigen; PCNA	NM_002592	+2.91	+2.56	_
Calmodulin 1;CALM1	NM_006888	+2.73	+2.09	_
Cyclin-dependent kinase 2; CDK2	NM_001798	+2.68	+2.07	_
Cyclin-dependent kinase inhibitor 1A (p21, Cip1); CDKN1A	NM_000532	+2.60	+2.01	_
Mitotic Arrest-Deficient 2; MAD-2	NM_006341	+2.57	+2.03	_
Cyclin-dependent kinase inhibitor 1C (p57, Kip2); CDKN1C	NM_000076	+2.00	+2.52	+3.05
Interleukin-1 receptor-associated kinase 1; IRAK-1	NM_001569	_	_	+2.18
B-cell CLL/Lymphoma 2; BCL-2	NM_000657	−4.46	−3.06	_
Receptors				
Transforming Growth factor-beta receptor, type II; TGFBR-2	NM_001024847	+5.82	+4.17	+3.32
Bone Morphogenetic protein receptor, type II; BMPR-2	NM_001204	+5.02	+4.32	+3.01
Discoidin domain receptor family, member 1; DDR-1	NM_013993	+4.56	+2.64	+2.00
Retinoic acid receptor, beta; RARB	NM_000965	_	−2.01	−2.71
Signal transduction/protein amino acid phosphorylation				
Mitogen-activated protein kinase kinase kinase 1; MAP3K-1	XM_042066	+5.61	+4.78	+3.43
Dual-Specificity Tyrosine Phosphorylation-regulated kinase 1A; DYRK1A	NM_001396	+4.21	+3.40	+2.63
Mitogen-activated protein kinase phosphatase-3; MKP-3	NM_001946	+4.09	+2.65	+2.00
Polo-like kinase 3; PLK-3	NM_004073	+3.89	+2.02	_
Janus kinase 1; JAK-1	NM_002227	+2.97	+2.31	+3.83
Casein Kinase II, beta; CSNK2B	NM_001320	+2.95	+2.42	+3.55
Casein Kinase I, gamma 3; CSNK1G3	NM_004384	+3.62	+3.00	+2.45
Protein Tyrosine Kinase 9-Like; PTK9L	NM_007284	+3.44	+2.71	+2.20
Mitogen-activated protein kinase phosphatase-1; MKP-1	NM_004417	_	+2.02	+3.01
Polo-like kinase 2; PLK-2	NM_006622	+2.67	+2.07	_
Metabolic/catabolic process				
Insulin-induced gene 1; INSIG-1	NM_005542	+5.58	+4.75	+2.14
Cytochrome P450,subfamily 3A; CYP3A4	NM_017460	+5.67	+4.35	+6.69
Ornithine Decarboxylase 1; ODC-1	NM_002539	+4.53	+3.64	+3.01
Dihydropyrimidine dehydrogenase; DPYD	NM_000110	+2.29	_	+3.24
Propionyl-CoA carboxylase, beta subunit; PCCB	NM_000532	+2.01	_	+3.77
Pyruvate kinase muscle 2; PKM-2	NM_002654	−2.07	_	_
Immune/inflammation				
Macrophage-Inhibiting cytokine-1; MIC-1	NM_004864	+4.51	+3.83	+5.65
Interleukin 8; IL-8	NM_000584	+3.04	+2.49	+4.60
Interleukin1, beta ; IL1B	NM_000576	_	_	+4.54
Cyclooxygenase 2; COX-2	NM_000963	+2.92	+2.47	+4.09
Suppressor of cytokine signalling 5; SOCS-5	NM_014011	+3.88	+2.79	+2.20
Chemokine, cc motif, ligand 2, CCL2; MCP-1	NM_002982	+2.99	+2.45	+3.87
Cytoskeleton/motility				
Oncogene RAF-1	NM_002880	+5.17	+3.35	+2.03
Annexin A2; ANXA2	NM_004039	+4.34	+3.02	+3.55
Vimentin; VIM	NM_003380	+4.28	+3.43	+2.78
Protein phosphatase 4; PP4C	NM_002720	+3.53	+2.95	+2.43
Keratin 19; KRT-19	NM_002276	+2.49	+2.99	+2.01
Fibrillin; FBN	NM_000138	+2.92	+2.01	_
Tubulin, beta; TUBB	NM_178014	−2.78	_	_
Secreted protein, acidic, cysteine-rich (osteonectin); SPARC	NM_003118	−2.66	−2.00	_
Tubulin,alpha-2; TUBA2	NM_006001	−2.07	_	_
Thrombospondin; THBS-1	NM_003246	_	_	−2.03

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Table 3 *Continued*

Biological function gene name	Gene bank	TRL fold change		
		Butter	ROO	VEFO
Transcription factors activity/DNA-binding proteins				
Activator protein 1; AP-1	NM_002228	+4.02	+2.11	+5.18
General transcription factor IIH; GTF2H1	NM_005316	+4.97	+4.54	+4.00
Transcription Factor SP-1	NM_138473	+4.60	+4.01	+3.75
Nuclear factor-kappa B; NF-κB	L_26267	+4.42	+2.60	+3.46
E2F Transcription Factor 1; E2F-1	NM_005225	+2.59	+2.08	_
Small mother against decapentaplegic; SMAD-2	NM_005901	+2.71	_	+2.04
Zinc Finger Protein 91; ZNF-91	NM_003430	+2.33	_	_
POU domain, class 2, transcription factor 1; POU2F-1	NM_002697	−2.67	−2.11	_
Signal transduction/oxidative stress				
Stress-activated protein kinase 3; SAPK-3	NM_002969	+3.15	+2.41	+4.14
Stress-activated protein kinase 2A; SAPK2A	NM_001315	+4.02	+3.11	+6.51
Hormones/growth factors				
Epidermal growth factor; EGF	NM_001963	+3.76	+2.31	_
Stanniocalcin 1; STC-1	NM_003155	+2.96	+2.00	_
Vascular endothelial growth factor B; VEGFB	NM_003377	+2.64	+2.06	_
Fibroblast growth factor; FGF-5	NM_004464	+2.53	+2.01	_
Proteolysis/extracellular matrix				
Matrix metallopeptidase 2; MMP-2	NM_004530	+2.97	_	_
Matrix metalloproteinase 1; MMP-1	NM_002421	+2.66	_	_
Tissue Inhibitor of Metalloproteinase 1; TIMP-1.	NM_003254	−2.37	_	_
Other or unknown				
Cell division cycle 2-like protein kinase 6; CDC2L6	AB028951	+3.06	+2.02	+3.85
ATP-binding cassette, subfamily B, member 6; ABCB6	NM_005689	+3.25	+2.70	+3.99
Post-meiotic segregation increased 2-like 1; PMS2L1	NM_002679	+2.28	-	+2.65
Post-meiotic segregation increased 2-like 2; PMS2L2	NM_002679	+2.01	_	+2.75

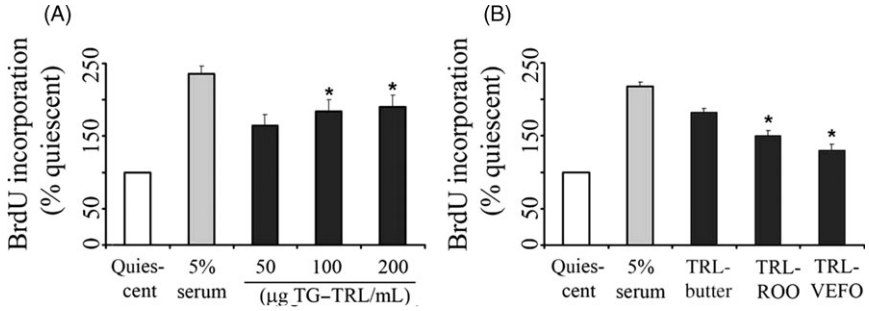

Figure 2 TRL (triglyceride-rich lipoproteins) induced CASMC (coronary artery smooth muscle cells) proliferation. BrdU (bromodeoxyuridine) incorporation was used as a measure of DNA synthesis in CASMC and in (*A*), the dose-response effect of TRL on BrdU incorporation in quiescent cells is shown (white). The cells treated with serum (grey) or the indicated concentrations of TG–TRL (black) for 48 h. *Significantly different from 50 μg TG–TRL ($P < 0.05$). (*B*) BrdU incorporation in cells exposed to 100 μg/mL of TRL-butter, TRL-ROO or TRL-VEFO (black) for 48 h. *$P < 0.005$ TRL-butter vs. TRL-ROO or TRL-VEFO. Values are normalized to the BrdU incorporation in quiescent cells and the bars indicate the SD as calculated from three different experiments.

TRL-ROO, and at 12, 18, and 24 h they were higher than those induced by TRL-VEFO (*Figure 4B*). Cell cycle progression may occur when complexes of D-type cyclins/CDK4/CDK6 phosphorylate Rb. Hyperphosphorylation of Rb was significantly higher after stimulation of TRL-butter, while no significant differences were found after exposure to TRL-ROO and TRL-VEFO (*Figure 4C*). In conjuction, these findings indicated that proliferation and entry into the S-phase were strongly induced by TRL-butter, and to a lesser extent by TRL-ROO and TRL-VEFO. Therefore, these data suggests that proliferation of CASMC can be induced by TRL in a lipid-dependent manner.

3.6 Activation of NF-κB and AP-1 by triglyceride-rich lipoproteins in coronary artery smooth muscle cells

To confirm whether NF-κB and AP-1 were activated in CASMC, cells were incubated with TRL for 2 h, and NF-κB and AP-1 binding were determined by EMSA. Low levels of basal NF-κB binding activity were detected in quiescent cells (*Figure 5A*) that were weakly activated following stimulation with TRL-ROO. Quantification of the data by phosphoimager analysis showed that the differences in NF-κB binding activity following TRL-VEFO and TRL-ROO stimulation were not significant. TRL-butter produced the

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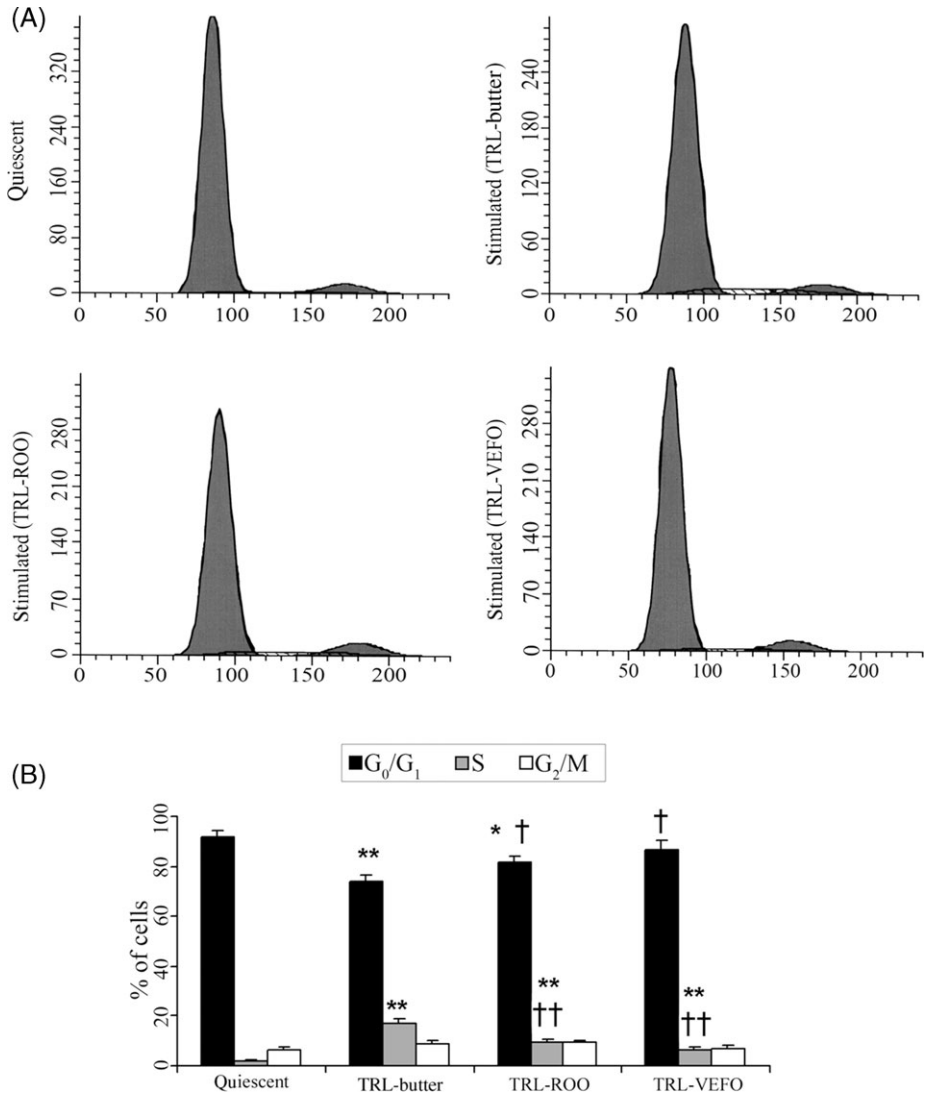

Figure 3 Differential effects of TRL (triglyceride-rich lipoproteins) on FACS-based cell cycle analysis. (*A*) Cell cycle distribution was determined by propidium iodide incorporation after cells were rendered quiescent (G_0/G_1-phase), and after a 12-h incubation with 100 μg TG/mL of TRL-butter, TRL-ROO, or TRL-VEFO. The G_0/G_1-phase is represented by the first peak, S-phase in the cross-bar, and G_2/M by the second peak. (*B*) Results expressed as the percentage of total cells in G_0/G_1 (black), S (grey), or G_2/M (white)-phase of the cell cycle. Significant differences from quiescent cells. $^*P < 0.05$, $^{**}P < 0.01$ and from TRL-butter: $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$. The bars show SD of three different experiments.

strongest activation of NF-κB (*Figure 5B*). The ability of TRL to influence AP-1 activity was particularly strong in cells exposed to TRL-VEFO (*Figure 5C*). In contrast, the weakest activation was detected after exposure to TRL-ROO, quantification of AP-1 binding demonstrated significant differences between each of the TRL tested (*Figure 5D*). Competition with a 50-fold excess of unlabelled oligonucleotides (specific competitor) abolished binding of the labelled probe, and binding to the oligonucleotide probe was still observed in the presence of a 50-fold excess of the mutant oligonucleotides, confirming the specificity of the binding (*Figures 5A* and *C*).

3.7 Triglyceride-rich lipoproteins oxidation by coronary artery smooth muscle cells

TBARS levels increased 4.3-fold from time 0 to 24 h in medium incubated with TRL-butter (0.07 ± 0.02 vs. 0.30 ± 0.09), while a 4.2-fold increase was observed in the medium incubated with TRL-VEFO (0.06 ± 0.02 vs. 0.25 ± 0.07) and a 3.3-fold increase in the medium with TRL-ROO (0.07 ± 0.03 vs. 0.23 ± 0.08). Levels of LDL resulted in a 15-fold increase over time (0.07 ± 0.3 vs. 1.05 ± 0.08). There were no significant differences between each of the TRL tested. However, LDL oxidability significantly increased compared with TRL ($P < 0.001$). Values are means for three experiments and expressed as nM malondialdehyde/mg of TG–TRL or LDL-protein.

4. Discussion

TRL are independent risk factors of CAD[1] and even though they are found in atherosclerotic plaques,[2] the direct effects on SMC are almost unknown. Several studies have shown that dietary fats modulate the fatty acid composition of postprandial TRL and that they have a regulatory influence on the postprandial haemostasis.[6,7] Hence, we hypothesized that postprandial TRL of different fatty acid compositions (SFA, MUFA, and PUFA) would be capable of

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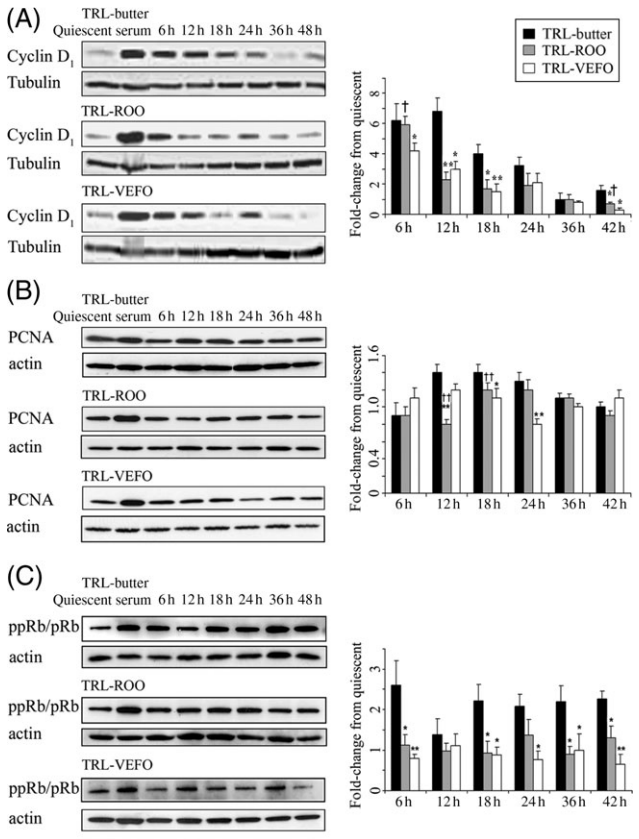

Figure 4 Differential effects of TRL (triglyceride-rich lipoproteins) on cell-cycle protein expression. The left part of the figure shows the representative autoradiograms corresponding to the cyclin D1 (*A*), PCNA (*B*) and ppRB (*C*) immunoreactivity from whole cell extracts of quiescent cells, treated with serum and TRL for 6, 12, 18, 24, 36, and 48 h. Right column shows the densitometric comparisons of protein expression in cells exposed to TRL-butter (black), TRL-ROO (refined olive oil) (grey), TRL-VEFO (vegetable and fish oils) (white). The values are expressed as the change with respect to protein expression in quiescent cells, significantly different to TRL-butter: $^{*}P < 0.05$, $^{**}P < 0.001$, and to TRL-VEFO: $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$. The data represent three independent experiments for each protein.

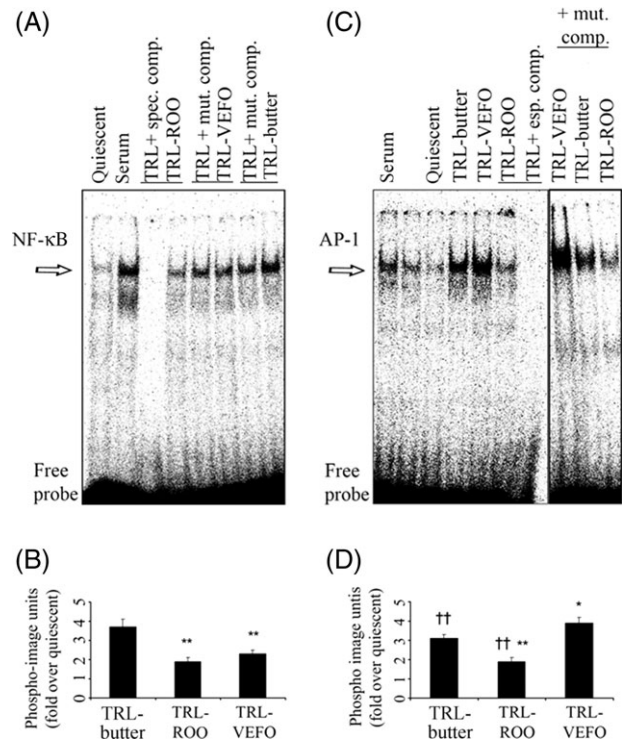

Figure 5 Electrophoretic mobility shift assay showing the effects of TRL (triglyceride-rich lipoproteins) on NF-κB and AP-1 activation. Induced (*A*) NF-κB and (*C*) AP-1 DNA binding activities in CASMC (coronary artery smooth muscle cells) treated with TRL for 2 h. Densities of (*B*) NF-κB and (*D*) AP-1 DNA binding as evaluated by PhosphoImager analysis and expressed with respect to the results from quiescent cells. Significantly different from TRL-butter: $^{*}P < 0.05$, $^{**}P < 0.001$, and TRL-VEFO (vegetable and fish oils): $^{\dagger\dagger}P < 0.01$. The bars indicate SD as calculated from three independent experiments. The arrows indicate the position of specific complexes, 'spec.comp' indicates the specific competitor oligonucleotides and 'mut.comp' the mutant competitor oligonucleotides.

differentially modifying gene expression in CASMC. In addition, we extended our investigations to explore the influence of TRL on CASMC proliferation.

4.1 Triglyceride-rich lipoprotein affects differential gene expression

We performed cDNA microarray analysis to examine the molecular basis of the effect of TRL in CASMC. We compared the effect of TRL-butter, TRL-ROO or TRL-VEFO on the expression profiles of 4376 known genes and compared these profiles with their steady state in serum-starved cells. A Correspondence analysis demonstrated that TRL induced a different gene profile in CASMC depending on their fat origin. Functional classification according to Gene Ontology (http://www.geneontology.org/) revealed the dominance of genes related to cell cycle, proliferation, and immune responses. The array data was then validated by real-time PCR for seven selected genes. Additionally, the differential expression of cyclin D1, PCNA, and pRB was verified by Western blotting, and the activation of AP-1 and NF-κB was assessed by EMSA.

Cell cycle regulatory molecules control eukariotic cell proliferation. The microarray data indicates that TRL stimulation provokes the up-regulation of several positive regulators of the cell cycle. All the TRL tested up-regulated cyclins D1 and E1, while the genes encoding CDK2, RB, and PCNA were only induced by TRL-butter and TRL-ROO. TRL also up-regulated genes that have a negative effect on cell cycle progress such as p57 and p21. While this might appear contradictory, the simultaneous induction of both cell cycle activators and suppressors has also been observed in ox-LDL stimulated cell proliferation.[16]

The role of lipoproteins in the development of atherosclerosis may result partly from their capacity to induce the intracellular signalling of protein kinases pathways. We have previously shown that TRL activates the extracellular signal-regulated kinases ERK1 and ERK2 in SMC.[13] Microarray analysis revealed that TRL-butter induced several protein kinases and phosphatases (MAP3K1, DYRK1A, PLK-3) that are important regulators of cell proliferation. However, MKP-1 was only up-regulated by TRL-ROO and TRL-VEFO which is in agreement with the fact that MKP are known to oppose proliferative stimuli.[17]

Several transcription factors and binding proteins known to control cellular proliferation and inflammatory responses were regulated by TRL. TRL-butter induced the strongest up-regulation of NF-κB, E2F-1, and SP-1, while AP-1 mRNA expression was strongly up-regulated by TRL-VEFO. TRL from hypertriglyceridemic subjects induce a pro-inflammatory response in the endothelium.[18] However, the selective

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effect of TRL according to their fatty acid composition has yet to be demonstrated. TRL-VEFO increased mRNA expression of inflammatory cytokines, chemokines, and adhesion molecules. Expression of the IL-8 gene was up-regulated by all the TRL, although it was strongly activated by TRL-VEFO. The higher LA content in TRL-VEFO could account for this, since LA but not OA up-regulates production of IL-8 in SMC.[19] IL1B and COX-2, both genes that are related to inflammation, were strongly up-regulated by TRL-VEFO. However, TRL-ROO diminished the levels of inflammatory gene expression in CASMC. Since TRL-ROO does not contain antioxidant compounds, their anti-inflammatory effects could have been mediated by OA. Indeed, MUFA-rich diets decrease the expression of inflammatory genes in endothelial cells.[20] Inhibitors of cytokine signalling such SOCS-5 were strongly up-regulated by TRL-butter, which is interesting since SOCS are negative regulators of cytokine signalling and they also have a clear effect on proliferation.[21]

Other proliferation-related genes that were up-regulated by TRL-butter, and to a lesser extent by TRL-ROO and TRL-VEFO include, TGFBR-2, BMPR-2, and INSIG-1. Growth factors involved in SMC proliferation, such as EGF, VEGFB, and FGF-5, were only up-regulated by TRL-butter and TRL-ROO, suggesting that the presence of n-3 PUFA could impede their induction by TRL-VEFO.[22] Other functional group include genes that encode heat shock proteins, all TRL strongly up-regulated HSPA1A while HSPA1L was only up-regulated by TRL-VEFO and TRL-butter. Failure of TRL-ROO to activate the expression of this gene is in agreement with its potential role in attenuating inflammation, since HSPA1L influences the concentrations of cytokine in plasma.[23]

Finally, TRL up-regulated expression of macrophage-inhibiting cytokine-1 (MIC-1) by more than five-fold. MIC-1 may constitute a marker of atherosclerosis as it is detected in the serum of individuals suffering from myocardial infraction.[24] MIC-1 is expressed in human atherosclerotic carotid arteries and is induced by ox-LDL in human macrophages.[25] Hence, it is significant that for the first time, we have shown that TRL can up-regulate MIC-1 expression in CASMC.

4.2 Effects of triglyceride-rich lipoproteins on cell proliferation and transcriptional activation

TRL can force cells to enter S-phase of the cell cycle and induce CASMC proliferation in the absence of any other mitogenic factor. Proliferation was dose-dependent up to a concentration of 100 μg TG–TRL/mL and it was affected by the nature of the TRL. TRL-butter had the strongest effect on CASMC proliferation while the presence of moderate amounts of n-3 PUFA in TRL-VEFO was able to attenuate proliferation, although the decrease was not significant when compared with TRL-ROO. The presence of SMC distinguishes progression-prone from progression-resistant lesions and in fact, progression-resistant lesions contain few SMC. TRL-butter increased the expression of cyclin D1 and hyperphosphorylated pRb, while TRL-ROO and TRL-VEFO diminished their expression, probably due to the presence of OA or EPA and DHA,[26] respectively. Similarly, both human and animal diets rich in SFA have been shown to produce greater lymphocyte proliferation than diets rich in OA, LA, or fish oil.[27,28] For the first time, our results suggest that the mitogenic effect of human TRL on CASMC depends on their fatty acid composition.

We confirmed that TRL differentially modulated AP-1 and NF-κB DNA binding activity in a lipid-dependent fashion in CASMC. TRL-butter induced the highest DNA binding capacity of NF-κB and indeed, postprandial studies showed that the intake of butter but not olive oil-enriched meals induced activation of NF-κB in human monocytes.[29] We have shown that postprandial TRL obtained after ROO ingestion did not completely inhibit but rather attenuated NF-κB-DNA binding in CASMC when compared with the butter meal. TRL could influence NF-κB by releasing fatty acids from core TG and while OA has little or no effect, SFA as well as n-6 and n-3 PUFA appear to activate NF-κB in endothelial cells.[30,31] Hence, the higher amount of OA could explain the low NF-κB transcriptional activation found after TRL-ROO incubation. Furthermore, we suggest that OA could reduce Cyclin D1 promoter activity by decreasing NF-κB-binding. While TRL-VEFO provoked the highest AP-1 activation, the role of n-6 and n-3 PUFA on AP-1 activation is controversial. Although n-6 PUFA appears to be most effective in activating AP-1 and in contributing to an inflammatory response,[31] n-3 PUFA may induce either AP-1 inhibition[32] or activation.[30] We suggest that the elevated ratio of n-6 to n-3 PUFA in TRL-VEFO may be a significant factor in mediating AP-1-DNA binding activity, and in the increase in inflammatory gene expression found in CASMC. Additionally, the resistance of TRL to oxidation did not depend on the fatty acid composition of the diet. Furthermore, TRL were more resistant to oxidation when compared with LDL, which may suggest that the fatty acid composition of TRL may be more important than their oxidation state in their atherogenicity. Accordingly, remnant TRL induces SMC proliferation regardless of its oxidative stress.[33]

4.3 Limitation of the study

We assessed the effect of TRL on the regulation of gene expression in human coronary artery SMC (CASMC) using a cell-based *in vitro* model. Extrapolation of the results to the human clinical situation remains uncertain.

In conclusion, the pathophysiological contribution of TRL to the development of atherosclerosis and the stability of the atherosclerotic plaque may depend on the fatty acid composition of TRL, indicating that the quality of the plaque rather than the quantity may determine the clinical consequences of atherosclerosis. Since enhanced SMC proliferation contributes to early lesion development, as does secretion of proinflamatory cytokines by SMC, TRL-butter could be considered a major determinant of plaque instability. TRL-VEFO reduced proliferation of CASMC, which could possibly lead to progression-resistant lesions, whilst inducing moderate gene expression of inflammatory cytokines. Ingestion of diets with a lower ratio of omega-6/omega-3 fatty acids could therefore offer protection against atherosclerosis. TRL-ROO induced moderate proliferation and a less atherogenic gene profile, which could improve atherosclerotic-plaque stability and support the prescription of olive oil enriched-diets in secondary prevention of cardiovascular disease. The results also suggest a role of MIC-1 in coronary artery cardiovascular events.

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Conflict of interest: none declared.

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