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Role of HDL in neutralizing the VLDL effect on endothelial dysfunction

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ABSTRACT

Objective: It has been reported that LDL inhibits endothelium-dependent relaxation (EDR) and that HDL can neutralize this effect. However, the atherogenic properties of VLDL have been so far difficult to demonstrate. Studies on VLDL are controversial, and nothing is known about the role of HDL on potential VLDL vascular actions. We examined the effect of human VLDLs on EDR, and the role of HDL in this system.

Methods: VLDL (n = 14) and LDL (n = 6) were isolated from volunteer subjects. Normal HDL was obtained from one healthy donor. VLDL ability to inhibit ACh-induced vasorelaxation (10^{-9}–10^{-5} mM) on aortic rings previously precontracted by noradrenaline (10^{-8} mM) was measured in the presence and absence of HDL.

Results: ACh-induced maximal relaxation (R%) was mildly, but not significantly attenuated in the presence of VLDL (72 ± 7%), while LDL caused a significant inhibition (60 ± 10%, $p < 0.05$) when compared to incubation in the absence of lipoproteins. VLDLs were subdivided into 2 groups depending on their cholesterol/triglyceride ratio: 0.18–0.22 (n = 8) was considered typical and 0.10–0.15, rich in triglycerides ($VLDL_{RT}$, n = 6). Typical VLDL had no effect on EDR ($p = 0.38$), however R% from $VLDL_{RT}$ was lower (54 ± 7%, $p < 0.01$) similar to the one obtained with LDL ($p = 0.32$). HDL showed favorable effects on EDR inhibition induced by the presence of $VLDL_{RT}$ ($p < 0.05$.).

Conclusion: Although typical VLDL did not cause endothelial dysfunction, triglyceride-enriched VLDL had inhibitory effect on EDR. It is proposed that alterations in VLDL composition would increase its atherogenic capacity. Moreover HDL appears to protect endothelium from VLDL action.

Introduction

For decades, hypertriglyceridemia has been associated with cardiovascular risk; however, it is still trying to be elucidated whether triglycerides are a risk factor or a biomarker of cardiovascular disease (Miller et al., 2011).

The increase in triglyceride levels reflects the accumulation of triglyceride-rich lipoproteins, among which VLDL particles are the most numerous and potentially atherogenic (Karpe et al., 1995; Rutledge et al., 2000).

The controversies about the relationship between triglycerides and vascular disease may be partly due to the heterogeneity of the triglyceride-rich lipoprotein particles, each of them with a different atherogenic impact. We have recently reported, according to other authors, that in insulin resistance states, VLDL particles are often larger and triglyceride over-enriched, contributing to a more atherogenic lipoprotein profile (Adiels et al., 2005; Lucero et al., 2012).

Moreover, as triglyceride-rich lipoprotein particles are lipolysed along the arterial surface, the potentially toxic products produced, could alter the endothelial function (Schwartz and Reaven, 2012). Endothelial dysfunction occurs before disruption of the vascular structure, constituting an early step in atherosclerosis development (Bonetti et al., 2003).

It has been clearly established that LDL causes endothelial dysfunction through the inhibition of endothelium-dependent relaxation, by decreasing nitric oxide bioavailability (Wang W et al., 2011). Regarding an effect of VLDL, reports are limited and controversial. Some authors postulate that VLDL may alter the endothelial function (Lewis et al., 1997) whereas others do not agree with this hypothesis (Lundman et al., 2001). These discrepancies could be due to a lack of characterization of the VLDL used in the assays.

On the other hand, it is well known that HDL protective actions expand beyond reverse cholesterol transport. Among its antiatherogenic roles HDL, seems to have a direct action on vascular endothelial function (Nofer et al., 2004). Even though it has been demonstrated that HDL antagonizes the deleterious vascular effects of oxidized LDL (Matsuda et al., 1993), little is known about the role of HDL on the potentially injurious vascular actions of VLDL.

Our aim in the present study was to evaluate the effects of human VLDLs on endothelium-dependent relaxation and the role of HDL in this setting.

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Methods

Samples

Lipoproteins were isolated from sera obtained from 18 volunteer subjects, who attended the University Clinic Hospital (Buenos Aires). The only exclusion criterion was to be under hypolipidemic treatment or using any other drug known to modify lipid metabolism. A healthy volunteer was selected as unique HDL donor. Written informed consent was obtained from each volunteer before blood was drawn.

After a 12-h overnight fast, blood samples were collected into dry tubes and serum was separated, kept at 4 °C and used within 24 h for lipoprotein isolation. Addition of EDTA (1 mg/ml) and sodium azide (0.1 mg/ml) was required to minimize lipid peroxidation and microorganism proliferation.

Lipoprotein isolation

Fresh sera were centrifuged 30 min at 15,000 rpm in order to discard the potential presence of chylomicrons. Lipoproteins were isolated from the infranatant by sequential preparative ultracentrifugation in an XL-90 Beckman ultracentrifuge, with a type 90 Ti rotor. VLDL [density (δ): 0.95–1.006 g/ml] and LDL [δ: 1.019–1.063 g/ml] were obtained after running at 105,000 ×*g*, for 18 h, at 15 °C. In parallel, HDL [δ: 1.063–1.210 g/ml] was obtained from the unique healthy donor, also by ultracentrifugation. The isolated HDL fraction was washed once, at the same density, in order to minimize albumin contamination. All buffers used for density adjustment contained EDTA 1 mM. Purity of lipoprotein fractions was tested by agarose gel electrophoresis, and albumin content was tested by immunoturbidimetry (Roche Diagnostics, Mannheim, Germany). Albumin in lipoprotein fractions was in no case higher than 2% of total protein. To preserve lipoproteins from modifications, all isolated fractions were used within the same day in the incubation assay.

VLDL composition

VLDL percentage chemical composition was assessed. Cholesterol and triglycerides were measured using commercial enzymatic methods (Roche Diagnostics, Mannheim, Germany) in a Hitachi 917 autoanalyser; inter-assay CV <3% for both parameters. Phospholipids were determined following Bartlett (Barlett, 1959), inter-assay CV <3.1%, and total protein content was quantified using the Lowry method (Lowry and Rosebrough, 1951), inter-assay CV <4.2%.

Cholesterol/triglyceride ratio in VLDL was calculated as an indicator of lipid proportion in lipoproteins, Table 1. Four VLDL samples were not included in the bioassay due to their scarce volume.

Reagents

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO., USA). All chemicals used for preparing Krebs solution were analytical grade.

Table 1
Percentage chemical composition of isolated VLDL.

Lipoprotein	Percentage chemical composition				Cholesterol/triglycerides
	Cholesterol	Triglycerides	Phospholipids	Total protein	
VLDL (n = 18)	10.3 ± 3.1	62.7 ± 8.5	12.2 ± 6.2	14.8 ± 6.1	0.18 (0.10–0.22)

Percentages are presented as mean ± SD. Cholesterol/triglycerides ratio is presented as median (range). VLDL: very low density lipoprotein.

In vitro assays

Preparation of aortic rings

The thoracic aorta from male Sprague–Dawley rats (200–250 g) was excised, transferred to a dish filled with Krebs–Henseleit buffer (composition in mM: 118 NaCl, 4.7 KCl, 1.8 KH_2PO_4, 1.15 Cl_2Mg, 1.13 NaH_2PO_4, 2.5 Ca Cl_2, 0,027 EDTA, 11 glucose, 25 $NaHCO_3$ and 0.11 ascorbic acid). The adhering tissue was immediately removed and the vascular segments were cut into 3 mm long rings. Aortic rings were suspended in organ baths containing Krebs solution constantly bubbled with a carbogenic mix (5% CO_2, 95% O_2) at 37 °C. Preparations were maintained at a basal tension of 1 g and allowed to stabilize for 1 h before the addition of any drug. Isometric force transducer (model FT03 B, Grass Instrument Company), coupled to a polygraph (model RPS 7C8, Grass Instrument Company), was used to record aortic ring contraction and relaxation.

Animal care and experimental procedures were in accordance with local and international guidelines concerning the use of laboratory animals for biomedical research (Guide, 1996). The animals had free access to a standard commercial diet and water, and were kept in rooms maintained at 22 ± 1 °C with a 12-h light/dark cycle.

Organ bath experimental protocol

The aortic rings were pre-contracted with noradrenaline (10^{-8} mM). Once the plateau was attained, concentration–response curves for acetylcholine (ACh; 10^{-9}–10^{-5} mM) were obtained. Ach-induced relaxation was expressed as the percentage of the pre-contracted tone obtained with noradrenaline. Maximal relaxation (R%) and pD2 were calculated.

Following the control curve, rings were thoroughly washed for 30 min, after which the vascular segments were incubated with isolated lipoproteins as described below. The relaxation response following incubation with each characterized lipoprotein was always compared to its corresponding preceding control response curve performed on the same vascular segment. The difference in maximal relaxation percentage between both curves was considered as the inhibitory effect of the lipoprotein on endothelium-dependent relaxation.

Effect of VLDL on endothelial function

VLDL was added to the organ bath to give a final concentration of 0.15 mg protein/ml. Artery rings were incubated in the lipoprotein-enriched solution for 120 min. In the continuous presence of VLDL, cumulative concentration–relaxation curves were repeated with the addition of ACh. Under the same conditions, LDL (0.15 mg protein/ml) was used as a positive control, given its known deleterious effect on endothelial function (Wang W et al., 2011).

In order to estimate the oxidative status in the incubation medium, lipid peroxide content was measured at 0 min and at 120 min after the incubation with aorta rings by means of thiobarbituric acid reactive substances (TBARS), as previously described (Schreier L et al., 1996). The calibration curve was prepared with a standard stock solution of malonaldehyde (MDA) and results are expressed as nmol MDA/mg VLDL protein.

VLDL and HDL co-incubation

In order to evaluate the effect of VLDL on endothelial function in the presence of HDL, both lipoproteins were co-incubated, as previously described. Concentration of HDL was selected after previous assay testing increased concentration of HDL proteins: 0.075, 0.15 and 0.30 mg/ml (data not shown). A final concentration of 0.15 mg protein/ml was selected, which represents the minimum concentration of HDL that exerts effect.

The resulting combined action on maximal relaxation percentage and the inhibitory effect on endothelium-dependent relaxation were determined.

In parallel, the effect of isolated HDL per se on ACh-induced relaxation was also assessed, as described for the other lipoproteins. To

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determine whether HDL had a protective effect on vascular function, the individual results obtained for each lipoprotein – VLDL or HDL – were considered. The percentage of inhibition of ACh-induced relaxation was obtained for each case. The sum of these effects was taken as the theoretical or "expected" value, which was compared to the actual or "observed" value, obtained after co-incubation with both lipoproteins.

The following calculation was applied:

$$\text{Protective effect of VLDL} + \text{HDL} = (\text{expected inhibition \% value} - \text{observed inhibition \% value}).$$

Expected value: the sum of the inhibition % exerted by isolated VLDL and HDL.

Observed value: the inhibition % exerted by co-incubated VLDL plus HDL.

Statistical analysis

Results were expressed as mean ± S.E.M. unless otherwise indicated. Concentration–response curves were plotted and experimental data were adjusted by the non-linear curve fitting program. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Tukey test. Paired *t*-test was applied for oxidation evaluation. P values less than 0.05 were considered statistically significant (Graph Pad Prism 5 version 5.03 for Windows).

Results

Effect of VLDL on endothelium-dependent relaxation

Fig. 1 exemplifies recorded polygraph plots of different lipoproteins. From these curves the concentration–response curves were constructed. Fig. 2 represents the mean concentration–response curve to ACh obtained in the absence of lipoproteins and in the presence of VLDL ($n = 14$) or LDL ($n = 6$) as a positive control lipoprotein-induced endothelial dysfunction. Maximal relaxation obtained with ACh was mildly attenuated in the presence of VLDL (72 ± 7%) in comparison to the control curve in the absence of lipoproteins (95 ± 2%); however this difference was not statistically significant while LDL, as a positive control, showed a significant inhibition of vasorelaxation (60 ± 10%, $p < 0.05$).

Taking into account the heterogeneity of VLDL particles, VLDLs were subdivided into two groups depending on their cholesterol/triglyceride ratio (chol/TG). VLDL chol/TG ratio showed a bimodal distribution: from 0.18 to 0.22 were considered typical VLDLs ($n = 8$), whereas from 0.10 to 0.15 were considered VLDL rich in triglycerides ($VLDL_{RT}$, $n = 6$). Data analysis was repeated with this two subpopulations; Fig. 2B shows the mean concentration–response curves to ACh obtained for typical and $VLDL_{RT}$. Typical VLDL did not show inhibition of endothelium-dependent relaxation, R% was similar to the control curve ($p = 0.380$). On the other hand, $VLDL_{RT}$ showed a reduction in relaxation that was significantly different from the effect of typical VLDL ($p < 0.05$), being in turn, similar to the R% obtained for LDL. No changes were observed in the sensitivity to ACh, since pD2 values were not affected (table 2).

With the aim of evaluating oxidative status, TBAR concentrations at 0 min and at 120 min after incubation were compared for both, typical VLDL and $VLDL_{RT}$. While no difference in TBAR concentrations after the incubation was observed with typical VLDL, TBARS in $VLDL_{RT}$ samples showed a tendency to an increase, delta (Δ) ± S:E:M: 0.32 ± 0.51, $p = 0.57$ for typical VLDL, and 1.27 ± 0.52, $p = 0.08$ for $VLDL_{RT}$.

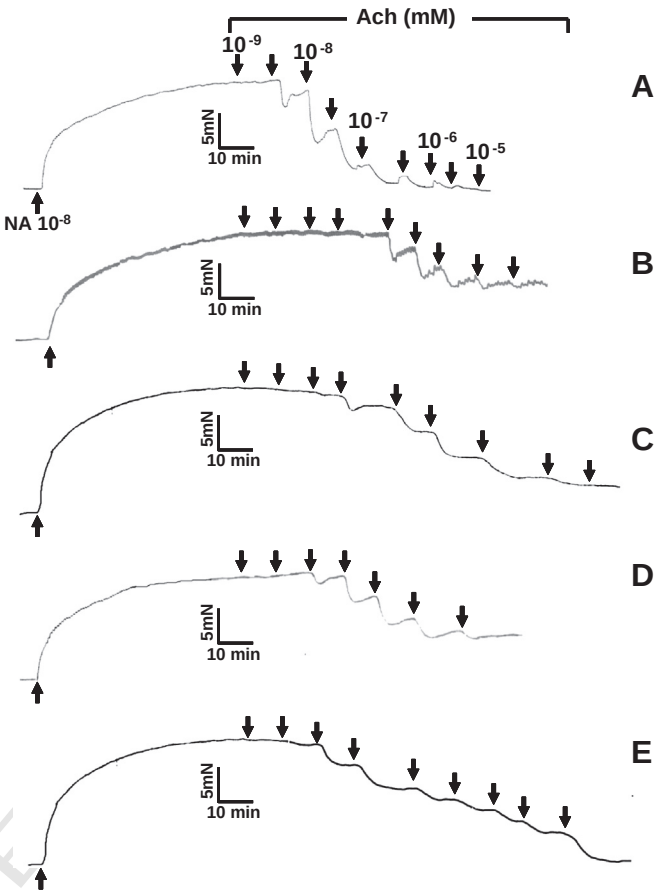

Fig. 1. Tracings show the acetylcholine-induced endothelium-dependent relaxation in the absence of lipoproteins (A) and in the presence of (B) LDL, (C) VLDL, (D) $VLDL_{RT}$ and (E) typical VLDL. The aortic rings were pre-contracted with noradrenaline (10^{-8} mM). Lipoproteins were added to the organ bath in a final concentration of 0.15 mg protein/ml and were incubated for 120 min. Ach: acetylcholine; NA: noradrenaline; $VLDL_{RT}$: VLDL rich in triglycerides.

Effect of VLDL in the presence of HDL on endothelium-dependent relaxation

Concentration–response curves to ACh obtained in the absence and presence of $VLDL_{RT}$, HDL and VLDL combined with HDL, are shown in Fig. 2C. As was previously shown in Fig. 2B, $VLDL_{RT}$ exerted a clear inhibitory effect on vasorelaxation; however, in the presence of HDL the vascular relaxation response was higher (72 ± 4%), when compared with the effect of $VLDL_{RT}$ alone ($p < 0.05$). Furthermore, HDL per se has also evidenced an attenuated relaxation response to ACh in comparison to control curve (76 ± 2% vs 95 ± 2%, $p < 0.05$).

To further analyze the inhibitory effect of HDL on VLDL vascular action, the inhibition % of endothelium-dependent relaxation exerted by VLDL in the absence and presence of HDL was compared. Fig. 3 shows the inhibition % of the "expected" value of the co-incubation, compared with the actual "observed" value, which was significantly lower ($p < 0.01$). In all cases, the interaction between VLDL and HDL caused a decrease in the inhibitory action on endothelium-dependent relaxation, compared to the expected additive effect.

Discussion

The effect of characterized VLDL on endothelium-dependent relaxation has been evaluated showing that triglyceride-enriched VLDL evidenced an inhibitory effect similar to that observed with LDL, whereas typical VLDL did not alter endothelial function.

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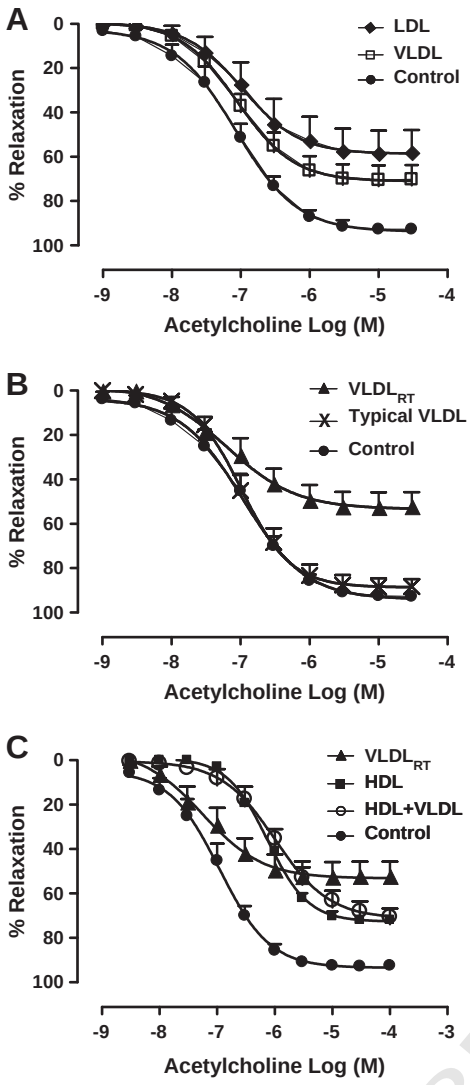

Fig. 2. Effect of lipoproteins on acetylcholine-induced endothelium-dependent relaxation of rat aortic rings. Mean concentration–response curves to acetylcholine obtained in the absence of lipoproteins (closed circles), and in the presence of (A) VLDL (open squares; 0.15 mg protein/ml), and LDL (closed diamond; 0.15 mg protein/ml). (B) Typical VLDL (asterisks; chol/TG 0.18–0.22; 0.15 mg protein/ml) and VLDL$_{RT}$ (closed triangles, chol/TG 0.10–0.15; 0.15 mg protein/ml). (C) VLDL$_{RT}$ (closed triangles; 0.15 mg protein/ml.), HDL (closed squares; 0.15 mg protein/ml.), and VLDL + HDL (open circles; 0.15 mg protein/ml each). Results are presented as mean and S.E.M. VLDL$_{RT}$: VLDL rich in triglycerides.

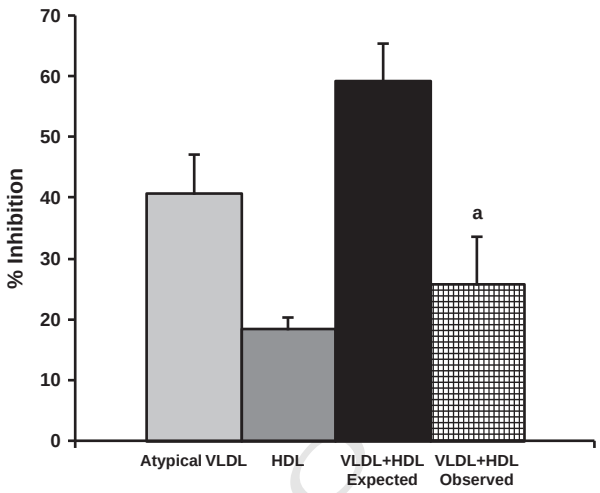

Fig. 3. Inhibitory effect of isolated lipoproteins and effect of VLDL–HDL interaction, observed and expected, on endothelium-dependent relaxation induced by increasing doses of acetylcholine in aortic rings pre-contracted with noradrenaline. [a]$p < 0.01$ vs VLDL + HDL expected.

Furthermore, HDL was able to partially revert the inhibition of vasorelaxation caused by VLDL rich in triglycerides.

Decreased endothelium-dependent vasorelaxation is an abnormality often observed at early stages of the development of atherosclerosis (Bonetti et al., 2003). Previous reports suggest that native lipoproteins could impair vasorelaxation (Lewis et al., 1997; Zheng and Liu, 2007). Most of this evidence points out to LDL; this lipoprotein contributes to the atherosclerotic process by producing endothelial dysfunction, among other deleterious actions (Steinberg, 2009).

However, the effect of VLDL on endothelial function has been scarcely studied, generally with controversial results. For instance, Lundman et al, working on a system similar to ours, demonstrated that while a triglyceride-rich fat emulsion and free fatty acids impaired endothelium-dependent relaxation, human VLDL did not affect endothelial function (Lundman et al., 2001). Results reported by other authors, also indicate that VLDL, does not exert endothelial dysfunction, but its remnants do (Doi et al., 1998).

Conversely, other studies have demonstrated that VLDL particles from healthy volunteers have inhibitory effects on in vitro endothelium-dependent relaxation (Lewis et al., 1997; Perségol et al., 2000). Furthermore, subsequent in vivo studies, that evaluated the endothelium function status, have reported that endothelium-dependent relaxation mediated by acetylcholine is impaired in hypertriglyceridemic patients with normal LDL cholesterol levels (Lewis et al., 1999).

In the present study, aortic rings have been incubated with human VLDL, and VLDL induced a mild inhibition of endothelium-dependent relaxation. However, when the VLDL fraction was subdivided according to particle lipid composition (chol/TG ratio), our results evidenced that VLDL rich in triglycerides (chol/TG < 0.18) produced endothelial dysfunction, unlike typical VLDL (chol/TG > 0.20).

Additionally, these triglyceride-enriched VLDLs have been described to be larger in size. These types of VLDL particles are often secreted in insulin-resistant states, as a consequence of a hepatic triglyceride overproduction, and are frequently precursors of an atherogenic lipoprotein profile (Adiels et al., 2008; Lucero et al., 2012).

Therefore, the controversial results reported in relation to VLDL effects on vasorelaxation, could be partly due to the heterogeneity of VLDL particles. Our finding of the inhibitory effect of VLDL rich in triglycerides suggests that alterations in VLDL composition would increase their atherogenic capacity.

In a previous work, we have observed a higher lipolysis by lipoprotein lipase on triglyceride-enriched VLDL particles (Schreier et al., 2002). This hydrolysis is likely to generate smaller remnants that are by themselves atherogenic because they can infiltrate the vascular wall and directly interact with macrophages, leading to the formation of foam cells. In turn, a variety of toxic products are released when VLDL undergoes lipolysis, like oxidized free fatty acids, phospholipids

Table 2
Effect of lipoproteins (0.15 mg protein/ml) on maximal relaxation response and potency (neg.log M EC50) to acetylcholine (mean ± S.E.M.).

	% Relaxation	pD2
Absence of lipoproteins (Control)	95 ± 2	7.1 ± 0.1
VLDL$_{RT}$	54 ± 7[a]	7.2 ± 0.2
Typical VLDL	91 ± 4[c]	6.9 ± 0.1
LDL	60 ± 10[b]	6.8 ± 0.2

VLDL: very low density lipoprotein. LDL: low density lipoprotein. VLDL$_{RT}$: VLDL rich in triglycerides. %Relaxation: maximal relaxation response. pD2: potency.
[a] $p < 0.01$.
[b] $p < 0.05$ vs control curve.
[c] $p < 0.05$ vs VLDL$_{RT}$.

and preformed mediators of oxidative stress that induce vascular cell inflammation and injury (Schwartz and Reaven, 2012; Wang L et al., 2009).

In the present study we couldn't demonstrate an increase in oxidative status in the medium during the incubation with VLDL; however, $VLDL_{RT}$ showed a tendency to an increase in TBAR production. It is expected that implementing a more sensitive method for the evaluation of oxidation, significant results could be obtained.

Since ACh-induced relaxation was demonstrated to be nitric oxide-dependent, the interpretation of our results suggests that VLDL rich in triglycerides, through non-specific cytotoxic compounds, may reduce nitric oxide bioavailability, leading to impairment of endothelium-dependent relaxation.

These results may explain in part why it has not still been conclusively determined whether triglycerides are atherogenic or not. Subgroup analyses in intervention trials such as BIP and ACCORD, suggest that triglyceride-rich lipoproteins may not be injurious in all metabolic circumstances (BIP study, 2000; Ginsberg et al., 2010). Beyond confounding factors, VLDL subtype could be determinant in endothelial dysfunction.

On the other hand, it has been extensively demonstrated that HDL plays a key protective role in atherogenesis, mainly by means of promoting cholesterol reverse transport (Rosenson et al., 2012). HDL has multiple additional endothelial and antithrombotic actions that can provide cardiovascular protection. HDL particles are likely to counteract the deleterious effect of ox-LDL on vascular relaxation by inhibiting the LDL oxidation process (Navab et al., 2000; Zago et al., 2004) and stimulating NO production (Tso et al., 2006). Nevertheless, few attempts have been made to elucidate the effect of HDL–VLDL interaction on endothelium-dependent relaxation.

Rutledge et al. showed that in an artery perfused with fluorescently-labeled VLDL, the lipolysis of VLDL increased by more than 2-fold its ability to cross through the endothelium and deposit lipids into the subendothelial space. However, when the artery was perfused together with HDL, decay in the subendothelial fluorescence intensity was detected. Therefore, HDL prevented VLDL lipid accumulation in the vessel wall (Rutledge et al., 2000).

The inhibition of vasorelaxation evidenced with isolated HDL was previously observed by other authors and it may depend on the phospholipid content (Lewis et al., 1997). In the present study, results showed that HDL per se can impair endothelial function. However, when co-incubated with VLDL, HDL partially reverted VLDL-induced inhibition of vasorelaxation, indicating that HDL particles were capable of counteracting the deleterious effect of VLDL on vascular relaxation.

A possible explanation for this effect may be the fact that HDL scavenges lipolysis products that may injure the endothelium and increase its permeability, similar to what was described for LDL protection (Matsuda et al., 1993). To our knowledge, this is the first report that has been able to demonstrate that HDL can protect endothelial function against triglyceride-enriched VLDL action.

It is to be taken into account that, even though these results highlight that VLDL particles with different compositions may impact differently on endothelium function, a characterization based solely on chol/TG ratio is a first attempt to evaluate the lipid composition of these particles. A complete characterization of VLDL subfractions, in composition and size, would be necessary for a deeper interpretation of these results. Studies are currently ongoing in our laboratory to address this issue, using size exclusion HPLC to assess the whole VLDL spectrum. Further studies are necessary to better account for the mechanism underlying the endothelial dysfunction caused by VLDL. On the other hand, in the co-incubation assays of VLDL/HDL, the concentration of HDL used was below its physiological levels, given that HDL protein concentration has been selected based on the criterion of the minimum concentration that can protect against vascular relaxation inhibition exerted by VLDL. Therefore, assays maintaining a VLDL/HDL physiological ratio still remain to be performed.

In conclusion, in the current study we provide evidence of an especially interesting finding; the fact that VLDL behavior on endothelium-dependent relaxation is not the same across different VLDL compositions. Triglyceride over-enriched VLDLs inhibited endothelium-dependent vasorelaxation, and HDL particles neutralized the VLDL deleterious effect on vascular function. This may contribute to the assessment of VLDL atherogenicity.

Author contributions

Zago Valeria: performed the experiments and participated in the experimental design, data analysis and writing of the manuscript; Gorzalczany Susana: participated in experimental procedures and writing of the manuscript; Lucero Diego: carried out sample collection and analytical procedures; Taira Carlos: participated in data research and analysis; and Schreier Laura: participated in the experimental design, scientific and technical supervision, data analysis and in revising the manuscript. The authors declare that they have no conflicts of interest.

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