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ORIGINAL ARTICLE

The influence of high-density lipoprotein (HDL) and HDL subfractions on insulin secretion and cholesterol efflux in pancreatic derived β-cells

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Abstract

Background High-density lipoprotein (HDL) is considered a complex plasma-circulating particle with subfractions that vary in function, size, and chemical composition. We sought to test the effects of HDL, and HDL subfractions on insulin secretion and cholesterol efflux in the β-cell line MIN-6.

Methods We used total HDL and HDL subfractions 2a, 2b, 3a, 3b, and 3c, isolated from human plasma, to test insulin secretion under different glucose concentrations as well as insulin content and cholesterol efflux in the insulinoma MIN-6 cell line.

Results Incubation of MIN-6 cells with low glucose and total HDL increased insulin release two-fold. Meanwhile, when high glucose and HDL were used, insulin release increased more than five times. HDL subfractions 2a, 2b, 3a, 3b, and 3c elicited higher insulin secretion and cholesterol efflux than their respective controls, at both low and high glucose concentrations. The insulin content of the MIN-6 cells incubated with low glucose and any of the five HDL subclasses had a modest reduction compared with their controls. However, there were no statistically significant differences between each HDL subfraction on their capacity of eliciting insulin secretion, insulin content, or cholesterol efflux.

Conclusions HDL can trigger insulin secretion under low, normal, and high glucose conditions. We found that all HDL subfractions exhibit very similar capacity to increase insulin secretion and cholesterol efflux. This is the first report demonstrating that HDL subfractions act both as insulin secretagogues (under low glucose) and insulin secretion enhancers (under high glucose) in the MIN-6 cell line.

Keywords Cholesterol efflux · HDL · HDL subfractions · Insulin secretion · MIN-6 cells · β-cells

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Introduction

The coexistence of insulin resistance and impaired insulin secretion are key features in type 2 diabetes (T2D) patients. Individuals with T2D elicit a progressive decline in β-cell function and impaired insulin secretion ~ 10 to 12 years before T2D onset, along with a nearly 50% decrease of the normal islet function at the time of the diagnosis [1]. Some of the well-known factors linked to β-cells dysfunction are lipotoxicity, glucotoxicity, increase of proinflammatory cytokines, and islet cell amyloid [1, 2]. Lipotoxicity could result from a chronic excess of free fatty acids [3], triglycerides, and cholesterol within the β-cell [4]. Evidence suggests that high-density lipoprotein (HDL) particles have an important role in glucose homeostasis, as well as directly on insulin secretion in β-cells [5]. In turn, epidemiological studies show that a low concentration of plasma HDL-cholesterol (HDL-c) is an independent risk factor for T2D [6–10]. In addition, a better HDL functionality measured by HDL-c efflux normalized to apoA-I is inversely associated with the development of T2D, even after adjustment for several risk factors [11, 12]. Along these lines of evidence, HDL-mediated cholesterol efflux is lower in patients with T2D as compared with controls [13], and a favorable HDL activity has been linked to the preservation of β-cell function in these patients, putatively through increased cholesterol efflux and the antioxidative capacity of HDL [14]. On the other hand, experiments performed in β-cell lines and animal models have shown that HDL particles and lipid-free apoA-I promote insulin synthesis and secretion [15, 16]. Mainly, apoA-I contributes to increase the number of insulin-docked granules through its internalization in the β-cell leading to an enhancement of insulin secretion [17]. Also, apoA-I increases the expression of a β-cell survival gene (*Pdx1)* and insulin production genes (*Ins1* and *Ins2*) [15, 18]. Nevertheless, HDL is considered a heterogeneous group of particles varying in composition and size [19]. It is well-known that HDL subpopulations have distinctive biological activities, such as anti-inflammatory, antioxidative, and vasoactive actions [20, 21]. The maturation of HDL produces particles that vary in size, density, and composition [22–24], from the smallest and more dense (HDL3) to the largest and less dense (HDL2), classifying HDL subfractions as 2a, 2b, 3a, 3b, and 3c. The protein component is higher in the smallest HDL subfractions when considering the weight percentage, ranging from 3c to 2a. Moreover, it has been shown that the function and composition of HDL subpopulations, mainly the proteome and lipidome, can be altered in the early stages of various diseases, including acute myocardial infarction, ischemic or valvular heart disease, which implies a putative dysfunction of their biological activities [25, 26]. Due to the heterogeneity of the HDL, their properties and functions cannot be directly inferred from HDL-c plasma levels, and as a consequence, a more detailed characterization is needed.

Thus, the objective of this study was to elucidate the effect of total HDL, and five HDL subpopulations isolated from plasma of healthy subjects, on insulin secretion under different glucose concentrations as well as on cholesterol efflux in the insulinoma cell line MIN-6.

Material and methods

Cell culture

MIN-6 cells at the passage 20–35 (AddexBio, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L of glucose (Thermo Fisher Scientific, USA) equilibrated with 5% CO_2 and 95% air. The medium was supplemented with 15% fetal calf serum (heat-inactivated), 2 mM L-glutamine (Sigma-Aldrich, USA), and 1% penicillin (Sigma-Aldrich, USA).

Isolation of HDL subfractions from human plasma

Peripheral venous blood samples from four healthy normolipemic male and four female volunteers were collected into sterile tubes (Vacutainer) containing K_3EDTA after 12-h fasting. None of the donors was receiving any drug known to affect lipoprotein metabolism or plasma concentrations. After blood collection, plasma was separated by centrifugation at 4 °C for 10 min. Then plasma lipoproteins were isolated from pooled samples by isopycnic density gradient ultracentrifugation corresponding to the well-defined density of each subfraction: HDL2b (d = 1.063–1.091 g/mL), HDL2a (d = 1.091–1.110 g/mL), HDL3a (d = 1.110–1.133 g/mL), HDL3b (d = 1.133–1.156 g/mL) and HDL3c (d = 1.156–1.179 g/mL), as previously described [27, 28], using a rotor (Beckman SW41 Ti) at 40,000 rpm for 44 h in a ultracentrifuge (Beckman XL70) at 15 °C. Total HDL was reconstituted at equivalent plasma concentrations of all the subfractions [25]. Low-density lipoprotein (LDL) particles were obtained with the former method at a density between 1.018 and 1.065 g/mL [25, 27, 28]. All lipoproteins were dialyzed against phosphate-buffered saline (PBS) for 24 h at a temperature of 4 °C in the dark, as previously stated [27, 28].

Chemical composition of HDL subfractions

We measured the total cholesterol (TC), free cholesterol (FC), phospholipid (PL), and triglyceride (TG) concentrations of each of the five HDL subfractions (2a, 2b, 3a, 3b, and 3c) using commercially available enzymatic assays (Wako Diagnostics and DiaSys, USA). Total protein (TP) was measured using the BCA assay (Thermo Fisher Scientific, USA). Cholesteryl esters (CE) were calculated by multiplying the difference between total and free cholesterol (expressed in mg/dL) by 1.67 [28, 29]. The chemical characterization of the five-isolated HDL was as we expected; the total protein content showed a trend to increase in parallel with the decrease of major lipid classes, measured as mg/dL (Fig. 1a), and weight percentage (Fig. 1b), from the smallest HDL (3c) to the largest HDL2b and 2a.

Insulin secretion assay

MIN-6 cells plated at 3×10^5 density per well (24 wells) were initially incubated at 37 °C with DMEM in normal glucose (5.5 mM) for 24 h. Then the cells were incubated with Hanks' balanced salt solution (HBSS) containing $CaCl_2$, $MgCl_2 \cdot 6H_2O$, $MgSO_4 \cdot 7H_2O$, KCl, KH_2PO_4, $NaHCO_3$, NaCl, Na_2HPO_4 with 0.1% (w/v) bovine serum albumin (BSA) and 2.8 mM glucose; after 1 h in HBSS, the media was substituted by HBSS containing 2.8 mM, 5.5 mM, or 25 mM glucose, with or without total HDL (protein concentration 0.5, 0.75 or 1 mg/mL) or each of the five HDL subfractions (HDL2b, HLD2a, HDL3a, HDL3b, or HDL3c, total protein concentration 1 mg/mL). After one hour, we collected the supernatant and centrifuged for 10 min at 10,000 rpm, and the released as well as the intracellular content of insulin was measured by radioimmunoassay (RIA) as stated elsewhere [30]. Trypan blue was used for assessed cell viability. All the cell protein was extracted and quantified using the Bradford method (Bio-Rad, USA) to normalize the results.

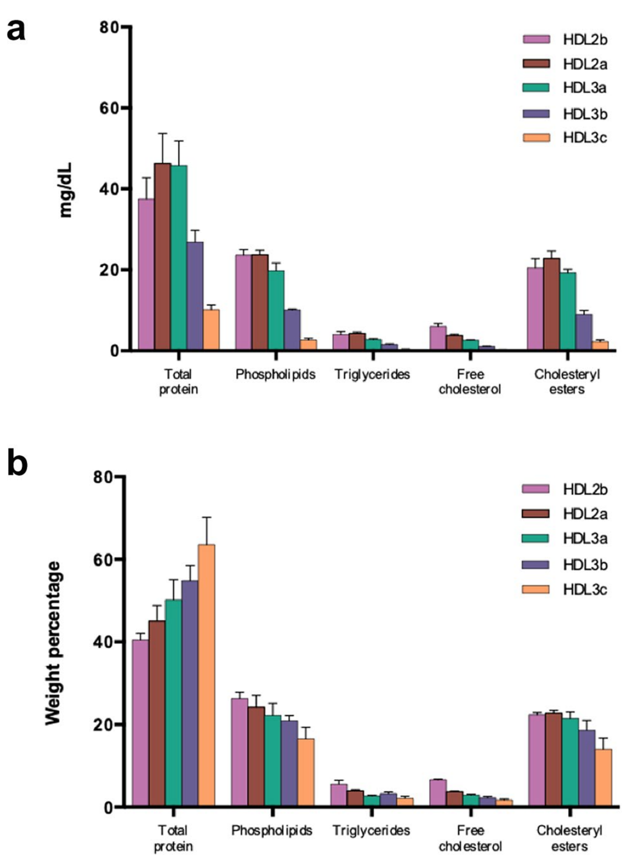

Fig. 1 Chemical composition of HDL2b, HDL2a, HDL3a, HDL3b and HDL3c subpopulations from 4 male and 4 female healthy donors, expressed as mg/dL (**a**) and as weight percentage of total mass (**b**). Data shown are mean ± SEM of three different experiments, each carried out in triplicate

Cholesterol efflux assay

Cholesterol efflux assays were performed using total HDL, or each of the HDL subfractions: 2b, 2a, 3a, 3b, and 3c, obtained from the plasma of eight healthy volunteers (4 men and 4 women), as we stated above. Efflux assays were performed as previously described [29]. Briefly, MIN-6 cells were seeded 3 x 10^5 cells/well in 24 well plates for 24 h. Then cells were labeled for 24 h with 1 μCi/mL ^{3}[H]-cholesterol in acetylated LDL (0.5 mL well, DMEM, 1% bovine serum albumin). The next day, cellular cholesterol pools were equilibrated with DMEM medium with 1% bovine serum albumin (0.5 mL/well) for 24 h. After that, cells were washed with PBS, and cholesterol efflux assay performed during 4 h with total HDL or each HDL subfraction (protein concentration of 20 μg/mL). PBS was used as a control, at the same volume as each HDL subfraction. Then the media was collected, and cell detritus were removed by centrifugation. The cell monolayer was washed with PBS, and cellular lipids were extracted with 3:2 hexane:isopropanol (v/v). Liquid scintillation counting was used to quantify ^{3}[H]-cholesterol concentration in the medium and cells. Cholesterol efflux capacity was calculated as: percent cholesterol efflux = [^{3}H]-cpm medium/([^{3}H]-cpm medium + [^{3}H]-cpm cells) × 100.

Statistical analysis

Experimental results are presented as mean ± standard error of the mean (SEM). Multiple comparisons were analyzed for continuous variables as appropriate by one-way ANOVA with post-test Bonferroni corrections for multiple testing. A *P* value < 0.05 was considered significant. GraphPad Prism version 6.0 was used for analyzing the present experimental data.

Results

Effect of HDL on insulin secretion under low, physiologic, and high glucose concentrations

To evaluate the effect of HDL from the human plasma on insulin secretion, we incubated MIN-6 cells under low (2.8 mmol/L glucose), physiologic (5.5 mmol/L glucose) or high glucose concentration (25 mmol/L glucose), without HDL or with HDL at 0.5 mg/mL, 0.75 mg/mL or 1 mg/mL (Fig. 2). Incubation for 1 h with 2.8 mmol/L glucose and 0.5 mg/mL of HDL released more insulin to the medium as compared to the control cells without HDL (101 ± 4 vs. 55 ± 7 ng insulin/mg of cell protein, $P < 0.01$). A similar trend was observed for insulin secretion at a glucose concentration of 5.5 mmol/L with 0.5 mg/mL of HDL vs. 5.5 mmol/L of glucose without HDL (146 ± 14 vs. 87 ± 9 ng insulin/mg of cell protein, respectively, $P < 0.05$). When MIN-6 cells were incubated in the presence of 25 mmol/L glucose for 1 h, the insulin concentration of the medium increased from 55 ± 7 to 283 ± 30 ng/mg cell protein (Fig. 2). Total HDL from human plasma (0.5 mg/mL of protein concentration) further increased the glucose-stimulated insulin secretion (GSIS) reaching a higher final concentration of insulin (393 ± 22 ng/mg cell protein) than the control (283 ± 30 ng/mg cell protein, $P < 0.05$). Similar results were observed when the cells were incubated with HDL at 0.5, 0.75, or 1 mg/mL in the presence of physiologic or high concentrations of glucose. These results showed HDL particles are able to trigger insulin secretion under low, physiologic, and high glucose conditions.

Effect of HDL subfractions on insulin secretion under high glucose concentrations in MIN-6 cells

To ascertain whether the capacity of HDL subfractions is different between them to increase insulin secretion under high glucose concentrations, MIN-6 cells were incubated with 25 mmol/L for 1 h. Incubation with 25 mmol/L glucose and 1 mg/L of HDL protein increased insulin release from 245 ± 22 to 410 ± 22 ng/mg of cell protein ($P < 0.001$) (Fig. 3a). Similarly, HDL subfractions 2a, 2b, 3a, 3b, and 3c increased insulin secretion at a higher level than controls ($P < 0.05$). Again, no statistical differences were observed between the effect of total HDL and the HDL subfractions.

Effect of HDL subfractions on insulin secretion and intracellular content of insulin under low glucose concentrations in MIN-6 cells

According to our findings, the stimulus with total HDL from human plasma increased insulin secretion in a stronger fashion than glucose alone: hence we further assessed the contribution of each HDL subfraction: 2b, 2a, 3a, 3b, and 3c on insulin secretion, taking into account the highest HDL concentration (protein concentration of 1 mg/mL) under low glucose (2.8 mmol/L) for 1 h in MIN-6 cells. Figure 3b shows that all HDL subfractions and total HDL exhibited higher insulin secretion than their respective controls ($P < 0.001$). Although no statistically significant differences were found between the HDL subfractions, there was a trend towards subfractions 2b and 2a showing lower insulin secretion compared with the HDL 3a, 3b, and 3c subfractions. Regarding intracellular insulin content of the MIN-6 cells incubated with total HDL or each of the subfractions, the insulin content was also lower in the cells that were

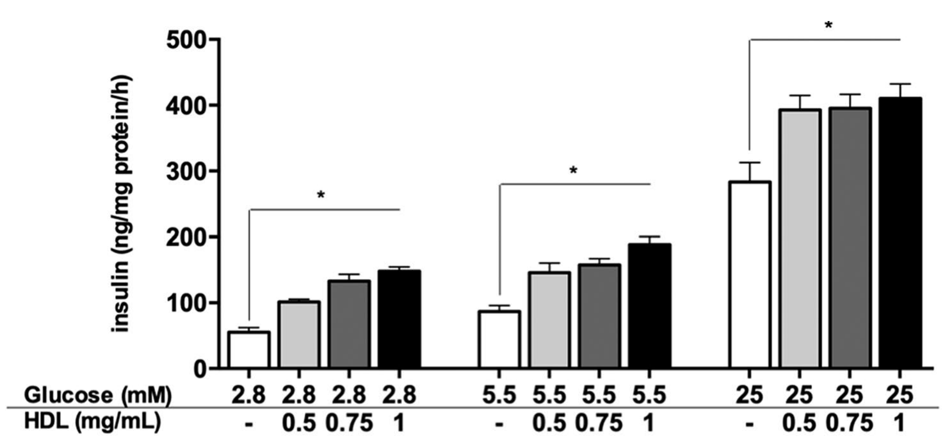

Fig. 2 Effect of human HDL on insulin secretion from MIN-6 cell line. MIN-6 cells were incubated during 1 h with 2.8, 5.5, or 25 mmol/L of glucose, in the absence (white bars, control cells) or the presence of HDL with 0.5 mg/mL (grey bars), 0.75 mg/mL (grey dark bars), or 1 mg/mL (black bars). All the results were normalized with total cellular protein concentration. Results represent the mean ± SEM of four experiments carried out in quadruplicate, *$P < 0.05$ compared with control cells

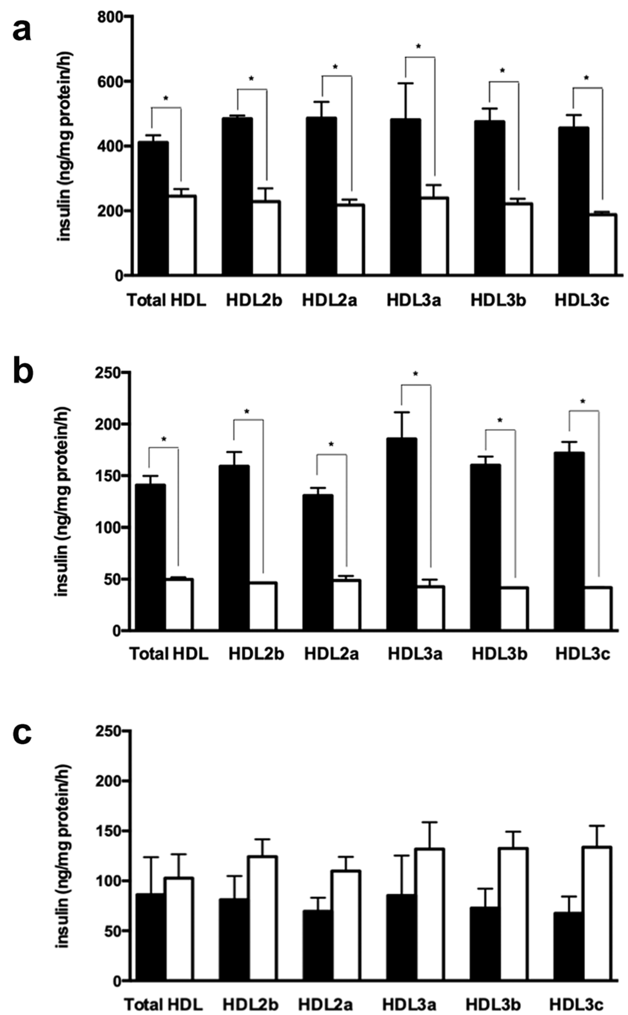

Fig. 3 Effects of HDL subfractions on insulin secretion and intracellular content of insulin in MIN-6 cell line. MIN-6 cells were incubated during 1 h with 25 mmol/L (**a**) or 2.8 mmol/L (**b**, **c**) of glucose either in the presence of 1 mg/mL total HDL or their subfractions 2b, 2a, 3a, 3b and 3c (black bars) or absence of HDL (white bars). Insulin levels of the medium (**a**, **b**) and intracellular insulin (**c**) were measured by RIA. All results were normalized with total cellular protein concentration. Results represent the mean ± SEM of 4 experiments carried out in triplicate, **P* < 0.05 compared with their respective control

incubated with each of the five HDL subclasses and low glucose concentrations as compared with their respective controls, however, no statistical differences were observed (Fig. 3c).

Effect of HDL subfractions on cholesterol efflux in MIN-6 cells

We assessed total HDL and HDL subfractions role in cholesterol efflux in β-cells (MIN-6). We evaluated the cholesterol efflux elicited by total HDL and HDL subfractions 2b, 2a, 3a, 3b, and 3c and compared them with their respective

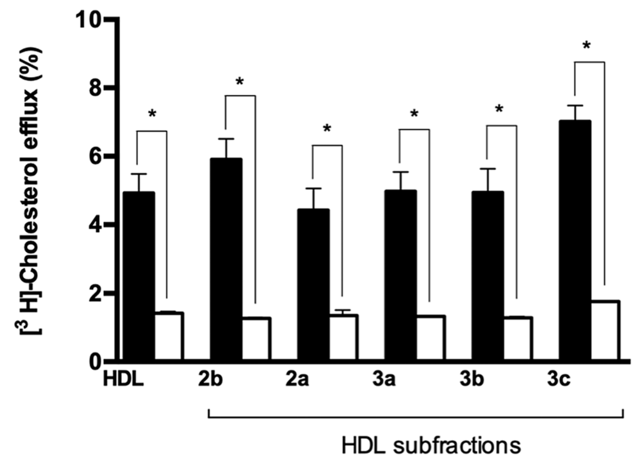

Fig. 4 Cholesterol efflux capacity of total HDL and HDL subfractions in MIN-6 cell line. MIN-6 cell line was incubated during 24 h with 1 μCi/mL 3[H]-cholesterol in LDL acetylated, then equilibrated with DMEM medium, afterwards the cholesterol efflux assay was performed either with total HDL or their subfractions 2b, 2a, 3a, 3b or 3c. Results represent the mean ± SEM of 3 experiments carried out in duplicate, **P* < 0.05 compared with their respective control

controls. All the HDL subfractions showed higher cholesterol efflux than their controls ($P < 0.05$). HDL3c subfraction elicited the largest cholesterol efflux (7.0%), and HDL2a subfractions evoked the smallest one (4.4%), but the difference between these two subfractions did not reach statistical significance ($P = 0.07$) (Fig. 4).

Discussion

In the present study, we assessed the role of native HDL and HDL subfractions on insulin secretion and cholesterol efflux in the insulinoma pancreatic cell line MIN-6. Our results show that HDL particles isolated from healthy subjects promote insulin secretion in the MIN-6 β-cell line in vitro, as they displayed a larger effect on insulin secretion than glucose alone. It has been documented that HDL works in two ways: by enhancing the effect of glucose as well as insulin secretagogues [5, 15, 16].

We observed that glucose promotes insulin secretion in a glucose concentration-dependent manner. However, when total HDL was present, insulin release increased further, regardless of the level of glucose. Importantly, in the present work we used HDL isolated from healthy subjects, as these particles putatively retain all physiological functions. Our results show that native HDL has the ability to increase insulin release in a glucose independent fashion. Under high glucose concentrations, HDL enhances glucose-stimulated insulin secretion (GSIS), presumably via the classic secretory pathway, dependent of the K_{ATP} channel activation [31]. However, under low glucose conditions, we also found that

HDL increased insulin secretion when compared with controls, consistent with the results by Fryirs et al. where they observed that the main proteins of the HDL, apoA-I, and apoA-II, were involved in the activation of cAMP, and consequently insulin release [15]. The insulin content was also lower in the cells that were incubated with each of the five HDL subclasses and low glucose concentrations compared with their respective controls. Even though the result was not statistically significant, it suggests that more insulin was released to the medium, and putatively there was an increase in insulin production due to the effect of the HDL. Similar results have been published for the long-term incubation with lipid-free apoA-I and apoA-II [15]. However, we did not specifically measure each apolipoprotein concentration, it is known the most abundant proteins in HDL are apoA-I (70%) and apoA-II (20%) [22].

In addition, we tested different concentrations of HDL to evaluate whether the insulin release increased accordingly; however, we did not find any difference when using increasing HDL concentrations. These results suggest that the effect of HDL on insulin release has maximum efficiency at any given HDL concentration (i.e., a ceiling effect).

On the other hand, it has been suggested that total HDL-c plasma concentration may not be a good biomarker for HDL functionality, as it does not reflect the complexity and putative differential functions of HDL subfractions [32]. It is of interest that when we used each one of the five HDL subfractions, we did not find significant differences in insulin secretion. A putative explanation is that we used the same concentration of HDL protein in all the evaluated subfractions. Hence, it is possible that by adjusting the protein concentration when assessing the effect of different HDL subfractions, we had missed a potential differential effect based on protein content. As it has been demonstrated before, the protein portion (mainly apoA-I) is an important regulator of insulin secretion [15, 18]. However, despite not having found significant differences, we did observe a trend in the small and dense HDL subfraction showing the most potent effect on insulin secretion under low glucose concentrations and the HDL3a displaying the strongest secretagogue capacity.

In the same line of evidence, even though we did not find significant differences among the HDL subpopulations on cholesterol efflux, we observed a trend towards the subpopulation 3 displaying the largest effect on cholesterol efflux. Importantly, this observation is consistent with earlier studies in other cell types such as macrophages, or hepatocytes derived cell lines showing a high cholesterol efflux mediated by the 3c subfraction [29, 33]. The HDL3 subfraction is considered the most active subpopulation in terms of cholesterol efflux mediated by ABCA1, probably due to the conformational differences in the apolipoprotein apoA-I between the small and dense particles (HDL3) compared with the larger ones (HDL2) [33]. Thus, although the HDL3c accounts for less than 15% of the total HDL, it has a potent effect against oxidation caused by LDL particles [24], a result that has been directly associated with the protein apoA-I [34].

Interestingly, beneficial roles of HDL particles in humans have been proven after the infusion of reconstituted HDL (rHDL) in patients with T2D in a double blind, placebo-controlled study. At the end of the four hours, plasma insulin levels were higher, and insulin sensitivity increased in the rHDL group, with the consequent decrease in plasma glucose levels [5]. Thus, the effects of the HDL particles can be seen not only as secretagogues of insulin but also as modulators of glucose metabolism [34, 35], increasing glucose uptake by human myocytes [34] and as mediators of the release of adiponectin, an adipocyte hormone that increases insulin sensitivity [36]. Therefore, HDL molecules have been shown to improve β-cells function, both by in vitro and in vivo studies.

This is the first study assessing the role of native HDL and HDL subfractions isolated from healthy individuals both on insulin secretion and cholesterol efflux in a pancreatic β-cell line MIN-6. Although we were not able to prove any differential effects for each HDL subfractions regarding insulin secretion or cholesterol efflux, we showed that HDL subfractions displayed a dual role both as insulin secretion enhancers as well as insulin secretagogues. However, it would be interesting to further characterize the protein and lipid content of each subfraction, as well as to assess the role of native HDL and HDL subfractions from patients with different metabolic conditions such as T2D and cardiovascular disease.

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Author contributions A-OG designed, performed experiments, analyzed data and wrote the manuscript. D-GQ, L-MH, AG, ED-D, RR-G, and IBM-A designed and/or performed experiments. OP-M, AZ-D and CAA-S designed, supervised the research and edited the manuscript. MTT-L designed, supervised the research and wrote the manuscript MTT-L is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Data Availability Statement The data will be shared by direct request to the corresponding author.

Compliance with ethical standards

Conflict of interest The authors declare that there are no competing conflicts of interest.

Ethics approval All procedures perfomed in human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments ethical standars. The study was approved by The Committee of Ethics and the Institutional Review Board of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ).

Informed consent All the participants provided written informed consent before inclusion in the study. Participants did not receive any stipend for taking part in the study.

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