Effects of angiopoietin-like protein 3 deficiency on postprandial lipid and lipoprotein metabolism

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Abstract The consequences of angiopoietin-like protein 3 (ANGPTL3) deficiency on postprandial lipid and lipoprotein metabolism has not been investigated in humans. We studied 7 homozygous (undetectable circulating ANGPTL3 levels) and 31 heterozygous (50% of circulating ANGPTL3 levels) subjects with familial combined hypolipidemia (FHBL2) due to inactivating ANGPTL3 mutations in comparison with 35 controls. All subjects were evaluated at fasting and during 6 h after a high fat meal. Postprandial lipid and lipoprotein changes were quantified by calculating the areas under the curve (AUCs) using the 6 h concentration data. Plasma changes of β-hydroxybutyric acid (β-HBA) were measured as marker of hepatic oxidation of fatty acids. Compared with controls, homozygotes showed lower incremental AUCs (iaAUCs) of total TG (−69%, P < 0.001), TG-rich lipoproteins (−90%, P < 0.001), apoB-48 (−78%, P = 0.032), and larger absolute increase of FFA (128%, P < 0.01). Also, homozygotes displayed attenuated postprandial lipemia, but the difference was significant only for the iaAUC of apoB-48 (−28%; P < 0.05). During the postprandial period, homozygotes, but not heterozygotes, showed a lower increase of β-HBA. Our findings demonstrate that complete ANGPTL3 deficiency associates with highly reduced postprandial lipemia probably due to faster catabolism of intestinally derived lipoproteins, larger expansion of the postprandial FFA pool, and decreased influx of dietary-derived fatty acids into the liver. These results add information on mechanisms underlying hypolipidemia in FHBL2.

In observational studies, plasma concentrations of TGs in both fasting and fed state are associated with risk of coronary heart disease (1, 2). Therefore, in searching for new therapeutic strategies to prevent cardiovascular disease, the investigation of factors regulating plasma TGs has received a great deal of interest in recent years (3). In particular, two angiopoietin-like proteins, angiopoietin-like protein 3 (ANGPTL3) and angiopoietin-like protein 4 (ANGPTL4), have been intensively studied (4–8). Both ANGPTLs have been characterized as potent inhibitors of LPL (4, 9, 10). LPL hydrolyzes TG in TG-rich lipoproteins (TRLs) derived from gut (chylomicrons) and liver (VLDLs). By its action, remnant particles are formed and taken up by the liver while FFAs are released into the circulation (11). Animals overexpressing ANGPTL3 and ANGPTL4 exhibit reduced circulating TG concentrations with improved insulin sensitivity (12, 13).

Abbreviations: ANGPTL3, angiopoietin-like protein 3; ANGPTL4, angiopoietin-like protein 4; AUC, area under the curve; CETP, cholesterol ester transfer protein; FABP4, fatty acid binding protein-4; FHBL2, familial combined hypolipidemia; FM, fat mass; FFM, free fat mass; FPLC, fast-performance liquid chromatography; HDL-C, HDL cholesterol; β-HBA, β-hydroxybutyric acid; iAUC, incremental area under the curve; LDL-C, LDL cholesterol; LOF, loss-of-function; MET, metabolic equivalent; OFTT, oral fat tolerance test; PLTP, phospholipid transfer protein; PON1, paraoxonase 1; REE, resting energy expenditure; RP, retinyl palmitate; TRL, TG-rich lipoprotein.

This study was presented in part at the 17th International Symposium on Atherosclerosis, Amsterdam, The Netherlands, May 23–26, 2015.

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The online version of this article (available at http://www.jlr.org) contains a supplement.
ANGPTL4 show lower LPL activities and hypertriglyceridemia (12), while those lacking ANGPTL3 have reduced levels of TGs and FFAs and increased LPL activity (10, 12).

Progress in the elucidation of ANGPTL3 function in lipid metabolism in humans has been made by the identification of individuals carrying inactivating mutations in the ANGPTL3 gene (13). These individuals present a distinct lipid phenotype characterized by marked reduction of all major plasma lipoprotein classes (VLDL, LDL, and HDL). This disorder was designated as familial combined hypolipidemia (FHBL2; OMIM #605019). The cause of low VLDLs in FHBL2 has been attributed to a reduced production of VLDLs (13) and this, in turn, has been hypothesized to be due to a reduced influx of TG-derived fatty acids into the liver (14, 15). On the other hand, mechanisms underlying low LDL and HDL levels in FHBL2 remain elusive. It has been proposed that low LDL might be due to reduced VLDL to LDL conversion (14), even though an increased clearance rate of LDL particles has been observed in homozygous individuals (13). Reduced HDL has been explained by elevated endothelial lipase activity in the absence of ANGPTL3 (16), but the direct in vivo demonstration of this mechanism in FHBL2 subjects is lacking (17). Therefore, the full effect of ANGPTL3 on lipoprotein metabolism is likely to be very complex and is not yet fully understood.

We have recently identified a large cohort of individuals carrying the p.S17* loss-of-function (LOF) mutation in the ANGPTL3 gene presenting the FHBL2 phenotype (18). Plasma levels of ANGPTL3 were undetectable in homozygous and about half of normal in heterozygous carriers, thus allowing us to explore the clinical and metabolic consequences of ANGPTL3 deficiency in humans. In particular, we found that, compared with noncarriers, homozygotes, but not heterozygotes, displayed a 3-fold higher postheparin LPL activity. Moreover, we also observed that the homozygotes had reduced fasting plasma levels of FFAs and lower homeostasis model assessment of insulin resistance compared with control subjects (17). Based on these findings, it is reasonable to postulate that complete ANGPTL3 deficiency would cause accelerated postprandial clearance of TRLs, an effect that might be potentially implicated in FHBL2 hypolipidemia. However, no direct demonstration of this effect has been provided so far. In addition, the mechanism underlying lower FFA levels in FHBL2 is also poorly understood. Previous human studies have documented that postprandial chylomicron fatty acid spillover is a significant source of FFAs, contributing to 20–30% of the plasma FFA pool (19). Therefore, the investigation of FFA changes during the post absorptive period may shed light on this peculiar phenotypic consequence of ANGPTL3 deficiency. Due to the link between HDL remodeling and TRL catabolism (20), we sought to determine whether plasma levels and composition of HDL particles were differently affected in FHBL2 compared with control individuals during the postprandial period. Finally, results of experimental studies indicated that ANGPTL3 and ANGPTL4 respond differently to fasting and refeeding. In fact, ANGPTL4 is maximally expressed in fasted state, while plasma levels of ANGPTL3 are only modestly altered by fasting and refeeding (6). Unfortunately, no data are available in humans so that the coordinate regulation of ANGPTL4 and ANGPTL3 during the postprandial period remains unexplored.

Therefore, we have carried out the present study to investigate, in carriers of inactivating mutations in ANGPTL3 as compared with controls, the dynamic response of plasma lipids and lipoproteins to an oral high fat load. Additionally, we have also evaluated the postprandial variation in plasma levels of ANGPTL3 and its cognate, ANGPTL4, according to the ANGPTL3 deficiency status.

MATERIALS AND METHODS

Study subjects

The study population was comprised of 38 carriers of ANGPTL3 mutations [5 homozygotes and 29 heterozygotes for the p.S17* (18) and 2 compound heterozygotes and 2 heterozygotes for the p.E94* and p.N121Kfs*3]; they were selected from 22 families and 17 of them were related. Thirty-five unrelated subjects not carrying any ANGPTL3 variant served as controls. None of subjects were consuming special diets or taking medication affecting lipid metabolism.

In the morning after an overnight fast, all participants were invited to undergo clinical evaluation, blood drawings, and body composition and indirect calorimetry measurements, and to receive an oral fat tolerance test (OFTT). Anthropometric and clinical characteristics of study subjects were assessed as previously described (18). All participants completed a 3 day dietary record and their dietary intakes were calculated using food analysis software according to the Italian Food Composition tables (21). Alcohol consumption was estimated by a semi-quantitative scale and subjects were classified as abstainers (zero glasses per day), users (one glass per day), and heavy drinkers (greater than or equal to two glasses per day). The level of physical activity was assessed using the International Physical Activity Questionnaire (short version), which was administered by face-to-face interview (22). Results were expressed as metabolic equivalents (METs) per minutes per week.

The Ethical Committee of Sapienza University of Rome approved the study protocol and all subjects provided their informed consent.

Body composition

Body composition was assessed by bioelectrical impedance analysis using a single-frequency 50 kHz analyzer, STA-BIA (AKERN Bioresearch SRL, Pontassieve, Florence, Italy). Measurements were taken following standardized procedures (23). Estimations of fat mass (FM) and fat-free mass were obtained using gender-specific bioelectrical impedance analysis prediction equations, as indicated by Sun et al. (24).

OFTT

Participants were challenged with a mixed meal type of oral fat load. The test meal, represented by a muffin prepared with olive oil, eggs, ricotta cheese, nuts, cocoa, and wheat flour, and served with 100 g skinned milk, was ingested within 10 min between 8:00 and 8:30 AM. The test meal provided 75 g fat, mostly constituted by long chain fatty acids, 52 g carbohydrate, 22 g protein, and 145 mg cholesterol (~950.2 kcal total calories), according to the
available recommendations (25, 26). A standard dose (100,000 UI) of vitamin A (retinyl palmitate (RP)) was added to the test meal to evaluate RP as a marker of chylomicron metabolism (27). During the test, small amounts of water were allowed and participants remained inactive. The test meal was well-tolerated by all subjects.

Venous blood samples were taken before and every 2 h up to 6 h after consumption of the test meal in EDTA-containing tubes and plasma was recovered within 10 min by low speed centrifugation in a refrigerated centrifuge. One aliquot was maintained at 4°C for plasma lipid and lipoprotein analysis and others were immediately frozen at −80°C for subsequent measurements. Postprandial response of lipids and other biochemical parameters was calculated as the 6 h area under the curve (AUC) and incremental AUC (iAUC) with the use of the trapezoidal rule.

Indirect calorimetry

Resting energy expenditure (REE) was assessed in the fasting state by indirect calorimetry using an open-circuit ventilated canopy measurement system (Quark RMR; COSMED, Rome, Italy). Prior to the measurements, the Quark RMR was calibrated following the manufacturer’s instructions. Measurements were performed while participants were resting in a supine position in a quiet room (22–25°C). Expired respiratory gas was collected for 30 min at baseline and immediately analyzed with a medical gas analyzer. During the period between measurements, participants remained inactive, allowed only to perform quiet activities. REE (kilocalories per day) was calculated from oxygen consumption (VO₂, liters per minute) and carbon dioxide production (VCO₂, liters per minute), as suggested (28–30). The assumption that protein metabolism was 15% of resting metabolic rate was used for the estimation of urinary nitrogen measurements (28). For the calculation of REE, values of expired respiratory gas were taken from a steady state period when consecutive value differences in measurements were <5% (30).

Laboratory measurements

Plasma levels of lipids, glucose, insulin, apoA-I, and apoB-100 were measured as reported (18). TRL fractions d < 1.019 g/ml (chylomicrons, VLDLs, and remnants) were obtained by sequential ultracentrifugation, as indicated elsewhere (31). Major lipoprotein classes were also separated by high-performance liquid chromatography (FPLC), as previously described (18).

Plasma levels of apoB-48 and apoC-III were determined by ELISA methods (Shibayagi Co., Gunma, Japan and Cloud-Clone Corp., Houston, TX, respectively). Plasma levels of ApoE were measured by ELISA method using the R107 polyclonal rabbit antibody (DAKO, Glostrup, Denmark). Standard curve was prepared using a standardized serum (Daichi, Tokyo, Japan). RP concentrations in plasma were determined by reverse-phase HPLC as previously described (32). Plasma FAs were measured by an enzymatic colorimetric assay (Wako Chemicals GmbH, Neuss, Germany). Plasma levels of β-hydroxybutyric acid (β-HBA) were analyzed by a cyclic enzymatic method (Wako Chemicals GmbH, Neuss, Germany). Commercially available ELISA kits were used to measure plasma concentration of ANGPTL3 and ANGPTL4 (R&D Systems, Minneapolis, MN), adiponectin (Affiymetrix, eBioscience, San Diego, CA), TNFs (R&D Systems), and fatty acid binding protein-4 (FABP4) (Laboratori Medica a.s., Brno, Czech Republic). Activities of paraoxonase 1 (PON1), phospholipid transfer protein (PLTP), and cholesterol ester transfer protein (CETP) were determined as reported (33–35).

The ability of human plasma samples to generate preβ-HDL was analyzed by incubating plasma for 6 h at 37°C in the presence of LCAT inhibitor (1 mM iodoacetate). The amount of the formed preβ-HDL particles was quantified by resolving the postincubation plasma samples with two-dimensional crossed immune-electrophoresis, as previously reported (36). Briefly, the crossed immunoelectrophoresis consisted of an agarose electrophoresis in the first dimension for separation of lipoproteins with their preβ, β, and α mobility, followed by electrophoresis in the second dimension agarose gel containing 7.5% (v/v) rabbit anti-human apoA-I antiserum. For visualization, the gel was dried and stained with Coomassie brilliant blue R250 and the areas under the preβ-HDL and α-HDL peaks were calculated. The preβ-HDL area was expressed as a percentage of the sum of α-HDL and preβ-HDL areas.

It is known that variants in ApoE and LPL genes may affect the magnitude of postprandial lipemia (37). Therefore, ApoE genotypes were determined (18) and common LOF (p.D9N and p.N291S) and gain-of-function (p.S447*) LPL variants were screened by using an allele-specific method or quantitative real-time RT-PCR, as described elsewhere (38).

Statistical analysis

Variables are presented as mean (SD) or median (interquartile range) as appropriate. Differences between groups were assessed with the Mann-Whitney test or unpaired t-tests for quantitative factors and with chi-square test for categorical factors. Bonferroni’s correction for multiple testing was performed. Traits that were not normally distributed were log transformed before analysis to achieve uniformity of variances. Comparisons were adjusted for age, gender, and BMI by using the general linearized model. Differences in the time-dependent changes during the postprandial lipemia were compared with the use of repeated-measures three-factor (time and genotype with repeated measures on subject) ANCOVA, which included age, sex, and body fat as covariates. Spearman’s correlations and linear regression models with adjustments for age, sex, and BMI were used to establish the association between metabolic parameters. Student’s t-test was performed to test for significant differences in PLTP, CETP, PON1, and preβ-HDL values. All analyses were conducted with the use of the SPSS software (version 19) and a two-sided P < 0.05 was set as the level of statistical significance.

RESULTS

Subjects characteristics

FHBL2 subjects and controls were comparable for age, sex, prevalence of menopause, dietary intake, level of physical activity, and use of anti-inflammatory medications (Table 1). FHBL2 subjects showed significantly higher body FM and this was particularly remarkable among males. Three of them reported a history of diabetes mellitus, but all were investigated when under optimal glycemic control (HbA1c <6%). Homozygotes showed significantly higher prevalence of hypertension, while smoking habits were comparable between the three groups. None of study subjects showed impairment of renal function. When groups were compared for apoE alleles or common LOF or gain-of-function LPL variants, no differences were noted.

Energy and macronutrient intake and physical activity level were not significantly different between groups. Indirect calorimetry revealed that basal REE was not significantly different between the three study groups, neither in absolute terms (1,545 ± 132 kcal/day, 1,832.7 ± 62.7 kcal/day, and 1,678.1 ± 582 kcal/day) nor after adjustment for fat-free mass (data not shown).
In controls. In homozygotes, baseline TG values were 47.0 ± 17.9 mmol/l and peaked to 58.9 ± 15.3 mg/dl at 4 h (P < 0.001 compared with controls at the peak time) (Fig. 1A). In heterozygotes, baseline TG values were 99.4 ± 56.3 mg/dl and showed a more rapid return to baseline levels (116.0 ± 53.7 mg/dl at 6 h; P = 0.028 compared with controls). TG-iAUC was significantly lower in homozygotes compared with controls (52.9 ± 36.0 mg/dl h⁻¹ vs. 361.2 ± 333.5 mg/dl h⁻¹; P < 0.001) (Fig. 1A). Conversely, TG response in heterozygotes did not show significant difference when compared with controls. A similar pattern was observed when the postprandial response of TG in the TRL fraction (d < 1.019 g/ml) was evaluated (Fig. 1B). In particular, TG-iAUC was 6-fold lower in homozygotes than in controls. Plasma levels of LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C) did not show remarkable variations during the postprandial period (Fig. 1 C, D).

Because postprandial TGs are mainly transported in apoB-48-containing chylomicron particles (40), we subsequently focused on postprandial apoB-48 changes (Fig. 2A). After the high fat meal, plasma apoB-48 levels increased from 2.15 ± 1.91 mg/dl up to 5.15 ± 6.19 mg/dl at 4 h in Controls (n = 35)

Table 2 reports fasting biochemical parameters of study subjects. Compared with controls, homozygous FHBL2 carriers showed very low plasma concentrations of all lipids and lipoprotein classes (all P < 0.001), while heterozygotes presented intermediate levels. Concentration of apoB-48 was reduced by 67% in homozygous subjects (P = 0.029) and by 28% in heterozygotes (not significant). No difference between groups was seen in plasma concentration of apoC-III, an inhibitory protein of LPL activity (39). ANGPTL3 was undetectable in plasma of homozygotes, while its concentration followed gene-dose response, being 51% (P < 0.001) lower in heterozygotes, compared with controls. Conversely, no difference between the three groups was observed in fasting plasma concentration of ANGPTL4. Compared with controls, plasma FFAs were reduced by 36% in homozygous and unchanged in heterozygous FHBL2 carriers. Indices of insulin sensitivity as well as plasma levels of TNFα, FABP4, apoE, and adiponectin did not differ between groups.

**Table 2.** Clinical characteristics of FHBL2 subjects and controls

| Variables                        | FHBL2 (n = 38) | Controls (n = 35) | P-value
|----------------------------------|----------------|------------------|-----------------------|
| Age, years (range)               |                |                  |                       |
| Sex, n (male/female)             |                |                  |                       |
| Menopause, n (%)                 |                |                  |                       |
| BMI (kg/m²)                      |                |                  |                       |
| WHR                             |                |                  |                       |
| Male                             |                |                  |                       |
| Female                           |                |                  |                       |
| Body fat (%)                     |                |                  |                       |
| Male                             |                |                  |                       |
| Female                           |                |                  |                       |
| Diabetes mellitus, n (%)         |                |                  |                       |
| Hypertension, n (%)              |                |                  |                       |
| Smoking, n (%)                   |                |                  |                       |
| Total energy intake (%)          |                |                  |                       |
| Carbohydrate intake (%)          |                |                  |                       |
| Protein intake (%)               |                |                  |                       |
| Fat intake (%)                   |                |                  |                       |
| Alcohol consumption              |                |                  |                       |
| Abstainers                       |                |                  |                       |
| <1 glass/week, n (%)             |                |                  |                       |
| <2 glass/day, n (%)              |                |                  |                       |
| >2 glass/day, n (%)              |                |                  |                       |
| Physical activity (MET minimum per week) | 1,549.6 ± 1,838.2 | 2,232.2 ± 3,563.9 | 2,751.7 ± 4,881.8 |
| Medications                      |                |                  |                       |
| Anti-inflammatory agents, n (%)  |                |                  |                       |
| Aspirin, n (%)                   |                |                  |                       |
| Antihypertensive drugs, n (%)    |                |                  |                       |
| ApoE, allelic frequency (%)      |                |                  |                       |
| e2                               |                |                  |                       |
| e3                               |                |                  |                       |
| e4                               |                |                  |                       |
| LPL, n (%)                       |                |                  |                       |
| p.D9N                            |                |                  |                       |
| p.S291S                          |                |                  |                       |
| p.S447*                          |                |                  |                       |
| p.*447*                          |                |                  |                       |
| Data are reported as mean ± SD. WHR, waist-hip ratio; MET, metabolic equivalent. |
| aP < 0.05 for comparison between homozygous subjects and controls. |
| bP < 0.001 for comparison between homozygous subjects and controls. |
| cP < 0.05 for comparison between heterozygous subjects. |
| dP < 0.05 for comparison between homozygous and heterozygous subjects. |

**TRL changes in response to the oral fat load**

In response to the high fat meal, TGs rose from 110.9 ± 48.7 mg/dl to a peak value of 195.7 ± 104.1 mg/dl at 4 h in controls. In homozygotes, baseline TG values were 47.0 ± 17.9 mmol/l and peaked to 58.9 ± 15.3 mg/dl at 4 h (P < 0.001 compared with controls at the peak time) (Fig. 1A). In heterozygotes, baseline TG values were 99.4 ± 56.3 mg/dl and showed a more rapid return to baseline levels (116.0 ± 53.7 mg/dl at 6 h; P = 0.028 compared with controls). TG-iAUC was significantly lower in homozygotes compared with controls (52.9 ± 36.0 mg/dl h⁻¹ vs. 361.2 ± 333.5 mg/dl h⁻¹; P < 0.001) (Fig. 1A). Conversely, TG response in heterozygotes did not show significant difference when compared with controls. A similar pattern was observed when the postprandial response of TG in the TRL fraction (d < 1.019 g/ml) was evaluated (Fig. 1B). In particular, TG-iAUC was 6-fold lower in homozygotes than in controls. Plasma levels of LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C) did not show remarkable variations during the postprandial period (Fig. 1 C, D).
controls and from 0.75 ± 0.70 mg/dl up to 1.49 ± 0.68 mg/dl in homozygotes. A similar increase was seen in heterozygotes, even though apoB-48 peaked earlier (1.54 ± 0.93 mg/dl and 2.65 ± 1.44 mg/dl at 2 h) and returned faster to baseline values as compared with controls (P = 0.035). This resulted in lower postprandial iAUCs of apoB-48 in homozygotes than in controls (2.71 ± 1.55 mg/dl/h \(^{-1}\) vs. 9.34 ± 19.84 mg/dl/h \(^{-1}\), respectively; P < 0.05); heterozygous subjects showed values of 6.72 ± 7.42 mg/dl/h \(^{-1}\), that was significantly lower when compared with controls (P < 0.05). In response to the fat load, plasma levels of apoB-100 remained almost unchanged (data not reported). When changes in plasma levels of apoB-containing lipoproteins were evaluated as the apoB-48/apoB-100 ratio, it was further evident that the postprandial elevation of intestinally derived particles was attenuated in FHBL2 subjects (Fig. 2B). Finally, we found that the plasma curve of RP mimicked that of apo48, as RP-AUC was lowest in homozygous subjects and intermediate in heterozygous subjects, even though the differences in AUCs between groups did not reach statistical significance (Fig. 2C).

When postprandial changes in lipoprotein fractions were analyzed by FPLC (supplementary Fig. 1), we observed, in both controls and heterozygous FHBL2 subjects, a 70% elevation in TRL-TG at 2 h and 4 h. In homozygous FHBL2 subjects, conversely, there was a markedly blunted postprandial change in these lipoprotein fractions, which was in line with apoB-48 and RP results. In all groups, LDL and HDL levels did not exhibit substantial changes during the postprandial phase. Similar changes were observed when cholesterol concentration in FPLC-isolated lipoproteins was measured (supplementary Fig. 2). In the whole group of study subjects, TRL-AUC was positively and significantly correlated with baseline levels of plasma insulin (r = 0.264; P = 0.026), ANGPTL3 (r = 0.239; P = 0.042), apoC-III (r = 0.462; P < 0.001), apoE (r = 0.356; P = 0.031), and TNFα (r = 0.237; P = 0.047). With the exception of plasma levels of insulin, apoE, and TNFα, apoB-48-AUC displayed similar correlations. The positive correlation with baseline total cholesterol, TG, HDL-C, and apoA-I might be only the reflection of the association between FHBL2 status and postprandial TGs changes.

**ANGPTL3, ANGPTL4, and apoC-III changes in response to the oral fat load**

During the postprandial period, plasma ANGPTL3 showed a steady reduction in heterozygotes and controls with the lowest values detected at the 6 h time point (Fig. 3). Overall, it decreased by 24% in heterozygotes (P < 0.001) and by 17% in controls (all P < 0.001). Conversely, no variation in plasma concentration of ANGPTL4 was observed. No significant differences were seen between groups in postprandial changes of apoC-III.

**PLTP, CETP, PON1, and preβ-HDL responses to the oral fat load**

We analyzed CETP, PLTP, and PON1 activities during the postprandial phase (supplementary Fig. 3A–C). No significant changes between groups were observed in CETP, PON1, and PLTP activities. We also analyzed preβ-HDL levels in pooled plasma samples in each group. Baseline levels of these particles were significantly lower (P < 0.01) in homozygotes as compared with controls at fasting and at each postprandial time point (supplementary

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**TABLE 2. Baseline biochemical parameters of FHBL2 and control subjects**

|                      | FHBL2 (n = 38) | Controls (n = 55) |
|----------------------|---------------|------------------|
|                      | Homozygotes (n = 7) | Heterozygotes (n = 31) | Controls (n = 35) |
| **Plasma lipids and apoproteins (mg/dl)** | | | |
| Total cholesterol    | 86.1 ± 9.9 \(^{+}\,^{+}\) | 190.1 ± 40.2 \(^{-}\) | 210.6 ± 41.7 |
| Total TGs            | 47.0 ± 17.9 \(^{+}\,^{+}\) | 99.4 ± 56.3 | 110.7 ± 49.7 |
| HDL-C                | 25.1 ± 7.6 \(^{+}\,^{+}\) | 50.4 ± 11.6 \(^{-}\) | 59.9 ± 12.9 |
| LDL-C                | 56.4 ± 12.7 \(^{+}\,^{+}\) | 123.2 ± 32.3 | 130.3 ± 32.0 |
| apoB-100             | 57.7 ± 11.2 \(^{+}\,^{+}\) | 98.6 ± 28.4 | 100.0 ± 27.8 |
| apoA-I               | 60.2 ± 19.0 \(^{+}\,^{+}\) | 123.5 ± 24.7 | 140.6 ± 33.0 |
| apoB-48              | 0.7 ± 0.7 \(^{+}\,^{+}\) | 1.5 ± 0.9 | 2.1 ± 1.9 |
| apoC-III             | 7.50 ± 1.32 | 9.14 ± 2.99 | 9.97 ± 4.32 |
| apoE                 | 3.1 ± 0.2 | 3.6 ± 0.8 | 3.5 ± 0.8 |
| Glucose (mg/dl)      | 98.7 ± 14.9 | 102.3 ± 13.0 | 95.3 ± 8.5 |
| Insulin (U/l)        | 9.4 ± 3.4 | 10.6 ± 3.8 | 9.6 ± 1.7 |
| HOMA\(_{IR}\)        | 2.3 ± 0.8 | 2.71 ± 1.0 \(^{-}\) | 2.3 ± 1.7 |
| ANGPTL3 (ng/ml)      | 0.81 ± 0.7 | 104.9 ± 61.6 \(^{+}\,^{+}\) | 212.7 ± 77.5 |
| ANGPTL4 (ng/ml)      | 79.5 ± 38.0 | 92.9 ± 42.5 | 87.0 ± 22.2 |
| FFA (mmol/l)         | 0.18 ± 0.10 | 0.30 ± 0.12 | 0.28 ± 0.23 |
| Adiponectin (μg/ml)  | 80.8 ± 75.7 | 51.6 ± 54.1 | 65.8 ± 47.8 |
| Leptin (ng/ml)       | 18.5 ± 12.7 | 10.1 ± 4.2 | 13.7 ± 9.5 |
| TNFα (pg/ml)         | 0.81 ± 0.7 | 0.71 ± 0.4 | 0.66 ± 0.3 |
| FABP4 (ng/ml)        | 19.7 ± 4.8 | 23.6 ± 4.1 | 20.0 ± 5.7 |
| Creatinine (mg/dl)   | 0.83 ± 0.11 | 0.94 ± 0.14 | 0.92 ± 0.14 |

Data are means ± SD. HOMA\(_{IR}\), homeostasis model assessment of insulin resistance.  
\(^{+}\)P < 0.001 for comparison between homozygous and heterozygous subjects.

\(^{+}\)P < 0.001 for comparison between homozygous subjects and controls.

\(^{+}\)P < 0.01 for comparison between heterozygous subjects and controls.

\(^{-}\)P < 0.05 for comparison between heterozygous subjects and controls.
Nevertheless, it is worth pointing out that the rate of decline in plasma glucose during the final 2 h of the fat load was greater in heterozygotes and homozygotes, consistent with an increased glucose disposal.

Postprandial hepatic oxidation of FFAs

To evaluate changes in hepatic oxidation of FFAs, we measured postprandial plasma levels of \( \beta \)-HBA (41, 42). FHBL2 subjects started with fasting levels of 0.11 ± 0.04 mmol/l (homozygotes) and 0.097 ± 0.03 mmol/l (heterozygotes), which were not significantly different from those observed in controls, 0.093 ± 0.01 mmol/l. Both FHBL2 and control subjects had a \( \beta \)-HBA peak at 6 h [homozygotes: 0.132 ± 0.06 mmol/l and heterozygotes: 0.143 ± 0.09 mmol/l vs. controls subjects: 0.155 ± 0.08 mmol/l (NS for comparison between groups)] (Fig. 4D). However, absolute median as well as percent increase in \( \beta \)-HBA concentration at peak were lower in homozygotes (0.033 ± 0.063 mmol/l and 36%, respectively) than in heterozygote FHBL2 (0.046 ± 0.085 mmol/l and 49%, respectively) and controls (0.062 ± 0.076 mmol/l and 68%, respectively), and this difference was statistically significant (\( P < 0.05 \)).
Fig. 2. Postprandial response of apoB-48 (A), apoB-48/apoB-100 ratio (B), and RP (C) in homozygous (light gray triangles) and heterozygous (dark gray diamonds) FHBL2 subjects and in controls (black circles). In the insets, bars represent the total AUCs and iAUCs for each subgroup. iAUC for apoB-48 and AUC for RP are expressed in milligrams per deciliter per hour$^{-1}$ and micrograms per deciliter per hour$^{-1}$, respectively. Data are means ± SE. *$P < 0.05$ for comparison between homozygous subjects and controls; †$P < 0.05$ for comparison between homozygous and heterozygous subjects; and ‡$P < 0.05$ for comparison between heterozygous subjects and controls.
DISCUSSION

In the present study, we have examined the effect of ANGPTL3 deficiency on postprandial lipid metabolism by investigating FHBL2 subjects carrying an inactivating mutation in the ANGPTL3 gene. Our data demonstrate that, compared with controls, postprandial elevation of TRLs was markedly attenuated in homozygous FHBL2 subjects, whereas it was modestly changed in heterozygotes. Our findings are in full agreement with previous investigations in transgenic animals (10, 12), as well as with our observation that homozygous, but not heterozygous, FHBL2 individuals have significantly increased post-heparin LPL activity (17). In addition, our findings also agree with the recent observation that mice injected with a blocking monoclonal anti-ANGPTL3 antibody showed a minimal increase of TG levels in response to a fat challenge (43). Taken together, the present observations are in line with the view that ANGPTL3 modulates postprandial metabolism of TRLs in humans via its action on LPL function. The almost abolished postprandial increase of plasma apoB-48 in homozygotes could raise the question whether ANGPTL3 deficiency might cause reduced absorption of intestinal lipid. This seems unlikely, as we have observed that FHBL2 subjects did not show any changes in markers of intestinal cholesterol absorption (18). Therefore, our results are more consistent with the model in which the deficiency of ANGPTL3, by increasing the lipolytic rate of TRLs generated during the postprandial phase, accelerates their conversion into remnant particles and, thereby, their removal by the liver (11).

Based upon postprandial changes of plasma TG levels, one could conclude that in vivo ANGPTL3 does not act in a gene/dose-dependent manner, as only complete ANGPTL3 deficiency (homozygous FHBL2) produces a remarkable attenuation of postprandial lipemia. However, this effect is not confirmed when postprandial changes of apoB-48 levels are considered. In fact, heterozygotes also showed lower increases in apoB-48 levels, which were intermediate between those in homozygotes and controls. We do not have any straightforward explanation for this observation. One could speculate that the postprandial removal rate of chylomicron remnants (containing apoB-48) is faster than that of VLDL remnants (containing apoB-100), thus affecting more plasma levels of apoB-48 than total TGs. Alternatively, it might be that the chylomicron assembly pathway in enterocytes is sensitive to an ANGPTL3 level that does not seem to affect TG secretion in chylomicron particles in heterozygous subjects. More experimental evidence is needed to clarify this observation.

Animal and human studies have consistently reported that ANGPTL3 deficiency is connected to lower FFA concentration (10, 17), even though the underlying mechanism is not well-established. When we evaluated the dynamics of FFA change in response to the fat load, the major finding was that homozygotes displayed a much larger increase of plasma FFA when compared with heterozygous and control subjects. As it has been documented that chylomicron fatty acid spillover represents a significant source of postprandial plasma FFA (19), this finding might be the reflection of a more rapid TG-chylomicron lipolysis due to higher LPL activity. From our data, it is difficult to explain why homozygous subjects showed lower fasting FFA levels. It is well-known that the rate of adipose fatty acid release remains the primary driver of postabsorptive FFA concentration (44). Therefore, the lower fasting FFA levels in homozygotes might be a consequence of the concomitant
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More detailed tracer studies are needed to clarify the role of ANGPTL3 in fatty acid metabolism. Our postprandial findings may add some information regarding the mechanisms underlying hypolipidemia in FHBL2. In fact, the attenuated postprandial lipemia together with reduced flux of FFAs directed to the liver may explain the lower VLDL secretion rate and fasting TG reported in homozygous FHBL2 (13) and recently confirmed during antibody-mediated inhibition of ANGPTL3 (48). Moreover, the faster hepatic removal of remnants may decrease the fraction that is converted in circulation into LDL, thereby lowering LDL-C levels.

It is well-known that HDL particles are generated during the postprandial period throughout the lipolytic degradation of TRLs that gives rise to poorly lipidated apoA-I-containing preβ-HDL particles (49). In pooled samples of control subjects, we detected a slight postprandial elevation of preβ-HDL, thus supporting this view. However, we were not able to detect any significant changes, neither in postprandial total HDL levels nor in PLTP activity. Based on these findings, we might conclude that the postprandial

Fig. 4. Changes of plasma FFA (A), glucose (B), insulin (C), and hydroxybutyric acid (HBA) (D) in homozygous (light gray triangles) and heterozygous (dark gray diamonds) FHBL2 subjects and in controls (black circles) during the postprandial period. In the insets, bars represent the total AUCs and iAUCs for each subgroup. iAUCs for FFAs and AUCs for glucose are expressed in milligrams per deciliter per hour$^{-1}$; AUCs for insulin are expressed in units per liter per hour$^{-1}$, respectively. Data are means ± SE. *$P < 0.01$ and †$P < 0.05$ for comparison between homozygous subjects and controls; ‡$P < 0.01$ for comparison between homozygous and heterozygous subjects; and §$P < 0.05$ for comparison between heterozygous subjects and controls. In (D), *$P < 0.05$ for comparison between homozygous subjects and controls.
HDL metabolism does not play a significant role in regulating fasting levels of total HDL in FHBL2. Additional investigations are required to clarify this issue.

Finally, we observed that, in both controls and heterozygous individuals, plasma ANGPTL3 showed a steady decline during the 6 h postprandial period, while ANGPTL4 remained almost unchanged. Even though this latter result was not surprising, as ANGPTL4 is expressed maximally in fasted state (5), the postprandial decrease in ANGPTL3 was in contrast with the current assumption that this protein is only modestly altered by fasting and refueling (6). The mechanism underlying this reduction is unknown. As ANGPTL3 is activated upon proteolysis by proprotein convertases (50), it can be speculated that its postprandial decline could be due to increased degradation. Alternatively, liver production of ANGPTL3 could be reduced during the postprandial period. The postprandial rise of insulin might mediate this effect, as we have found in human cultured hepatocytes that insulin decreases the expression and secretion of ANGPTL3 in a dose-dependent manner (15, 51). Additional studies looking for metabolic signals controlling ANGPTL3 production are warranted.

In summary, by investigating subjects with FHBL2, we have demonstrated that ANGPTL3 deficiency causes markedly reduced postprandial lipemia, probably due to faster catabolism of intestinally derived TRLs, larger postprandial expansion of the FFA pool, and a decreased influx of dietary-derived fatty acids into the liver. These findings predict that the pharmacological inhibition of ANGPTL3 might determine broader changes in lipid metabolism, which could be beneficial in several cardiometabolic derangements (e.g., insulin resistance, metabolic syndrome, hypertriglyceridemia), all associated with a high postprandial TG response (52). However, only clinical trials using pharmacological intervention aimed at blocking ANGPTL3 may definitively prove this possibility.

The authors thank Dr. Laura D’Erasmo, Dr. Giovanni Pigna, Dr. Paola Coletta, and Dr. Elvira Grieco for their help in the clinical evaluation of study subjects and execution of OFTT, Dr. Cristina Prisco for sample preparation, and Sari Nutunen for her help in the lipid analysis of FPLC fractions. Finally, the authors express their sincere thanks to the study participants, to whom they are indebted for their patience and commitment to the study protocol.

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