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Four days high fat diet modulates vitamin D metabolite levels and enzymes in mice

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Abstract

Obesity is classically associated with low serum total and free 25(OH)D. Hypotheses have been advanced to explain this observation but mechanisms remain poorly understood, and notably priming events that could explain such association. We investigated the impact of short-term high fat (HF) diet to investigate early events occurring in vitamin D metabolism. Male C57BL/6J mice were fed with a control diet (control group) and HF diet for 4 days. HF fed mice displayed similar body weight to control mice but significantly increased adiposity, together with a decrease of free 25(OH)D concentrations, which could be explained at least in part by a decrease of *Cyp2r1* and *Cyp3a11* expression in the liver. An increase of 1,25(OH)₂D concentration was also observed and could be explained by a decrease of *Cyp24a1* expression observed in the kidney. In white adipose tissue (WAT), no modification of vitamin D metabolites quantity detected by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Nevertheless, an increase of *Cyp2r1* and *Cyp27a1* mRNA expression and a decrease of *Cyp27b1* mRNA expression could suggest a possible storage of 25(OH)D in WAT at long-term. Our data are supportive of an active role of HF diet in mediating a priming effect leading the well-established perturbation of the vitamin D metabolism associated with obesity, including a decrease of free 25(OH)D and modulation of expression of genes involved in vitamin D metabolism.

Key Words

- ▶ vitamin D
- ▶ metabolism
- ▶ adipose tissue
- ▶ free 25(OH)D
- ▶ short-term high fat diet

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Introduction

Low levels of total circulating 25-hydroxy-vitamin D (25(OH)D) are strongly associated with obesity and more specially with increased fat mass and BMI (Vilarrasa *et al.* 2007, Earthman *et al.* 2012, Landrier *et al.* 2016). Recently it has been shown that the free form of 25(OH)D was also low in obesity (Walsh *et al.* 2016), as well as 1,25 dihydroxy-vitamin D (1,25(OH)₂D). Several hypotheses have been emitted to explain the observations. Bell *et al.* reported that hepatic 25-hydroxylation was inhibited by 1,25(OH)₂D and parathyroid hormone (PTH) (Bell 1985). Wortsman *et al.*

suggested a possible sequestration of vitamin D in white adipose tissue (WAT) caused by a passive phenomenon due to the hydrophobic nature of vitamin D (Wortsman *et al.* 2000). Drincic *et al.* proposed that 25(OH)D was simply diluted in a higher volume in obese and hypothesized a volumetric dilution (Drincic *et al.* 2012). Indeed, these authors showed an inverse correlation between plasma 25(OH)D concentration and total volume (lean and fat mass), which could be the best predictive marker than fat mass. Wamberg *et al.* suggested that vitamin D metabolism

in WAT is modified by obesity and reported a modification of *Cyp2j2* expression in biopsies of obese compared to lean patients (Wamberg *et al.* 2013).

In mice, Park *et al.* reported that high fat diet-induced obesity influenced vitamin D metabolizing enzymes expression in liver, kidney and WAT, which could provide a possible mechanism for altered vitamin D metabolism in obesity (Park *et al.* 2015). Roizen *et al.* and Aatsinki *et al.* recently reported an impact of a high fat diet and obesity/diabetes on hepatic 25 hydroxylase activity in mice liver, that could explain the low 25(OH)D level (Aatsinki *et al.* 2019, Roizen *et al.* 2019). In addition, we showed recently that adipose tissue could play an important active role in the modulation of vitamin D metabolism observed during obesity. Indeed, we found a decrease of free 25(OH)D, strongly associated with the induction of *Cyp2r1* in adipose tissue, which could be responsible for the active production and storage of 25(OH)D highlighted in adipose tissue by direct quantification (Bonnet *et al.* 2019a).

It is noteworthy that all these observations have been made in obese mice models or in obese subjects and it is presently unclear if modulation of expression of genes involved in vitamin D metabolism have a priming effect in vitamin D metabolites modifications (total and free 25(OH)D and 1,25(OH)₂D), or are just consequences of obesity.

The objective of this study was to identify early events that could explain the modulation of 25(OH)D (total and/or free) and 1,25(OH)₂D levels observed in obesity. To this aim, a short term high fat diet (4 days) was implemented to initiate obesity and to study early modifications of vitamin D status. In parallel, a profile of expression of genes coding for enzymes involved in vitamin D metabolism was established in the liver, kidney and WAT in order to bring mechanistic keys.

Materials and methods

Reagents

TRIzol reagent, random primers, and Moloney murine leukemia virus reverse transcriptase were obtained from Life Technologies (Courtaboeuf, France). SYBR Green reaction buffer was purchased from Eurogentec (Liege, Belgium).

Animal experiments

The protocol received the agreement of the local ethics committee and by the French Ministry of Research (APAFIS#2595-2016091911217758). Six-week-old male C57BL/6J mice were obtained from Janvier (Le Genest

Saint Isle, France), fed with control food (chow diet A04 from Safe-diets) *ad libitum* during the 1-week acclimation period and with full access to drinking water. The animals were maintained at 22°C under a 12 h light:12 h darkness cycle a 20% humidity level. Mice were divided into control diet group (control: chow diet A04 from Safe-diets, *n*=10) or high fat diet group (HF group: 60% energy from lipids from Test Diet 58Y1, *n*=10). The cholecalciferol concentration was 900 UI/kg for the control diet and 1300 UI/g for the high fat diet. The daily food intake of mice has been determined by giving them a known (i.e. pre-weighed) amount of food in their cage and weigh the remaining food once per day. We used adapted containers that limit the risk of spillage and hoarding. The intake of cholecalciferol is calculated by multiplying the food intake but the concentration of cholecalciferol in the food. After 4 days, mice were fasted overnight and blood was collected by cardiac puncture anesthesia, serum was isolated by centrifugation at 350 g for 15 min at 4°C and was stored at -80°C. Cervical dislocation was used to scarify animals and tissue (kidney, liver and WAT (epididymal (eWAT), subcutaneous and retroperitoneal pads)) were collected, weighted and stored at 80°C.

Free 25(OH)D, albumin and VDBP quantification

To determinate the free 25(OH)D and VDBP quantity in mice plasma, specific ELISA kits were used (mice free form of 25(OH)D, DAsources, Louvain-La-Neuve, Belgique, Mouse VDBP DuoSet ELISA, R&S systems, Minneapolis, USA, respectively) according to manufacturer protocol. The direct assay for free 25(OH)D has been recently validated (Heureux *et al.* 2017). Albumin concentration in plasma was determined using BCG method (Biolabo, Maizy, France). The free 25(OH)D calculation was performed as previously reported (Bikle *et al.* 2017).

Cholecalciferol, 25(OH)D and 1,25(OH)₂D quantification in plasma and eWAT

All quantifications were performed using LC-MS/MS according to the protocol as previously reported (Bonnet *et al.* 2018, 2019b) and detailed in Supplementary data (see section on [supplementary materials](#) given at the end of this article).

RNA extraction and real-time PCR

Total RNA was extracted from the liver, kidney and eWAT or from cells using TRIzol reagent (Life Technologies).

One microgram of total RNA was used to synthesize cDNAs using random primers and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Courtaboeuf, France). Real-time quantitative PCR analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, USA) as previously described (Karkeni *et al.* 2017a). For each condition, expression was quantified in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (CT) method (Livak & Schmittgen 2001). The sequences of the primers used in this study are reported in Supplementary Table 1.

Statistical analysis

The data are communicated as the mean $\pm$ S.E.M. Significant differences between the control and treated group were determined using Student's *t*-test with $P < 0.05$ was considered statistically significant.

Results

Morphological data of 4 days high fat diet fed mice

The effect of a high fat diet (60% energy from fat, HF group) compared to control diet (control group) was evaluated in WT C57BL/6J male mice for 4 days. Body weight and liver weight were not modified by the diet (Table 1). Adiposity index (sum of epididymal, inguinal and retroperitoneal adipose tissue mass relative to total body mass), as well as fat pads weight of subcutaneous, epididymal and peritoneal WAT and ratio of organ, weigh on body weight of these three organs, were increased in HF group (1.58-fold for adiposity index, Table 1). Energy intake was increased in HF group (1.2-fold, Table 1). No difference was observed in vitamin D intake between groups (Table 1).

Table 1 Mice morphologic parameters.

	Control	HF
Body weight (g)	21.45 $\pm$ 0.28	22.06 $\pm$ 0.45
Liver (mg)	875 $\pm$ 28.7	850 $\pm$ 29.59
Subcutaneous AT (mg)	36.3 $\pm$ 5.06	71 $\pm$ 3.7 ^a
Epididymal AT (mg)	187.4 $\pm$ 17.7	282.2 $\pm$ 18.2 ^a
Retroperitoneal AT (g)	31.7 $\pm$ 3.9	63.7 $\pm$ 5.5 ^a
Adiposity index	1.185 $\pm$ 0.10	1.87 $\pm$ 0.09 ^a
Energy intake (kcal/g)	14.38 $\pm$ 0.44	17.28 $\pm$ 0.40 ^a
Vitamin D intake (UI/g/day)	4.46 $\pm$ 0.24	4.52 $\pm$ 0.08

Values are presented as means $\pm$ S.E.M. For unpaired Student's *t*-test. ^a $P < 0.05$ between control and high fat group (HF) for each condition.

Plasma concentrations of cholecalciferol and metabolites parameters of 4 days high fat diet fed mice

Plasma cholecalciferol concentration was decreased in HF group compared to control group (0.68-fold, Table 2) as well as measured plasma free form of 25(OH)D concentration (0.31-fold, Table 2) and VDBP plasma level (0.3-fold, Table 2). Surprisingly the calculated free 25(OH)D was increased in HF group compared to control group (1.4-fold, Table 2). Conversely, no difference was observed in plasma total of 25(OH)D concentrations between control group (27.9 $\pm$ 0.9 ng/mL) and HF group (29.9 $\pm$ 0.9 ng/mL). Plasma 1,25(OH)₂D concentration was increased in HF group (2.78-fold, Table 1). Interestingly, we observed a negative correlation between measured plasma free 25(OH)D concentrations and adiposity index ($R^2=0.4002$, $P=0.0085$; Fig. 1E) and a positive correlation between adiposity index and plasma 1,25(OH)₂D concentrations ($R^2=0.335$, $P=0.012$; Fig. 1G).

Effect of a 4 days high fat diet on expression of genes coding for enzymes of the vitamin D metabolism in mice

Expression of genes coding major actors of vitamin D metabolism were studied in the liver, kidney and eWAT of mice (all data presented in supplemental Table 2). We observed a significant decrease of mRNA expression of *Cyp2r1* and *Cyp3a11*, involved in 25-hydroxylation (0.15 and 0.76-fold, respectively, Fig. 2A), in the liver. In kidney, a decrease of *Cyp24a1* and *Cyp27b1* expression decreased in HF group (0.42 and 0.34-fold, respectively, Fig. 2B). In eWAT, relative RNA expression of *Cyp27a1* and *Cyp2r1* were significantly increased in HF group compared to control group (1.48- and 1.98-fold, respectively, Fig. 2C). Conversely, expression of *Cyp27b1* was decreased in HF group (0.78, Fig. 2C).

Quantification of vitamin D and metabolites in eWAT of 4 days high fat diet fed mice

Vitamin D and metabolites were quantified in eWAT by LC-MS/MS. No difference of 1,25(OH)₂D concentration was observed whereas concentrations of cholecalciferol and 25(OH)D were decreased in HF group (0.5- and 0.25-fold, respectively, Table 2). Quantities of cholecalciferol, 25(OH)D and 1,25(OH)₂D in eWAT were not modified by the short-term high fat diet (Table 2).

Table 2 Plasma and epididymal white adipose tissue vitamin D metabolites and VDBP quantification.

	Control	HF
Plasma cholecalciferol concentration (ng/mL)	1.196 ± 0.148	0.387 ± 0.098 ^a
Plasma total 25(OH)D concentration (ng/mL)	27.986 ± 0.998	29.987 ± 0.925
Plasma free 25(OH)D concentration (pg/mL)	10.879 ± 0.48	7.46 ± 0.60 ^a
Plasma 1,25(OH) ₂ D concentration (ng/mL)	0.548 ± 0.051	1.524 ± 0.137 ^a
Plasma VDBP quantification (µg/mL)	114 ± 10.6	83 ± 8.5 ^a
Plasma albumin concentration (g/dL)	3.85 ± 0.09	5.46 ± 0.28 ^a
Calculated free 25(OH)D (pg/mL)	2.43 ± 0.14	3.48 ± 0.21 ^a
eWAT cholecalciferol quantity (ng)	174.1 ± 28.6	122.8 ± 12.5
eWAT 25(OH)D quantity (ng)	13.1 ± 1	15.3 ± 0.8
eWAT 1,25(OH) ₂ D quantity (ng)	14.1 ± 1.4	17.7 ± 2.5
eWAT cholecalciferol concentration (ng/g of tissue)	949.2 ± 193.3	471.9 ± 73.9 ^a
eWAT 25(OH)D concentration (ng/g of tissue)	76 ± 6.1	57.1 ± 3.5 ^a
eWAT 1,25(OH) ₂ D concentration (ng/g of tissue)	68.4 ± 6.8	63.1 ± 7.8

Values are presented as means ± S.E.M. For unpaired Student's *t*-test. ^a*P* < 0.05 between control and high fat group (HF) for each condition.

Discussion

In this present study, we report for the first time the effect of a short-term high fat diet on vitamin D and metabolites plasma and eWAT levels as well as expression of genes involved in vitamin D metabolism in the liver, kidney and WAT.

In order to mimic a food imbalance state associated with weight gain, we have used a short-time (4 days) high fat diet. This model allows to study the early effects of an unbalanced diet on weight gain and/or fat mass gain (Voigt *et al.* 2013). It is thus used to study the causal events that are concomitant with the initiation of weight gain, in contrast to a classical model of high fat diet-induced (generally 10 weeks or more) which mainly reflect consequences of weight gain. As expected, this diet did not affect body weight but increased adiposity index of HF fed mice.

It is also important to note that the two diets used in this protocol brought the same quantity of cholecalciferol, which is a crucial point for this experiment. To this aim, we increased cholecalciferol content of HF diet, to account for the lower food consumption that occurs during HF feeding. Despite similar vitamin D supplementation it is intriguing that cholecalciferol plasma levels were strongly reduced in HF group. The origin of such discrepancy is presently not well understood, but could be due to a reduced ability of intestine to absorb cholecalciferol, as a consequence of deregulation of proteins involved in vitamin D uptake (Reboul *et al.* 2011), such assumption will require further investigations.

In these experimental conditions, we did not observe any modification of the total 25(OH)D serum levels, which is consistent with previous reports that adjusted

the cholecalciferol content in chow and HF diets (Park *et al.* 2015, Seldeen *et al.* 2017, Bonnet *et al.* 2019a). Such observation is not in the favour of the classical hypothesis of passive sequestration proposed by Wortsman *et al.* (2000) which suggests that the 25(OH)D plasma decrease observed during obesity is the direct result of adipose tissue expansion, since we observed an increase of adiposity index without modification of total 25(OH)D concentration.

In HF group, we observed an increase of serum 1,25(OH)₂D concentration compared to control group, that was correlated to adiposity. Such increase has already been reported (Park *et al.* 2015), and could be linked to the downregulation of *Cyp24a1* in kidney of HF group, leading to a lower catabolism of 1,25(OH)₂D, which in turn lead to the decrease of *Cyp27b1* mRNA levels in HF fed mice kidneys through a well-established negative feedback mechanism (Bikle *et al.* 1975, 1986). Interestingly 1,25(OH)₂D has been demonstrated to repress hepatic 25-hydroxylation (Bell *et al.* 1984). Such observation is consistent with the observed decrease of *Cyp2r1* and *Cyp3a11* mRNA levels in the liver of HF fed mice. Note that similar hepatic repression of *Cyp2r1* and *Cyp3a11* have previously been reported in long-term HF diets (Park *et al.* 2015, Aatsinki *et al.* 2019, Roizen *et al.* 2019). Surprisingly, the downregulation of *Cyp2r1* mRNA levels is not strictly associated with reduction of 25(OH)D plasma levels as reported by Park *et al.* (2015) and the present study, whereas Aatsinki *et al.* (2019) and Roizen *et al.* (2019) reported a decrease in 25(OH)D plasma level in parallel to a strong decrease of *Cyp2r1*. The origin of such discrepancy is presently not understood, but could be due to the different types of regimens used and will deserve further investigations. Concerning our experimentation,

Figure 1

Relationship between adiposity index, body weight and serum concentration of vitamin D metabolites in mice fed with high fat diet. Relationship between cholecalciferol and adiposity index (A) or body weight (B), total 25(OH)D and adiposity index (C) or body weight (D), free 25(OH)D and adiposity index (E) or body weight (F), 1,25(OH)₂D and adiposity index (G) or body weight (H), in mice fed with control or high fat diet. For unpaired Student's *t*-test, one-tailed probability.

we have to keep in mind that only mRNA levels were reported and the decrease was relatively low notably for *Cyp2r1*, whereas Roizen *et al.* and Aatsinki *et al.* reported stronger downregulation of the *Cyp2r1* mRNA levels (Aatsinki *et al.* 2019, Roizen *et al.* 2019). Nevertheless, our observation suggests that the downregulation of *Cyp2r1* could precede the decrease of the 25(OH)D level and is thus in agreement with a causal role of HF diet on *Cyp2r1* expression with consequences on 25(OH)D levels.

Even if we did not observe modification of the total 25(OH)D, we reported an impact of short-term HF diet

Figure 2

Effect of high fat diet for 4 days on gene expression of vitamin D metabolism in eWAT in mice. Expression of genes coding proteins of vitamin D metabolism relative to 18S ribosomal RNA in liver (A), kidney (B) and eWAT (C) of mice fed with control diet (control) or high fat diet 60% (HF) for 4 days ($n = 10$ per group). Values are presented as means $\pm$ s.e.m. For unpaired Student's *t*-test, $*P < 0.05$.

on the VDBP plasma level and liver mRNA coding for VDBP (*Gc* mRNA). Such effect was not observed in a recently published article (Elkhwanky *et al.* 2020) but could be due not to the reduction of total 25(OH)D as suggested (Carpenter *et al.* 2013), but to the reduction of free 25(OH)D observed. This last assumption will require further investigations. In addition, we reported a reduced measured free 25(OH)D plasma level under short term HF diet, as previously reported in long-term

HF diet (Bonnet *et al.* 2019a), and in agreement with published data in obese subjects (Walsh *et al.* 2016). Surprisingly, when calculating the free 25(OH)D, we did not find a correlation between measured and calculated free 25(OH)D as previously reported (Bikle *et al.* 2017). This observation could be due to the fact that such correlations were observed in normal subjects. We cannot exclude that in other pathophysiological situations (including high fat diet), affinity constants of VDBP and/or albumin regarding 25(OH)D are modified resulting in erroneous calculations. Such assumption will require further experimentations. Nevertheless, it is noteworthy that such reduction of measured free 25(OH)D could participate in the amplification of obesity and/or metabolic inflammation (Marcotorchino *et al.* 2012, 2014, Karkeni *et al.* 2015, 2017b, Marziou *et al.* 2020). Interestingly the reduction of free 25(OH)D was inversely correlated to adiposity but not to body weight, suggesting a specific role of adipose tissue accretion in the level of free 25(OH)D. In agreement, we reported in eWAT of HF fed mice, an increase of mRNA levels of two enzymes involved in 25-hydroxylation, *Cyp2r1* and *Cyp27a1* (Schuster 2011). This data related to the expression of *Cyp2r1*, which encodes a major enzyme of 25-hydroxylation (Zhu *et al.* 2013), under short-term effect of HF diet is innovative. Similarly to data generated in long-term HF diet, it suggests an increased ability of adipose tissue to store 25(OH)D due to the enhanced expression of 25-hydroxylation enzymes (Wamberg *et al.* 2013, Park *et al.* 2015, Bonnet *et al.* 2019a). In addition, mRNA expression of *Cyp27b1* was decreased in HF group, suggesting that at term 1,25(OH)₂D will decrease locally, even if not yet observed (no modification of quantity and concentration of 1,25(OH)₂D in eWAT). Altogether, these data suggested that short-term HF diet induces the ability of adipose tissue to synthesis and to store 25(OH)D. Nevertheless, such assumption is not yet supported by the LC-MS/MS vitamin D metabolites quantifications in eWAT. Indeed, no cholecalciferol and metabolites quantity modifications were observed between control and HF groups, whereas cholecalciferol and 25(OH)D concentration decreased in HF group. Such decrease of concentration is simply due to the increase of adipose tissue quantity in absence of metabolite quantity modification. In fact, we hypothesized the modulation of genes expression reported in eWAT could precede the modulation of cholecalciferol and metabolites accumulations in eWAT. It could thus be considered as a priming event, induced by HF diet at an early stage, that could lead to the active storage of cholecalciferol and

25(OH)D in eWAT, via an active mechanism, as previously depicted (Bonnet *et al.* 2019a). Note that all quantification of gene expression and metabolites have been performed only in eWAT, which could be considered as a limitation to the present work, since we cannot exclude that other fat pads would have different responses.

To conclude, short-term HF is able to modulate vitamin D metabolism and vitamin D metabolites content in plasma and in eWAT. Our data are supportive of an active role of HF diet in mediating a priming effect leading the well-established perturbation of the vitamin D metabolism associated with obesity (a decrease of free 25(OH)D) and modulation of expression of genes involved in vitamin D metabolism (*Cyp* in the liver and in eWAT).

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-20-0198>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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