Brief Communications

Knockdown of angiopoietin-like 2 mimics the benefits of intermittent fasting on insulin responsiveness and weight loss

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Impact statement

Intermittent fasting is an efficient diet pattern to prevent weight gain and improve insulin sensitivity. It is, however, a difficult regimen to follow and compliance is expected to be very low. In this work, we demonstrate that knockdown of ANGPTL2 in mice fed ad libitum mimics the beneficial effects of intermittent fasting on weight gain and insulin sensitivity in wild-type mice. ANGPTL2 is a cytokine positively associated with fat mass in humans, which inactivation in mice improves resistance to a high-fat metabolic challenge. This study provides a novel pathway by which IF acts to limit obesity despite equivalent energy intake. The development of a pharmacological ANGPTL2 antagonist could provide an efficient tool to reduce the burden of obesity.

Abstract

Angiopoietin-like 2 (ANGPTL2) is an inflammatory adipokine linking obesity to insulin resistance. Intermittent fasting, on the other hand, is a lifestyle intervention able to prevent obesity and diabetes but difficult to implement and maintain. Our objectives were to characterize a link between ANGPTL2 and intermittent fasting and to investigate whether the knockdown of ANGPTL2 reproduces the benefits of intermittent fasting on weight gain and insulin responsiveness in knockdown and wild-type littermates mice. Intermittent fasting, access to food ad libitum once every other day, was initiated at the age of three months and maintained for four months. Intermittent fasting decreased by 63% ($p < 0.05$) gene expression of *angptl2* in adipose tissue of wild-type mice. As expected, intermittent fasting improved insulin sensitivity ($p < 0.05$) and limited weight gain ($p < 0.05$) in wild-type mice. Knockdown mice fed ad libitum, however, were comparable to wild-type mice following the intermittent fasting regimen: insulin sensitivity and weight gain were identical, while intermittent fasting had no additional impact on these parameters in knockdown mice. Energy intake was similar between both wild-type fed intermittent fasting and ANGPTL2 knockdown mice fed ad libitum, suggesting that intermittent fasting and knockdown of ANGPTL2 equally lower feeding efficiency. These results suggest that the reduction of ANGPTL2 could be a useful and promising strategy to prevent obesity and insulin resistance, although further investigation of the mechanisms linking ANGPTL2 and intermittent fasting is warranted.

Keywords: Energy metabolism, obesity, diabetes, mice

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Introduction

Among adult men and women, the prevalence of obesity in the United States reaches 35% and an epidemic burst of obesity is occurring worldwide.[1] Obesity and high body fat are associated with a higher risk of metabolic diseases such as type-2 diabetes (T2D)[2] but the mechanisms underlying this association are not clear. Angiopoietin-like 2 (ANGPTL2), a pro-inflammatory circulating glycoprotein mainly secreted by adipose tissue, has been proposed to be a potential key mediator linking obesity to insulin resistance.[3–5] Indeed, circulating ANGPTL2 levels increase and correlate with insulin resistance in obese humans,[3] they are associated with the development of T2D in a general Japanese population[4] and with deleterious cardiovascular events in a French cohort of diabetic patients.[6] In contrast, ANGPTL2-deficient mice fed with a high-fat diet have a

lower body fat mass and a better insulin sensibility when compared to wild-type (WT) mice.[3,7]

Intermittent fasting (IF), a lifestyle intervention involving alternative cycles of fasting and eating,[8,9] improves weight and fat loss and insulin sensitivity both in humans[10] and in animal models.[11,12] However, IF is difficult to implement and maintain, and alternative strategies are needed. Although there is no known link between IF and ANGPTL2, we aimed to investigate whether IF lowers ANGPTL2 and if the knockdown (KD) of ANGPTL2 reproduces the benefits of IF on weight loss and insulin responsiveness in mice.

Methods

Animal experiments

Three-month-old C57BL/6 ANGPTL2 KD male mice (25.2 $\pm$ 0.5 g) and their WT littermates (27.6 $\pm$ 0.6 g) from our colony were used, as previously described.[7] Negligible levels of *angptl2* mRNA were detected in liver and adipose tissues from KD mice.[7] All animals were housed individually. A standard diet (2019S; Harlan Laboratories, Madison, WI, US) was given for four months either ad libitum (AL) or through IF (AL access every other day from 5:30 PM to 5:30 PM). Mice were sacrificed at the age of seven months by exsanguination under anesthesia (100 mg/kg ketamine and 10 mg/kg xylazine i.p.) following 15 h of fasting. Plasma, liver and adipose tissues were collected and kept at -80°C until further analysis. Body weight and food consumption were measured weekly (Figure 1). All animal experiments were performed in accordance with the "Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care" and were approved by the Montreal Heart Institute Ethics Committee.

Real-time quantitative polymerase chain reaction

Total RNA was extracted from adipose tissue using the RNeasy lipid tissue mini-kit as specified (Qiagen, Canada). Quantitative polymerase chain reaction (qPCR) for *angptl2* gene was performed using the EvaGreen qPCR Mastermix (Mastermix-LR; Applied Biological Materials Inc., Richmond, BC, Canada). The $\Delta\Delta$CT method was used for analysis of relative ANGPTL2 expression using cyclophilin A as the housekeeping gene with the following primers: ANGPTL2 forward (5′-GATCCA GAGTGACCAGAATC-3′), ANGPTL2 reverse (5′-TCTC AGGCTTCACCAGGTAG-3′), cyclophilin A forward (5′-CCGATGACGAGCCCTTGG-3′), cyclophilin A reverse (5′-GCCGCCAGTGCCATTATG-3′).

Oral glucose tolerance test

Oral glucose tolerance test (OGTT) was performed at 9:00 AM after 15 h of fasting by an oral administration of glucose (2 g/kg); blood was collected in the saphenous vein after 0, 30, 60, 90 and 120 min to measure blood glucose levels. Total area under the curve (AUC) was calculated with Prism 6 (La Jolla, CA, USA).

Insulin tolerance test

Insulin tolerance test (ITT) was performed at 1:00 PM after 4 h of fasting following an intra-peritoneal injection of insulin (0.6 U/kg); blood was collected in the tail vein after 0, 30, 60, 90 and 120 min to measure blood glucose levels and total AUC calculated.

Statistical analyses

The sample size required for this study was estimated by performing power calculations (5% level of significance with 84% power) using preliminary results from our laboratory. Data are expressed as mean $\pm$ SEM. Data normality was analyzed using D'Agostino and Pearson omnibus normality test. Two-way ANOVA with genotype (*angptl2*-KD and WT mice) and frequency of the diet (AL and IF) as fixed factors and Tukey's post hoc multiple comparison tests were performed with Prism 6 (La Jolla, CA, USA) to assess differences between groups. Statistical significance was set at $p < 0.05$.

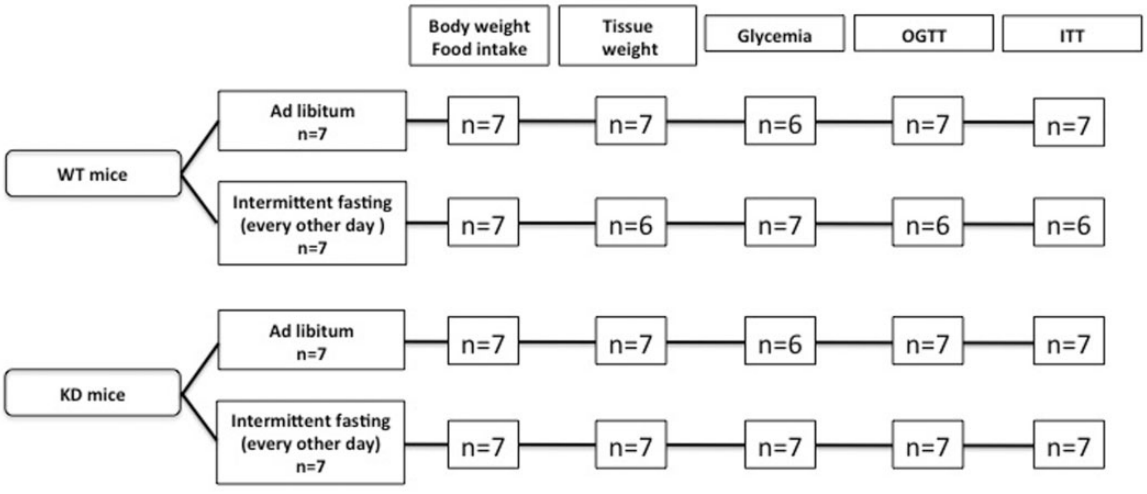

Figure 1. Experimental study design. From the age of three months, wild-type (WT) and ANGPTL2 knockdown (KD) mice were fed ad libitum or with intermittent fasting (every other day). Then, at the age of seven months, all the mice were sacrificed and plasma and tissues (liver, white adipose tissue) were collected. Insulin sensitivity (ITT) and oral glucose tolerance test (OGTT) were measured before sacrifice. Weight gain was monitored weekly during the four months of treatment.

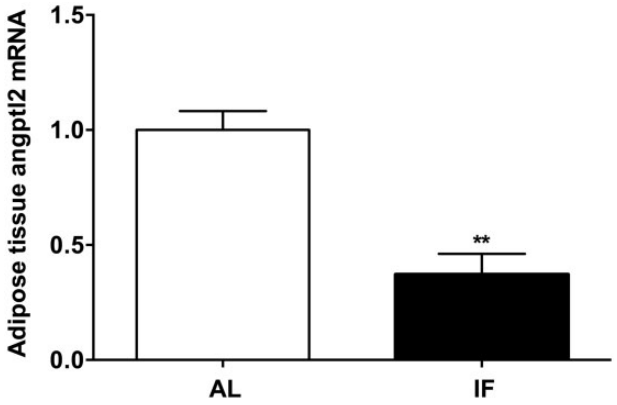

Figure 2. Expression of *angptl2* mRNA in adipose tissue from wild-type mice fed ad libitum (AL; n = 4) or exposed to intermittent fasting (IF; n = 7). T-test was performed to assess differences between frequencies. Results are mean ± SEM. **p < 0.01: mice fed IF versus AL.

Results

IF reduces *angptl2* mRNA expression in adipose tissue

IF for four months reduced by 63% ($p < 0.05$) *angptl2* mRNA expression in adipose tissue from WT mice (Figure 2).

ANGPTL2 KD increases insulin sensitivity and mimics IF

IF did not significantly reduce fasting glycemia (Figure 3 (a)). In the insulin tolerance test (ITT), IF decreased the AUC for ITT in WT, but not in KD mice (Figure 3(b)). AUC for ITT was similar between WT mice fed IF and KD mice fed AL (Figure 3(b)), suggesting that insulin responsiveness was improved by IF and that ANGPTL2 KD mimics IF with no further additive effect. In the

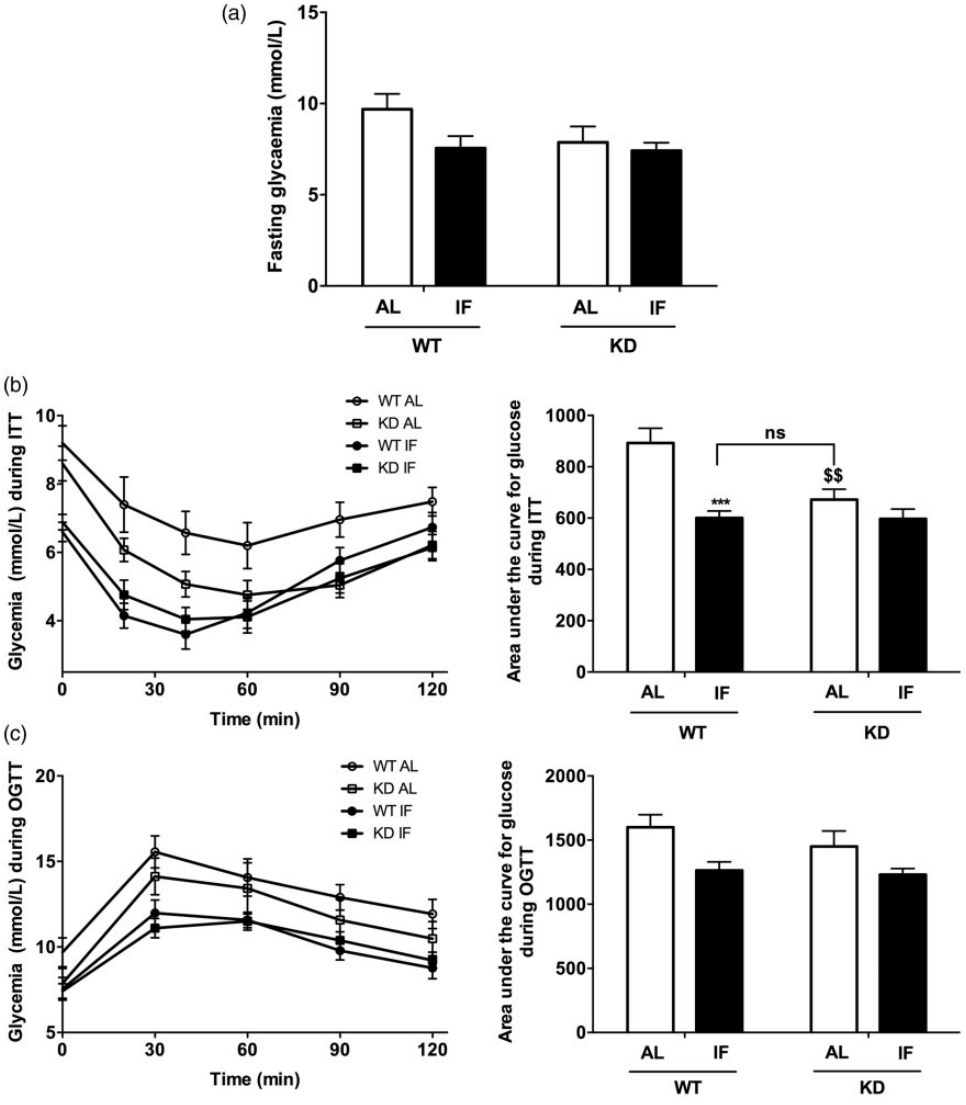

Figure 3. Fasting glucose levels (a), insulin tolerance test (ITT) and total area under the curve for glucose ($AUC_{glucose}$) of ITT (b) and oral glucose tolerance test (OGTT) and total area under the curve for glucose ($AUC_{glucose}$) of OGTT (c) in wild-type (WT) mice and ANGPTL2 knockdown (KD) mice fed AL or exposed to IF. Results are mean ± SEM. Two-way ANOVA and Tukey's multiple comparison tests post hoc analyses were performed to assess differences between groups; 6–7 animals per group. ***$p < 0.001$: mice fed IF versus AL; $^{\$\$}p < 0.01$, KD versus WT mice; ns: not significant.

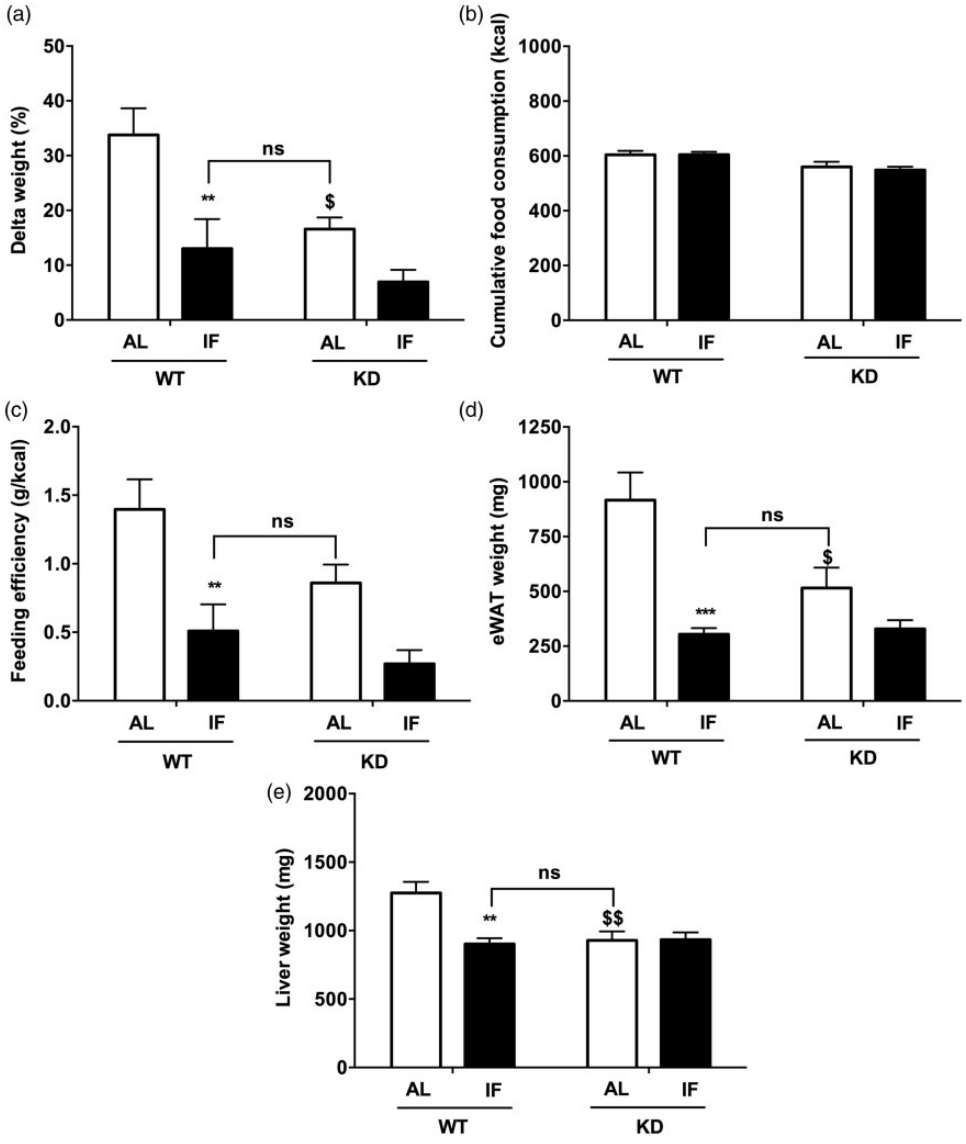

Figure 4. Weight gain (a), cumulative food consumption (b) and feeding efficiency (c), adipose tissue (d) and liver (e) weights of wild-type (WT) and ANGPTL2 knockdown (KD) mice fed ad libitum (AL) or exposed to intermittent fasting (IF). Results are mean ± SEM. Two-way ANOVA and Tukey's multiple comparison tests post hoc analyses were performed to assess differences between groups; 6–7 animals per group. $^{**}p < 0.01$, $^{***}p < 0.001$: mice fed IF versus AL; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$: KD versus WT mice; ns: not significant.

OGTT, IF did not significantly reduce the AUC for OGTT in both WT and KD mice (Figure 3(c)). Altogether, these results suggest that ANGPTL2 KD mimics the beneficial effects of IF on insulin responsiveness.

ANGPTL2 KD lowers weight gain, feeding efficiency and mimics IF

As expected, IF for four months significantly reduced weight gain in WT mice compared to mice fed AL (Figure 4(a)). KD mice fed AL have a 51% lower weight gain than WT mice fed AL (Figure 4(a)). IF did not significantly potentiate weight loss in ANGPTL2-KD mice. Thus, weight gain was similar in WT mice fed IF diet and in KD mice fed AL (Figure 4(a)), suggesting that ANGPTL2 KD mimics IF benefits on weight gain. Despite this significant difference in body weight gain between WT and KD mice, food consumption and thus calorie intake was similar between all four groups (Figure 4(b)). This suggests that both IF and KD of ANGPTL2 reduce feeding efficiency (body weight gain/kilocalories consumed): indeed, as observed for weight gain, IF and ANGPTL2 KD similarly reduced feeding efficiency (Figure 4(c)). Altogether, these data demonstrate that ANGPTL2 KD reproduces the benefits of IF observed in WT mice.

ANGPTL2 KD lowers liver and adipose tissue weights

As expected from the reduction in weight gain induced by both IF and ANGPTL2 KD, IF reduced epididymal white adipose tissue (eWAT) weight in WT mice and eWAT weight was similar between WT fed IF and KD mice fed AL (Figure 4(d)). The same pattern was observed for liver weight (Figure 4(e)). Therefore, IF decreases eWAT and liver weight and KD of ANGPTL2 reproduces the benefits of IF observed in WT mice.

Discussion

The novel findings of this study are that IF, which limits weight gain and improves insulin sensitivity, reduces *angptl2* gene expression in adipose tissue from WT mice, suggestive of a potential link between ANGPTL2 and IF. Importantly, the KD of ANGPTL2 reproduces the benefits of IF on insulin responsiveness, weight gain, feeding efficiency, and eWAT and liver weight.

Despite similar calorie intake between WT and ANGPTL2-KD mice, KD mice fed AL achieved similar targets in terms of insulin responsiveness and body, liver and adipose tissue weights than WT mice fed IF. IF in KD mice neither further increases insulin responsiveness nor further limited weight gain, suggesting that rather than being synergistic, IF and ANGPTL2-KD may share common pathways. Since cumulative food consumption was similar between groups, one possibility is that both IF and ANGPTL2-KD improve metabolic fuel use. Indeed, it is known that lipid oxidation provides more energy than carbohydrates oxidation, but it requires more oxygen consumption.[13] Therefore, higher fat oxidation rate should result in an increase in whole-body energy expenditure that could lead to fat mass loss even if energy intake remains constant. Interestingly, it has been reported that fatty acid oxidation in the liver and muscle are increased by IF.[14,15] In addition, ANGPTL2$^{-/-}$ mice fed with a high-fat diet had a better insulin responsiveness than WT mice and preferentially used lipids over carbohydrates for oxidation to create basal energy.[3] Increased fat oxidation may therefore be a common mechanism between IF and KD of ANGPTL2 to explain their benefits on insulin responsiveness and weight loss. This hypothesis remains to be validated in our mouse model using indirect calorimetry to quantify lipid and carbohydrate oxidation rates.

In conclusion, the present study shows that IF lowers adipose tissue *angptl2* mRNA expression and that KD of ANGPTL2 reproduces the benefits of IF on weight loss, reduced feeding efficiency and increased insulin sensitivity. Higher fatty acid oxidation rates may be the mechanism shared by IF and ANGPTL2 KD, a hypothesis that requires further investigations. Altogether, these results suggest that the reduction of ANGPTL2 could be a useful and promising strategy to prevent obesity and insulin resistance.

Authors' contributions: CM and AP equally contributed to this work. ET designed the research; CM, AP, NT-T analyzed the data; AP wrote the paper; CM, AP, AMB, M-AG, XL conducted the experiments; NT-T, AP, CM, ET reviewed the manuscript; ODM contributed to discussion.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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