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REVIEW OF THE DOCTORAL DISSERATION OF MARIKA OLGA ZIĘTAK PREPARED AT THE INSTITUTE OF ANIMAL REPRODUCTION AND FOOD RESEARCH OF POLISH ACADEMY OF SCIENCES IN OLSZTYN UNDER THE SUPERVISION OF PROF. LESLIE P. KOZAK

The aim of this study was to examine the effect of reduced ambient temperature on energy balance and gut microbiota in mice fed high-fat diet. In addition, it was examined if transplantation of microbiota from mice kept at reduced environmental temperature to germ-free mice has any effect on the development of diet-induced obesity. B6 mice were fed either standard diet (11% fat) or high-fat diet (58% fat) for 4 weeks at were kept at 29^{O}C, 17 ^{O}C or 12 ^{O}C. Food intake, body weight, fat and lean mass, energy expenditure and metabolic efficiency were monitored throughout the experiment. After 4 weeks of feeding, intraperitoneal glucose tolerance test and insulin tolerance test were performed after 4 hours of fasting. The expression of selected genes involved in insulin signaling, glucose transport, gluconeogenesis, bile acid synthesis, metabolism and signaling, and brown fat thermogenesis was measured by qRT-PCR in adipose tissue, liver and small intestine. The expression of some selected respective proteins was also examined by Western blotting. In addition, the composition of gut microbiota was evaluated by sequencing of DNA isolated from fecal samples. Bile acid concentrations were measured in plasma using UPLC-mass spectrometry and concentrations of short-chain fatty acids were measured in coecal content by gas chromatography. In the second part of the experiments, coecal microbiota was transplanted from high-fat diet fed mice maintained at 29^{O}C or 12^{O}C for 4 weeks to germ free mice and the recipient mice were monitored for 6 weeks regarding development of high fat diet-induced obesity and the respective signaling mechanisms.

In the first set of the experiments it has been demonstrated that reduction of ambient temperature to 17^{O}C decreased and to 12^{O}C completely blunted the development of high fat diet-induced obesity. Mice fed the high fat diet and maintained at reduced temperatures exhibited lower fat mass and, to a lesser extent, lean mass. Reduced ambient temperatures increased food intake and total energy expenditure and decreased metabolic efficiency (percent of delivered energy stored in adipose tissue) in mice fed either standard or high fat diet. Neither high fat diet nor reduced ambient temperature had any effect on baseline glucose

concentration. However, reduced temperature increased glucose tolerance (decreased area under the curve of plasma glucose concentration after ip. glucose challenge) in mice fed high fat diet to the level observed in mice fed standard diet. In addition, reduction of ambient temperature to 17°C improved glucose-lowering effect of insulin at 120 min after its peritoneal injection. Interestingly, there was no difference in insulin-induced glucose lowering effect between STD and HFD groups at either 29°C or 17°C. Reduction of ambient temperature to either 17°C or 12°C increased Glut4 expression in brown adipose tissue at the mRNA and protein levels in mice fed high fat diet but not in animals fed standard diet. The expression of insulin receptor substrate-1 did not differ between HFD and STD groups at 29°C. Interesting, reduction of ambient temperature increased Irs-1 expression in BAT in animals fed standard diet but not in those fed high fat diet. Reduction of temperature to either 17°C or 12°C increased the expression of rate-limiting enzyme of gluconeogenesis, Pepck, in the liver in both STD and HFD groups. Similarly, glucose 6-phosphatase in the liver was up-regulated by reduced temperature irrespectively of the diet. These results indicate that low temperature stimulates hepatic gluconeogenesis. In addition, low temperature tended to increase Pepck and glucose 6-phosphatase expression in the intestine, but this effect was not significant. Importantly, reduction of temperature to either 17°C or 12°C increased Ucp1 expression in brown adipose tissue in both STD and HFD groups. Ucp-1 mRNA was undetectable at 29°C in the inguinal white fat but was induced at 17°C or 12°C although still remained much lower than in BAT. Decrease in ambient temperature increased the expression of beta3-adrenergic receptor and peroxisome proliferator activated receptor coactivator (PGC-1) in brown adipose tissue irrespectively of the diet. The results suggest that hepatic lipogenesis was suppressed at 12°C because the expression of Srebp-1c and fatty acid synthase were reduced. The expression of carbohydrate response element-binding protein in the liver was stimulated in HFD group by lowering temperature to 17°C. Reduction of temperature to 12°C increased AMPK phosphorylation and phosphorylation of its target, acetyl-CoA carboxylase (ACC), in the liver of mice fed the high fat diet. The expression of CPT-1 at the mRNA and protein level was higher at 12°C than at 29°C suggesting increase in fatty acid oxidation.

The effect of high fat diet on intestinal microbiome was characterized by reduced bacterial richness and diversity with decreased abundance of Bacteroitides, Lactobacillus and Clostridium coccoides. Reduction of ambient temperature increased bacterial diversity. In

particular, reduction of ambient temperature increased the abundance of Bifidobacterium, Clostridium coccoides and Clostridium leptum.

Reduction of ambient temperature increased the expression of enzymes involved in bile acid synthesis in the liver including Cyp7a1, Cyp8b1, Cyp27a1 and Cyp7b1, the enzymes involved in taurine synthesis (cysteine sulfinic acid decarboxylase and cysteine dioxygenase) and bile acid conjugation (bile acid-CoA synthase and bile acid transferase). Concomitantly, the increase in the ratio of conjugated to unconjugated bile acids was observed and plasma concentration of secondary bile acids was increased. Furthermore, the results suggest that low temperature augmented bile acid signaling in brown adipose tissue as evidenced by greater expression of TGR5, type 2 deiodinase and Fgf21. Neither high fat diet nor reduced ambient temperature had any significant effect on acetate and propionate concentrations in the cecal content. However, high fat diet reduced butyrate concentration and this effect was reversed by reduced ambient temperature.

In the second set of experiments cecal content was transplanted from mice fed the high fat diet at either 29°C or 12°C and the recipient animals were fed high fat diet and monitored. Transplantation of cecal content from animals maintained at 12°C had no effect on body weight or lean mass but reduced fat mass and adiposity. Ucp1 and deiodinase-2 expression in brown adipose tissue was increased. The mRNA level of carbohydrate response element binding protein in the liver was lower and CPT-1 mRNA was higher in mice receiving cecal content of animal kept at 12°C, suggesting lower fatty acid synthesis and increased fatty acid oxidation; this conclusion being supported by greater AMPK and ACC phosphorylation. However, fatty acid synthase and Srebp-1c were not different. In addition, microbiome transplantation from "12°C" mice increased glucose tolerance while having no effect on Glut4 expression in skeletal muscles or brown adipose tissue. The expression of some (Cyp8b1 and Cyp7b1) bile acid-synthesizing enzymes was increased. Microbiome transplantation from "12°C mice" increased the expression of cysteinesulfinate decarboxylase but had no effect on cysteine dioxygenase or taurine transporter. The ratio between conjugated and unconjugated bile acids was higher in mice receiving cecal content transplant from animals kept at 12°C. Taken together, the results indicate that reduction of ambient temperature induces favorable changes in the composition of gut microbiota which result in altered bile acid metabolism ultimately leading to the increase in brown fat thermogenesis.

The dissertation has a typical layout. It is presented on 95 printed pages and consists of Introduction, Aim of research and its justification, Materials and Methods, Results and Discussion. The Introduction presents a brief but informative overview of the topics related to this study. The methods are sophisticated and clearly described. The Results section is logically divided into subsections focused on specific topics which greatly facilitates following the results. The results are described in the text and presented on 16 figures and one table. Conclusions are presented at the end of the Discussion. In addition, list of abbreviations, list of figures and list of tables are provided. Polish and English summaries are included. References are presented in the alphabetical order.

The topic addressed in this study is very important and interesting. The role of gut microbiome in the pathogenesis of obesity and related complications is now a very hot topic. The original aspect of this study is to address the relationship between reduced ambient temperature and intestinal microbiome. The results represent an important progress in our understanding of this field and have also potential translational implications regarding reduction of ambient temperature and/or microbiome transplantation as the potential new treatment strategies.

There are some concerns which, however, do not reduce the high impact of this study

1) As many aspects related to this study are covered in the Introduction, it would be convenient for the reader to include some figures presenting, for example, the major regulatory mechanisms of BAT thermogenesis or brief overview of bile acid synthesis, metabolism and signaling.
2) Mice kept at reduced temperatures exhibited lower lean mass than animals kept at $29^{O}C$. This issue should be discussed. It could be unbeneficial effect associated with decrease in muscle or bone mass.
3) In many figures statistical significance was examined only between respective groups kept at different temperatures and some potential differences between mice fed standard and high fat diets at the same temperature are not indicated. For example, on Fig. 2A food intake seems to be higher in high fat fed mice kept at $29^{O}C$ than in standard diet fed mice kept at $29^{O}C$ but it is unclear if this difference is significant. If it is significant, the relative increase in food intake at reduced temperatures seems to be lower in high fat than in standard diet group because food intake at reduced

temperatures seems to be similar irrespectively of the diet. It should be discussed why cold-induced relative increase in food intake is lower in high fat than in standard diet group. Similarly, on figure 2B total energy expenditure at 29^{O}C seems to be lower in HFD than in STD group but it is unclear if this difference is significant or not. On Fig. 2C metabolic efficiency at 29^{O}C seems to be higher in HFD than in STD group; if this difference is significant, the effect should be discussed. The results shown on Fig. 3C suggest that reduced temperature decreased area under the curve of glucose concentration in glucose tolerance test also in standard diet-fed group but is this effect significant? Fig. 4D and 4E: the expression of Pepck and G6pc at 29^{O}C seems to be higher in HFD vs. STD group; is it significant? Fig. 6B and 6C: is AMPK and ACC phosphorylation at 29^{O}C significantly different between STD and HFD groups?

4) Page 32: increase in hypoglycemic effect of insulin induced by temperature lowering is interpreted as the increase in insulin-induced glucose uptake. However, insulin tolerance test was performed after 4 hours of fasting. The effect of insulin during fasting state is mainly to inhibit gluconeogenesis and hepatic glucose output rather that stimulation of glucose uptake. In addition, it would be convenient to measure plasma insulin concentration during both glucose tolerance and insulin tolerance tests. Reduced ambient temperature can affect insulin absorption after its ip. injection and/or insulin metabolism (for example by modulating hemodynamics). Thus, some effects could be mediated by altered insulin pharmacokinetics in addition to improvement of insulin signaling.
5) To explain the mechanism through which temperature reduction increased the effect of insulin, GLUT4 and IRS-1 expression was measured. High fat diet-induced impairment of insulin signaling is often associated with posttranslational modifications of IRS-1 (such as enhanced phosphorylation by serine/threonine protein kinases which impairs its phosphorylation at tyrosine residues by insulin receptor beta-subunit). This mechanism is often more important than total amount of IRS-1 protein.
6) UCP-1 expression in brown adipose tissue at the protein level is presented separately for 17^{O}C (Fig. 5B) and 12^{O}C (Fig. 5C); why? In addition, UCP-1 expression at 29^{O}C seems to be different on Fig. 5B and Fig. 5C (on Fig. 5B it seems to be higher in HFD than in STD group whereas on Fig. 5C is similar in these groups). This discrepancy should be explained.

7) It is concluded that fatty acid synthesis in the liver was reduced and fatty acid oxidation was stimulated at low temperatures. There is no definite evidence of this since the respective fatty acid fluxes were not measured. Only effects on gene expression of some proteins involved in lipogenesis (SREBP-1c, fatty acid synthase), fatty acid oxidation (CPT-1) and phosphorylation of AMPK were measured.
8) Why the effect of transplantation on food intake and metabolic efficiency was not reported? In addition, it would be of interest to examine microbiome composition of mice receiving the transplant from 29°C and 12°C mice.
9) While cecal content transplantation from 12°C mice had some beneficial metabolic effects in the recipient animals, some effects observed in donor mice kept at 12°C are not observed in the recipient mice. Thus, the effects of reduced ambient temperature are only partially mediated by changes in gut microbiome. This issue should be discussed. What other mechanism, independent of microbiome, are involved in the effect of reduced temperature?
10) Page 11, line 4 from the bottom: *Dio2* is "iodothyronine type 2 deiodinase", not iodothyronine type 2"
11) Fig. 6A is not easily readable, bars are too small to present significant differences.
12) .Page 54, Table 5: composition of cecal short chain fatty acid pool is shown without SEM/SD values which makes any statistical analysis impossible.
13) Page 60, paragraph 4.7.4: it is stated that "Csd is unchanged" which is not correct; Csd was reduced and Cdo was unchanged.

In addition, there are some concerns related to six conclusions. Conclusions #1 and #2 are not the primary conclusions from this study because these effects have been described previously by others. Conclusions #3, 4, 5 and 6 are the most innovative and important and should be presented first. In addition, the phrase "reduction of ambient temperature increased glucose and insulin sensitivity" (conclusion #2) is not optimal; it is unclear what "glucose sensitivity" means. Although the results suggest that reduced ambient temperature could increase fatty acid oxidation, there is no definite evidence of this since fatty acid metabolic fluxes have not been studied. Therefore, this sentence should be removed from conclusion #2. The conclusion #6 that bile acids mediated the effect of reduced ambient temperature and of modified gut microbiome is too strong because no attempts have been made to block the effect of reduced temperature on bile acid metabolism/signaling to clearly demonstrate their

involvement. While bile acids could be the mediator, other mechanisms such as changes in short chain fatty acids and their signaling cannot be excluded.

Despite these minor concerns, the dissertation of Marika Olga Ziętak is very interesting and well-written. The study has significant scientific impact and has translational implications. The results also open some new areas for future research. I have a great honor and pleasure to recommend the Council of the Faculty of Medicine with the Division of Dentistry and Division of Medical Education in English of the Medical University of Białystok to proceed to the next steps of doctoral procedure.

Lublin 16.05.2016

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