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Fasting and sampling time affect liver gene expression of high-fat diet-fed mice

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Several physiological and biological variables are known to affect peroxisome proliferator-activated receptor (PPAR)-α-dependent signaling pathway and plasma biochemical profiles. However, less is known about the effect of these variables on high-fat diet-fed mice. In a 5-week study, C57BL/6 mice were divided into control (C) and high-fat diet-fed (H) groups, whereby before dissection, each group was subdivided into non-fasted (nC and nH) and a 15-h fasted mice (fC and fH) killed in the early light cycle, and a 15-h fasted mice (eC and eH) killed in the late phase of the light cycle. Liver and blood from the vena cava were collected. Non-fasted nC and nH mice have a marginal difference in their body weight gain, whereas significant differences were found for fasted mice. In nH mice, PPAR-α, acyl-CoA oxidase and insulin-like growth factor-binding protein expressions were significantly elevated, in contrast to fatty acid synthase (Fasn), stearoyl CoA-desaturase (SCD)-1, and elongase (ELOVL)-6 expressions. Fasn was profoundly induced in fH mice, while decreased sterol regulatory-binding protein-1 and SCD-1 were found only in eH mice. Different from the gene expression profiles, plasma total cholesterol level of the eH mice was higher than controls, whereas nH mice have increased plasma non-esterified fatty acids. Only glucose level of the fH mice was higher than that observed for controls. Results showed that fasting and sampling time have significantly affected liver gene expression and plasma biochemical indices of the high-fat diet-treated mice. An overlook in these aspects can cause serious discrepancies in the experimental data and their interpretations.

Keywords: fasting, gene expression, high-fat diet, mice, sampling time

Implications

This study showed that when high-fat diet-fed mice were killed in a fed state, their expression of genes related to obesity and their plasma biochemical profiles differed significantly from mice killed in a fasted state. Also, differences in the timing of sample collection have greatly affected these results. Therefore, it is important to standardize the procedures of sample collection. This is to avoid 'untrue' discrepancies in the data obtained, thus resulted in wrong prediction or conclusion on certain biological and physiological status.

Introduction

Prolonged fasting causes a shift in the use of carbohydrate and fat as the whole-body fuel to mainly fat. The dramatic changes in metabolism occur primarily in the liver. Fasting promotes the release of fatty acids from adipose tissues. Fatty acids are taken up by the liver and are oxidized in the mitochondria (Kersten *et al.*, 1999). *Peroxisome proliferator-activated receptor (PPAR)-α*, a ligand-activated transcription factor, is a key regulator of genes for mitochondrial and peroxisomal fatty acid oxidation as well as for ketogenesis (Cheon *et al.*, 2005). *Acyl-CoA oxidase* (*ACOX*), a *PPAR-α* target gene, is required for fatty acid β-oxidation in liver peroxisomes (Tugwood *et al.*, 1992). Suppressed *PPAR-α* expression poses a risk of developing obesity. Besides fasting, high-fat diet is another physiological factor that may affect the *PPAR-α*-dependent signaling pathway (Patsouris *et al.*, 2006). Other pre-analytical and biological variables that have been found to influence lipid measurements include the length of fasting period, the posture of an individual and blood sampling time (Sundvall *et al.*, 2008).

High-fat diet feeding in animal is a widely used method to identify *PPAR-α* agonist. But, physiological and biological variables that may affect the observations are seldom being considered. This may lead to inaccurate interpretation of the results and the adoption of inappropriate treatment. Therefore, the objective of this study was to investigate the effect of fasting and sampling time on the expression of

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PPAR-α, *ACOX* and selected lipogenic genes in high-fat diet-fed mice. Plasma levels of glucose, triglycerides (TGL), total cholesterol (T-CH), non-esterified fatty acids (NEFAs) and ketone bodies such as acetoacetate (AcAc) and hydroxybutyrate (OHBA) were also measured.

Material and methods

Animals

Male C57BL/6 mice (Japan SLC, Shizuoka, Japan) were housed five per cage in a temperature-controlled room (23 ± 1°C), and allowed free access to water and a standard diet (D12450B; Research Diets Inc., NJ, USA) for 10 days before treatments. Mice were 12 weeks of age at the beginning of the experiment. Animal experiments were approved by the University of Shizuoka Animal Usage Ethics Committee. Mice were divided into two groups: controls (C, $n = 15$), which were continuously fed with the standard diet and mice given a high-fat diet (H, $n = 15$). The high-fat diet contained 45 kcal percentage fat (D12451; Research Diets Inc., NJ, USA). Body weight and food intake were recorded twice a week for a period of 5 weeks.

Before dissection on the fifth week, each group of mice was further divided into three subgroups ($n = 5$ each): non-fasted (nC and nH) and a 15-h fasted (fC and fH) groups, which were killed at 0900 h; and a 15-h fasted group, killed at 1700 h (eC and eH). Blood was collected from the vena cava into capiject tubes (Terumo Medical Corp., Somerset, NJ, USA) and centrifuged at 3000 r.p.m. for 10 min to separate out the plasma, which was stored at −80°C. Liver tissues of approximately 30 mg were transferred into a 1 ml of RNA*later*® solution (Ambion Inc., Austin, TX, USA).

Plasma biochemical analysis

Selected plasma biochemical indices, including glucose, NEFAs, TGL, T-CH, AcAc, and OHBA were determined using an automated analyzer (TBA-120FR, Toshiba Corp., Tokyo, Japan).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Liver tissues of 15 mg were homogenized with a digital-handheld homogenizer. The medium used for homogenization and the subsequent procedures for total RNA extraction were as described in the RNA tissue kit SII (Fujifilm Corp., Tokyo, Japan). The concentration of the extracted RNA was estimated from optical densities set at 260 and 280 nm (Amersham, Biochrom Ltd., Cambridge, England, UK).

Complementary DNA (cDNA) was synthesized from 300 ng of total RNA according to the protocol by Applied Biosystems (ABI, Foster City, CA, USA). The synthesized cDNA was PCR amplified using the 2× TaqMan® Gene Expression Master Mix and various set of primers obtained from ABI. All transcripts were amplified in an ABI 7500 machine followed the recommendations of the manufacturer. The value of amplification was normalized against glyceraldehyde 3-phosphate dehydrogenase, which acted as an internal control of each sample.

Statistical analysis

All data, except that of food intake, are presented as mean ± s.d. Differences between the control and the high-fat diet-treated mice were determined by the Student's *t*-test. For the plasma biochemical analysis, comparisons were also made among controls by using ANOVA, followed by a post hoc Tukey–Kramer Multiple Comparisons test. Differences were considered significant when P was <0.05.

Results

High-fat diet feeding increased food intake and body weight of fasted fH and eH mice by 22% and 35% (*v.* fC and eC, respectively) at the end of the 5-week treatment. However, non-fasted nH mice which have consumed similar calories of food, as compared to fH and eH, have identical body weight when compared with nC (Figure 1).

In nH mice, *PPAR-α*, *ACOX* and *insulin-like growth factor-binding protein (IGFBP)-1* levels were 1.4- to 2.2-fold higher than the control. However, expression of lipogenic genes such as *fatty acid synthase (Fasn), stearoyl CoA-desaturase (SCD)-1* and *elongation of very long-chain fatty acids (ELOVL)-6* were lowered by 71%, 58% and 80%, respectively (nH *v.* nC) (Figure 2a). Except for *Fasn*, there was no significant difference between fH and fC in the expression of all the other genes. *Fasn* in fH mice was markedly upregulated (+3.7-fold *v.* fC). eC and eH mice have comparable levels of *Fasn*. Meanwhile, although *SCD-1* expression was not significantly different between fH and fC, its level of expression was significantly reduced when liver samples were collected in the evening. In eH mice, the expression of transcription

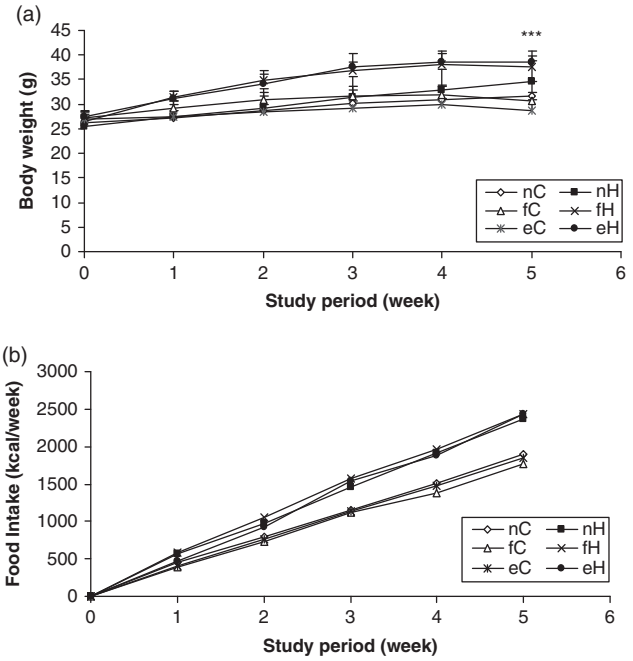


Figure 1 Effects of fasting and sampling time on body weight gain (a) and food intake (b) of mice fed with a standard (C) or high-fat (H) diet. Values are mean ± s.d. ***$P < 0.001$ between fC and fH, and between eC and eH mice, as determined by the Student's *t*-test.

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receptor *sterol regulatory-binding protein (SREBP)-1* and *SCD-1* were decreased by 21% and 63%, respectively.

As shown in Table 1, the 5-week treatment with high-fat diet has significantly increased plasma glucose level of the fasted fH mice (+15% *v.* fC). There was no similar observation for the fasted eH mice, because its control exhibited a glucose level that was 21% higher than fC. The glucose level of eC was also comparable to nC mice. Plasma NEFAs of eC mice was 1.5- to 3.8-fold higher than fC and nC mice. However, it was only nH mice that have profoundly higher NEFAs than its control (+31% *v.* nC). Plasma TGL of fC mice was 34% to 54% higher than nC and eC mice. Nonetheless, there was no significant difference between the control and the high-fat diet-fed mice for TGL measurement. Despite an increase in TGL, T-CH of fC mice was identical to nC and eC mice. Somehow, T-CH levels of fH and eH mice were significantly elevated when compared to controls (+18% and +10%, respectively). Plasma AcAc and OHBA of both the fC and fH mice were distinctly higher than all the other mice.

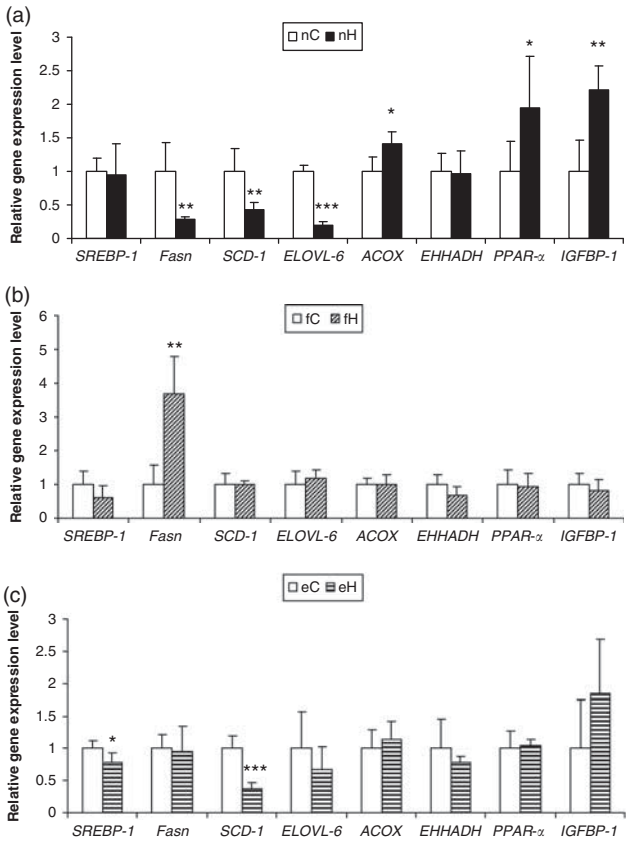


Figure 2 Liver gene expression of the control mice (C) and mice treated with a high-fat diet (H) when they were killed under these conditions: (a) early phase of the light cycle in a non-fasted state; (b) early phase of the light cycle after a 15-h fasting; and (c) late phase of the light cycle after a 15-h fasting. Values are mean ± s.d. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$, as analyzed using the Student's *t*-test. *SREBP-1 = sterol regulatory binding protein-1*; *Fasn = fatty acid synthase*; *SCD-1 = stearoyl CoA-desaturase-1*; *ELOVL-6 = elongation of very long chain fatty acids-6; ACOX = acyl-CoA oxidase*; *EHHADH = enoyl-CoA hydratase*; *PPAR-α = peroxisome proliferator-activated receptor-α*; *IGFBP-1 = insulin-like growth factor-binding protein-1*.

Discussion

High-fat diet feeding is a commonly used method to induce obesity. Despite that, it is not known that before dissection, continuous food intake can mask the effect of high-fat diet on inducing body weight gain. Mice without fasting, nC and nH, not only failed to show differences in body weight gain at the end of the 5-week treatment, their liver gene expression and plasma biochemical profiles differed significantly from those of the fasted mice.

In mice treated with a high-fat diet, genes responsible for fatty acid catabolism and ketone body synthesis are upregulated, whereas downregulation is found for genes involved in lipogenesis and cholesterol production (Kim *et al.*, 2004). Besides that, when C57BL/6 mice fed with a high-fat–high-sucrose diet are killed in a non-fasted state in an early phase of the light cycle, *PPAR-α* and *ACOX* expressions are significantly upregulated (Matsuzaka *et al.*, 2007). These observations concurred with our data of non-fasted mice (Figure 2a). It is suggested that the inductions of *PPAR-α* and *ACOX* are likely to be an adaptive response. On the other hand, *IGFBP-1* is known to be negatively related with hypertension, high-density lipoprotein cholesterol, body mass index, waist-to-hip ratio, insulin, proinsulin and TGL levels. Low circulating *IGFBP-1* concentration is correlated with impaired glucose tolerance (Heald *et al.*, 2002). With regard to the properties of *PPAR-α*, it was logical to see that the expression of *IGFBP-1* associated well with that of the *PPAR-α* in nH mice.

Table 1 *Plasma biochemical indices of the different groups of mice*

	Glucose (mg/dl)	NEFAs (μEq/l)	TGL (mg/dl)	T-CH (mg/dl)	AcAc (mg/dl)	OHBA (mg/dl)
nC	253.2 ± 30.8^{a,b}	307.8 ± 39.8^{a}	91.0 ± 8.3^{a}	175.8 ± 22.3^{a}	6.1 ± 4.2^{a}	65.0 ± 17.2^{a}
nH	289.2 ± 37.3	443.5 ± 73.0**	87.4 ± 25.6	176.3 ± 10.3	0.4 ± 0.2*	74.5 ± 30.3
fC	210.6 ± 13.3^{a}	776.4 ± 63.0^{b}	138.6 ± 18.1^{b}	187.6 ± 14.4^{a}	28.6 ± 3.8^{b}	1661.3 ± 243.8^{b}
fH	248.8 ± 31.8*	703.0 ± 72.5	134.4 ± 16.6	229.5 ± 18.5**	10.5 ± 5.6***	1352.0 ± 72.6*
eC	267.5 ± 10.0^{b}	1162.5 ± 217.9^{c}	63.1 ± 14.8^{c}	170.8 ± 3.8^{a}	5.2 ± 6.9^{a}	271.1 ± 17.4^{a}
eH	262.5 ± 18.0	1079.5 ± 86.5	67.5 ± 13.1	190.6 ± 6.9***	0.5 ± 0.2	337.4 ± 149.7

NEFAs = non-esterified fatty acids; TGL = triglycerides; T-CH = total cholesterol; AcAc = acetoacetate; OHBA = hydroxybutyrate.
Different superscript letters (a, b, c) indicate significant differences at $P<0.05$, as calculated by ANOVA and post hoc test.
$^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$, as calculated by the Student's *t*-test.

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Liver gene expression profiles of the fasted mice (Figure 2b) were completely different from those observed for the non-fasted mice. This was especially interesting for *Fasn*. Even in the fasted mice *per se* (Figures 2b and 2c), different sampling time has greatly affected their gene expression levels. The pattern of *Fasn*, *SCD-1*, and *ELOVL-6* expressions appeared to be contrasting between liver samples collected in the early and late phases of the light cycle. eH mice showed lower lipogenic gene levels than fH mice. The differences were likely to be due to changes in hormonal levels that follow the circadian system. As shown by Cano *et al*. (2008), plasma corticosterone level of the high-fat diet-fed rat fluctuates throughout the 24-h period, and is lower from 1700 to 2100 hours than in the early light cycle.

Because pineal melatonin increases significantly from 1700 to 2100 hours (Cano *et al*., 2008), it is conceivable that melatonin is also involved in regulating lipogenic gene expression, although the exact mechanism is not clear. The significantly decreased *SCD-1* expression, as well as low levels of *Fasn* and *ELOVL-6* in eH, but not in fH mice, may occur as a result of elevated melatonin, because melatonin prevents hepatosteatosis (Hu *et al*., 2009). It is interesting to point out that the expression pattern of *SCD-1* and *ELOVL-6*, as observed in eH, was very similar to that of the nH mice. Comparison made between the present findings with that reported by Hu *et al*. (2009), which indicate that melatonin has no effect on hepatic expression of lipogenic genes, suggested further that the discrepancies are due to differences in the sampling time.

Data obtained for plasma glucose level have probably the greatest impact on metabolic syndrome-related studies, as glucose is a commonly measured parameter. It is generally known that plasma glucose level is lower in a fasted than in a non-fasted state. The differences between these two are valid for samples collected either in the early (shown in this study) or in the late phase of the light cycle (Nakai *et al*., 2008). However, we may not be aware that even if fasting samples are collected from all subjects, different sampling time significantly affects the readings (fC *v.* eC). This can explain the distinctive differences found between fC and fH, but not between eC and eH mice.

NEFAs are increased in the liver during fasting (Song *et al*., 2002). The released fatty acids may act as endogenous ligands for *PPAR-*α (Cheon *et al*., 2005). In this study, plasma NEFAs of both the fasted fC and eC mice were significantly higher than nC mice. The control levels of *PPAR-*α and *ACOX* were not compared since the values have been normalized. However, induction of β-oxidation can be seen by referring to the plasma ketone bodies. As expected, OHBA was distinctly elevated in fC and eC mice, whereas AcAc was significantly increased in fC mice. Meanwhile, fH mice have lower levels of ketone bodies when compared to fC. With *Fasn* expression being distinctly upregulated, these results implied that high-fat diet feeding suppresses β-oxidation. On the other hand, NEFAs were significantly higher in nH than in nC mice. The increased NEFAs corresponded well with the high expressions of *PPAR-*α and *ACOX*.

In human, serum TGL reaches a minimum level after a 7 to 8 h of fasting, where the later the sample is collected (between 1100 and 1800 hours) would mean the longer the fasting period (Sundvall *et al*., 2008). Therefore, it was reasonable to suggest that the significantly decreased TGL in eC mice, the lowest among the three study groups, was due mainly to the late sampling time. Besides that, it is reported that TGL increases rapidly postprandial before being lipolyzed by lipoprotein lipase. Hence, a 9- to 12-h fasting is recommended in order to obtain a sample that represents the metabolic steady state (Stein and Myers, 1995). A very long fast will cause the serum TGL to increase transiently (Zech *et al*., 1979), and this could be the reason of high TGL in fC mice. With the same fasting period being introduced to both eC and fC, current findings suggested that TGL is very much affected by circadian rhythm. eH mice, which showed low TGL, have also shown a reduction in lipogenic gene expression, especially the *SCD-1*.

It is common that both fasting and non-fasting samples are collected for T-CH measurement (Sundvall *et al*., 2008), which suggest that food has no effect on plasma T-CH level. However, this may only be true in mice given a normal diet. In this study, T-CH level of all the controls (nC *v.* fC *v.* eC) were comparable, whereas significant increase in T-CH was shown in fasted fH and eH mice. Non-fasted nC and nH mice showed identical T-CH levels. These results also suggested that plasma T-CH level is not affected by circadian rhythm. Elevated T-CH in fH mice was accompanied by enhanced *Fasn* expression and decreased β-oxidation. Although eH mice exhibited high T-CH levels, differences between eC and eH in the plasma AcAc and OHBA were not statistically significant.

Conclusion

We knew that apart from plasma biochemical indices, gene expression, either in the liver or adipose tissues, has always been used to provide insight for mechanisms that lead to obesity or diabetes. However, as shown above, fasting plasma collected in the early light cycle is needed for plasma biochemical analysis, but non-fasting samples may be more suitable when liver gene expression is studied. Of course, these data cannot fully represent the situation in human, especially the turnover of TGL and T-CH levels, but a diet-induced obese model does indeed reproduce human obesity better than genetically developed obese animal models (Aoki *et al*., 2007). In conclusion, because gene expression is affected by fasting and sampling time, physiological and biological variables need to be considered during the interpretation of experimental data.

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References

Aoki F, Honda S, Kishida H, Kitano M, Arai N, Tanaka H, Yokota S, Nakagawa K, Asakura T, Nakai Y and Mae T 2007. Suppression by Licorice flavonoids of abdominal fat accumulation and body weight gain in high-fat diet-induced obese C57BL/6J mice. Bioscience Biotechnology Biochemistry 71, 206–214.

Cano P, Jimenez-Ortega V, Larrad A, Toso CFR, Cardinali DP and Esquifino AI 2008. Effect of a high-fat diet on 24-h pattern of circulating levels of prolactin, luteinizing hormone, testosterone, corticosterone, thyroid-stimulating hormone and glucose, and pineal melatonin content, in rats. Endocrine 33, 118–125.

Cheon Y, Nara TY, Band MR, Beever JE, Wallig MA and Nakamura MT 2005. Induction of overlapping genes by fasting and a peroxisome proliferator in pigs: evidence of functional PPARα in nonproliferating species. American Journal of Physiology – Regulatory, Integrative and Comparative Physiology 288, R1525–R1535.

Heald AH, Siddals KW, Fraser W, Taylor W, Kaushal K, Morris J, Young RJ, White A and Gibson JM 2002. Low circulating levels of insulin-like growth factor binding protein-1 (IGFBP-1) are closely associated with the presence of macrovascular disease and hypertension in type 2 diabetes. Diabetes 51, 2629–2636.

Hu S, Yin S, Jiang X, Huang D and Shen G 2009. Melatonin protects against alcoholic liver injury by attenuating oxidative stress, inflammatory response, and apoptosis. European Journal of Pharmacology 616, 287–292.

Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B and Wahli W 1999. Peroxisome proliferator-activated receptor α mediates the adaptive response to fasting. The Journal of Clinical Investigation 103, 1489–1498.

Kim S, Sohn I, Ahn JI, Lee KH and Lee YS 2004. Hepatic gene expression profiles in a long-term high-fat-diet-induced obesity mouse model. Gene 340, 99–109.

Matsuzaka T, Shimano H, Yahagi N, Kato T, Atsumi A, Yamamoto T, Inoue N, Ishikawa M, Okada S, Ishigaki N, Iwasaki H, Iwasaki Y, Karasawa T, Kumadaki S, Matsui T, Sekiya M, Ohashi K, Hasty AH, Nakagawa Y, Takahashi A, Suzuki H, Yatoh S, Sone H, Toyoshima H, Osuga J and Yamada N 2007. Crucial role of a long-chain fatty acid elongase, Elovl6, in obesity-induced insulin resistance. Nature Medicine 13, 1193–1202.

Nakai Y, Hashida H, Kadota K, Minami M, Shimizu K, Matsumoto I, Kato H and Abe K 2008. Up-regulation of genes related to the ubiquitin-proteasome system in the brown adipose tissue of 24-h-fasted rats. Bioscience Biotechnology Biochemistry 72, 139–148.

Patsouris D, Reddy JK, Muller M and Kersten S 2006. Peroxisome proliferator-activated receptor α mediates the effects of high-fat diet on hepatic gene expression. Endocrinology 147, 1508–1516.

Song HW, Nara TY and Nakamura MT 2002. Delayed induction of delta-6 and delta-5 desaturases by a peroxisome proliferator. Biochemical and Biophysical Research Communications 299, 832–838.

Stein EA and Myers GL 1995. National Cholesterol Education Program recommendations for triglyceride measurement: executive summary. The National Cholesterol Education Program Working Group on Lipoprotein Measurement. Clinical Chemistry 41, 1421–1426.

Sundvall J, Laatikainen T, Hakala S, Leiviska J and Alfthan G 2008. Systematic error of serum triglyceride measurements during three decades and the effect of fasting on serum triglycerides in population studies. Clinica Chimica Acta 397, 55–59.

Tugwood JD, Issemann I, Anderson RG, Bundell KR, McPheat WL and Green S 1992. The mouse peroxisome proliferator-activated receptor recognizes a response element in the 5′flanking sequence of the rat acyl CoA oxidase gene. The EMBO Journal 11, 433–439.

Zech LA, Grundy SM, Steinberg D and Berman M 1979. Kinetic model for production and metabolism of very low density lipoprotein triglycerides. Evidence for a slow production pathway and results for normolipidemic subjects. The Journal of Clinical Investigation 63, 1262–1273.

https://doi.org/10.1017/S1751731109991583 Published online by Cambridge University Press