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Possible role of triacylglycerol-rich lipoproteins in the down-regulation of adipose Obese mRNA expression in rats re-fed a high-fat diet

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Summary

The large amount of absorbed dietary lipid after feeding a high-fat diet is mainly transported as triacylglycerol (TG)-rich lipoproteins (TRL) in the post-prandial blood and is subsequently distributed to peripheral tissues including adipose and muscle tissues. An *in vivo* and an *in vitro* study were conducted to investigate the possible role of post-prandial TRL after high fat feeding in the regulation of obese (*ob*) gene expression. Adult male Wistar rats were fasted for 48 h and re-fed either a fat-free/high-carbohydrate diet or a high-fat diet for 2, 4, or 8 h and plasma glucose, insulin, TG, and leptin as well as *ob* mRNA expression in epididymal fat pads were examined. Rats re-fed the high-fat diet had significantly higher plasma TG ($p < 0.05$) and lower plasma leptin and adipose *ob* mRNA ($p < 0.05$) than those fed the fat-free/high-carbohydrate diet; however, plasma glucose and insulin concentrations were not significantly different between the two groups. Plasma lipid analysis found large amount of TRL in rats fed with high-fat diet; however, only very small amount of the TRL was found in rats fed with fat-free/high-carbohydrate diet. We speculated that TRL might involve in regulation of *ob* gene expression. To further examine the regulation of TRL on *ob* mRNA expression, differentiated 3T3-L1 adipocytes were treated with TRL collected from rats fed 5 ml soybean oil by gastric intubations. TRL down-regulated *ob* mRNA not only in a dose and time dependent manner but also in the presence of insulin in 3T3-L1 adipocytes. These results suggest a possible role of TRL in the down-regulation of adipose *ob* mRNA expression and may account, at least in part, for the previous observations that short-term high fat feeding resulted in lower plasma leptin.

Introduction

The mouse *ob* gene and its human homologue have been cloned by positional cloning. The *ob* gene encodes a 16 kd protein hormone, leptin, which is secreted primarily from white adipose tissue [1]. Leptin acts as a satiety factor to control appetite and energy expenditure. The action of leptin on food intake and weight loss is mediated by interaction of the hormone with the long form receptor and activation of a JAK-STAT signaling pathway in the hypothalamus [2–4]. Thus, failure to produce adequate amounts of leptin to its central actions would result in the overeating and development of obesity. Administration of recombinant leptin reduces food intake, increases energy expenditure and produces weight loss in *ob/ob* mice [4, 5].

Given the importance of leptin in energy balance, the expression of the *ob* gene is highly

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sensitive to nutritional status. Fasting led to a substantial fall in *ob* mRNA in the epididymal adipose of rats, which was rapidly reversed on refeeding [6]. The decrease in *ob* mRNA in the fasted rats is restored by insulin administration, indicating that insulin is an important regulator of the effects of food intake on *ob* gene expression [6]. *In vitro* study showed that insulin increased *ob* mRNA levels in fully differentiated adipocytes [7, 8]. The reduction of *ob* gene expression in the fasting state is probably through the action of cAMP signaling pathway, which stimulates lipolysis in adipocytes [9].

In addition to feeding status, the expression of *ob* gene appears to be influenced by the amount of fat in the diet. Levels of circulating leptin were significantly increased when mice fed with a high-fat diet [10, 11]. Masuzaki et al. [12] demonstrated an increase of *ob* mRNA level in adipose tissues of rats fed with a high-fat diet for 2 weeks. However, conflicted results were observed by Havel et al. [13] that 24-h circulating leptin concentrations were lower in healthy women consumed a single high-fat, low carbohydrate meal than those who had consumed a low-fat, high carbohydrate meal. Similarly, Ainslie et al. [14] reported that short-term feeding with high-fat diet resulted in lower plasma leptin concentrations in rats and proposed that the effects of dietary fat on *ob* gene expression is dependent on the length of the dietary treatment. Nevertheless, none of these short-term high fat feeding studies have examined the *ob* gene expression in the adipose tissue. How *ob* gene expression is regulated in accordance with the short or long-term feeding of high-fat diet thus remains unclear.

Shortly after the consumption of a fatty meal, the absorbed fat is incorporated into chylomicrons in intestinal enterocytes and transports to peripheral tissues, such as adipose and muscle. The triacylglycerol (TG) in chylomicrons is hydrolyzed by lipoprotein lipase located on the surface of capillary endothelium, the remnants of chylomicrons are uptaken by hepatocytes through a receptor-mediated pathway and degraded in lysosomes. In the liver, the fatty acids are converted to TG and packed into very-low-density lipoprotein (VLDL) and secreted into the circulation. Both chylomicrons and VLDL are TG-rich lipoproteins (TRL), and their concentrations are high in post-prandial blood after a fatty meal. To test whether these post-prandial TRL may involve in adipose *ob* gene expression through transporting substantial amount of lipids to adipose tissue, this study first measures the levels of adipose *ob* mRNA and plasma leptin at 2, 4, and 8 h after feeding with a high-fat or a fat-free/high-carbohydrate diet in rats. To further confirm this effect *in vitro*, TRL obtained from post-prandial plasma of rats fed a fat bolus were employed to treat 3T3-L1 adipocytes, and the *ob* mRNA expression was examined.

Materials and methods

Animals and treatment

Male Wistar rats (9-weeks-old; 180–200 g), obtained from the Animal Center of the National Taiwan University (Taipei, Taiwan), were housed in groups of 3 in stainless cages on a 12-h light cycle (0700–1900 h), temperature and humidity controlled room with free access to Purina rat chow and tap water for 3 days prior to the experiments. Rats were fasted for 48 h and were fed a fat free or a high-fat diet (28% lard) for 30 min at 9 a.m., and sacrificed in a CO_2 chamber at 0, 2, 4, or 8 h after re-feeding. Semisynthetic high-fat diet and fat-free/high-carbohydrate diet were prepared as described [15]. The high-fat diet contained (g/kg diet) casein, 150; lard, 280; cornstarch, 235; sucrose, 235; vitamin mixture (AIN-76), 10; mineral mixture (AIN-76), 35; methylcellulose, 50; methionine, 3 and choline, 2. The fat-free/high-carbohydrate diet contained same composition except that the fat was replaced with 140 g of cornstarch and 140 g of sucrose. Blood samples were collected in EDTA-containing tubes. The epididymal white adipose tissues (WAT) were removed, immediately frozen in liquid nitrogen, and stored at −70 °C. All animal experimental procedures were conducted followed the "Guide for the Care and Use of Laboratory Animals, National Science Council, Taiwan".

Blood lipids and glucose measurements

Plasma was isolated by low speed centrifugation at 4 °C. TG and glucose concentrations were measured by using commercially available colorimetric enzyme assays (RANDOX, USA). Levels of plasma insulin and leptin were measured by ELISA kits from Mercodia (STATE, Sweden) and Assay

Designs (STATE, USA), respectively. Protein concentrations were determined by the Bradford method [16] with bovine serum albumin as the standard.

TRL isolation

Male rats were fed 5 ml soybean oil by gastric intubations. Three hours after soybean oil gavage, blood was aspirated and collected through portal vein. Plasma was immediately isolated by low speed centrifugation at 4 °C. The TRL was isolated by discontinuous ultracentrifugation according to the procedure of Havel et al. [17]. Briefly, plasma was layered under a salt solution of density 1.006 g/ml and centrifuged for 30 min at 30,000 rpm (40,000 × *g*) at 11 °C. After centrifugation, the white layer at top was collected. Analysis of the lipoprotein fraction using agarose gel electrophoresis (Paragon system, Beckman, Palo Alto, CA) had shown the existence of both VLDL and chylomicron bands. Thus, the lipoprotein fraction was called TRL fraction. TG concentration in TRL was determined as described above.

3T3-L1 adipocyte cultures

Mouse 3T3-L1 preadipocytes were obtained from ATCC (ATCC, Rockville, MD, USA) and cultured as described [18]. Briefly, cells were initially maintained in Dulbècco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Life Technologies, Rockville, MD, USA) under an atmosphere of 5% CO_2 at 37 °C. One day after the cells reached confluence, cells were induced to differentiate by replacing the culture medium for 2 days with DMEM supplemented with 125 nM indomethasin (Sigma), 10 μg/ml insulin (Sigma), and 10% FBS (Gibco BRL), followed by the same medium without indomethasin for another 2 days. Cells were then maintained in DMEM containing 10% FBS for further 8 days, medium were changed every 2 days. To study the effect of TRL, the cells were changed to serum-free DMEM containing 0.1% BSA for 24 h, then treated with various amount of TRL as indicated.

Analyses of ob mRNA

Total cellular RNA in adipose tissue and 3T3-L1 adipocytes were isolated according to the method of Chomczynski and Sacchi [19]. RNA concentrations were estimated from the absorbance at 260 nm. For Northern blot analysis, 15 μg of total RNA was electrophoresed on a 1% agarose–formaldehyde gel, transferred onto nylon membrane, and hybridized with ^{32}P-labeled cDNA probes for rat or mouse *ob* in animal studies and 3T3-L1 cell line studies, respectively. The blot was then hybridized with a probe for β-actin or 18S RNA to check the amount of RNA loaded on the gel. The cDNA fragments were labeled with ^{32}P-dCTP (NEN Life Science Products, Boston, MA, USA) and used as probe for Northern blot analyses. The signals of *ob*, β-actin or 18S RNA were quantified by a UVP AutoChemi Image System. The amount of *ob* mRNA was expressed as arbitrary units relative to the amount of β-actin or 18S RNA.

Statistical analysis

Data were analyzed by two-way ANOVA using a SAS Software (SAS version 8.2, SAS Institute, Cary, NC), and differences between mean values were evaluated by the Duncan's multiple range test. Some data were analyzed by Student's *t*-test. Differences with p values < 0.05 were considered significant.

Results

Plasma TG, glucose, insulin, and leptin of rats

Plasma TG, glucose, insulin, and leptin levels were relatively low in rats fasted for 48 h. Two, 4, and 8 h after refeeding resulted in significant increases of glucose, insulin, and leptin level (Table 1). Levels of plasma glucose were comparable in rats fed with the fat-free/high-carbohydrate diet or the high-fat diet. Levels of plasma insulin were not statistical different between the two dietary groups ($p = 0.0795$). However, plasma leptin concentrations were significantly lower ($p = 0.0018$) in rats fed with the high-fat diet than in rats fed with the fat-free/high-carbohydrate diet. Re-feeding with the high-fat diet resulted in 380, 820 and 290% increase of plasma TG at 2, 4, and 8 h. In contrast, plasma TG was not significantly changed in rats that were re-fed with the fat-free/high-carbohydrate diet (Table 1).

Table 1. Plasma glucose, triacylglycerol (TG), insulin, and leptin concentrations of the rats.

		Fat-free/High-carbohydrate diet			High-fat diet			Two-way ANOVA		
	F48	2 h	4 h	8 h	2 h	4 h	8 h	Time	Diet	Time × Diet
Glucose(mmol/l)	5.0 ± 0.7[1]	14.4 ± 1.7*	14.0 ± 0.6*	12.4 ± 0.6*	13.2 ± 0.4*	13.3 ± 0.6*	13.1 ± 0.4*	0.5295	0.4962	0.4664
TG(mmol/l)	0.31 ± 0.02	0.38 ± 0.01^{c}	0.30 ± 0.02^{c}	0.58 ± 0.07^{bc}	1.51 ± 0.06^{*ab}	2.87 ± 0.44^{*a}	1.23 ± 0.09^{*abc}	0.6054	0.0466	0.1750
Insulin(pmol/l)	35.3 ± 6.5	163.0 ± 26.3^{*a}	150.2 ± 9.4^{*ab}	98.2 ± 16.6^{*bc}	113.4 ± 14.1^{*abc}	86.5 ± 19.6^{c}	86.5 ± 9.4^{*c}	0.1377	0.0795	0.3797
Leptin(ng/ml)	1.2 ± 0.1	2.8 ± 0.5^{*bc}	3.8 ± 0.5^{*ab}	4.3 ± 0.3^{*a}	1.9 ± 0.2^{*c}	2.7 ± 0.5^{*bc}	2.5 ± 0.5^{*bc}	0.0263	0.0018	0.6101

[1]Values are mean ± SEM (n = 3).
* Significantly different from the mean of 48-h starved control (F48) analyzed by Student's t test, $p < 0.05$.
[abc] Within a row, values with different superscript letters are significantly different at $p < 0.05$. evaluated by Ducan's multiple range test.

Changes of ob mRNA in WAT in response to fasting and re-feeding

Deprived of diet for 48 h led to a decrease of the *ob* mRNA in the epididymal WAT compared to normal fed control (Figure 1a). The levels of *ob* mRNA in epididymal WAT increased gradually after re-feeding with the fat-free/high-carbohydrate diet or the high-fat diet (Figure 1a and b). A significant increase of 2 fold and 2.5 fold increase in *ob* mRNA were detected 4 or 8 h after re-feeding

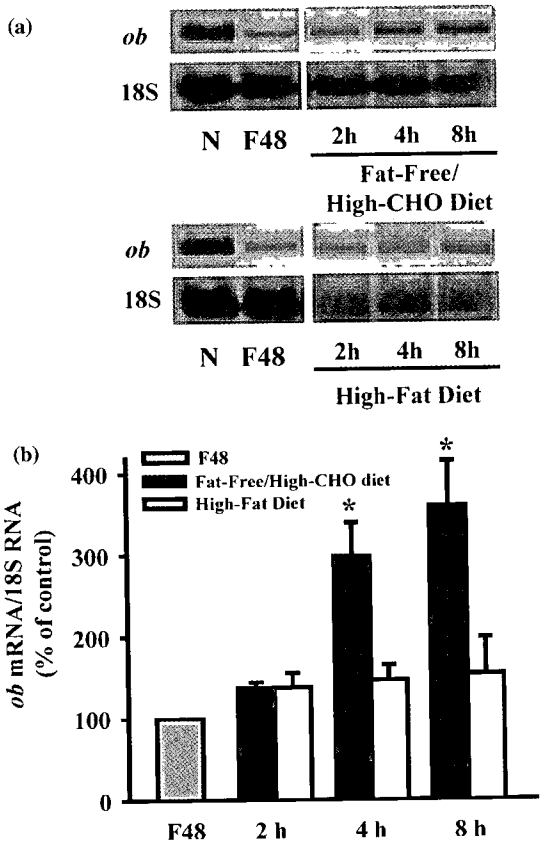

Figure 1. Effects of a fat-free or a high-fat diet on *ob* mRNA expression in rat adipose tissue. Rats were starved for 48 h, then refed with a fat-free/high-carbohydrate (fat-free/high-CHO) or a high-fat diet for 30 min, and sacrified at 0, 2, 4, and 8 h. Epididymal white adipose tissues RNA were isolated and *ob* mRNA was detected by Northern blotting as described in the Materials and Methods. Upper panel (a): representative Northern blot results. Lower panel (b): *ob* mRNA levels normalized to those for 18S mRNA and expressed relative to those in the F48 control (relative value = 100). N: rats consumed Purina chow diet were used as normal control. Values are the means ± SEM (n = 3). * $p < 0.05$ compared to the corresponding high-fat diet group evaluated by Duncan's multiple range test.

with the fat-free/high-carbohydrate diet; however, there was only 50% increase of *ob* mRNA at 4 or 8 h in rats re-fed with the high-fat diet. The effects of the fat-free/high-carbohydrate and the high-fat diet on *ob* mRNA levels were significantly different ($p = 0.001$, ANOVA test).

TRL treatment reduces ob mRNA levels in 3T3-L1

Analysis of plasma lipoproteins using agarose gel electrophoresis revealed clear both VLDL and chylomicron bands in the plasma of rats fed with the high-fat diet; however, only a light VLDL band, but no chylomicron band was seen in the plasma of rats fed the fat-free/high-carbohydrate diet (Figure 2). Isolation of lipoproteins by ultracentrifugation revealed that 55% plasma TG was found in the isolated TRL fraction in rats fed with the high-fat diet, while only about 10% plasma TG was found in the isolated TRL fraction in rats fed with the fat-free/high-carbohydrate diet. These results suggested that TRL may play a role in the decrease of plasma leptin and *ob* mRNA in epididymal fat pads in rats re-fed the high-fat diet. We then tested whether TRL could regulate *ob* gene expression in 3T3-L1 adipocytes. When 3T3-L1 adipocytes were treated with TRL containing 100 μg/ml TG for 12 or 24 h, *ob* mRNA levels were decreased by 26 and 45% ($p < 0.05$), respectively, and 73% decrease ($p < 0.05$) was detected when treated with 300 μg/ml TG of TRL for 12 or 24 h (Figure 3).

TRL reduces the effect of insulin on ob mRNA expression in 3T3-L1

Since insulin is a major regulator of *ob* mRNA expression, we tested whether TRL could down-regulate *ob* mRNA expression in the presence of insulin. When 3T3-L1 adipocytes were treated for 12 h with various concentrations of insulin (1–4 nM), *ob* mRNA levels increased in a dose-dependent manner (Figure 4), with a 86 and 216 % increase ($p < 0.05$) by using 2 or 4 nM of insulin, respectively. However, a significant 13% decrease in *ob* mRNA was detected when cells were co-treated with 2 nM insulin and 300 μg/ml TRL (Figure 4).

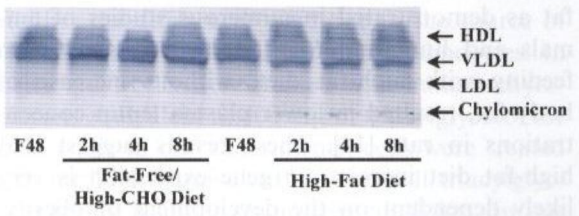

Figure 2. Plasma lipoproteins analysis by agarose gel electrophoresis. Five μl of plasma was loaded on each well and electrophoresis was carried out as described in the Materials and Methods. The locations of chylomicrons, VLDL, LDL and HDL were marked.

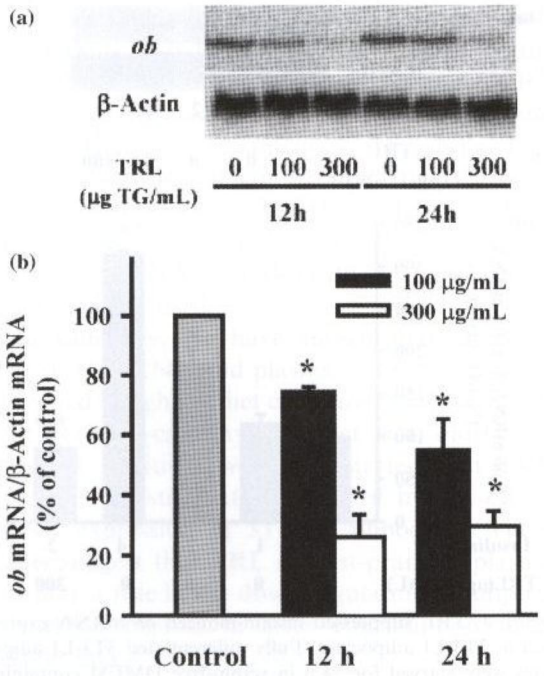

Figure 3. Dose and time course effects of TRL on *ob* mRNA expression in 3T3-L1 adipocytes. Fully differentiated 3T3L-1 adipocytes were starved in serum-free DMEM containing 0.1% BSA for 24 h, then TRL were added to a final of 0 (control, C), 100, or 300 μg/ml TG and cultured for another 12 or 24 h, levels of *ob* mRNA were detected by Northern blotting as described in the Materials and methods. Upper panel (a): representative Northern blot results. Lower panel (b): *ob* mRNA levels normalized to those for β-actin mRNA and expressed relative to those in the control (relative value = 100). Values are the mean ± SEM of three independent experiments. * $p < 0.05$ compared to the control.

Discussion

The present study shows that high-fat diet lowered levels of plasma leptin and adipose *ob* mRNA in rats 2 to 8 h after consumed a high fat compared to those consumed a fat-free/high-carbohydrate

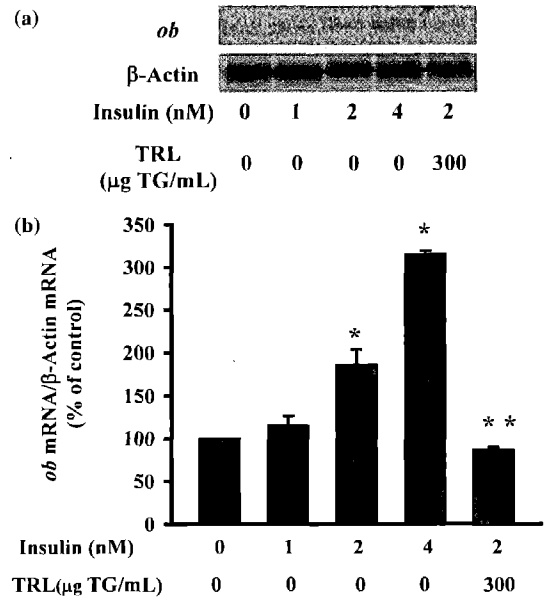

Figure 4. TRL suppressed insulin-induced *ob* mRNA expression in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were starved for 24 h in serum-free DMEM containing 0.1% BSA, then insulin were added for 24 h in a final concentration of 0 (control), 1, 2, or 4 nM, or 2 nM insulin were added in combination with 300 μg/ml TG of TRL for another 24 h. The levels of *ob* mRNA were detected by Northern blotting as described. Upper panels (a): representative Northern blot results. Lower panels (b): *ob* mRNA levels normalized to those for β-actin mRNA and expressed relative to those in the control (relative value = 100). Values are the mean ± SEM of three independent experiments. *$p < 0.05$ compared to the control; ** $p < 0.05$ compared to 2 nM insulin treated cells.

diet. The result supports the observations of Havel et al. [13] that levels of 24-h circulation leptin were significantly lower in subjects after consumption of high fat meals. In post-prandial state, insulin has been demonstrated to be a potent stimulator of *ob* gene expression [6–8, Figure 4]; however, change of plasma leptin was not parallel to the change of insulin in plasma has been shown by Havel et al. [13]. Furthermore, we observed significant increase of plasma TG and TRL in rats fed with the high-fat diet but not in rats fed with the fat-free/high-carbohydrate diet. It is known that TRL (chylomicrons and VLDL) are responsible for the transport of TG to peripheral tissues, mainly adipose tissue, heart, and muscle. Moreover, chylomicrons activate the generation of acylation-stimulating protein (ASP) in adipose tissue, which is a stimulator of adipocyte TG synthesis, and hence increase pool of TG in adipose tissue. [20, 21]. We therefore hypothesized that TRL may play a role in regulating *ob* gene expression in adipose tissue, and test the effect of TRL on *ob* gene expression in 3T3-L1 adipocytes. As shown in Figure 3, addition of TRL resulted in a time and dose-dependent decrease of *ob* mRNA in 3T3-L1 adipocytes. Besides, TRL reduced stimulatory effect of insulin on *ob* gene expression (Figure 4). These results indicate that TRL in post-prandial plasma are involved in the regulation of *ob* gene expression. Nevertheless, it remains unknown how *ob* mRNA expression is regulated by TRL. Arai et al. [22] demonstrated that treatment with triacsin, an acyl-CoA syhthase inhibitor, or 2-bromopalmitate, a poorly metabolizable palmitate analog, reduced *ob* mRNA expression in 3T3-L1 adipocytes, and suggested that *ob* gene expression is regulated by intracellular free fatty acid pool. It is possible that when TRL were hydrolyzed by lipoprotein lipase, fatty acids were released and delivered to adipocytes, and thus increased intracellular free fatty acid pool and reduced *ob* mRNA expression. The expression of *ob* mRNA has also been shown to be influenced by the type of dietary fatty acids. Several studies have reported that adipose tissue *ob* mRNA levels were higher after PUFA feeding [23–25]. However, the mechanism by which TRL reduces *ob* gene expression is not clear and merit further investigation.

Conflicting results regarding the differences of dietary fat on *ob* gene expression are not fully understood so far. Levels of leptin were significantly increased when mice were fed a high-fat diet, and were positively correlated with body weight [10, 11]. Masuzaki et al. [12] demonstrated that rats fed a high-fat diet for 2 weeks developed moderate degree of obesity and increase of *ob* mRNA levels in adipose tissues. The increase in blood leptin or adipose *ob* mRNA levels in these studies could be attributed to the increase in body fat as demonstrated in numerous studies of animals and humans [26–28]. However, short-term feeding with high-fat diet, without increase of body fat, resulted in lower plasma leptin concentrations in rats [14]. These results suggest that high-fat diet increase *ob* gene expression is very likely dependent on the development of obesity. Thus, the effect of dietary fat on *ob* gene expression can be different between before and after development of obesity. One likely explanation is that, before development of obesity, high-fat diet

reduces circulating leptin led to less suppression of appetite, resulted in higher energy intake, and lead to a gradual increase of body weight. Decreases of plasma leptin were reported to increase hunger sensations, and led to higher energy intake [29]. Blundell et al. [30] have shown a weak action of dietary fat on satiation, and could induce a positive energy balance. According to these reports, we propose that high fat increase of *ob* gene expression and plasma leptin occurs only after consumption of high-fat diet for a period of time that is accompanied by the development of obesity. However, Asakuma et al. [31] suggested that it is leptin level within the cerebrospinal fluid reflects characteristics of feeding behavior but not leptin level in plasma. Whether dietary fat also regulates leptin level in cerebrospinal fluid requires further investigation.

Unlike in mouse adipose tissue, the level of *ob* mRNA in 3T3-L1 differentiated by the methylisobutylxanthine/dexamethasone/insulin protocol is relatively low that RT-PCR was required for detection [32–34]. Slieker et al. [34] demonstrated that the expression level of *ob* mRNA in adipocytes is dependent on differentiation protocol. When 3T3-L1 adipocytes are differentiated by indomethacin/insulin the expression of *ob* mRNA increased to a level that can be detected by Northern blot analysis. Using the indomethacin/insulin differentiation protocol, we were able to obtain over 95% of the cells differentiated into adipocytes and detect *ob* mRNA by Northern blot analysis. We showed down-regulation of *ob* mRNA by TRL with or without the presence of insulin in this study [Figure 3 and 4]. Arai et al. [22] reported that treatment with both chylomicrons and triacsin C caused a 50% decrease of *ob* mRNA while triacsin C alone caused only 20% decrease; however, chylomicrons alone has no effect on the level of *ob* mRNA. It is not clear whether the differences were due to the source of TRL or the method to differentiate adipocytes. In their study, cells were treated with human chylomicrons equivalent to 400 mg/dl triacylglycerol for 48 h, while in our experiments, cells were treated with rat TRL (equivalent to 30 mg/dl triacylglycerol) for 24 h under serum free condition. In our study, cells were differentiated by indomethacin/insulin method, whereas cells were differentiated by traditional methylisobutylxanthine/dexamethsone/insulin protocol in Arai's study. Using the indomethacin/insulin differentiation protocol, Slieker et al. [34] have shown no change of the levels of aP2, C/EBPα, PPARγ, LPL and UCP2. We showed increased of *ob* mRNA responding to 1–4 nM insulin treatment in these cells (Figure 4), which is similar to that in 3T3–442A adipocytes reported by Leroy et al. [35]. These results suggest that TRL down-regulate the expression of *ob* mRNA is unlikely due to the differentiation method used.

In summary, we have shown that levels of adipose *ob* mRNA and plasma leptin are lower in rats re-fed a high-fat diet compared to those re-fed a fat-free/high-carbohydrate diet in a short-term study. In addition, we demonstrated that TRL decreased the stimulatory effect of insulin on *ob* mRNA expression in 3T3-L1 adipocytes. These results suggest that TRL in post-prandial plasma may play a role in the down-regulation of adipose *ob* mRNA expression after consumption of a high-fat diet.

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