

Transgenic Expression of *n*-3 Fatty Acid Desaturase (*fat-1*) in C57/BL6 Mice: Effects on Glucose Homeostasis and Body Weight

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ABSTRACT

The *fat-1* gene, derived from *Caenorhabditis elegans*, encodes for a fatty acid *n*-3 desaturase. In order to study the potential metabolic benefits of *n*-3 fatty acids, independent of dietary fatty acids, we developed seven lines of *fat-1* transgenic mice (C57/BL6) controlled by the regulatory sequences of the adipocyte protein-2 (aP2) gene for adipocyte-specific expression (AP-lines). We were unable to obtain homozygous *fat-1* transgenic offspring from the two highest expressing lines, suggesting that excessive expression of this enzyme may be lethal during gestation. Serum fatty acid analysis of *fat-1* transgenic mice (AP-3) fed a high *n*-6 unsaturated fat (HUSF) diet had an *n*-6/*n*-3 fatty acid ratio reduced by 23% ($P < 0.025$) and the *n*-3 fatty acid eicosapentaenoic acid (EPA) concentration increased by 61% ($P < 0.020$). Docosahexaenoic acid (DHA) was increased by 19% ($P < 0.015$) in white adipose tissue. Male AP-3-*fat-1* line of mice had improved glucose tolerance and reduced body weight with no change in insulin sensitivity when challenged with a high-carbohydrate (HC) diet. In contrast, the female AP-3 mice had reduced glucose tolerance and no change in insulin sensitivity or body weight. These findings indicate that male transgenic *fat-1* mice have improved glucose tolerance likely due to increased insulin secretion while female *fat-1* mice have reduced glucose tolerance compared to wild-type mice. Finally the inability of *fat-1* transgenic mice to generate homozygous offspring suggests that prolonged exposure to increased concentrations of *n*-3 fatty acids may be detrimental to reproduction. J. Cell. Biochem. 107: 809–817, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: *fat-1* OMEGA-3 FATTY ACID DESATURASE; ADIPOSE-TISSUE-SPECIFIC TRANSGENIC MICE; GLUCOSE HOMEOSTASIS; BODY WEIGHT; GESTATIONAL LETHALITY

Fish oil is recommended for cardiovascular health [Bhatnagar and Durrington, 2003], whereas diets rich in saturated fatty acids (FAs) and a high ratio of dietary *n*-6 to *n*-3 polyunsaturated fatty acids (PUFAs) may contribute to the increasing prevalence of heart disease and diabetes [Levinson et al., 1990; Adler et al., 1994; Leaf and Kang, 2001; La Guardia et al., 2005]. The most potent *n*-3 fatty acids with healthful properties are the long-chain PUFAs, including eicosapentaenoic acid (EPA-20:5*n*-3) and docosahexaenoic acid (DHA-22:6*n*-3), which locate in the C₂-position of triglycerides in marine fish oil. In contrast, animal derived fats contain mostly saturated FAs, monounsaturated FAs, and the *n*-6 PUFA arachidonic acid (AA-20:4*n*-6); also, common vegetable oils are rich in *n*-6 PUFAs, particularly linoleic acid (LE-18:2*n*-6). The *n*-3 fatty acid desaturase required for synthesizing the beneficial *n*-3

FAs from linoleic acid exists only in some plants, *Caenorhabditis elegans* and primitive organisms such as sea plankton, which is the original source of fish oil *n*-3 PUFAs from the food chain.

The *n*-3 desaturase encoded by the gene *fat-1* of *C. elegans* is an integral membrane protein, whose substrate is *n*-6 fatty acids at the 2-position of phospholipids in cellular membrane [Pugh and Kates, 1977; Spsychalla et al., 1997]. The enzyme converts LE-18:2*n*-6 and AA-20:4*n*-6 to α -linolenic acid (ALN-18:3*n*-3) and EPA-20:5*n*-3, respectively [Spsychalla et al., 1997] (Fig. 1). The shorter chain *n*-3 fatty acid ALN may not be efficiently converted to EPA and DHA, as the required elongases and desaturases are not highly active in carnivores [German et al., 1996]. In mammals, the conversion of EPA into DHA occurs through the Sprecher pathway consisting of two elongations (to produce a 24-carbon fatty acid) followed by

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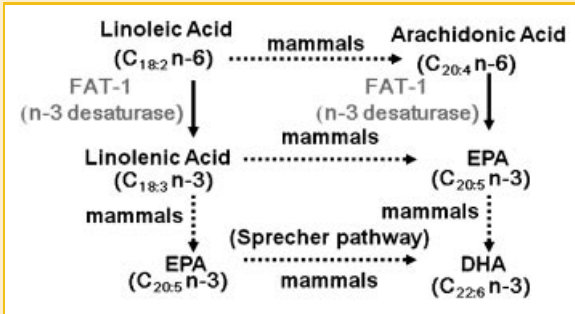


Fig. 1. The expected pathway of converting *n*-6 and *n*-3 fatty acids to EPA and DHA via the *fat-1* desaturase enzyme combined with mammalian enzymes. The dotted arrow denotes that the shorter chain *n*-3 fatty acid ALN may not be efficiently converted to EPA and DHA, as the required elongases and desaturases are not highly active in carnivores [German et al., 1996]. Also, in mammals the conversion of EPA into DHA occurs through the Sprecher pathway consisting of two elongations (to produce a 24-carbon fatty acid) followed by a desaturation with delta(6)-desaturases, and finally a β -oxidation step (to shorten the chain length by 2-carbons) [Voss et al., 1991].

a desaturation with delta(6)-desaturases (D6D), and finally a β -oxidation step (to shorten the chain length by 2-carbons) [Voss et al., 1991]. A transgenic mouse model made with a CMV (enhancer)/ β -actin promoter expressing the *fat-1* cDNA increased the ratio of *n*-3 and *n*-6 fatty acids in various tissues [Kang et al., 2004]. These same *fat-1* transgenic mice had a reduction of melanoma formation and growth [Xia et al., 2006], reduced colitis-associated colon cancer [Jia et al., 2008], and were protected from diseases with excessive inflammatory responses [Bhattacharya et al., 2006; Hudert et al., 2006; Schmocker et al., 2007]. However, to our knowledge, no study of glucose homeostasis has been reported from these mice. While increasing the ratio of dietary *n*-3 to *n*-6 PUFAs is suggested to be beneficial for the prevention and treatment of dyslipidemia, coronary heart disease [Middaugh, 1990; La Guardia et al., 2005], obesity, insulin resistance [Storlien et al., 1987; Jucker et al., 1999; Neschen et al., 2002], impaired glucose tolerance, and diabetes [Storlien et al., 1987; Adler et al., 1994], it is difficult to ascertain the contribution of certain *n*-3 PUFAs per se, without the potential confounding effects of other dietary components. Diet-based studies evaluating intake of fish and fish oil suffer from the potentially confounding effects concerning the fish proteins, and the heterogeneity and possible modification of the fatty acids contained in the oils. Also, in both human and animal studies, the major sources of *n*-3 and *n*-6 PUFA are from fish oil and vegetable oil, respectively. Such sources are rich in 20- and 22-carbon *n*-3 PUFAs or 16- and 18-carbon *n*-6 PUFAs, yet FAs with varying chain-length and unsaturation are not freely converted among each other [Burdge et al., 2002a, 2003; Burdge and Wootton, 2002b]. Indeed, while it is generally accepted that fish oil improves the serum lipid profile, it is controversial whether dietary flaxseed oil (containing mostly ALN-18:3n-3) has a similar effect [Prasad, 1997; Pfrommer et al., 2006].

To study the effect of *n*-3 fatty acids in adipose tissue on glucose homeostasis, we developed seven *fat-1* transgenic C57BL/6 mouse lines controlled by promoter sequences of the adipocyte protein-2

(aP2) for adipocyte expression (AP-lines, including AP-2 and -3). Using the adipose-tissue-specific promoter our study extends the results by Kang et al. [2004] where their promoter presumably resulted in ubiquitous expression of *fat-1*, although that was not reported. We consistently detected *fat-1* mRNA levels in the target organs. We performed serum and adipose tissue fatty acid analysis *fat-1* transgenic mice fed a high-unsaturated fat (HUSF) diet, which provided substrates for the FAT-1 enzyme. Additionally, glucose and insulin tolerance were examined in the *fat-1* transgenic mice challenged with a high-carbohydrate (HC) diet, which induces impaired glucose tolerance in the wild-type (WT) C57BL/6 mice.

MATERIALS AND METHODS

TRANSGENE CONSTRUCT WITH ADIPOCYTE-SPECIFIC PROMOTER CONTROLLING *fat-1*

The *fat-1* cDNA derived from *C. elegans* in the pCE8 clone was used previously by Kang et al. [2001] in their adenoviral vector with successful expression. The *fat-1* cDNA clone contained a 1205 bp coding sequence, preceded by a 12 bp 5' untranslated region (UTR) and followed by a 72 bp 3' UTR. The 3' UTR has no consensus poly (A) signal sequence (AATAAA or ATAAA) but only a variant form (cATAAAg) which, as far as we know, has not been proven to function in mammalian cells. Since only a stretch of 19 poly(A) is present at the end of 3' UTR of *fat-1* cDNA in the plasmid pCE8, a SV40 late poly(A) signal sequence provided in the vector pGL3-Basic (Promega) was added. The 5.4 kb aP2 promoter-enhancer [Ross et al., 1990] was removed from the plasmid pSKII+ (Stratagene) with *KpnI* and *SmaI* and ligated into the *KpnI* and *EcoRV* sites in the cloning vector pZero2 (Invitrogen Life Technologies, Inc.) to yield a plasmid pZero2-aP2. Construction of the plasmid pGL3-aP2-*fat-1* and excision of the aP2-*fat-1* expression cassette is illustrated (Fig. 2).

PRODUCTION AND HUSBANDRY OF *fat-1* TRANSGENIC MICE

The 8 kb *Sall*—*Sall* fragment of aP2-*fat-1* and was purified for microinjection into fertilized eggs to generate transgenic mice on the C57BL/6Tac (B6; Taconic, Inc.) background according to

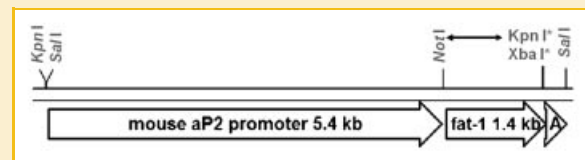


Fig. 2. Construction of pGL3-aP2-*fat-1*, and excision of the aP2-*fat-1* expression cassette. The 1.4 kb *NotI*-*KpnI* (blunt-ended) fragment with 1.2 kb *fat-1* coding sequence followed by its native 3' UTR and poly(A)19, as presented in pCE8 is indicated as a double arrow ("←→"). The 5.4 kb *KpnI*-*NotI* fragment of mouse aP2 promoter from pZero2-aP2 and this 1.4 kb fragment were ligated into a *KpnI*-*XbaI* (blunt-ended) vector pGL3-Basic (Promega). The blunted sites are shown with a star ("*"). The expression cassette aP2-*fat-1* is excised from the vector via two *Sall* sites flanking the cassette for microinjection.

standard protocols. All transgenic mice in the experiments were the fourth generation of a male offspring from the founder mice backcrossed to female wild-type B6. All control mice were transgene negative B6 siblings of the transgenic mice. Breeders, weaned pups, and stock mice were fed a standard rodent diet (Purina RMH2500, with macronutrient contents in terms of percentage energy as carbohydrate 58%, fat 13%, and protein 29%). A high-unsaturated fat (HUSF) diet was composed of the basal components plus total fat of 62% calories composed of safflower oil (77% linoleic acid, LE-18:2*n*-6 and 13% oleic acid, 18:1*n*-9). This HUSF diet, Teklad TD 03387, had macronutrient contents expressed as of percentage energy with carbohydrate 15%, fat 62%, and protein 23% was produced, stored under nitrogen to avoid fatty acid oxidation. A HC diet (Teklad TD 03389) with macronutrient contents in terms of percentage energy as carbohydrate 64%, fat 13%, and protein 23% had starch and fructose (50:50) added to the base ingredients as a substitute for the equivalent quantity of fat calories found in the HUSF diet. The mice were housed in restricted access facility using sterilized caging and water. Weaned pups were switched to either HUSF diet or HC diet at 4- to 5-week-old and maintained on that diet prior to being sacrificed for the fatty acid distribution at 12- to 13-week-old (after being fed the HUSF diet for 8 weeks) or entry into the 3-week glucose/insulin tolerance experiments at 11- to 13-week-old. Upon completion of the glucose/insulin tolerance experiments, mice were sacrificed at the age of 15- to 17-week old (after being fed the HC diet for 11–13 weeks).

We attempted to obtain homozygous transgenic mice in the two lines (AP-2 and -3) in order to have relatively higher mRNA levels from the *fat-1* transgene. There were two litters produced from hemizygous matings of each of these two lines. Homozygosity was tested by mating transgene positive offspring with a wild-type (WT) mate. True homozygous mice would transmit the transgene 100% of the time, which was tested in two litters per test mating pair.

GENOTYPING BY PCR

Founder pups were genotyped by PCR for presence *fat-1* cDNA using a pair of primers with sequence corresponding to the 5' and 3' ends of the *fat-1* cDNA: *fat-1-5'* (ATATTCTAGACAAGTTTGAGGTA-TGGTCGC) and *fat-1-3'* (ATATACTAGTAAGAGTTATGGCTTTAT-GCA).

SOUTHERN BLOT ANALYSIS

Southern blot was performed using standard methods and the probe was first generated by PCR amplification using the plasmid containing the *fat-1* cDNA and the above mentioned primer pair (*fat-1-5'* and *fat-1-3'*). A positive control in the Southern blot analysis consisted of the plasmid pGL3-aP2-fat-1 in a range of copy number equivalents mixed with the same amount of C57BL/6 genomic DNA. This was used to estimate the copy number of transgene constructs integrated into the initial founder mouse lines.

REAL-TIME QUANTITATIVE RT-PCR (qRT-PCR) ANALYSIS

Steady state concentrations of *fat-1* mRNA in target tissues were evaluated by real-time qRT-PCR. First strand cDNA was generated from 1 µg of RNA in 20 µl volume reaction containing both oligo-(dT) and random hexamers (Invitrogen First Strand Synthesis Kit)

according to the manufacturer's instructions. Real-time quantitative RT-PCR was carried out in a 20 µl reaction volume containing 10 µl Supermix UDG (Invitrogen Life Technologies, Inc.), 2 µl cDNA, 0.3 µM LUX *fat-1* primer forward (GTACACCCGATGATGTCGCT-GAGGTG[FAM]AC, FAM labeled Invitrogen Life Technologies, Inc.), 0.3 µM LUX *fat-1* primer reverse (CGATGGTTTGGG-TTTGTCCA), 0.05 µM 18S RNA primer forward, and 0.05 µM 18S RNA primer reverse (JOE labeled Invitrogen Life Technologies, Inc.). Cycling conditions included incubation at 50°C for 2 min, a 2 min 95°C denaturing step, followed by 45 cycles of 95°C denaturation for 15 s, an annealing step at 56.5°C for 45 s (followed by plate reading), an extension step at 72°C for 30 s (followed by plate reading again). There was a final extension at 72°C for 30 s. Melting curve was read from 55 to 95°C every 0.5°C (hold 1 s).

FATTY ACID ISOLATION AND FAME ANALYSIS

Fatty acids from plasma were isolated and methylated according to Moser and Moser [1991]. Briefly, 100–250 µl of plasma was mixed with 1 ml methanol:dichloromethane (3:1, v/v). After addition of internal standard (50 nmol of heptadecanoic acid), 200 µl acetyl chloride was added with vortexing, and the sample was incubated at 75°C for 1 h. After cooling, the reaction solution was neutralized with 4 ml of 7% K₂CO₃ and the lipids were extracted into hexane. The hexane fraction was washed with acetonitrile and concentrated under nitrogen. The fatty acid methyl ester (FAME) mixture was then resuspended in hexane and analyzed by gas chromatography–mass spectroscopy (GC–MS) [Batal et al., 2007].

ADIPOSE TISSUE EXTRACTION

Frozen tissue, weighing between 0.05- and 0.3 g, was thawed while on ice and wet weight recorded to the nearest 0.1 mg. Tissue was then placed in homogenization buffer, (phosphate buffered saline containing 20 mg/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamide), and homogenized using a Fisher PowerGen 125 Homogenizer equipped with a 10 mm × 195 mm sawtooth generator. A 250 µl aliquot was then removed and extracted as described above for plasma samples. Tissue FAME were then resuspended in hexane and quantified by GC–MS.

GLUCOSE TOLERANCE TEST (GTT) AND INSULIN TOLERANCE TEST (ITT)

Intraperitoneal (IP) GTT was performed twice in 11- and 13-week-old mice fed the HC diet for ~7 and 9 weeks, respectively. At 18 h prior to GTT, mice were food deprived with water overnight. After the mice were weighed, a baseline blood sample was obtained by tail bleed and additional blood samples were obtained for glucose at 15, 30, 60, and 120 min following IP glucose administration at 2.0 mg/g body weight with 50% glucose. The GTT data were summarized as both the area-under-the-curve (AUC) of a GTT and the “partial-GTT (P-GTT),” which indicates the excursion of glucose concentrations from basal concentrations by subtracting GTT-AUC with the “area under the base line.” Insulin tolerance testing (ITT) was performed twice in 12- and 14-week-old mice after a 1-week recovery period following GTT. The mice were fasted for 4 h and blood glucose concentrations were measured at 0, 10, 30, and 60 min after IP injection (0.75 U/kg body weight) of recombinant human insulin

(Humulin R, 100 U/ml stock using a 1.5:1,000 dilution in saline). The ITT data were summarized as both AUC and the reversed AUC (R-ITT). The former takes into account the basal glucose concentrations following a 4 h fast, whereas the latter measures only the glucose-lowering effect of the insulin load.

STATISTICAL ANALYSES

The unpaired Student's *t*-test, the Chi-square test for analysis of variance, and linear regression analysis were used to determine *P* values ($\alpha < 0.05$).

RESULTS

TRANSGENIC LINES PRODUCED

Following microinjection of transgene constructs into fertilized eggs of C57BL/6Tac, seven AP founder lines were identified among 22 pups, as detected by PCR screening of the *fat-1* transgene constructs in mouse tail DNA. PCR screening results were initially confirmed by Southern blot analysis, then PCR was used exclusively following that validation for additional lines. We found copy numbers of the transgene construct of 40 and 50 in lines AP-2 and -3, respectively.

TISSUE-SPECIFIC EXPRESSION OF *fat-1* mRNA

Using the same amount of total RNA, the *fat-1* mRNA levels in the white adipose tissue (WAT) and brown adipose tissue (BAT) in male and female mice of the AP-2 and -3 lines were consistently detected by RT-PCR at about $2.8 \pm 0.3\%$ and $2.0 \pm 0.4\%$ (mean \pm SE), respectively, of that in total RNA from *C. elegans* used as a positive control (data not shown). Although there are gender-specific differences in body fat, we found no gender-specific differences in expression of the *fat-1* transgene using the AP-2 promoter (data not shown). In Figure 3A, *fat-1* mRNA levels in WAT and BAT of male and female AP-3 mice were indicated as 4,000- to 6,000- fold of the background signal in corresponding tissues of WT control mice. Also, *fat-1* mRNA levels in heart, skeletal muscles, liver, spleen, brain, and kidneys in male and female AP-3 mice were not different from that in WAT of WT control mice (Fig. 3B), suggesting that the transgene expression is adipose-tissue-specific.

HOMOZYGOSITY

As indicated from the combined data of the two highest expressing lines AP-2 and -3, there were no homozygous pups produced in the four test litters (Table I). Also, assuming that homozygosis of the transgene was lethal, then there were the predicted number of hemizygous and WT pups produced in such mating, suggesting that a haploid dose of the transgene did not affect germ-line transmission.

FATTY ACID PROFILES

In serum of AP-3 transgenic mice, as shown in Figure 4A, the ratios of *n*-6 to *n*-3 fatty acids were significantly lower, with a mean ratio of 4.9:1 versus 6.4:1, or a 23% reduction in the ratio of total *n*-6 to total *n*-3 fatty acids ($P = 0.025$). There was a 61% increase in EPA-20:5*n*-3 in AP-3 mice versus WT mice ($P = 0.02$, 109 vs.

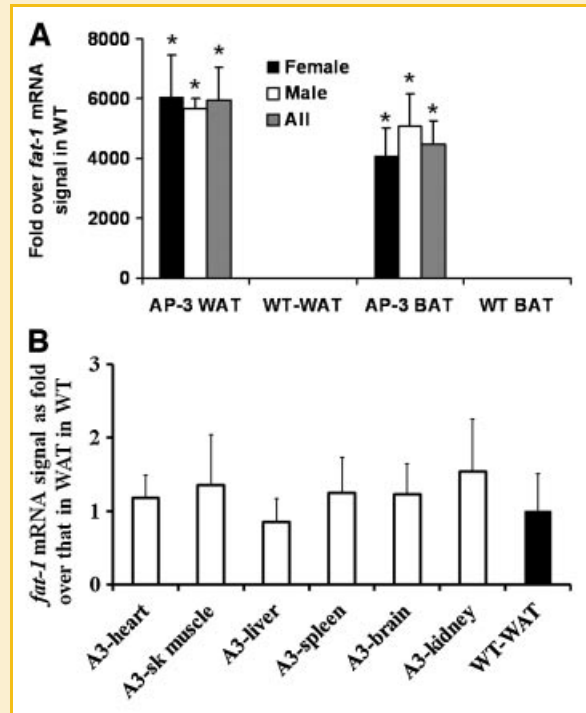


Fig. 3. Real-time quantitative RT-PCR analysis of total RNA of WAT and BAT in AP-3 transgenic mice. AP-3 mice female ($n = 6$) and male ($n = 5$) and WT ($n = 6$) mice at 12- to 13-week-old were food-deprived for 6 h before sacrifice. A: Mean levels of *fat-1* mRNA in WAT and BAT of WT controls were arbitrarily set to 1, and the fold over this background is displayed. The data are expressed as mean \pm SE ($*P < 0.0001$ compared to WT values). B: Mean levels of *fat-1* mRNA in WAT of WT controls were arbitrarily set to 1, and the fold over this background is displayed. The data are expressed as mean \pm SE.

68 nmol/ml, Fig. 4B). In adipose tissues of AP-3 mice, there was a 19% increase in the most abundant *n*-3 PUFA, DHA-22:6*n*-3 (2.6 vs. 2.2 nmol/g, Fig. 4C, $P = 0.015$).

METABOLIC PHENOTYPES: BODY WEIGHT, VISCERAL FAT PAD WEIGHT, AND FASTING BLOOD GLUCOSE

The male AP-3 mice had lower body weight (26.0 g vs. 27.6 g in control mice, $P < 0.01$) at 3 months old (following an 18 h fast) with no difference in epididymal fat pad weight upon sacrifice at 4 months old. In contrast, both body weight and visceral fat weights were not altered in the female AP-3 mice (data not shown). Also, there were no changes in fasting blood glucose levels in both male and female AP-3 mice (data not shown).

GLUCOSE TOLERANCE AND INSULIN TOLERANCE

In previous studies the HC diet caused significant weight gain and impaired glucose tolerance in the male B6 mice (L. Tian and P.A. Wood, unpublished work). During GTT, there were lower glucose concentrations at 60 and 120 min, and lower AUC values of GTT and P-GTT in male AP-3 mice (Fig. 5A,B). This was also true for the male AP-2 mice (data not shown). In contrast, the glucose concentrations at 30 and 60 min during GTT, and AUC values of GTT in female AP-3 mice (Fig. 5C,D) were higher than control concentrations. We

TABLE I. Testing for Homozygosity of *fat-1* Transgene (Tg+) With Lines AP-2 and -3 Tg+/- (M) × Tg+/- (F), Two Litters Tested From Each Line

Pups born	Tg+/+	Tg+/-	Tg-/- (WT)	Total pups
Observed (expected)	0* (9)	25* (18)	11* (9)	36
Observed (expected) if Tg+/+ lethal	0 (0)	25 (24)	11 (12)	36

* $P < 0.05$ by Chi-square test of variance.

obtained a similar finding in the female AP-2 mice (data not shown). Therefore, while male AP-2 and -3 mice had better glucose tolerance, there was reduced glucose tolerance in female AP-2 and -3 mice.

Since glucose concentrations during GTT are affected by both insulin sensitivity and insulin concentrations we performed ITT to investigate whether there was reduced insulin sensitivity in the peripheral tissues or reduced glucose-stimulated insulin response. During ITT in both AP-3M and -3F mice, the glucose concentrations at all time points, and the AUC values of ITT and R-ITT were not different from control concentrations (Fig. 6A–D), suggesting no change in insulin sensitivity. Similar results were found in the AP-2 male and AP-2 female mice (data not shown).

DISCUSSION

The *fat-1* mRNA levels were uniformly low in seven lines of our transgenic mouse model using an adipose-specific (ap2) promoter when compared to relative expression found in *C. elegans* when normalized to total RNA. Kang et al. [2004] suggested that “humanizing” the worm’s “preferred codon usage” increases translation of the *fat-1* mRNA in mouse. However, since the mRNA levels of their transgenic mice were not compared to *C. elegans*, although they reported an increase in *n-3* PUFA and decrease in *n-6* PUFA [Lai et al., 2006], it is unclear whether the use of “humanized codons” per se rather than simple variation in transgene mRNA expression among different transgenic mouse lines is the reason for

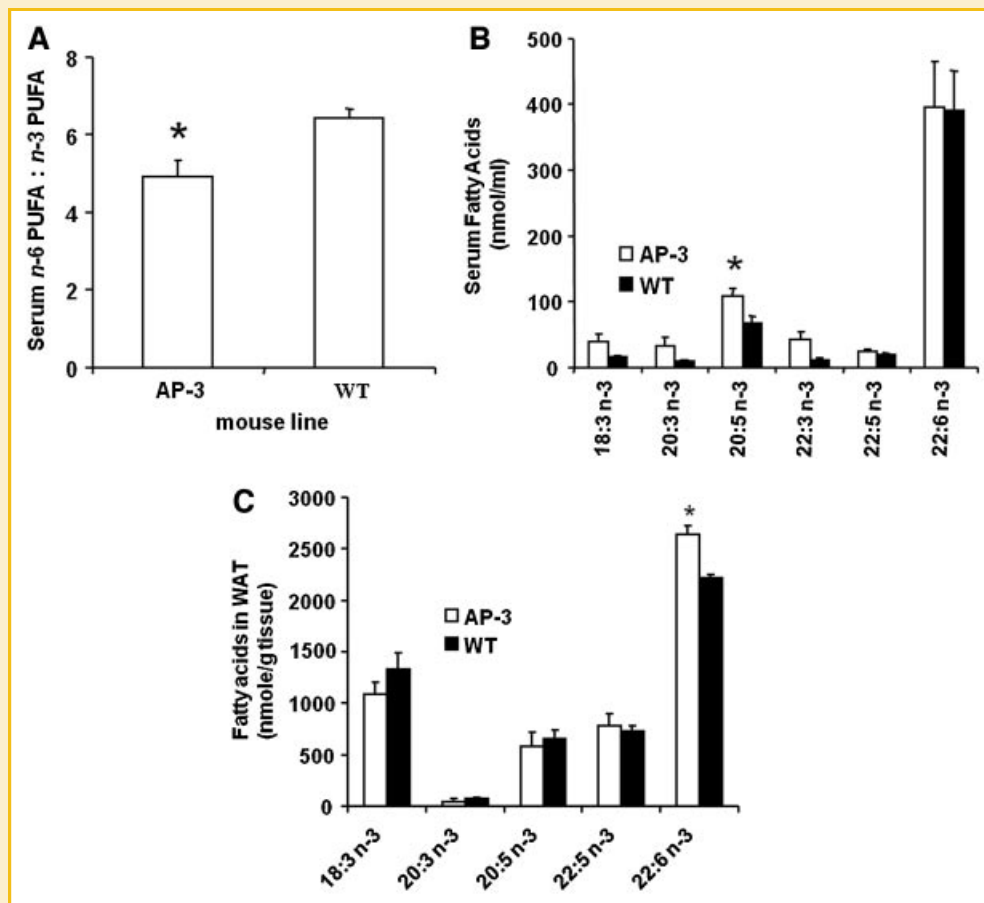


Fig. 4. Fatty acid profiles in male and female AP-3 transgenic mice on HUSF diet. A: The ratio of total *n-6* to *n-3* fatty acids in serum samples ($n = 5$); (B) the concentrations of each *n-3* fatty acids in serum ($n = 5$); and (C) the levels of each *n-3* PUFA in WAT were indicated. The data are expressed as mean \pm SE (* $P < 0.05$ vs. WT values).

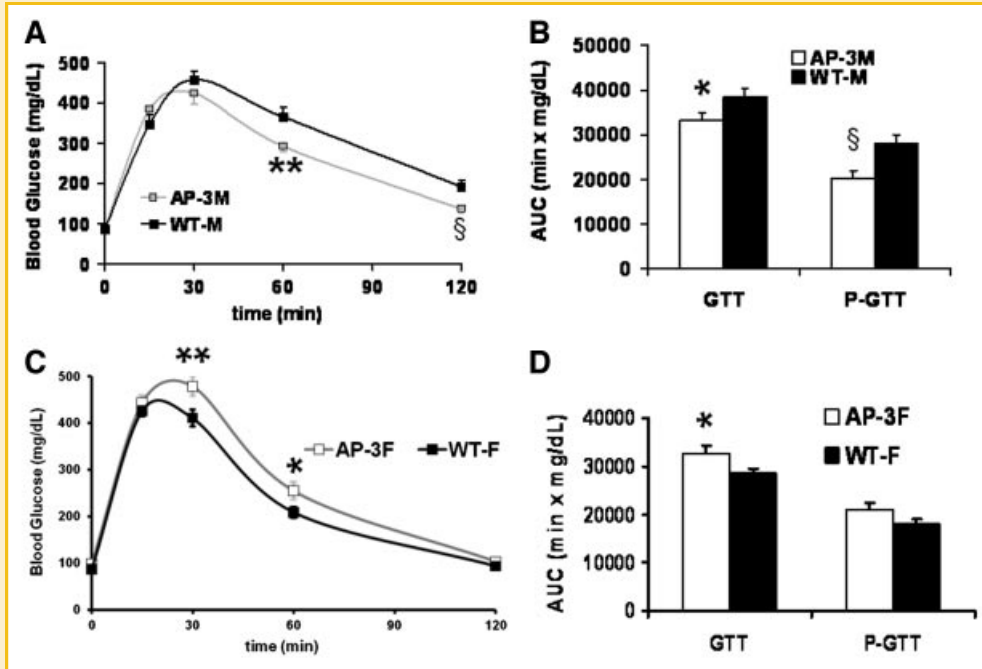


Fig. 5. Glucose tolerance test (GTT). Duplicate GTT experiments were performed in mice at 1 week before and 1 week after the age of 3 months. A,C: Glucose levels during GTT experiments in the AP-3M (male) mice ($n = 4$, gray line) and in the AP-3F (female) mice ($n = 6$, gray line) were compared to those in the WT-M (wild-type male) mice ($n = 7$, black line) and the WT-F (wild-type female) mice ($n = 15$, black line), respectively. B,D: Corresponding values of area-under-the-curve (AUC) and partial AUC of the GTT experiments in (A) and (C) were indicated as "GTT" and "P-GTT," mean \pm SE, * $P < 0.05$, ** $P < 0.01$, $^{\S}P < 0.005$.

reversal of the $n-6$ to $n-3$ fatty acid ratio in their *fat-1* transgenic mouse model. A second possibility is that a functionally active *fat-1* transgene might be detrimental to a mouse embryo that does not normally express it; therefore only those lines that express the *fat-1* transgene at relatively low levels survive to birth. This possibility is consistent with the fact that we could not produce homozygous transgenic lines using the two highest expressing lines (AP-2 and -3, Table I). However, live-borne homozygous *fat-1* transgenic mice using the CMV promoter were produced previously [Kang et al., 2004]. A possible explanation for this discrepancy is that there might not be enough *fat-1* enzyme activities in their homozygous fetus to be detrimental, however this would require direct comparison of $n-3$ fatty acids in fetuses between the two mouse lines.

Therefore, we postulate that *fat-1* transgene expression at relatively high levels may be lethal to the mouse embryo. Alteration in the ratio of $n-3$ to $n-6$ fatty acids in the diet affected reproduction [Wathes et al., 2007]. In one study [Wakefield et al., 2008], high $n-3$ PUFA concentrations in the diet during oocyte maturation and fertilization was associated with embryos with increased production of reactive oxygen species and decreased ability to develop to the blastocyst stage. Although increases in serum and tissue concentrations of $n-3$ fatty acids and the $n-3/n-6$ ratio were small in our transgenic mouse model, high levels of $n-3$ fatty acids in membrane phospholipids may be sufficient to disrupt reproduction. Other mechanisms of reduced reproduction may include changes in prostaglandin synthesis [Abayasekara and Wathes, 1999; Elmes et al., 2004], steroidogenesis [Stocco et al., 2005], activity of

transcription factors such as the peroxisome proliferator-activated receptor (PPAR) family, and the steroid response element binding protein (SREBP) family.

As part of our FAME analysis, we did evaluate the concentrations of linoleic acid (18:2, $n-6$) and arachidonic acid (20:4, $n-6$) in serum and WAT, and we saw no significant differences between (serum and WAT) samples from *fat-1* transgenic mice and WT mice. This could be due to saturating the capacity of the FAT-1 enzyme in our model by the abundant $n-6$ fatty acid provided from the diet such that a small reduction in $n-6$ fatty acids cannot be detected.

In *fat-1* transduced mammary epithelial cells, the increase in EPA concentration was much more pronounced in the phospholipids fraction versus cellular triacylglycerols [Morimoto et al., 2005]. The mechanisms of preferential partition of de novo synthesized $n-3$ fatty acids to phospholipid membrane fractions include the ability of $n-3$ PUFA to suppress phosphatidate phosphohydrolase [Marsh et al., 1987], acyl-coenzyme A:1,2-diacylglycerol acyltransferase [Rustan et al., 1988a], and acyl-CoA:cholesterol acyltransferase [Rustan et al., 1988b; Rustan and Drevon, 1989]. We speculate that there are relatively low rates of redistribution by phospholipid hydrolysis and re-acylation of $n-3$ fatty acids, as compared to the assembly and hydrolysis of fatty acids from triglycerides. Therefore we expect that transgenic expression of the FAT-1 enzyme results in a higher localized concentration of membrane-bound, $n-3$ fatty acids in adipose tissue compared to other tissues. Thus, equilibrium among different tissues may not be reached readily. We chose adipose-tissue-specific expression to achieve tissue-specific *fat-1* expression levels in live-borne mice to study the $n-3$ fatty acid

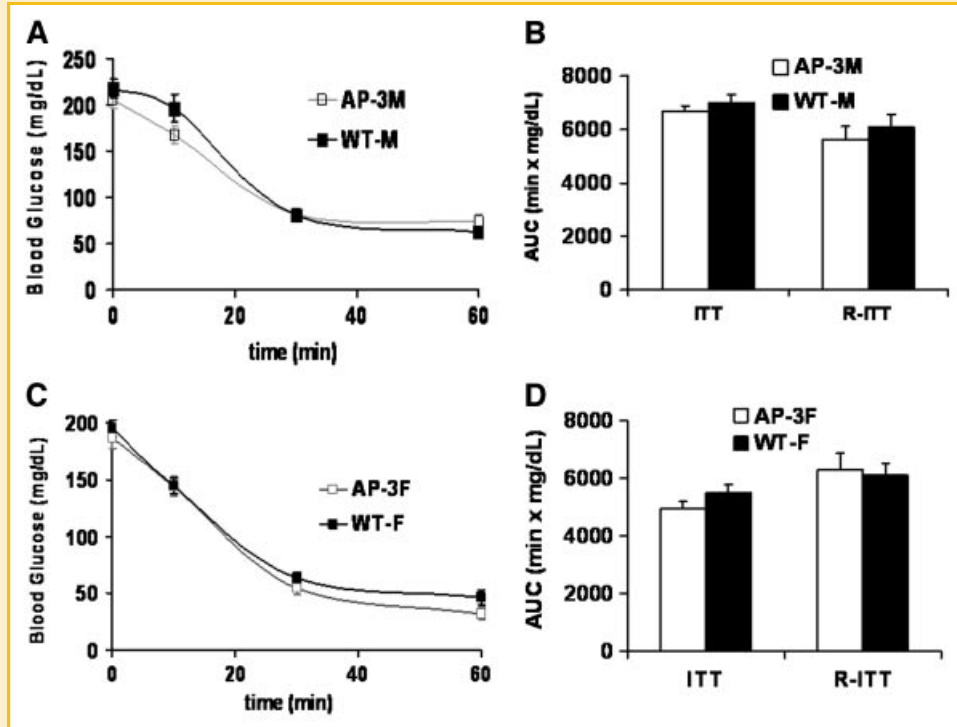


Fig. 6. Insulin tolerance test (ITT). Duplicate ITT experiments were performed in mice at 3.0 and 3.5 months of age. A,C: Glucose levels during ITT experiments in the AP-3M (male) mice ($n = 4$, gray line) and the AP-3F (female) mice ($n = 6$, gray line) were compared to those in the WT-M (wild-type male) mice ($n = 7$, black line) and the WT-F (wild-type female) mice ($n = 15$, black line). B,D: Corresponding values of AUC and reversed AUC of the ITT experiments in (A) and (C) were indicated as "ITT" and "R-ITT," respectively (mean \pm SE). There were no significant differences between the groups.

related mechanisms that affect adipose tissue and energy metabolism.

While unsaturated fatty acids (including both PUFA and monounsaturated fatty acids [MUFA]) reduce the proteolytic processing and maturation of SREBP-1a, -1c, and -2 proteins [Worgall et al., 1998], PUFA but not MUFA enhances decay of SREBP-1c mRNA [Xu et al., 1999, 2001]. PUFA suppress the nuclear content of SREBP-1c by reducing the stability of its mRNA [Xu et al., 1999], which is independent of PPAR- α activation [Clarke and Jump, 1997; Ren et al., 1997]. Moreover, PUFA inhibit the transcription of a number of hepatic lipogenic and glycolytic genes [Jump et al., 1994]. In vitro studies suggest both n -6 and n -3 PUFA are ligands for PPAR α , PPAR δ , and PPAR γ [Kliewer et al., 1997], liver X receptors, retinoid X receptor α , and hepatic nuclear factor 4 α [Jump, 2002]. However, in rodents, only dietary n -3 PUFA (e.g., fish oil) and not dietary n -6 PUFAs (e.g., safflower oil) induced hepatic peroxisome proliferation and fatty acid oxidation, reduces hepatic triglyceride (TG) content [Neschen et al., 2002], and prevents high-fat diet (HFD) induced hepatic insulin resistance and increase in hepatocyte diacylglycerol in a PPAR- α -dependent manner [Neschen et al., 2007], suggesting fish oil exerts beneficial effects in a HFD-dependent manner. Similarly, in female C57BL/6J mice fed either a regular diet or HFD, dietary supplementations with 1% n -3 enriched PUFA combined with 1% conjugated-linoleic acid (CLA) led to insulin resistance and hyperinsulinemia, however reduced glucose intolerance developed only in mice fed regular diet [Winzell

et al., 2006]. Therefore, fish oil effects on glucose homeostasis may be diet-dependent.

In the current study, the male AP-3 mice fed the HCD had improved glucose tolerance compared to WT. Since there is no change in insulin sensitivity, we hypothesize that there might be a better insulin response in the pancreatic β -cell. Because fish oil protects β -cells in mice from the toxicity of a low-dose streptozotocin [Linn et al., 1989], n -3 fatty acids may preserve insulin secretion by protecting β -cell in male mice fed the HCD. In contrast, reduction in insulin secretory response was suggested in female *fat-1* transgenic mice eating the HCD. Similarly, in rats made insulin resistant by high-saturated fat feeding for 1 month, hyperinsulinemia can be rapidly reversed via the dietary provision of small amounts of long-chain n -3 fatty acids in the absence of an acute improvement in insulin sensitivity and therefore at the expense of maintenance of glucose tolerance [Holness et al., 2004]. It is not clear why the female transgenic mice demonstrate glucose intolerance.

There is some experimental evidence in rodents that n -3 fatty acids lead to changes in energy balance and body weight, being less obesogenic than other fatty acids [Cunnane and McIntosh, 1994; Merzouk and Khan, 2003]. In the leptin deficient obese mice, Cunnane and McIntosh [1994] showed that, despite no significant change in food intake, there is less weight gain with a fish oil diet than when fed an iso-energetic diet with n -6 fatty acids diet [Cunnane and McIntosh, 1994]. In vivo activation of PPAR- γ by n -3

PUFA increased plasma adiponectin levels [Neschen et al., 2006], which are negatively associated with percent body fat, visceral fat, insulin, and leptin levels [Ryan et al., 2003]. Consistent with this, in our study, the male AP-3 mice had lower body weight.

In summary, we created the first transgenic mouse model with adipose-tissue-specific expression of *fat-1* enzyme. We used the native *fat-1* gene sequence controlled by the *aP2* promoter. There was an increase of *n-3* fatty acids in the serum and WAT of AP-3 mice. *Fat-1* expression improved glucose tolerance possibly by improving insulin secretion and reduced body weight in male AP-3 mice fed a HC diet; however, female AP-3 mice had reduced glucose tolerance. Finally, this is the first report of a potential reproduction problem in *fat-1* transgenic mice, as we were unable to produce homozygous transgenic lines and the lines analyzed all had a relatively low expression level implicating that excessive *fat-1* expression may be detrimental to a developing embryo or fetus.

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