Polyunsaturated Fatty Acids Suppress Hepatic Sterol Regulatory Element-binding Protein-1 Expression by Accelerating Transcript Decay*

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The reduction in hepatic abundance of sterol regulatory element binding protein-1 (SREBP-1) mRNA and protein associated with the ingestion of polyunsaturated fatty acids (PUFA) appears to be largely responsible for the PUFA-dependent inhibition of lipogenic gene transcription. Our initial studies indicated that the induction of SREBP-1 expression by insulin and glucose was blocked by PUFA. Nuclear run-on assays suggested PUFA reduced SREBP-1 mRNA by post-transcriptional mechanisms. In this report we demonstrate that PUFA enhance the decay of both SREBP-1a and -1c. When rat hepatocytes in monolayer culture were treated with albumin-bound 20:4(n-6) or 20:5(n-3) the half-life of total SREBP-1 mRNA was reduced by 50%. Ribonuclease protection assays revealed that the decay of SREBP-1c mRNA was more sensitive to PUFA than was SREBP-1a, i.e. the half-life of SREBP-1c and -1a was reduced from 10.0 to 4.6 h and 11.6 to 7.6 h, respectively. Interestingly, treating the hepatocytes with the translational inhibitor, cycloheximide, prevented the PUFA-dependent decay of SREBP-1. This suggests that SREBP-1 mRNA may need to undergo translation to enter the decay process, or that the decay process requires the synthesis of a rapidly turning over protein. Although the mechanism by which PUFA accelerate SREBP-1 mRNA decay remains to be determined, cloning and sequencing of the 3'-untranslated region for the rat SREBP-1 transcript revealed the presence of an A-U-rich region that is characteristic of a destabilizing element.

Dietary (n-6) and (n-3) polyunsaturated fatty acids (PUFA) lower blood triglycerides, decrease intra-muscular lipid droplet size, improve insulin sensitivity, and enhance nonhepatic glucose utilization (1–5). PUFA control these metabolic changes in size, improve insulin sensitivity, and enhance nonhepatic glucose utilization (1–5). PUFA control these metabolic changes in size, improve insulin sensitivity, and enhance nonhepatic glucose utilization (1–5). PUFA control these metabolic changes in size, improve insulin sensitivity, and enhance nonhepatic glucose utilization (1–5). PUFA control these metabolic changes in size, improve insulin sensitivity, and enhance nonhepatic glucose utilization (1–5). PUFA control these metabolic changes in size, improve insulin sensitivity, and enhance nonhepatic glucose utilization (1–5). PUFA control these metabolic changes in size, improve insulin sensitivity, and enhance nonhepatic glucose utilization (1–5). PUFA control these metabolic changes in size, improve insulin sensitivity, and enhance nonhepatic glucose utilization (1–5). PUFA control these metabolic changes in size, improve insulin sensitivity, and enhance nonhepatic glucose utilization (1–5). PUFA control these metabolic changes in size, improve insulin sensitivity, and enhance nonhepatic glucose utilization (1–5).

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‡‡ The abbreviations used are: PUFA, polyunsaturated fatty acids; SREBP, sterol regulatory element-binding protein; ADD1, adipocyte differentiation and determination factor-1, BSA, bovine serum albumin.

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**Fatty Acid Regulation of SREBP-1 mRNA Decay**

**A. Protein**

**B. mRNA**

**EXPERIMENTAL PROCEDURES**

**Primary Hepatocyte Culture**—Male Harlan Sprague-Dawley rats (150–170 g) were fasted for 24 h prior to hepatocyte isolation. Hepatocytes were isolated and maintained in primary monolayer culture as previously described by Salati and Clarke (21). Briefly, isolated hepatocytes (9 × 10^6 cells) were plated onto 10-cm tissue culture plates that were previously coated with rat tail collagen (Becton Dickinson Labware). Cells were allowed to attach for 4 h in Waymouth MB 752/1 medium (Life Technology) supplemented with 0.4 mM alanine, 0.5 mM serine, 26 mM sodium bicarbonate, 100 mM insulin (Life Technologies Inc.), 100 mM dexamethasone (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. After the attachment period, medium was changed to a serum-free media and treated with 150 μM albumin-bound 20:4(ω-6), 20:5(ω-3), and 18:1(ω-9) at a fatty acid/albumin ratio of 4:1. Cells not treated with fatty acid received an amount of albumin equal to that provide with the fatty acid-albumin complex. The source of albumin for all assays was essentially fatty acid-free bovine serum albumin (Sigma).

**Nuclear Run-on Assay and mRNA Analyses**—The impact of dietary PUFA on the transcription of hepatic fatty acid synthase and SREBP-1 was determined using the nuclear run-on assay (11). A rat specific SREBP-1c/ADD1 cDNA (B. Spiegelman) was employed to quantify the amount of nascent SREBP-1 mRNA synthesized by the nuclei. The abundance of SREBP-1 and fatty acid synthase mRNA in cultured primary rat hepatocytes was determined using total RNA extracted by the phenol-guanidinium isothiocyanate procedure (22). For Northern analysis, total RNA (30 μg per lane) was size-fractionated on a 1% agarose/formaldehyde denaturing gel, and subsequently transferred to a Zeta-probe nylon membrane (Bio-Rad) (11). The mRNA abundance of the respective transcripts was estimated by sequentially hybridizing the same membrane to 32P-labeled cDNA probes for SREBP-1, fatty acid synthase, and glyceraldehyde-3-phosphate dehydrogenase. All probes were labeled with [α-32P]dCTP (PerkinElmer Life Sciences) using random prime labeling (Life Technologies). Hybridization and wash conditions have been described previously (23). Autoradiographic signals were quantified using Instant Imager (Packard). The effect of fatty acid on the abundance and decay of SREBP-1c and -1a was determined using a ribonuclease protection assay (24). A rat SREBP-1c cDNA fragment with sequence corresponding to exon 1 (specific to SREBP-1c) and part of exon 2 (common to both SREBP-1a and SREBP-1c) was determined by Northern analysis (B) using total RNA (30 μg/lane) extracted from hepatocytes immediately after treatment (initial) or from hepatocytes treated with BSA or 20:4(ω-6) for 48 h. The Northern blot is a representative experiment showing analyses for replicate plates.

SDS, 1% dithiothreitol plus protease inhibitors. The abundance of mature SREBP-1 (nuclear) and precursor SREBP-1 (microsomal) was determined by Western blotting following the procedure described by Xu et al. (11). Immuno-reactive SREBP-1 was identified by incubating the blot for 4 h with monoclonal anti-SREBP-1 (IgG-2A4) prepared from hybridoma cells (ATCC, CRL 2121), and the protein visualized using an enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech). Bands were quantified for relative intensity using the Ambis imaging system.

**Transcription and Translation Inhibition Assays**—PURA purification of SREBP-1a, -1c, and fatty acid synthase mRNA half-lives in primary hepatocyte was evaluated by an α-amanitin transcription inhibition assay (27). Hepatocytes were isolated from fasted rats and maintained in a serum-free media containing 28 mM glucose, and 150 μM albumin-bound 20:4(ω-6) or 37.5 μM BSA alone. The abundance of membrane bound precursor (nuclear) and precursor (microsomal) SREBP-1c protein (A) was determined using Western blot analysis using pooled (5–5 plates) protein extracts prepared from freshly isolated cells (initial), or cells treated for 48 h with 20:4(ω-6) or BSA. The effect of 20:4(ω-6) on SREBP-1, fatty acid synthase (FAS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA abundance was determined by Northern analysis (B) using total RNA (30 μg/lane) extracted from hepatocytes immediately after treatment (initial) or from hepatocytes treated with BSA or 20:4(ω-6) for 48 h. The Northern blot is a representative experiment showing analyses for replicate plates.

**SDS-PAGE**—SDS-PAGE was performed according to the method of Laemmli (25). After electrophoresis, proteins were transferred to nitrocellulose for Western blotting using antibodies against rat SREBP-1 (IgG-2a4) prepared from hybridoma cells (ATCC, CRL 2121), and the protein visualized using an enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech). Bands were quantified for relative intensity using the Ambis imaging system.

**Glyceraldehyde-3-phosphate dehydrogenase**—Glyceraldehyde-3-phosphate dehydrogenase was determined by Northern blot or ribonuclease protection procedures. To examine the possibility that mRNA translation was needed for SREBP-1 mRNA to undergo decay, the half-lives of SREBP-1a and -1c mRNA were determined in the presence of the translational inhibitor, cycloheximide. As described previously, SREBP-1 expression in isolated hepatocytes was induced by culturing hepatocytes in a media containing glucose and insulin. After 48 h in culture, the cells were treated with cycloheximide (5 μM final concentration) for 2 h, and subsequently treated with 150 μM albumin-bound 20:4(ω-6) or albumin alone for an additional 6 h. The abundance of SREBP-1a and -1c mRNA was quantified using the ribonuclease protection assay.

**Cloning of the 3′-Untranslated Region of Rat SREBP-1**—Total RNA was extracted from rats fed a high carbohydrate diet. The 3′-end of rat SREBP-1 mRNA was cloned using reverse transcription-polymerase chain reaction methodology. First strand synthesis was accomplished using murine leukemia virus reverse transcriptase and a poly(T) de- generating primer, 5′-TTTATGTCAGACTGAAACTCTCAGGGCTTTTTTTTTTTTTTTTAC/G/C/G/AC/T-3′ that was linked to an adaptor sequence of 5′-TCTATTGCGACTGAAACTCTCAGGGCG-3′.
After a 20-min 42 °C reaction, polymerase chain reaction amplification was conducted using the gene-specific primer at position 5′-GAGGAG-GGTCTTCTCCTACATGAGGC-3’ and the adaptor primer sequence 5′-TCTAGTUGACTGAATCTCTCGAGGCG-3’. The reaction conditions were comprised of an initial denaturation at 94 °C for 10 min followed by 5 cycles of 94 °C for 30 s and 72 °C for 1.5 min, 5 cycles of 94 °C for 30 s and 70 °C for 1.5 min, and finally 33 cycles of 94 °C for 30 s and 68 °C for 1.5 min. The resulting amplification product was sequenced by the dideoxy chain termination method.

RESULTS

SREBP-1 Gene Transcription Is Not Suppressed by 20:4(n-6)—Earlier work from our group suggested that the hepatic reduction in SREBP-1 mRNA abundance elicited by dietary polyunsaturated fatty acids was not accompanied by a reduced rate of gene transcription (11). However, this earlier work employed a hamster cDNA for SREBP-1 to quantify the synthesis of nascent SREBP-1 transcripts that occurred in rat liver nuclei (11). It is possible that the hamster SREBP-1 cDNA may have hybridized to a nonspecific nascent transcript produced in the nuclear run-on assay. Such nonspecific hybridization could have masked potential changes in SREBP-1 gene transcription. Therefore, the effect of dietary (n-6) and (n-3) polyunsaturated fatty acids on rat liver SREBP-1 gene transcription was re-evaluated using a cDNA specific for rat SREBP-1, i.e. adipose differentiation and determination factor-1 (ADD-1). Nuclear run-on assays (n = 4 per diet group) continued to indicate that SREBP-1 gene transcription was not inhibited by dietary fats rich in 18-carbon (n-6) or 20- and 22-carbon (n-3) fatty acids (data not shown).

20:4(n-6) Accelerates SREBP-1 Decay in Isolated Hepatocytes—The nuclear run-on results cited above led to the hypothesis that polyunsaturated fatty acids reduce SREBP-1 mRNA and protein abundance by accelerating the decay of the SREBP-1 transcript. Pursuit of this question required an hepatic cellular model that mimicked intact animal responses. Isolated rat hepatocytes maintained in primary culture fulfill this requirement. As observed in the intact animal (11–13), the amount of mature SREBP-1 found in the nuclei of hepatocytes was positively correlated with the hepatocyte content of fatty acid synthase mRNA (Fig. 1). Specifically, culturing hepatocytes isolated from 24-h fasted rats in medium containing insulin and 28 mM glucose resulted in a 3–4-fold increase in the amount of membrane (precursor) and nuclear (mature) SREBP-1 (Fig. 1A). The increase in hepatocyte SREBP-1 protein was paralleled by a comparable rise in the abundance of SREBP-1 mRNA (Fig. 1B). Most importantly, treating the hepatocytes with 150 μM albumin-bound 20:4(n-6) completely blocked the insulin-glucose dependent induction of SREBP-1 mRNA and protein (Fig. 1). An examination of the rapidity with which 20:4(n-6) exerted its influence on SREBP-1 expression revealed that a 5- and 10-h exposure to 20:4(n-6) reduced the hepatocyte content of SREBP-1 mRNA 50 and 85%, respectively (Fig. 1). Interestingly, there was at least a 2-h lag before 20:4(n-6) exerted its suppressive influence on SREBP-1 mRNA (Fig. 2). Finally, it is important to note that 20:4(n-6) had no effect on either the insulin-glucose induction or the steady state level of glyceraldehyde-3-phosphate dehydrogenase mRNA (Fig. 1B and Fig. 2). This indicates that the suppression of SREBP-1 and associated lipogenic genes by 20:4(n-6) is specific and comparable to the in vivo responses achieved with dietary (n-6) or (n-3) polyunsaturated fatty acids (11–13, 28).

The influence of 20:4(n-6) on the half-life of SREBP-1 mRNA was examined by pretreating rat liver cell monolayers for 3 h with albumin-bound 20:4(n-6) or albumin alone, and subsequently adding α-amanitin to inhibit gene transcription (Figs. 3-5). The abundance of glyceraldehyde-3-phosphate dehydrogenase was not affected by either 20:4(n-6) or α-amanitin (Figs. 3–5). On the other hand, Northern analyses revealed that 20:4(n-6) treatment of rat cell monolayers accelerated the rate
of SREBP-1 mRNA decay (Figs. 3–5). Regression analysis employing all measurements from 0 to 10 h indicated that 20:4(n-6) significantly \( (p < 0.05) \) shortened the half-life for total SREBP-1 mRNA from 11.6 h \( (r = 0.94, p < 0.01) \) in the absence of fatty acid to 6.4 h \( (r = 0.92, p < 0.01) \) in the presence of 20:4(n-6). Examination of the decay curve suggested that the decay of SREBP-1 mRNA occurred in two phases, i.e. a rapidly decaying pool \((0–4 \text{ h})\), and a more slowly decaying pool \((4–10 \text{ h})\). However, 20:4(n-6) accelerated the decay of both of these putative pools (Fig. 3). Because the cDNA probe utilized in the Northern analyses encoded a sequence that was common to both SREBP-1a and -1c, we hypothesized that the apparent

**FIG. 3.** 20:4(n-6) accelerated SREBP-1 mRNA decay in rat liver cell monolayers. Rat hepatocytes were isolated from 24-h fasted rat donors and maintained in a media containing insulin and 28 mM glucose. After 44 h in culture, cells were treated with 150 \( \mu \text{M} \) albumin-bound 20:4(n-6) (open circles) or 37.5 \( \mu \text{M} \) BSA alone (closed circles) for 3 h \((3)\) prior to the addition of the transcription inhibitor, \( \alpha \)-amanitin. A, the abundance of SREBP-1, fatty acid synthase (FAS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined by Northern analysis (30 \( \mu \text{g} \) of total RNA per lane). The logarithmic decay for SREBP-1 and FAS mRNA are depicted in B and C, respectively. The SREBP-1 mRNA decay equations for BSA and 20:4(n-6) are \( y = -0.026x + 1.376 \) and \( y = -0.047x + 1.274 \), respectively. A test of differences revealed a significant effect of 20:4(n-6) \( (p < 0.05) \). GAPDH mRNA did not decrease during the 10-h period. Consequently the abundance of SREBP-1 and FAS mRNA is expressed relative to the level of GAPDH. The regression equations for FAS mRNA decay are \( y = -0.026x + 1.655 \) and \( y = -0.04x + 1.636 \) for the BSA and 20:4(n-6) treated cells, respectively. Data are representative of two independent experiments.
existence of two decay rates for SREBP-1, as determined by Northern analyses, may represent differences in decay between the SREBP-1a and -1c transcripts. To examine this possibility, a ribonuclease protection assay was employed to separately quantify the effect of 20:4(n-6) on the decay of SREBP-1a and -1c mRNA (Fig. 4). The SREBP-1c/1a ratio found in isolated hepatocytes maintained in a media containing insulin and glucose was 3/1, which was not dissimilar from the 5/1 ratio observed in vivo (29). When hepatocytes were maintained in a fatty acid-free media, the half-life of SREBP-1c was similar to that of SREBP-1a, i.e. 10.0 h ($r = 0.77$, $p < 0.07$) and 11.6 h ($r = 0.71$, $p < 0.10$), respectively (Fig. 4). However, when the hepatocyte monolayers were treated with 20:4(n-6), the half-life of SREBP-1c was significantly ($p < 0.05$) reduced to 4.6 h ($r = 0.93$, $p < 0.01$). The half-life of SREBP-1a mRNA also appeared to be reduced by 20:4(n-6) but the decrease from 11.6 to 7.7 h ($r = 0.94$) was not statistically significant. Like 20:4(n-6), 20:5(n-3) accelerated the decay of SREBP-1c (Fig. 5). The decay of SREBP-1a appeared to be unaffected by 20:5(n-3), but this lack of significant decay likely reflects the fact that cells were harvested after 4 h of fatty acid treatment which was well below the 7.7 h half-life of the SREBP-1a transcript. Unlike the effect of PUFA, the monounsaturated fatty acid, 18:1(n-9) had no effect on the hepatocyte content of SREBP-1c and -1a mRNA. This observation was consistent with several dietary studies that have shown that SREBP-1 expression is only suppressed by (n-6) and (n-3) polyunsaturated fatty acids (8, 11, 23).

**Decay of SREBP-1c mRNA Requires Translation**—In an attempt to ascertain if the PUFA enhancement of hepatic SREBP-1 mRNA decay involves a short-half-life regulatory
protein, the influence of 20:4(n-6) on SREBP-1a and -1c mRNA decay was examined in the presence of the translational inhibitor, cycloheximide. Interestingly, cycloheximide treatment of the hepatocyte monolayers increased hepatocyte mRNA abundance (Fig. 6). Moreover, blocking mRNA decay was examined in the presence of the translational inhibitor cycloheximide. The abundance of SREBP-1a and -1c mRNA is expressed relative to 18S mRNA and represent the average of n = 3 plates per treatment. Asterisk (*) indicates 20:4(n-6) significantly (p < 0.01) reduced SREBP-1c mRNA; double asterisk (**) indicates a significant (p < 0.05) increase in SREBP-1c mRNA due to CHX treatment; B, representative ribonuclease protection assay for SREBP-1a and -1c.

DISCUSSION

Supplementing a high carbohydrate diet with oils rich in (n-6) and (n-3) PUFA results in a rapid (i.e. <3 h) and coordinate inhibition of hepatic gene transcription for a wide array of lipogenic enzymes including fatty acid synthase, acetyl-CoA carboxylase, citrate lyase, malic enzyme, and stearoyl-CoA desaturase (8, 11, 23, 30). Dose-response studies indicate that lipogenic gene transcription is suppressed 50% when the diet contains as little as 3–5% of its energy as PUFA (31). The coordinate regulation of gene transcription by PUFA suggested that PUFA may employ a “master switch” type mechanism. The hepatic nuclear factor SREBP-1 may serve this function (11–14). PUFA appear to reduce the nuclear content of SREBP-1 by interfering with the proteolytic release of mature SREBP-1 from its endoplasmic reticulum anchored precursor. In addition, PUFA reduces the amount SREBP-1 precursor by decreasing the abundance of SREBP-1 mRNA (11–14).

In vivo and cell culture studies indicate that insulin and glucose increase the hepatic content of SREBP-1 by inducing the SREBP-1 gene transcription (17, 32). In contrast, glucagon and cAMP suppress SREBP-1 gene transcription, and consequently block the rise in hepatic SREBP-1 mRNA and protein elicited by glucose and insulin (20). Like glucagon and cAMP, treating hepatocytes with 20:4(n-6) also blocked the insulin-glucose induction of SREBP-1 expression (Fig. 1). However, unlike glucagon and cAMP, PUFA govern SREBP-1 expression by post-transcriptional mechanisms (11). Such mechanisms may involve interference with mRNA processing (33) and/or acceleration of mRNA decay (34). In the case of SREBP-1, PUFA reduce the hepatic content of SREBP-1 mRNA by accelerating the rate of mRNA decay.

The liver expresses two forms of SREBP-1, 1a and 1c. SREBP-1 antibody recognizes both proteins. Consequently, the relative abundance of the two proteins cannot be quantified. However, due to differences in splicing, the 5’-end of the
SREBP-1c transcript is shorter than the SREBP-1a mRNA (29). This difference in size permits the use of a ribonuclease protection assay to examine the influence of PUFA on SREBP-1a and -1c mRNA abundance and decay. Interestingly, this assay revealed that the SREBP-1c transcript was more sensitive to PUFA regulation than was SREBP-1a. Specifically, PUFA reduced the half-life of SREBP-1c mRNA by 55% while it decreased the half-life of SREBP-1a by only 35%. Because the decay of SREBP-1c mRNA was more sensitive to 20:4(n-6) and 20:5(n-3) feedback, the ratio of SREBP-1a to SREBP-1c decreased from 3:1 to 1:1. The selective loss of SREBP-1c in response to PUFA is reminiscent of what has been observed in streptozotocin-diabetic rats (17, 32), but unlike glucagon and insulin which appear to regulate SREBP-1 gene transcription, PUFA exert their effects at the level of SREBP-1 mRNA decay.

The mechanisms by which PUFA enhance SREBP-1 decay remain to be established. Often times the rate-limiting step in mRNA decay is the shortening of the poly(A) tail (35). The rate of poly(A) tail shortening appears to depend upon the binding of specific proteins to "destabilizing" elements within the mRNA (35). One common destabilizing element is the A-U rich sequence, notably UUAUUUA(U/A)(U/A), located within the 3'-untranslated region or within the open reading frame that is targeted by PUFA control mechanisms. In this respect it is interesting to note that cycloheximide not only blocked the ability of PUFA to accelerate SREBP-1 decay, but actually increased the hepatocyte content of both SREBP-1c and -1a. These results suggest that SREBP-1 mRNA translation is required for mRNA decay, and/or that PUFA may modulate the activity of a rapidly turning over protein involved in SREBP-1 mRNA decay. One additional dilemma is why do the SREBP-1c and -1a transcripts differ in their response to PUFA-regulated decay. Presumably, SREBP-1c and -1a both contain the same 3'-untranslated region, but under the influence of PUFA, the rate of SREBP-1c mRNA decay is faster than is the decay of SREBP-1a. A key difference between the two transcripts is that they are produced by two different promoter sites. This results in the 5'-untranslated region of SREBP-1c being 45 nucleotides shorter than SREBP-1a. Thus, one possible explanation for the difference in decay rates in response to PUFA is that the 5'-untranslated region of SREBP-1c plays a regulatory role in the PUFA-mediated decay of SREBP-1.

In conclusion, dietary (n-6) and (n-3) PUFA have long been known to decrease the expression of hepatic lipogenic as well glycolytic genes (8). While recent data indicate that 18:2(n-6) and 18:3(n-3) must undergo desaturation by the 6-desaturase and 3-desaturase enzymes to exert their inhibitory effects (36), numerous studies have failed to link the PUFA control of gene expression to the production of eicosanoids (31). Moreover, PUFA exercise their effects in an insulin- and glucagon-independent manner (37). Despite our inadequate understanding of the signaling mechanisms employed by PUFA, SREBP-1 is emerging as a transcription factor that is pivotal to the overall understanding of PUFA regulation of hepatic lipogenic gene expression. In this story, PUFA exert two effects. First, PUFA inhibit the proteo-
lytic release of SREBP-1c from its endoplasmic reticulum anchored precursor which in turn results in an immediate suppression of lipogenic gene transcription (11, 14). Second, PUFA accelerate the decay of SREBP-1c mRNA which in turn lowers the hepatic content of SREBP-1 precursor. The outcome is a lower capacity for hepatic lipogenesis, and a decrease in hepatic triglyceride output (11–13). This ability of PUFA to suppress SREBP-1 expression and thereby exert a strong anti-lipogenic influence not only explains how PUFA function as hypolipidemic agents but may also offer a partial explanation for how PUFA improve glucose metabolism and insulin sensitivity (2–5, 38).

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