ORIGINAL

Nutritional status, feeding practice and incidence of infectious diseases among children aged 6 to 18 months in northern mountainous Vietnam

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Abstract : *Objective* : To assess the prevalence of undernutrition, incidence of infectious diseases and the situation of feeding practices to determine the risk factors for undernutrition among children aged 6 to 18 months in rural Vietnam. *Design* : A cross-sectional study was conducted among one hundred eighty-eight mother-child pairs in Bac Giang, Vietnam. Weight and height of the children were measured and referred to data from the WHO/CDC/NCHS. Incidence of infectious diseases was diagnosed based on the WHO Recommended Surveillance Standards. Data on socio-demographic variables and feeding practices were obtained through a structured questionnaire. *Result* : The prevalence of underweight, stunting and wasting was 19.7%, 23.4% and 5.3%, respectively. The incidence of diarrhea and acute respiratory infections (ARIs) during the last 14 days of the interview was 12.2% and 20.2%, respectively. Although 99% of the children were breastfed, the prevalence of exclusive breastfeeding in the first 4 mo was 21.3%. Non-exclusive breastfeeding in the first 4 month (OR 3.95, p=0.025) and low birth weight (OR 4.38, p=0.009) were associated with underweight in the children, while incidence of infectious disease was not (OR 1.16, p=0.734). *Conclusion* : Undernutrition is highly prevalent in the study site and non-exclusive breast feeding is one of the risk factors. J. Med. Invest. 57 : 45-53, February, 2010

Keywords : *undernutrition, exclusive breastfeeding, infectious disease, Vietnam, infant and child*

INTRODUCTION

Child deaths worldwide have decreased in number from 13·5 (13·4-13·6) million in 1980 to an estimated 9·7 (9·5-10·0) million in 2005 ; however, the decline is less than the target set by Millennium Development Goal 4 (MDG4) (1). Most of the child deaths were caused by preventable and treatable illness despite effective health interventions. At least half of the deaths are caused by undernutrition (2). UNICEF has developed a framework for the basic underlying causes of undernutrition and has demonstrated that a combination of insufficient nutrient intake and infection are the

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primary factors (3). When children consume insufficient nutrients, their immune systems deteriorate, resulting in greater incidence, severity and duration of disease. Disease in turn affects their nutrient intake and also interferes with nutrient absorption, further aggravating the undernutrition. As to the underlying causes of undernutrition, poverty plays a central role with environmental, economic, and sociopolitical factors.

In Vietnam, child undernutrition is one of the major national health problems. It has been reported that the nationwide prevalence of child undernutrition has been remarkably reduced during the last 2 decades : the prevalence of underweight among children aged under 5 y was 51.5% in 1985, but it was reduced to 25.2% in 2005 (4). However, the prevalence of undernutrition varied among ecological regions in Vietnam. A greater proportion of the population in mountanious and remote areas were undernourished than in urban areas. The child undernutrition problem was reported to occur from an early stage of their life in Vietnam. The prevalence of undernutrition increased remarkably during 6 to 18 mo and was sustained over the next 3 y (4). Previous studies indicated that premature complementary feeding (5, 6) and frequent infectious disease (7) were the primary causes. It has also been recognized that stunting occurred even before 6 mo of age : 6.3% of children aged under 6 mo were stunted (4). Poor health care and insufficient food intake during pregnancy were also suggested as high risk factors for childhood undernutrition.

Although approach for general deprivation and inequality would result in substantial reduction in undernutrition and should be global priority, major reduction in undernutrition can also be achieved through programmatic health and nutrition intervention (8-11). The present study was aimed at providing baseline information, including the prevalence of undernutrition, incidence of infectious disease and the situation in feeding practice among children aged 6 to 18 mo for future programs with which to combat child undernutrition in northern mountainous Vietnam.

METHODS

During May and June 2005, in the middle of the dry season, a cross-sectional survey was conducted in 4 communes in Yen The, Bac Giang, Vietnam. Bac Giang province is a rural mountainous region in Vietnam, located in the North-East region and 51 km from Hanoi. The province had a relatively high prevalence of childhood undernutrition among the 8 regions in Vietnam (4). Yen The district is one of the nine districts in the province. The district has a population of approximately 91,000 with 7,500 children under 5 y of age and 2,900 children under 2 y (Dec, 2004). There are 19 communes in the district, out of which four communes were selected. Extremely poor villages which belong to the government's "Phase One 10,000 Poorest Communities" Program and the villages that were already targeted in special intervention programs by the government were not included among the selected communes. All children aged 6 to 18 mo were recruited for the survey. A census was carried out to identify all children aged 6 to 18 mo and 191 eligible children were identified.

Anthropometric measurements comprising height and weight were performed among the children and mothers by four trained field workers using standard procedures (12). Briefly, children were weighed in light cloths on an infant scale. Recumbent length was measured to the nearest 0.1 cm with a portable infant measuring board. The anthropometric indicators for children, Z-scores of weight-for-age (WAZ), height-for-age (HAZ) and weight-for-height (WHZ), were calculated on the basis of growth references developed by the World Health Organization (WHO), the Center Disease for Control (CDC), and the National Center for Health Statistics (NCHS) (13) and were calculated also by using growth references recently developed by WHO (14). Most of the available data on the growth of children in Vietnam were calculated based on WHO/CDC/NCHS growth references at the time that the present study was conducted. Undernutrition was classified according to the cut-off indicated by WHO (15) : underweight was defined as WAZ<-2, stunting as HAZ<-2, wasting as WHZ<-2. Mothers were weighed in light clothes (to the nearest 100 g) on a scale. Height was measured to the nearest 0.1 cm. Chronic Energy Deficiency (CED) among mothers was classified as Body Mass Index (BMI) under 18.5 kg/m^2.

Morbidity data were collected from the mother's description of the infant's symptoms of diarrhea, acute respiratory infections (ARIs), measles and pertussis during the previous two weeks. The descriptions of the infectious diseases were based on the WHO recommended Surveillance Standards (16). Children who had a passage of 3 or more loose or watery stools in the past 24 hours were classified

as suffering from diarrhea. If the diarrhea lasted more than 2 weeks, these children were classified as suffering from chronic diarrhea. If the stool contained blood, these children were classified as suffering from dysentery. Children who had a cough or difficulty breathing were classified as suffering from ARIs. If the cough or difficult breathing was accompanied by frequent breathing (> 50 breath/min. for age 2 mo to < 1 y, > 40 breath/min. for age 1 to < 5 y), these children were classified as suffering from pneumonia. Measles was determined if a child had all of the following three symptoms : fever, maculopapular rash, and cough or coryza or conjunctivitis. Pertussis was determined if a child showed both of the following symptoms : cough for at least 2 wks with at least one of the following : paroxysms of coughing, inspiratory "whooping", post-tussive vomiting without other apparent cause.

In this survey, a face-to-face interview was conducted with mothers and children to collect information on socio-demographic status, feeding practices and coverage of local immunization and Vitamin A supplementation. Data collection concerning breast feeding practice was based on WHO guidelines (17) and on a previous study in Vietnam (5). The mothers were asked about their breastfeeding practices during the children's first 6 mo and also about the current breastfeeding status. The following operational definitions were used in the survey : breastfeeding referred to children who were receiving at the time of interview or had ever received breast milk. Breastfeeding status at 4 and 6 mo of age were classified as exclusive breastfeeding (only breast milk plus medical drops and syrups), almost exclusive breastfeeding (only breast milk and water plus medical drops and syrups), predominant breastfeeding (water, herbal teas or fruit juice in addition to breast milk), partial breastfeeding (breast milk plus other types of milk or foods) and weaned (no longer breastfed). All the questions were pre-tested at a study site and revised before initiating the survey. The interviewers, physicians from the NIN and local community health centers, were trained in standardized questionnaire administration and anthropometric measurements through lectures and practice in the field. Four investigation teams were established for the four communities, each one including at least eight members and a supervisor. During the survey, a check system was applied including checking in the field by interviewers, interviewer's checking each other, and checking by supervisors. Personnel in the local community centers and collaborators assisted in the organization of data collection and in the explanation of procedures to the study participants. The study participants were re-interviewed whenever transcription or logical questions arose or missing values were found. The apparatus and method for measurement were checked and/or adjusted for accuracy by the supervisors before each day's work. The precise age of each child was obtained from the Permanent Residence Registration where birth data are recorded.

Before the survey, all eligible mothers in the communities were informed of the procedures and purpose of the study, then informed consent was obtained. The protocol of this study was approved by the Scientific Board of the National Institute of Nutrition of Vietnam and the Ethical Committee of Tokushima University.

A database was established using Epi info version 6 (CDC, Atlanta, GA, USA). All data were checked for missing data and outliers, and cleaned before data analysis. Statistical analysis was performed using SPSS version 11.5J (Statistical Package for Social Science, Inc.). A one-sample Kolmogorov-Smirnov test was used to assess whether the data were normally distributed. Results were presented as mean and standard deviation (SD) or as median and 25th, 75th percentile. Z-scores between children aged 6 to 11.9 mo and those aged 12 to 17.9 mo were compared by unpaired t-test. Z-scores calculated by WHO/CDC/NCHS and WHO growth reference were compared by paired t-test. The proportion of children classified as underweight, stunted and wasted were compared by chi-square test. Logistic regression analysis was used to analyze the effects of infectious disease and exclusive breastfeeding status, as well as those of socioeconomic and demographic factors, on the nutritional status of children. We selected children's underweight for the analysis since acute malnutrition among children is a key indicator routinely used for describing the presence and magnitude of humanitarian emergencies (18).

RESULTS

Socio-demographic characteristics and coverage of some of local health services

A pair of twins and a child whose mother was suffering from tuberculosis were excluded and the data of 188 children were used for the analysis in the present study. Socio-demographic characteristics of the children are shown in Table 1. Ninety one percent

Table 1 Socio-demographic characteristics

Variable	All (n=188)
Gender	
Boys	111 (53.7%)[1]
Girls	87 (46.3%)
Age	
6-8.9 mo	64 (34.0%)
9-11.9 mo	34 (18.1%)
12-14.9 mo	30 (16.0%)
15-18.9 mo	60 (31.9%)
Birth weight, g	3000 (2700, 3375)[2]
Low Birth Weight	20 (10.6%)
Number of children aged under 5 y	
1 child	149 (79.3%)
≧2 children	39 (20.7%)
Family income, 1000VND/capita/d[3]	11.1 (6, 22.2)
Mother's age, y	25.0 (22, 28)
Mother's education level	
Primary school (1-5 y)	26 (13.8%)
Secondary school (6-9 y)	130 (69.1%)
More (> 10 y)	32 (17.0%)
Mother's BMI[4]	
6-11.9 mo postpartum, kg/m^2	19.7 (18.4, 20.8)
12-18.9 mo postpartum, kg/m^2	19.0 (17.8, 20.1)

Abbreviation : BMI, Body Mass Index
[1] Number, % in parentheses (all such values).
[2] Median, 25th and 75th percentile in parentheses (all such values).
[3] Exchange rate as of May-June 2007 : US1$=VND17,803.
[4] n=98 for 6-11.9 mo postpartum and n=90 for 12-18.9 mo postpartum

of children belonged to the "Kinh" ethnic group. As to the main family occupation, 55.3% were farmers or agricultural workers. Water from wells was used by 98.4% of the participant households and 99% of mothers answered that they boiled the water for drinking. The latrine types were mainly traditional pit latrine (76.1%) and ventilated improved pit latrine (13.3%). The family income per capita was < 17,800 VND, 17,800-35,600 VND and > 35,600 VND for 67.0%, 20.7% and 12.2% of the study participants, respectively. Coverage of immunization in the study site was 99.5% for BCG, 97.4% for DPT 3rd, 97.9% for OPV 3rd, 97.3% for Measles and 63.7% for Hepatitis B 3rd. The coverage of Vitamin A distribution among the children aged 6 to 36 mo was 96.7%.

Z-scores and the prevalence of undernutrition calculated by WHO/CDC/NCHS reference (13) among the children are shown in Table 2. The prevalence of underweight, stunting and wasting

Table 2 Z-scores and prevalence of undernutrition

	All	6-11.9 mo	12-18.9 mo
n	188	98	90
WAZ	-1.21 ± 1.01[1]	-0.89 ± 1.01	-1.57 ± 0.90 **
HAZ	-1.20 ± 1.06	-0.94 ± 1.04	-1.50 ± 1.01 **
WHZ	-0.52 ± 0.88	-0.21 ± 0.86	-0.99 ± 0.77 **
Underweight	37 (19.7%)[2]	14 (14.3%)	23 (25.6%) *
Stunting	44 (23.4%)	18 (18.4%)	26 (28.9%)
Wasting	10 (5.3%)	3 (3.1%)	7 (7.8%)

Abbreviation : WAZ, Weight-for-Age ; HAZ, Height-for-Age ; WHZ, Weight-for-Height
[1] Mean ± SD (all such values).
[2] Number, % in parentheses (all such values).
*P < 0.05 ; ** P < 0.01. vs. children aged 6-11.9 month (unpaired t-test)

was 19.7%, 23.4% and 5.3%, respectively. The WAZ, HAZ and WHZ among the children aged 12 to 18.9 mo were significantly lower than those among the children aged 6 to 11.9 mo (P < 0.05). When the Z-score was calculated by the recent WHO growth reference (14) for all the children (n=188), the mean WAZ, HAZ and WHZ were -0.91 ± 1.08, -1.21 ± 1.19 and -0.37 ± 1.09, respectively ; and the prevalence of underweight, stunting and wasting was 25.5% (n=48), 14.9% (n=28), 6.9% (n=13), respectively. Comparing these scores with the NCHS growth reference, the mean WAZ and WAZ were significantly lower (P < 0.001), the mean HAZ was similar (P = 0.772), and a higher proportion of children was classified as stunted (P < 0.001) and fewer children were classified as underweight (P < 0.001).

Table 3 shows the incidence of diarrhea and ARIs

Table 3 Incidence of infectious disease during the last 14 days of the survey

	Total	age group	
		6-11.9 mo	12-18.9 mo
n	188	98	90
Diarrhea			
acute diarrhea	23 (12.2%)[1]	10 (10.2%)	13 (14.4%)
dysentery	5 (2.7%)	2 (2.0%)	3 (3.3%)
chronic diarrhea	1 (0.5%)	1 (1.0%)	0 (0.0%)
Acute Respiratory Infectious disease			
cough and difficult breathing	38 (20.2%)	21 (21.4%)	17 (18.9%)
bronchial infection	5 (2.7%)	2 (2.0%)	3 (3.3%)
pneumonia	11 (5.9%)	8 (8.2%)	3 (3.3%)
throat infection	15 (8.0%)	8 (8.2%)	7 (7.8%)
nose infection	3 (1.6%)	4 (2.0%)	2 (1.1%)

[1] Number, % in parentheses (all such values).

during the last 14 days of the interview. The incidence of diarrhea and ARI was 12.2% and 20.2%, respectively. The incidence of diarrhea and ARIs did not differ for older children and younger children. There was no child with measles or pertussis during the time of the survey.

Ninety nine percent of the children had been breastfed or were being breastfed at the time of interview. The percentage of currently breastfed children was 99.0% among those aged 6 to 11.9 mo, while that was 73.3% among those aged 12 to 18.9 mo. Breastfeeding status in the first 4 and 6 mo is shown in Table 4. In the first 4 mo, 21.3% of the children were exclusively breastfed and 18.6% were almost exclusively breastfed. The prevalence of exclusively and almost exclusively breastfed children declined rapidly as at 6 mo, there was no child who had been exclusively breastfed and only 3.2% who were almost exclusively breastfed, while the percentage of partially breastfed children increased to 95.2%.

A logistic regression model was used to identify the risk factors related to a child's underweight (Table 5). As independent variables, incidence of infectious disease, exclusive breastfeeding in the first 4 mo, gender, age of children, low birth weight, number of children age under 5 y, family income, mother's age and mother's education level were applied. For the analysis, incidence of infectious disease was defined if the child had diarrhea or ARIs. Exclusive breastfeeding was defined if the child had exclusive breastfeeding or almost exclusive breastfeeding in the first 4 mo. Non-exclusive breastfeeding in the first 4 mo (OR 3.95, p=0.025) and low birth weight (OR 4.38, p=0.009) were associated with underweight in the children, while incidence of infectious disease was not (OR 1.16, p=0.734).

Table 4 Breastfeeding status (Total n=188)

	Breastfeeding at age 4 mo	Breastfeeding at age 6 mo
Exclusive	40 (21.3%)[1]	0 (0%)
Almost exclusive	35 (18.6%)	6 (3.2%)
Predominant	4 (2.1%)	2 (1.1%)
Partial	108 (57.4%)	179 (95.2%)
Artificial	1 (0.5%)	1 (0.5%)

[1] Number, % in parentheses (all such values).

Table 5 Odds ratio of the risk factors for underweight (Total n=188)

	Underweight		
	Odd Ratio	95% CI	*P*-value
Morbidity[1]			
No	1.00		
Yes	1.16	0.48, 2.81	0.734
Exclusive breast feeding at age 4 mo			
Yes	1.00		
No	3.95	1.19, 13.16	0.025
Gender			
Boys	1.00		
Girls	0.66	0.79, 3.96	0.329
Age			
6 to 11.9 mo	1.00		
12 to 18.9 mo	2.11	0.94, 4.75	0.072
Low Birth Weight			
Normal	1.00		
Birth weight< 2500 g	4.38	1.45, 13.24	0.009
Number of children aged under 5 y			
1 child	1.00		
2 children	2.27	0.82, 6.26	0.113
Family income			
more than 11,100 VND/capita/d	1.00		
under 11,100 VND/capita/d	0.86	0.37, 2.02	0.734
Mother's age at birth			
less than 25 y	1.00		
over 25 y	1.37	0.60, 3.14	0.451
Mother's education level			
Up to primary school	1.00		
More	0.69	0.19, 2.48	0.568

[1] Morbidity was defined as "Yes" if the child had the incidence of diarrhea or ARI during the last 14 days of the survey.

DISCUSSION

To our knowledge, this is the first study to assess the nutritional status, feeding practice and incidence of infectious diseases simultaneously in order to determine the risk of undernutrition among children aged 6 to 18 mo in northern mountainous Vietnam. In mountainous areas, the prevalence of undernutrition among children is remarkably higher compared to other regions (19). Growth faltering in Vietnam occurs early in their life and the prevalence of undernutrition accumulates with age; it starts from 3 to 4 mo of age and increases quickly from 6 to 12 mo of age, then obtains the highest level at age 24 mo (20); therefore, early prevention and control of undernutrition are expected. The present study indicated that high prevalence of undernutrition in terms of underweight, stunting and wasting among children aged 6 to 18 mo in northern mountainous Vietnam. Non-exclusive breastfeeding status in the first 4 mo and low birth weight were predictors for the child undernutrition, while incidence of infectious disease was not.

The prevalence of underweight and stunting in the study site were relatively lower compared to those nationwide as well as a previous report in Bac Giang province on children under 5 y (4). This was due to the fact that the prevalence of malnutrition was relatively higher among children older than 12 mo compared to younger children, which is consistent with a previous report of Vietnam (21), and that our study participants were relatively younger than those in the previous reports. Another report on Vietnamese children of the same age in a mountainous region demonstrated a similar prevalence of undernutrition (22). Additionally, using the recent WHO growth reference resulted both in a difference in mean Z scores for WAZ and WHZ and in changes in the prevalence of stunting and underweight. The differences in results between the WHO/CDC/NCHS growth reference and the recent WHO growth reference were similar to those in a previous report in Vietnam (23).

Since suitable and low-cost alternatives to breastfeeding are not available and non-breast milk food such as unhygienic water and low nutrient-density food has several problems in most of developing countries, WHO (24) has recommended giving only breast milk during the first 4 to 6 mo. The benefits of exclusive breastfeeding in the first 6 mo for the child's nutritional status were also reported both in industrialized countries and developing countries (25). In this study, non-exclusive breastfed children in the first 4 mo had 3.95 times higher incidence of underweight (P=0.025). The result supports the previous findings that Vietnamese children who were not exclusively breastfed or predominantly breastfed in the first 4 mo showed significantly lower anthropometric measurements at the age of 6 to 12 mo than the children who were exclusively or predominantly breastfed (5). It has been reported that an ideal method of identifying exclusive breastfeeding is a descriptive longitudinal and prospective study design with an indicator of "exclusively breastfeeding since birth" (26). In this study, we developed a standardized definition of exclusive breast feeding status following the definition that WHO has proposed (17), while we determined the status by retrospectively asking the time of introduction of non-breast milk food. Since this study has a cross-sectional design, the assessments might have retrospective bias. Although we could not draw a direct epidemiological inference for causality between exclusive breastfeeding and underweight, our findings indicate that advantages of the practice of exclusively breastfeeding would include amelioration of undernutrition among the children. Exclusive breastfeeding has been recommended through a childhood undrernutrition control program provided by the government of Vietnam since 2000 to the present and covering all communes in the country (27); however, the percentage of mothers giving exclusive breastfeeding in the first 4 mo is still low in Vietnam and even lower than the global rate : 51% (28). In the present study, chronic energy malnutrition among mothers was also highly prevalent and mothers complained that "the child appeared hungry just after breastfeeding" and gave this as the reason for non-exclusive breastfeeding (data not shown). Further efforts to scale-up exclusive breastfeeding, such as monitoring and evaluation with a feedback system that allow for periodic program corrections and continued innovation (29), are expected.

As to the other risk factors of underweight in this study, low birth weight was the strong predictor of underweight. This observation is consistent with the findings of other studies in Vietnam (21) and other Asian countries (30, 31). In Vietnam, thanks to the economic improvement during the last two decades the prevalence of low birth weight has been decreasing, especially in urban areas ; however, the prevalence of low birth weight remains high in rural area (32). It has been demonstrated that the identificatio

in an early stage and immediate direct intervention such as extra macro- and micro-nutrition can help infants of low birth weight catch up with their heavier contemporaries (33). It has been also shown that appropriate breastfeeding and the quality of breast milk are also important to catch up to the normal nutritional status (34).

Although a marked negative relationship between diarrhea and the physical growth of children has been demonstrated in clinical and epidemiological studies (35-38) and the relative risk of diarrhea mortality is significantly increased for malnourished children especially among children aged 6 to 11 mo (39), this relationship was not observed in the present study. Since the incidence of diarrhea has seasonal variation and the survey was conducted in May, colder and drier season with a lower incidence of diarrhea, a further prospective longitudinal survey is needed.

In addition, socioeconomic factors such as older age, male gender, higher number of children aged under 5 yrs, lower family income, young mothers' age at giving birth and mothers' poor education those observed association with childhood undernutrition in previous studies in Vietnam (19, 21) and also in other Asian countries (27, 28, 40-42) were assumed to be predictors of childhood underweight in the present study. However, we did not observe any association between these socioeconomic factors and children's underweight in the logistic regression model. For economic status, inequality of income was reported in Vietnam and it was the lowest in the northern mountainous area compared to other regions (43). The mean family income in the present study was similar to the reported income in northern mountainous areas : 10.9000 VND (40). Significant association between economic status and childhood undernutrition has been observed when across different economic areas are plotted (19) ; however, the present study was conducted in a particular rural mountainous area, and the difference of family income might be too small to show the significant association to childhood underweight. Mother's young age at birth was assumed to be a risk factor for underweight because of lack of experience in child care ; however, younger mothers in the study area usually lived with their parents and could have support from them. Therefore the mother's younger age at birth may not be a risk factor for the childhood underweight. Lower education is considered to create difficulties in accessing skills, information and health care services (44), and several previous studies have reported a relationship between the mother's education level and the childhood underweight ; however, the small sample size may limit the opportunity to observe such a statistical association in the present study.

Other factors such as vitamin A deficiency, zinc deficiency and anemia continue to be serious problem in the public health of preschool children, particularly in children aged under 2 y in mountainous areas, and contribute to underweight in this population (22, 45). A further research is needed to assess their micronutrient status and efficacy of nutritive intervention for children aged under 2 y to be considered in the health system in northern mountainous Vietnam.

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Polyunsaturated Fatty Acids Selectively Suppress Sterol Regulatory Element-binding Protein-1 through Proteolytic Processing and Autoloop Regulatory Circuit*

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Sterol regulatory element-binding protein (SREBP)-1 is a key transcription factor for the regulation of lipogenic enzyme genes in the liver. Polyunsaturated fatty acids (PUFA) selectively suppress hepatic SREBP-1, but molecular mechanisms remain largely unknown. To gain insight into this regulation, we established *in vivo* reporter assays to assess the activities of *Srebf1c* transcription and proteolytic processing. Using these *in vivo* reporter assays, we showed that the primary mechanism for PUFA suppression of SREBP-1 is at the proteolytic processing level and that this suppression in turn decreases the mRNA transcription through lowering SREBP-1 binding to the SREBP-binding element on the promoter ("autoloop regulatory circuit"), although liver X receptor, an activator for *Srebf1c* transcription, is not involved in this regulation by PUFA. The mechanisms for PUFA suppression of SREBP-1 confirm that the autoloop regulation for transcription is crucial for the nutritional regulation of triglyceride synthesis.

Polyunsaturated fatty acids (PUFA)[3] have been well established as negative regulators of hepatic lipogenesis (reviewed in Ref. 1). Allmann and Gibson (2) discovered that adding 2% linoleate to a high carbohydrate fat-free diet suppressed the rate of hepatic fatty acid biosynthesis and the activities of fatty-acid synthase and glucose-6-phosphate dehydrogenase by nearly 70% in mice. In contrast, supplementing the high carbohydrate diet with palmitate, oleate, or cholesterol had no effect on hepatic lipogenesis or the activity of lipogenic enzymes. Since then, a number of investigators have demonstrated that dietary PUFA of the *n*-6 and *n*-3 families suppress hepatic lipogenesis. This anti-lipogenic action of PUFA reflects decreases in mRNA levels of hepatic enzymes, including acetyl-CoA carboxylase, fatty-acid synthase, and stearoyl-CoA desaturase.

The fatty acid biosynthetic pathway, composed of some 25 enzymes, has been elucidated in detail (3). For the *de novo* synthesis of long chain saturated fatty acids, fatty-acid synthase, the main synthetic enzyme that catalyzes the condensation of malonyl-CoA to produce the 16-carbon saturated fatty acid palmitate, and acetyl-CoA carboxylase, which synthesizes malonyl-CoA from acetyl-CoA, are of particular importance. The regulation of these lipogenic enzymes has been revealed to be primarily controlled by a transcription factor sterol regulatory element-binding protein (SREBP)-1c (4, 5).

SREBPs are transcription factors that belong to the basic helix-loop-helix leucine zipper family and are considered to be profoundly involved in the transcriptional regulation of cholesterogenic and lipogenic enzymes (6, 7). Unlike other members of the basic helix-loop-helix leucine zipper family, SREBPs are synthesized as precursors bound to the endoplasmic reticulum and nuclear envelope. Upon activation, SREBPs are cleaved, and the N-terminal parts are released from the membrane into the nucleus as mature protein by a sequential two-step proteolytic processing. To date, three SREBP isoforms, SREBP-1a, -1c, and -2, have been identified and characterized. SREBP-1a and -1c are transcribed from the same gene, each by a distinct promoter, and the predominant SREBP-1 isoform in liver is 1c rather than 1a (8). It has been established by several lines of evidence, especially by those from transgenic and knock-out mouse models, that SREBP-1c controls hepatic lipogenesis, whereas SREBP-2 plays a crucial role in regulation of cholesterol synthesis (5, 9, 10).

In 1999, we and others reported that the PUFA-specific suppression of lipogenic enzymes is mediated by the reduction of nuclear SREBP-1c protein in the liver (11–14). Interestingly, PUFA selectively decreases SREBP-1, not affecting SREBP-2. The mechanism by which PUFA specifically suppresses SREBP-1c nuclear abundance, however, remains unclear, although several potential mechanisms have been implicated, including suppression of *Srebf1c* gene transcription

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[3] The abbreviations used are: PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; LXR, liver X receptor; LXRE, liver X receptor-binding element; SREBP, sterol regulatory element-binding protein; SRE, SREBP-binding element; pfu, plaque-forming unit; OLA, oleic acid ethyl ester; shRNA, short hairpin RNA; SCAP, SREBP cleavage-activating protein.

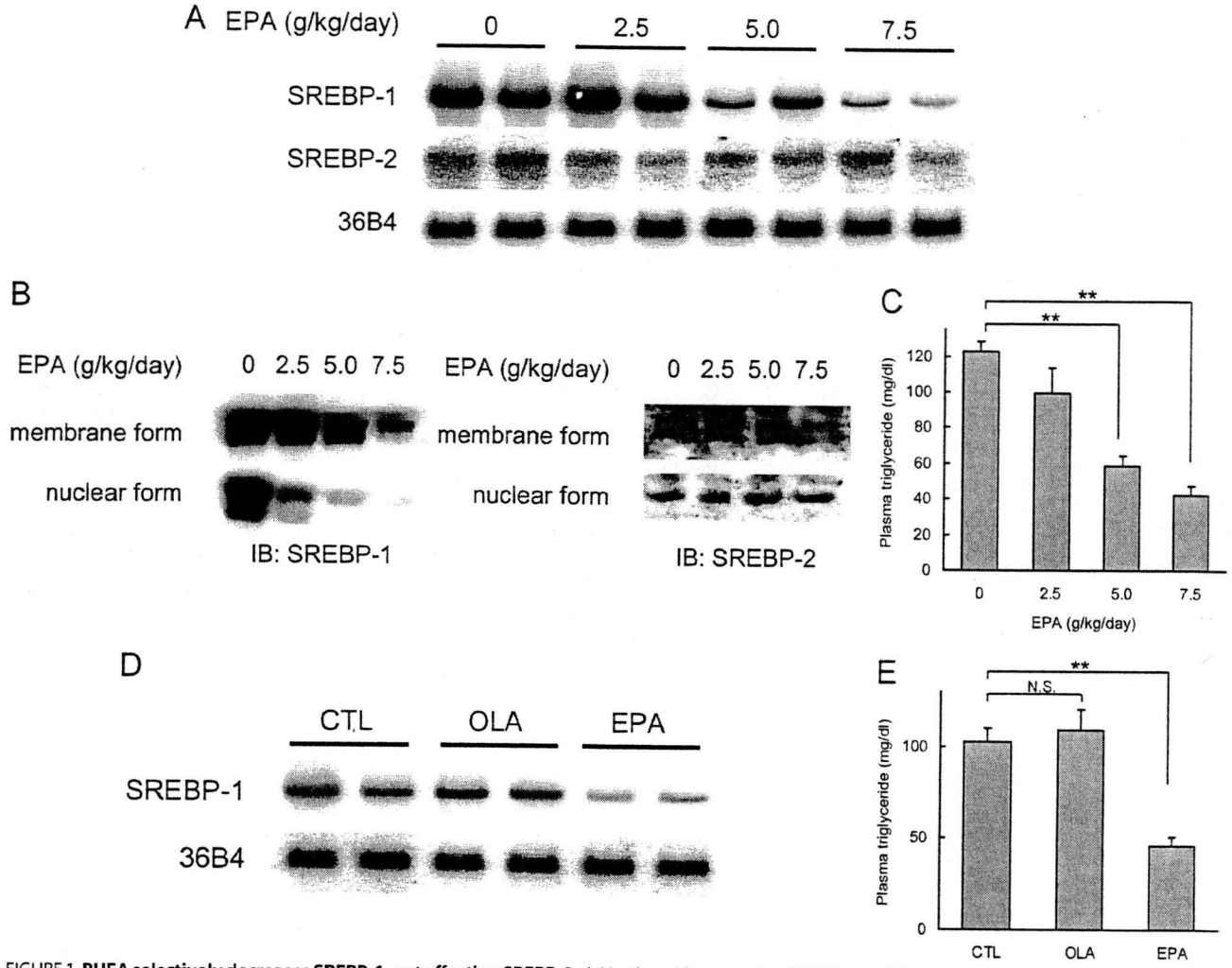


FIGURE 1. **PUFA selectively decreases SREBP-1, not affecting SREBP-2.** *A*, Northern blot analysis of SREBP-1 and SREBP-2 from livers. Total RNA (7.5 μg) from livers pooled equally from two mice for each group was subjected to Northern blotting to determine SREBP-1, SREBP-2, and 36B4 (used as a loading control) mRNA levels. ICR male mice were fed a high carbohydrate fat-free diet and treated orally with EPA at indicated doses once a day for 4 consecutive days. Control mice were treated orally with 7.5 g/kg water. Mice were sacrificed in a nonfasted state. *B*, immunoblot (*IB*) analysis of mature and precursor SREBP-1 and SREBP-2 proteins from livers. Aliquots of nuclear extracts (10 μg) and total proteins (50 μg) from livers pooled equally from four male mice for each group were subjected to immunoblot analysis. The primary antibodies used were polyclonal anti-mouse SREBP-1 and polyclonal anti-mouse SREBP-2. *C*, plasma triglycerides in EPA-treated mice. *D*, Northern blot analysis of SREBP-1 from livers. ICR male mice were fed a high carbohydrate fat-free diet and treated orally with 7.5 g/kg OLA or EPA once a day for 4 days. Total RNA (7.5 μg) from livers pooled equally from two mice for each group was subjected to Northern blotting to determine SREBP-1 and 36B4 (used as a loading control (*CTL*)) mRNA levels. *E*, plasma triglycerides in OLA- and EPA-treated mice. These data are representative of at least two independent experiments (n = 4 mice/group). Results are means ± S.E. **, $p < 0.01$; *N.S.*, not significant.

and proteolytic processing as well as enhancement of proteasomal degradation and mRNA decay (11, 15–18). As for the suppression of *Srebf1c* gene transcription by PUFA, we have previously identified liver X receptor-binding element (LXRE) and SREBP-binding element (SRE) on the *Srebf1c* promoter region by a series of promoter analyses (19, 20), and we have also suggested that PUFA can antagonize LXR in an *in vitro* setting (16).

These situations prompted us to clarify the molecular mechanism underlying the suppressive effect of PUFA on nuclear SREBP-1 abundance, especially in the *in vivo* setting. Because the inhibitory effect of fatty acids on SREBP-1 was specific and clear for PUFA in *in vivo* experiments, whereas many previous reports using *in vitro* system have failed to show this specificity (21, 22), we adopted an approach of *in vivo* reporter assays utilizing the *in vivo* imaging system (IVIS™; Xenogen, Alameda, CA). First, to examine the transcriptional mechanism, *in vivo* promoter analyses were performed, and the responsible cis-element on the *Srebf1c* promoter was located at SRE, not at LXRE. Next, the mechanism by which PUFA decreases the nuclear form of SREBP-1 was explored by another reporter system detecting proteolytic activity for the precursor form of SREBP-1, demonstrating that PUFA suppresses the maturation of SREBP-1 through proteolytic processes. From these experiments, we concluded that the primary mechanism for PUFA suppression of SREBP-1 expression is at the proteolytic processing level and that this suppression in turn decreases the *Srebf1c* mRNA transcription through lowering SREBP-1 binding to SRE on the promoter ("autoloop regulation" (19)).

EXPERIMENTAL PROCEDURES

Materials—Eicosapentaenoic acid (EPA) ethyl ester (95% grade) was provided from Mochida Pharmaceutical (Tokyo,

TABLE 1

Fatty acid composition in the liver

Fatty acid composition in the liver was analyzed by gas chromatography (n = 4 mice/group). Results are means ± S.E.

	EPA (g/kg/day)			
	0	2.5	5.0	7.5
	mg/g liver weight			
C12:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C14:0	0.22 ± 0.05	0.09 ± 0.03	0.05 ± 0.01[a]	0.07 ± 0.02[a]
C14:1*n*-5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C16:0	10.11 ± 1.07	8.06 ± 0.60	6.48 ± 0.40[a]	7.97 ± 0.58
C16:1*n*-7	2.06 ± 0.26	1.10 ± 0.18[a]	0.70 ± 0.04[b]	0.75 ± 0.13[b]
C18:0	3.12 ± 0.09	3.72 ± 0.17	3.13 ± 0.18	4.15 ± 0.18[b]
C18:1*n*-9	14.77 ± 2.96	7.76 ± 1.82[a]	3.70 ± 0.54[a]	4.88 ± 1.43[a]
C18:2*n*-6	1.76 ± 0.32	1.74 ± 0.23	1.92 ± 0.27	1.65 ± 0.30
C18:3*n*-6	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
C18:3*n*-3	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	0.00 ± 0.00
C20:0	0.04 ± 0.01	0.07 ±0.02	0.08 ± 0.02	0.08 ± 0.02
C20:1*n*-9	0.64 ± 0.17	0.22 ± 0.09	0.10 ± 0.02[a]	0.12 ± 0.04[a]
C20:2*n*-6	0.07 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	0.04 ± 0.00[a]
C20:3*n*-9	0.93 ± 0.06	0.18 ± 0.10[b]	0.07 ± 0.01[b]	0.08 ± 0.02[b]
C20:3*n*-6	0.44 ± 0.09	0.19 ± 0.02[a]	0.17 ± 0.03[a]	0.15 ± 0.01[a]
C20:4*n*-6	2.37 ± 0.33	1.79 ± 0.05	1.64 ± 0.15	1.54 ± 0.08[a]
C20:5*n*-3	0.12 ± 0.02	3.15 ± 0.31[b]	3.06 ± 0.17[b]	4.42 ± 0.35[b]
C22:0	0.09 ± 0.02	0.14 ± 0.03	0.16 ± 0.03	0.17 ± 0.04
C22:1*n*-9	0.04 ± 0.01	0.00 ± 0.00	0.03 ± 0.01	0.03 ± 0.01
C22:4*n*-6	0.11 ± 0.02	0.07 ± 0.01	0.06 ± 0.01[a]	0.06 ± 0.01[a]
C22:5*n*-3	0.05 ± 0.01	1.21 ± 0.33[a]	1.50 ± 0.23[b]	2.01 ± 0.44[b]
C22:6*n*-3	2.88 ± 0.23	3.60 ± 0.32	3.33 ± 0.34	3.65 ± 0.09[a]
C24:0	0.07 ± 0.01	0.10 ± 0.02	0.12 ± 0.01[a]	0.13 ± 0.02[a]
C24:1*n*-9	0.18 ± 0.01	0.18 ± 0.01	0.12 ± 0.02[a]	0.15 ± 0.01

[a] $p < 0.05$.
[b] $p < 0.01$.

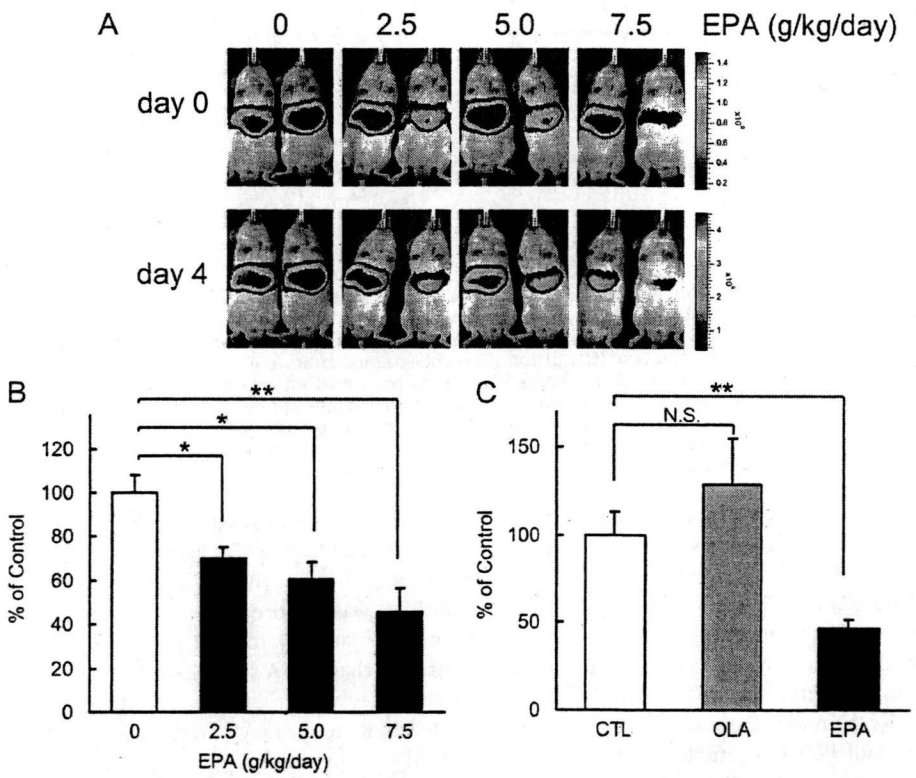


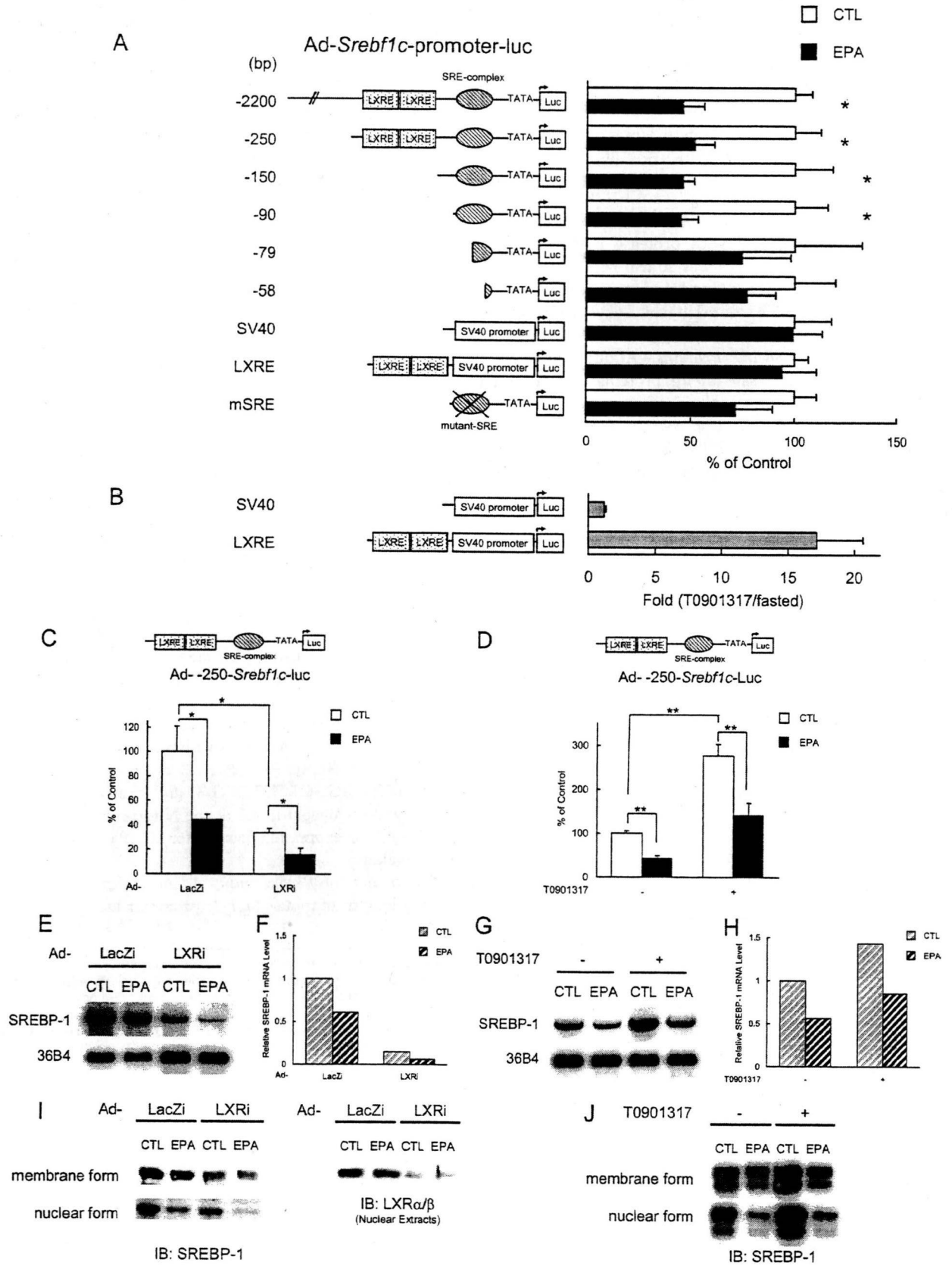
FIGURE 2. **PUFA suppresses *Srebf1c* promoter activity.** 2.2-kbp *Srebf1c*-Luc adenovirus (Ad-2.2k-*Srebf1c*-1c-Luc) (6.0×10^6 pfu/body) was intravenously injected into ICR male mice. After 4 days, mice (n = 4 for each group) were treated orally with EPA, OLA, or water (*CTL*) at indicated doses once a day for 4 days. *A*, on day 0 (before) and day 4 from the first treatment of EPA, luciferin was injected intraperitoneally in nonfasted mice, and the luminescence from liver was captured with IVIS. The *color overlay* on the image represents the photons/s emitted from the animal with a range of 1.5×10^5–1.5×10^6 photons/s (day 0), 5.0×10^5–5.0×10^6 photons/s (day 4), as indicated by the color scale next to the images. *B* and *C*, quantification of luciferase activity with LivingImage software. Fold changes of luciferase activity on day 4 *versus* day 0 are shown. These data are representative of at least two independent experiments (n = 4 mice/group). Results are means ± S.E. *, $p < 0.05$; **, $p < 0.01$, respectively. *N.S.*, not significant.

Japan) and GW532 (SCAP ligand) from GlaxoSmithKline (Les Ulis Cedex, France). The synthetic LXR agonist T0901317 was purchased from Cayman Chemical (Ann Arbor, MI). Standard laboratory diet (CRF-1, composed of 60% carbohydrate, 13% fat, and 27% protein on a caloric basis) and high carbohydrate fat-free diet (70% sucrose and 20% casein supplemented with methionine, vitamins, and minerals) were obtained from Oriental Yeast (Tokyo, Japan). Other materials were purchased from Sigma unless indicated otherwise.

Animals—Seven- to 9-week-old ICR male mice were purchased from CLEA (Tokyo, Japan). All animals were maintained in a temperature-controlled environment with a 12-h light/dark cycle and were given free access to standard laboratory diet and water. Four days before the start of indicated fatty acid administration, the basal diet was switched to a high carbohydrate fat-free diet. EPA or oleic acid ethyl ester (OLA) was administered orally once a day for 4 days. GW532 (0–15 mg/kg/day) or vehicle (0.9% carboxymethylcellulose, 9.95% polyethylene glycol 400, and 0.05% Tween 80) was administered orally once a day for 3 days. Mice were sacrificed in the light phase in a nonfasted state. All experiments were repeated at least twice. All animals studied were anesthetized and euthanized according to protocol approved by the Tokyo University Animal Care and Use Committee.

RNA Isolation and Northern Blotting—Total RNA from mouse liver was extracted using TRIzol reagent (Invitrogen), and a 7.5-μg RNA sample equally pooled among each group was run on a 1% agarose gel containing formaldehyde and transferred to a nylon membrane. The cDNA probes for mouse SREBP-1, SREBP-2, and 36B4 were cloned as described previously (11). The probes were labeled with [α-^{32}P]dCTP using Megaprime DNA labeling system (Amersham Biosciences). The membranes were hybridized with the radiolabeled probe in RapidHyb Buffer (Amersham Biosciences) and washed in 0.1× SSC, 0.1% SDS at 65 °C. Blots were exposed to BAS imaging plate for the BAS2000 BIO imaging analyzer (Fuji Photo Film). The quantification results obtained with the BAS2000 system were normalized to the signal generated from 36B4 mRNA.

Nuclear Protein Extraction from Liver—Nuclear extract protein from mouse or rat liver was prepared as described previously (23). Briefly, excised livers (0.5 g) were homogenized in a Polytron in 5 ml of buffer A, which consisted of 10 mM HEPES, pH 7.9, 25 mM KCl, 1 mM EDTA, 2 M sucrose, 10% glycerol, 0.15 mM



spermine, and 2 mM spermidine, supplemented with protease inhibitors (6 μg/ml *N*-acetyl-leucyl-leucyl-norleucinal (ALLN, Calbiochem), 2.5 μg/ml pepstatin A, 2 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride and 2.5 μg/ml aprotinin). Pooled homogenate was then subjected to one stroke of a Teflon pestle in a Potter-Elvehjem homogenizer, followed by filtration through two layers of cheesecloth, and layered over 10 ml of buffer A. After centrifugation at 24,000 rpm on a Beckman SW28 rotor for 1 h at 4 °C, the resulting nuclear pellet was resuspended in a buffer containing 10 mM HEPES, pH 7.9, 100 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol supplemented with protease inhibitors, after which 0.1 volume of 5 M NaCl was added. Each mixture was agitated gently for 30 min at 4 °C and then centrifuged at 89,000 rpm on a Himac S120AT2 rotor (Hitachi, Tokyo, Japan) for 30 min at 4 °C. The supernatant was used as nuclear extract.

Immunoblotting of SREBP Proteins—Aliquots of nuclear extract (10 μg) and total lysate (50 μg) proteins were subjected to SDS-PAGE. Immunoblot analysis was performed using the ECL Western blotting detection system (Amersham Biosciences) and exposed to XAR-5 film (Eastman Kodak Co.). The primary antibodies for SREBPs (rabbit polyclonal; number 931 for mouse SREBP-1 and number 528 for SREBP-2) were used as described previously (24). The primary antibody for LXRα/β (H-144; sc-13068) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Fatty Acid Composition of Liver—Fatty acid composition was measured as described previously (24). An aliquot (0.1 g) of liver samples snap-frozen by liquid nitrogen was homogenized in 1 ml of normal saline. The fatty acid composition was measured by gas chromatography at Bio-Medical Laboratories (Tokyo, Japan) (25) Briefly, total lipids in liver homogenates were extracted according to the Folch's procedure (26), followed by transesterification of fatty acids with boron trifluoride/methanol at 100 °C for 90 min. The methylated fatty acids were then extracted with hexane and analyzed using a GC-17A gas chromatograph (Shimadzu Corp., Kyoto, Japan) and BPX70 capillary column (0.25 mm inner diameter × 30 m, SGE International Ltd., Melbourne, Australia).

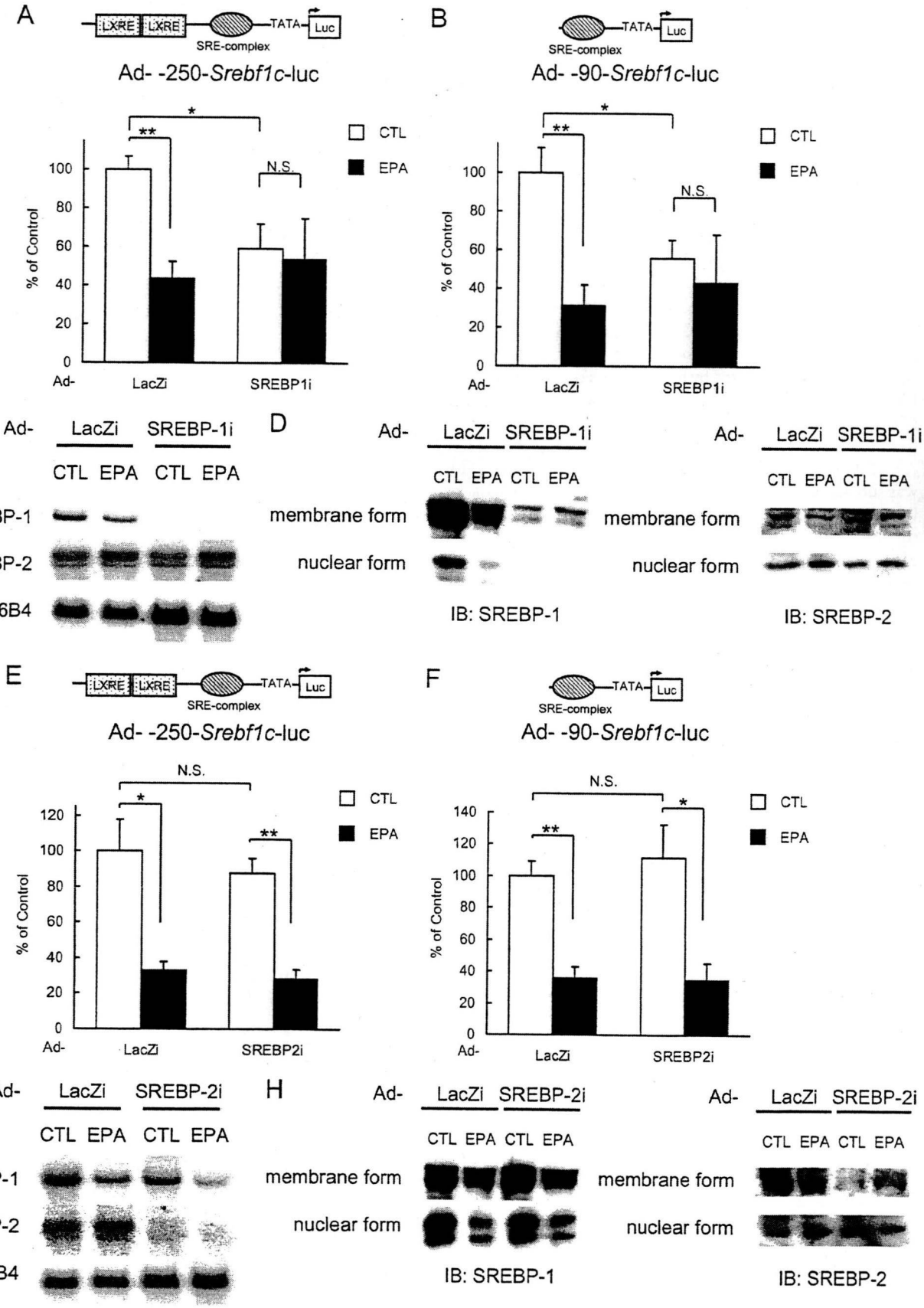
Transfection and Luciferase Assays—HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate supplemented with 10% fetal bovine serum. For luciferase assay, HEK293 cells were seeded in a 48-well plate and incubated until 80% confluent. The indicated amounts of expression plasmids, firefly luciferase reporter plasmid, and pSV40-*Renilla* luciferase plasmid were co-transfected into HEK293 cells using SuperFect transfection reagent (Qiagen) according to the manufacturer's protocol. Total amounts of transfected DNA were adjusted with empty vector. The luciferase activity in transfectants was measured on a luminometer. *Renilla* luciferase activities were used to normalize transfection efficiencies.

In Vivo Imaging of Luciferase Activity—In vivo imaging was performed as described previously (27). Mice were anesthetized with isoflurane/oxygen, and 3.0 mg of luciferin dissolved in 0.4 ml of phosphate-buffered saline (7.5 mg/ml) was injected into the intraperitoneal cavity. Mice were imaged from the ventral side using an In Vivo Imaging System (IVIS™, Xenogen) 15 min following the injection of luciferin. Relative photon emission over the liver region was quantified using LivingImage™ software (Xenogen).

Plasmid Construction—To construct expression plasmids for GAL4-DNA binding domain (GAL4-DBD) and VP16-transactivation domain (VP16-AD) fused to human SREBPs, VP16-AD from pACT vector (Promega) was inserted into pM vector (Clontech) with various lengths of DNA fragment of human SREBP-1c (amino acids 1–1123 ("FL" for full length), 1–436 ("Nuc" for N-terminal nuclear part), or 431–1123 ("Reg" for C-terminal regulatory domain) or human SREBP-2 (amino acids 14–1141 (FL) or 450–1141 (Reg)) retrieved by PCR from pTK-HSV-hSREBP-1c, pTK-HSV-hSREBP-2, and pcDNA3.1(+)-SREBP-1c (28, 29). Gal4-RE-Luc plasmid was described previously (30). For the construction of an expression plasmid for mouse Insig-1, cDNA fragment was amplified by PCR with primers 5′-GGATCCATGCCCAGGCTGCACGACCACG-3′ and 5′-CTCGAGTCAGTCACTGTGAGGCTTTTCCG-3′ and cloned into pcDNA3 vector with hemagglutin tag at the N terminus using BamHI and XhoI. The expression plasmid for SCAP is a kind gift from Dr. Nakakuki.

Preparation of Recombinant Adenoviruses—To construct various lengths of mouse *Srebf1c* promoter luciferase reporter

FIGURE 3. **PUFA suppresses *Srebf1c* promoter activity through SRE site.** *A*, adenoviruses encoding various lengths of *Srebf1c* promoter, as well as two LXRE sites on *Srebf1c* promoter (−239 to −165) with SV40 promoter and SRE mutant version of 90-bp *Srebf1c* promoter, attached with luciferase (*Luc*) (6.0×10^6 pfu/body) were injected intravenously into ICR male mice. After 4 days, mice ($n = 4–7$ for each group) were treated orally with 7.5 g/kg EPA or water (*CTL*) once a day for 4 days. On day 0 and 4 after the first treatment of EPA, luciferin was injected intraperitoneally, and the luminescence from liver was captured with IVIS. *B*, Ad-LXRE-Luc injected mice were administered orally with 50 mg/kg T0901317 or vehicle (0.9% carboxymethylcellulose, 9.95% polyethylene glycol 400, and 0.05% Tween 80) after fasting overnight. At 0 and 16 h following T0901317 treatment in fasted states, luciferin was injected intraperitoneally, and the luminescence from liver was captured with IVIS. Fold change of luciferase activity at 16 *versus* 0 h was presented. *C*, *E*, *F*, and *I*, knockdown of hepatic LXRα/β by adenoviral expression of shRNA. 250-bp *Srebf1c*-Luc adenovirus (Ad-250-bp- *Srebf1c*-Luc; 6.0×10^6 pfu/body) plus adenovirus expressing LXRα/β-specific or LacZ-specific shRNA (Ad-LXRi or Ad-LacZi, respectively; 2.5×10^8 pfu/body) were intravenously injected into ICR male mice. After 4 days, the mice ($n = 4$ for each group) were treated orally with 7.5 g/kg EPA or water (*CTL*) once a day for 4 days. On day 0 and 4 after the first treatment of EPA, luciferin was injected intraperitoneally, and the luminescence from liver was captured with IVIS. *D*, *G*, *H*, and *J*, inhibitory effect of PUFA on *Srebf1c* gene transcription is not affected by LXR agonist. 250-bp *Srebf1c*-Luc adenovirus (Ad-250bp- *Srebf1c*-Luc) (6.0×10^6 pfu/body) was intravenously injected into ICR male mice. After 6 days, the mice ($n = 4$ for each group) were treated orally with 7.5 g/kg EPA or water (*CTL*) once a day for 4 days. At day 3 of EPA treatment, the mice were administered orally with 10 mg/kg T0901317 or vehicle. On days 0 and 4 after the first treatment of EPA, luciferin was injected intraperitoneally, and the luminescence from liver was captured with IVIS. *C* and *D*, quantification of luciferase activity with LivingImage software. Fold changes of luciferase activity on day 4 *versus* day 0 are shown. *E* and *G*, Northern blot analysis of SREBP-1 from livers. Total RNA (7.5 μg) from livers pooled equally from mice of each group was subjected to Northern blotting to determine SREBP-1 and 36B4 (used as a loading control) mRNA levels. *F* and *H*, quantification of the data shown in *E* and *G*. The fold change is the relative ratio of each signal *versus* the control mice. *I* and *J*, immunoblot (*IB*) analysis of mature and precursor SREBP-1 proteins and LXRα/β in livers. Aliquots of nuclear extracts (10 μg) and total proteins (50 μg) from livers pooled equally from four male mice of each group were subjected to immunoblot analysis. The primary antibodies used were polyclonal anti-mouse SREBP-1 and polyclonal anti-mouse LXRα/β. These data are representative of at least two independent experiments ($n = 4–7$ mice/group). Results are means ± S.E. *, $p < 0.05$, and **, $p < 0.01$ *versus* controls.



plasmids, DNA fragments retrieved from pGL2 vectors constructed previously (19, 20, 31) were inserted into pGL3 basic vector plasmids (Promega). The fragments including promoter region linked to luciferase reporter gene were inserted into the Gateway entry vector pENTR4 (Invitrogen) and generated by homologous recombination between the entry vector and the pAd promoterless vector (Invitrogen). The fragments of human SREBPs GAL4-DBD and VP16-AD fusion protein from various GAL4-SREBP vectors were inserted into pENTR4 and generated by homologous recombination with the pAd/CMV/V5-DEST vector (Invitrogen). Adenoviruses encoding SREBP-1-specific and LacZ-specific shRNA for RNA interference (SREBP1i and LacZi, respectively) were described previously (32). Adenovirus construct encoding SREBP-2-specific shRNA (SREBP2i) targeting 5′-GGAGCAGTCTCAACGTCAACG-3′ sequence on SREBP-2 was subcloned into U6 entry vector (Invitrogen) and generated by homologous recombination with the pAd promoterless vector. Adenovirus construct encoding both LXRα and LXRβ shRNA (LXRi) targeting 5′-ACAGCTC-CCTGGCTTCCTA-3′ sequence on LXRα (33) and 5′-CTAC-AACCACGAGACAGAA-3′ sequence on LXRβ, respectively, was subcloned into U6 entry vector (Invitrogen) and generated by homologous recombination with the pAd promoterless vector. Recombinant adenoviruses were propagated in HEK293 cells and purified by CsCl gradient centrifugation as described previously (34).

RESULTS

PUFA Selectively Decreases SREBP-1, Not Affecting SREBP-2—In our first series of experiments, we verified the effects of PUFA on SREBP-1 and -2 expression in the liver. As expected, administration of EPA, one of the major *n*-3 PUFA in mammals, down-regulated SREBP-1 mRNA and protein expression (Fig. 1, *A, B, D,* and *E*), leading to a decrease in plasma triglycerides concentration (Fig. 1, *C* and *F*) as compared with control or oleate. The delivery of orally administered EPA to the liver was confirmed by gas chromatography analysis (Table 1). EPA administration did not affect the body weights of mice (data not shown). The dose-response relationship data suggested that the nuclear form is more sensitive to PUFA than the membrane form of protein or mRNA. In contrast, SREBP-2 mRNA and protein levels were not altered by PUFA.

PUFA Suppresses Srebf1c Promoter Activity through SRE Site—Next, we attempted to estimate the *Srebf1c* promoter activity in the liver with an *in vivo* luciferase reporter analysis. The luciferase reporter gene driven by the 2200-bp *Srebf1c* promoter was adenovirally transduced into mouse liver, and the transcriptional activity was assessed by measuring luciferase activity with the IVIS imaging system. The physiological activity of this promoter had previously been confirmed by transgenic mice (31). As shown in Fig. 2, EPA decreased the *Srebf1c* promoter activity in a dose-dependent manner.

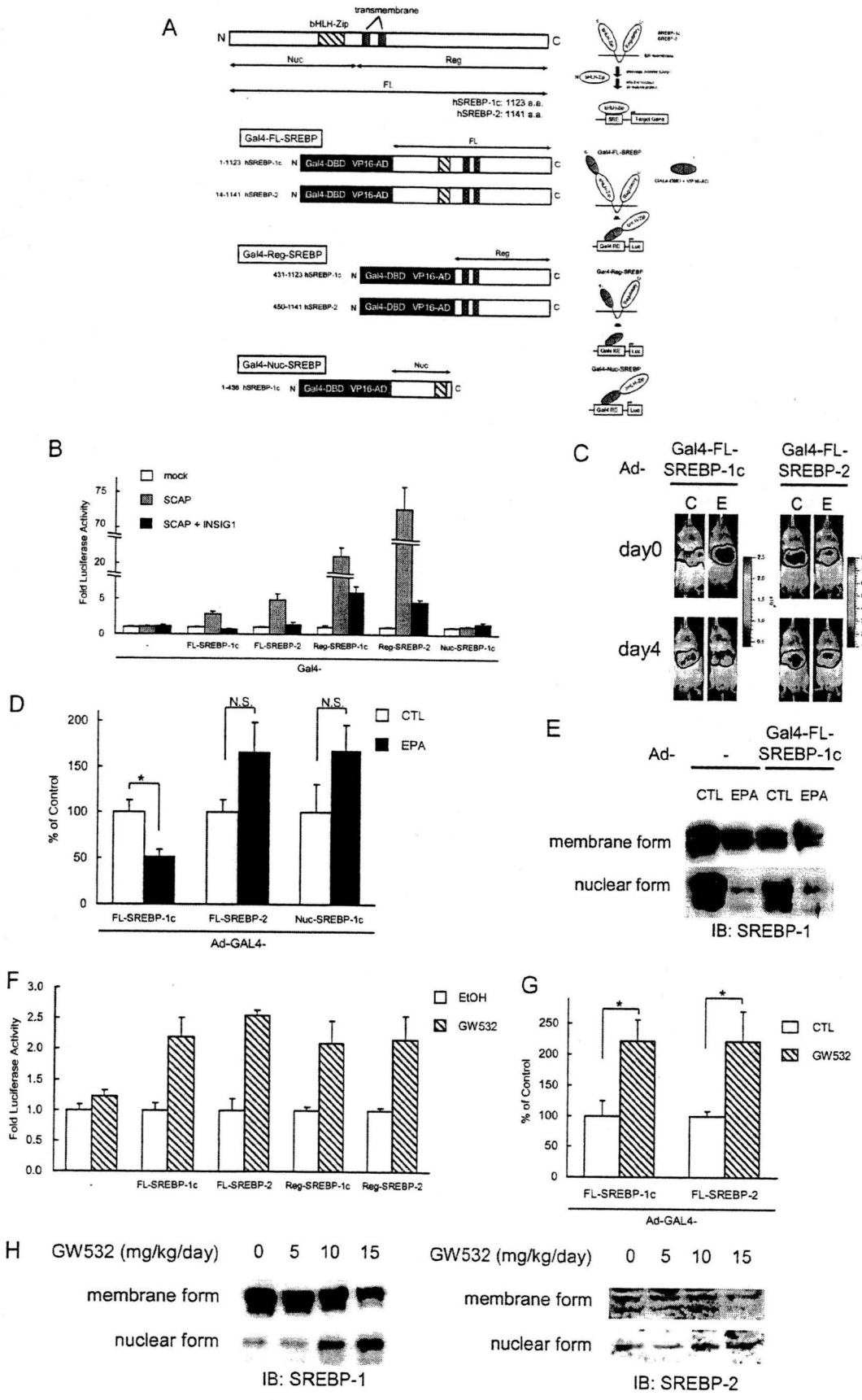
To determine the responsible region for PUFA suppression, a promoter deletion study was performed; six adenovirus constructs containing different lengths of the *Srebf1c* promoter (ranging from 2200 to 58 bp) were transduced into mouse livers, and the suppressive effect of EPA on promoter activity was assessed with IVIS (Fig. 3*A*). From this experiment, the responsible element was located at the SRE site within −90 to −60 bp upstream of the transcription start site. This result was confirmed by another experiment using a mutant SRE construct.

LXR Is Not Involved in the PUFA Suppression of Srebf1c Gene Expression in Vivo—Furthermore, it was clarified from the series of deletion studies that EPA did not suppress the promoter activity of a construct containing only LXRE (Fig. 3*A*), demonstrating that EPA does not antagonize LXR binding to LXRE on the *Srebf1c* promoter at least in the *in vivo* setting. Moreover, as shown in Fig. 3, *C, E, F,* and *I*, it was demonstrated that the simultaneous knockdown of both LXRα and -β did not affect the suppressive effect of PUFA on *Srebf1c* promoter activity. Furthermore, the stimulation of LXR by an LXR agonist T0901317 was also shown to be independent of the inhibitory effect of PUFA, as shown in Fig. 3, *D, G, H,* and *J*. Based on these findings, we concluded that LXR is not the direct target of PUFA regulation in the *in vivo* setting, although LXR is a determinant of the expression level of SREBP-1 mRNA.

Inhibitory Effect of PUFA on Srebf1c Gene Transcription Is Mediated through SREBP-1 Itself—To determine whether the trans-acting factor for SRE is SREBP-1 or -2 or both, SREBP-1/2 was knocked down by RNA interference. As shown in Fig. 4, *A* and *B*, the effects of PUFA administration and knocking down SREBP-1 were essentially equal, and when SREBP-1 was knocked down, the promoter activity of *Srebf1c* gene was reduced by half, and PUFA showed no additive effects. In contrast, knocking down SREBP-2 did not affect the promoter activity of *Srebf1c* as assessed by luciferase reporter expression (Fig. 4, *E* and *F*), demonstrating that SREBP-2 is not involved in the transcriptional regulation of *Srebf1c* gene expression. From these findings, it was concluded that the trans-acting factor for SRE on the *Srebf1c* promoter is SREBP-1 and not SREBP-2. This indicates that SREBP-1 constitutes an autoloop regulatory circuit.

PUFA Suppresses the Proteolytic Activation of SREBP-1, Not Affecting SREBP-2—SREBP-1 is synthesized as a precursor bound to the endoplasmic reticulum and nuclear envelope

FIGURE 4. **Inhibitory effect of PUFA on *Srebf1c* gene transcription is mediated through SREBP-1 itself.** *A–H,* knockdown of hepatic SREBP-1 (*A–D*) or SREBP-2 (*E–H*) by adenoviral expression of shRNA. 250- or 90-bp *Srebf1c*-Luc adenovirus (Ad-250bp- *Srebf1c*-Luc or Ad-90bp- *Srebf1c*-Luc; 6.0×10^6 pfu/body) plus adenovirus expressing SREBP-1-specific, SREBP-2-specific, or LacZ-specific shRNA (Ad-SREBP1i, Ad-SREBP2i, or Ad-LacZi; 2.5×10^8 pfu/body) were intravenously injected into ICR male mice. After 4 days, the mice ($n = 3–6$ for each group) were treated orally with 7.5 g/kg EPA or water (*CTL*) once a day for 4 days. On days 0 and 4 after the first treatment of EPA, luciferin was injected intraperitoneally, and the luminescence from liver was captured with IVIS. *A, B, E,* and *F,* quantification of luciferase activity with LivingImage software. Fold changes of luciferase activity on day 4 *versus* day 0 are shown. *C* and *G,* Northern blot analysis of SREBP-1 and -2 in the liver. Total RNA (7.5 μg) from livers pooled equally from mice for each group was subjected to Northern blotting to determine SREBP-1, -2, and 36B4 (used as a loading control) mRNA levels. *D* and *H,* immunoblot (*IB*) analysis of mature and precursor SREBP-1 and -2 proteins in the liver. Aliquots of nuclear extracts (10 μg) and total proteins (50 μg) from livers pooled equally from four male mice of each group were subjected to immunoblot analysis. The primary antibodies used were polyclonal anti-mouse SREBP-1 and polyclonal anti-mouse SREBP-2. These data are representative of at least two independent experiments ($n = 3–6$ mice/group). Results are means ± S.E. *, $p < 0.05$, and **, $p < 0.01$, respectively.



and is released from the membrane into the nucleus as a mature protein by a cleavage process (7). To explore the molecular mechanism by which PUFA decreases the nuclear form of SREBP-1, another reporter system to detect proteolytic activity for the precursor form of SREBPs was constructed (Fig. 5*A*). In this system, Gal4-DNA binding domain and VP16 activation domain were attached to the N terminus of SREBPs, and the nuclear entry of this N-terminal fragment was measured by the Gal4-UAS system to assess the proteolytic release of the SREBP N termini. Using this cleavage-detecting reporter system, the proteolytic activities for precursor forms of SREBP-1 or -2 were tested *in vitro* with SCAP and insulin-induced gene (INSIG)-1 overexpressions as an activator and an inhibitor for cleavage of SREBP, respectively. As expected, the SCAP overexpression drastically enhanced the cleavage of N-terminal fragments of both SREBP-1 and -2, and the co-expression of INSIG1 reversed the effect of SCAP overexpression (Fig. 5*B*), indicating that this reporter system reflects the physiological regulation of SREBP activation processes by proteolysis. Next, we transduced the reporter genes into mouse livers using adenoviruses and examined the effect of PUFA in the *in vivo* setting. As shown in Fig. 5, *C* and *D*, EPA suppressed only the SREBP-1 cleavage-detecting reporter but did not affect the SREBP-2 reporter. Additionally, EPA did not decrease the reporter activity from the construct that contains only the N terminus (designated as Nuc-SREBP-1c) and enters the nucleus without cleavage, demonstrating that EPA did not accelerate the degradation of reporter fragment. In contrast, GW532, a SCAP activator, enhanced the cleavage of both SREBP-1 and -2 in the *in vitro* (Fig. 5*F*) and *in vivo* (Fig. 5*H*) situations.

DISCUSSION

This study has clearly demonstrated that the primary mechanism of the inhibitory effect of PUFA is the suppression of the proteolytic activation of SREBP-1 and that the transcriptional regulation is secondary to this post-translational suppression of mature SREBP-1 that itself binds to the SRE site on the *Srebf1c* promoter (autoloop regulation (19)), whereas LXR is not involved in the transcriptional regulation by PUFA (Fig. 6).

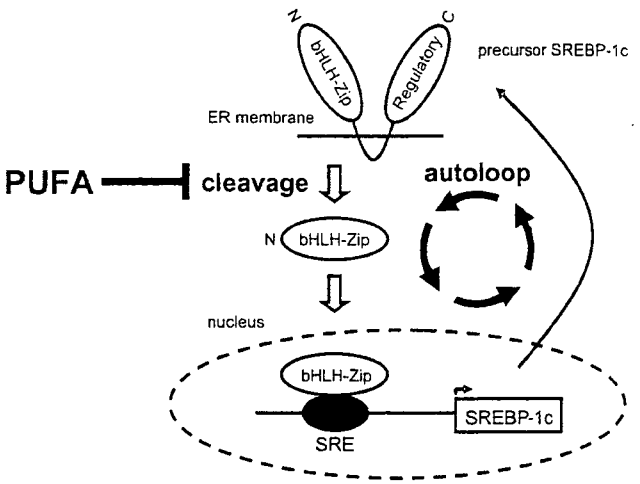


FIGURE 6. **Schematic representation of molecular mechanisms for inhibitory effects of PUFA on SREBP-1.** The primary mechanism for PUFA suppression of SREBP-1 is at the proteolytic processing level, and this suppression in turn decreases the mRNA transcription through lowering SREBP-1 binding to SRE on the promoter (autoloop regulatory circuit), although LXR is not involved in this regulation.

This is the first report that has clearly demonstrated the inhibitory effect of PUFA on the SREBP-1 proteolytic processing *in vivo*. This result is consistent with our previous report showing that PUFA decreases nuclear SREBP-1, although it does not affect the nuclear abundance of the truncated form of SREBP-1 expressed from a transgene, suggesting that PUFA

FIGURE 5. **PUFA suppresses the proteolytic activation of SREBP-1, not affecting SREBP-2.** *A*, various regions of human SREBP-1c (amino acids (*a.a.*) 1–1123 (FL), 431–1123 (Reg), 1–436 (Nuc)) and human SREBP-2 (amino acids 14–1141 (FL), 450–1141 (Reg)) were fused to Gal4-DNA binding domain and VP16-transactivation domain. *FL*, full length; *Nuc*, nuclear; *Reg*, regulatory; *bHLH-Zip*, basic helix-loop-helix leucine zipper. GAL4-VP16-SREBP fusion protein is attached to the endoplasmic reticulum (*ER*) similarly as endogenous SREBP protein. Upon activation, its N-terminal region is released from the membrane into the nucleus by a cleavage process. The GAL4-VP16 promotes the luciferase (*Luc*) reporter gene expression by binding to GAL4-RE containing eight copies of upstream activation sequence, Gal4-binding site. *B*, regulation of cleavage of GAL4-VP16 fusion SREBP protein by SCAP and INSIG-1. HEK293 cells in a 48-well plate were co-transfected with GAL4-RE-Luc plasmid (100 ng/well), expression plasmids of GAL4-VP16 fusion SREBP protein (100 ng/well), SCAP (250 ng/well), INSIG-1 (100 ng/well), and pSV40-Renilla plasmid (50 ng/well). Cells were harvested 24 h after transfection. The firefly luciferase activity was measured and normalized by *Renilla* luciferase activity. All experiments were performed in triplicate. *C–E*, regulation of cleavage of GAL4-VP16 fusion SREBP protein by EPA *in vivo*. GAL4-RE-Luc adenovirus (Ad-GAL4-RE-Luc, 2.0×10^8 pfu/body) plus adenovirus expressing GAL4-VP16 fusion SREBP protein (Ad-Gal4-FL-SREBP-1c, Ad-Gal4-FL-SREBP-2 or Ad-Gal4-Nuc-SREBP-1c, 1.0×10^8 pfu/body) were intravenously injected into ICR male mice. After 2 days, the mice (n = 7–9 for each group) were treated orally with 7.5 g/kg EPA or water (*CTL*) once a day for 4 days. *C*, 0 and 4 days after the fast treatment of EPA, luciferin was injected intraperitoneally, and the luminescence from liver was captured with IVIS. The color overlay on the image represents the photons/s emitted from the animal with a range of 4.0×10^5–2.5×10^6 photons/s (Ad-Gal4-FL-SREBP-1c) and 1.0×10^6–8.0×10^6 photons/s (Ad-Gal4-FL-SREBP-2), as indicated by the *color scale* next to the images. *D*, quantification of luciferase activity with LivingImage software. Fold changes of luciferase activity on day 4 *versus* day 0 are shown. *E*, immunoblot analysis of mature and precursor SREBP-1 proteins from livers. Aliquots of nuclear extracts (10 μg) and total proteins (50 μg) from livers pooled equally from male mice for each group were subjected to immunoblot analysis. The primary antibodies used were polyclonal anti-mouse SREBP-1. *N.S.*, not significant. *F–H*, SCAP ligand GW532 accelerates SREBP-1 cleavage. *F*, regulation of cleavage of GAL4-VP16 fusion SREBP protein by GW532. HEK293 cells in a 48-well plate were co-transfected with GAL4-RE-Luc plasmid (100 ng/well), expression plasmids for GAL4-VP16 fusion SREBP protein (100 ng/well), and pSV40-*Renilla* plasmid (50 ng/well). 3 h after transfection, GW532 (1 μM) or EtOH was added to media, and cells were harvested 24 h after transfection. The firefly luciferase activity was measured and normalized by *Renilla* luciferase activity. All experiments were performed in triplicate. *G* and *H*, GAL4-RE-Luc adenovirus (Ad-GAL4-RE-Luc, 2.0×10^8 pfu/body) plus adenovirus expressing GAL4-VP16 fusion SREBPs protein (Ad-Gal4-FL-SREBP-1c or Ad-Gal4-FL-SREBP-2, 1.0×10^8 pfu/body) were intravenously injected into ICR male mice. After 2 days, the mice (n = 8 for each group) were administered orally with GW532 or vehicle at indicated doses. 6 h after the last treatment, luciferin was injected intraperitoneally, and the luminescence from liver was captured with IVIS. *G*, quantification of luciferase activity with LivingImage software. Fold changes on day 3 *versus* day 0 are shown. *H*, immunoblot analysis of mature and precursor SREBP-1 and SREBP-2 proteins from livers. Aliquots of nuclear extracts (10 μg) and total proteins (50 μg) from livers pooled equally from four male mice for each group were subjected to immunoblot (*IB*) analysis. The primary antibodies used were polyclonal anti-mouse SREBP-1 and anti-mouse SREBP-2. These data are representative of at least two independent experiments (n = 3–8 mice/group). Results are means ± S.E. *, $p < 0.05$.

does not accelerate the degradation of nuclear SREBP-1 protein (11).

We have shown that two mechanisms are involved in the PUFA regulation on SREBP-1; one is at the proteolytic processing level, and the other is at the transcription level. What is the physiological role of this two-step regulation? As we have shown in Fig. 1, the proteolytic mechanism is high sensitive, although the transcriptional mechanism is low sensitive, consistent with the previous report by Ezaki and co-workers (35). The combination of these two steps of regulation with different sensitivity connected in series probably helps to achieve the broader responsive range of the amount of PUFA.

One of our conclusions is that LXR is not involved in the transcriptional regulation by PUFA, but controversy exists over this point; several previous reports suggested the involvement of LXR in this regulation (15, 16), whereas others did not (36). These previous studies have been all performed in *in vitro* settings, and therefore we evaluated the contribution of the LXR pathway in the *in vivo* setting for the first time, and we have concluded that the involvement of LXR is not detectable, although LXR is an important determinant of the SREBP-1 expression level.

Because an *in vitro* model is always only a small part of the whole *in vivo* system, how large or small the contribution of a regulatory pathway elucidated *in vitro* is in the whole *in vivo* system cannot be estimated until it is assessed in the in *vivo* setting. To address these issues, our approach of the extension of *in vitro* reporter assays to *in vivo* settings will be very useful in various situations.

We have demonstrated that PUFA selectively suppresses the proteolytic processing of SREBP-1, but the molecular mechanism underlying this SREBP-1-specific regulation is currently unknown. SCAP escorts both SREBP-1 and -2 to the Golgi, and SREBP-1-specific adaptor protein has not been reported yet. Recently, an endoplasmic reticulum membrane protein TRC8 (translocation in renal cancer from chromosome 8) has been documented to hamper endoplasmic reticulum-to-Golgi transport of SREBP-2/SCAP and reduce SREBP-2 cleavage specifically (37). Perhaps there might be some adaptor molecule that specifically interacts with SREBP-1 and mediates the suppressive effect of PUFA, although we have no evidence. If the molecular mechanism underlying this SREBP-1-specific effect of PUFA is clarified in the future, it will be a potential molecular target for new lipid-lowering drugs. In conclusion, the primary mechanism for PUFA suppression of SREBP-1 expression is at the proteolytic processing level and that this suppression in turn decreases the transcription of *Srebf1c* through lowering SREBP-1 binding to SRE on the promoter.

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