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Utility and Limitations of SP600125, an Inhibitor of Stress-Responsive c-Jun N-Terminal Kinase

Shuhei Tanemura[1,2], Tokiwa Yamasaki[1,2], Toshiaki Katada[2] and Hiroshi Nishina*,[1]

[1]*Department of Developmental and Regenerative Biology, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan*

[2]*Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

Abstract: Stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) belongs to the mitogen-activated protein kinase (MAPK) family and plays an important role in many biological contexts. JNK is deeply involved in several serious human disorders, including inflammation, obesity, diabetes, neuronal disease and cancer. Accordingly, JNK has been recognized as an appropriate target for the treatment of these diseases, and much effort has been expended over the past 15 years to isolate JNK inhibitors that can inactivate this kinase. In 2001, the compound SP600125 was reported as the first JNK-specific inhibitor. Many researchers have subsequently employed SP600125 in *in vitro* and *in vivo* models to evaluate whether certain disease-associated events are JNK-dependent. Indeed, more than 1300 studies citing the use of SP600125 as a JNK inhibitor in cell cultures and animal models have been reported. However, although SP600125 has been employed successfully to inhibit JNK in several situations, there have been questions about its specificity for JNK. SP600125 can bind to a broad range of protein kinases and inhibit some of them with similar or greater potency than JNK, confirming that many additional kinases may be targets of SP600125. In this article, we review both the usefulness of SP60125 as a JNK inhibitor and the limitations to its specificity.

Keywords: JNK, MAP kinase, SP600125, kinase inhibitor, mast cell, IgE, PI3K, allergy.

I. INTRODUCTION

The mitogen-activated protein kinases (MAPKs) are a family of kinases that transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli [1-7]. Upon stimulation, MAPKs phosphorylate their specific substrates at serine and/or threonine residues. Such phosphorylation events can either positively or negatively regulate the substrate's activity, and thus the activity of the entire signaling cascade. There are three "conventional" MAPK family members: extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38-MAPK. Each of these encompasses its own subfamily: ERKs (ERK1 and ERK2), JNKs (JNK1, JNK2, and JNK3), and p38-MAPKs (p38-MAPKα, p38-MAPKβ, p38-MAPKγ, and p38-MAPKδ) [1, 3-6, 8] (Fig. **1**).

Members of the JNK subfamily are activated both by cellular stresses such as UV irradiation, heat shock, cisplatinum, etoposide, thapsigargin and tunicamycin, and by inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα). Activation of a JNK enzyme requires phosphorylation of the Tyr and Thr residues located in the Thr-Pro-Tyr motif of the MAPK domain. Two kinases, MKK4 (also known as SEK1) and MKK7, are responsible for this phosphorylation [4, 5, 9]. Once activated, JNK phosphorylates a number of substrates, including the c-Jun component of the activator protein-1 (AP-1) transcription factor that regulates the expression of genes involved in stress responses.

*Address correspondence to this author at the Department of Developmental and Regenerative Biology, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan; Tel: +81-3-5803-4659; Fax: +81-3-5803-5829;
E-mail: nishina.dbio@mri.tmd.ac.jp

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II. STUDIES OF JNK USING GENE DISRUPTION IN MICE

Many approaches have been taken to study JNK function, including gene targeting in mice. This work has yielded much information on JNK's roles in embryogenesis, the immune system, the neuronal system, and metabolism. In the following subsections, we summarize JNK functions revealed through the examination of mice in which the JNK gene has been disrupted. The implication of the studies outlined below is that regulation of JNK activity may be an effective means of amelioriating certain immunologic, neuronal and metabolic disorders.

i. Physiological Role of JNK in Murine Embryogenesis

Despite the importance of JNK functions to diverse essential cellular processes, single mutant mice deficient for JNK1, JNK2 or JNK3 all survive and appear morphologically normal. These data indicate that JNK1, 2, and 3 likely have redundant or overlapping functions during embryonic development. Moreover, JNK1/JNK3 and JNK2/JNK3 double mutants are also viable. In contrast, JNK1/JNK2 double mutants show severe dysregulation of apoptosis during brain development that result in embryonic lethality. Thus, JNK1 and JNK2 play a redundant but critical role in the regulation of region-specific apoptosis during early brain development [10].

ii. Physiological Role of JNK in the Murine Neuronal System

In situ hybridization and Northern blot studies have shown that the *jnk1* and *jnk2* genes encoding JNK1 and JNK2 are expressed ubiquitously. In contrast, the *jnk3* gene

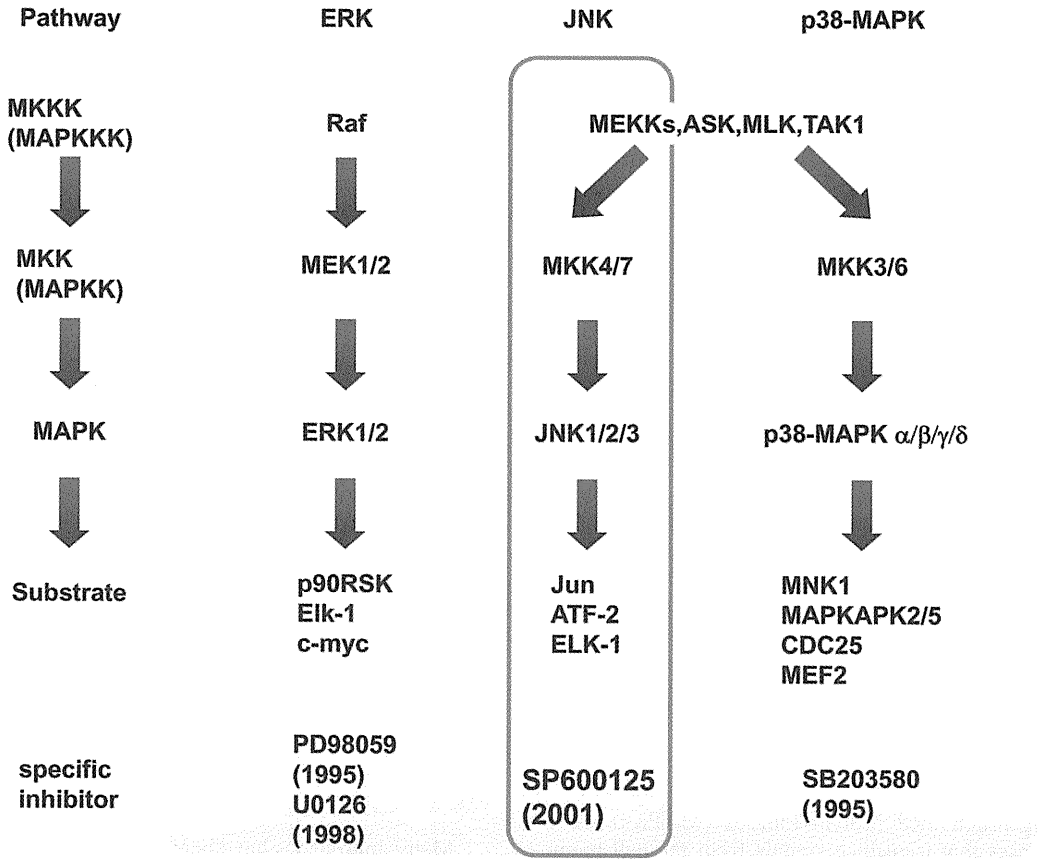

Fig. (1). The MAPK family of signaling pathways.

There are three major groups of MAPKs: ERK, JNK and p38-MAPK. Each of these is the central kinase in a pathway (left) characterized by an MAPK kinase-kinase (MKKK) and a MAPK kinase (MKK) that respond to different stimuli to activate the MAPK enzyme (ERK, JNK or p38-MAPK) and allow it to act on its substrate. Specific inhibitors (with their dates of discovery) are shown for each pathway.

has a more limited pattern of expression and is largely restricted to brain. Mice deficient in JNK3, or mice with mutations in the phosphorylation site of c-Jun, are resistant to the hippocampal neurotoxic events associated with administration of the glutamate receptor agonist kainic acid [11]. These results suggest that JNK3 is involved in glutamate excitotoxicity, an important component of ischemic neuronal organismal death. Targeted deletion of JNK3 has been shown to protect mice from brain injury after cerebral ischemia-hypoxia [12], leading to the proposal that JNK3 may be a potential target for neuroprotection therapy in stroke patients [9].

iii. Physiological Roles of JNK in the Murine Immune System

Although mice deficient for JNK1 or JNK2 appear to be morphologically normal, these mutants are immunodeficient due to severe defects in T cell function. JNK signaling activates the IL-2 promoter and thus plays an important role in T cell activation. In particular, mice lacking JNK1 or JNK2 exhibit deficits in $CD4^+$ T-helper cell function. In culture, JNK1-deficient $CD4^+$ T cells selectively differentiate into Th2 cells [13], and JNK2-deficient $CD4^+$ T cells also fail to differentiate into Th1 cells [14]. Intriguingly, JNK1 and JNK2 have differing roles in $CD8^+$ T cells [15, 16]. JNK1-deficient mice exhibit defective $CD8^+$ T cell expansion *in vitro* and *in vivo* and JNK1-deficient $CD8^+$ T cells show reduced expression of IL-2 and IFN-γ. In contrast, JNK2-deficient $CD8^+$ T cells express greatly increased amounts of IL-2 and IFN-γ. These data imply that JNKs play multiple roles in T cell-mediated immune responses, and that JNKs might be useful therapeutic targets. It has been suggested that controlled JNK inactivation might allow the selective modulation of effector T cell functions in diseases such as rheumatoid arthritis, asthma and chronic transplant rejection [9].

iv. Physiological Roles of JNK in Metabolism

Biochemical studies have established that JNK phosphorylates the insulin receptor substrate-1 (IRS-1) protein at its inhibitory site Ser-307 [17, 18]. JNK activation can therefore suppress signal transduction by the insulin receptor. These observations implicate the JNK signaling pathway in insulin resistance, metabolic syndrome, and type 2 diabetes. Indeed, ablation of the JNK pathway in mice can influence their susceptibility to obesity and diabetes [19] . For exam-

ple, knockout mice that lack expression of JNK1, or the JNK scaffolding protein known as JNK-interacting protein-1 (JIP1), are resistant to the effects of a high-fat diet and do not develop obesity or insulin resistance [20-22].

III. SP600125 AS A JNK INHIBITOR

Inhibitor studies are another important means by which enzyme functions can be revealed. The first inhibitors of MAPK activity targeted ERK and p38 MAPK were reported in 1995 (Fig. **1**), and many studies centered on the use of these inhibitors have been reported since then. In contrast, an inhibitor of JNK activity was developed only in 2001 [23]. Anthrax [1,9-cd] pyrazol-6 (2H)-one, known less formally as SP600125, was the first compound used to study the function of JNK both in cells and in whole animals. JNK inhibition by SP600125 was observed to be reversible and ATP-competitive, and showed IC_{50} values for JNK inhibition in the range of 40–90 nM. In addition, SP600125 exhibited >300-fold selectivity for JNK over ERK1 and p38-MAPK, and 10-100 fold selectivity for JNK over 14 other protein kinases. These results suggested that SP600125 bound specifically and with high affinity to residues in JNK's ATP-binding site [23].

At the level of cellular functions, SP600125 treatment prevents the expression of several anti-inflammatory genes in cell-based assays [23], the activation of AP-1 and expression of collagenase-3 in synoviocytes [24], the expression of type IV collagenase in ovarian cancer (OVCAR) cells [25], and the activation and differentiation of primary human $CD4^+$ cell cultures [23]. In animal studies, SP600125 blocks lipopolysaccharide-induced expression of TNFα and also inhibits anti-CD3-induced apoptosis of $CD4^+$ and $CD8^+$ thymocytes [23].

Since the initial studies of SP600125 conducted in 2001-2002, this inhibitor has become increasingly popular for two reasons. First, prior to the advent of SP600125, it was possible to study the contribution of JNK signaling to biological processes only *via* the introduction of active or dominant-negative JNK pathway components into cells, most often by transfection. Although much valuable information on the roles of JNK pathways in mammalian cells has been garnered from this method, the process of transfection itself alters the cell for many hours or days prior to the experiment of interest. This delay is a drawback that does not exist with chemical inhibitors, which can be rapidly applied to cells either *in vitro* or *in vivo*. For example, the inhibitors PD98059 and U0126 target the MEK1 and MEK2 kinases that act directly upstream of ERK [26]. Similarly, the inhibitor SB203580 and related pyridinylimidazole compounds directly inhibit p38 MAPK (reviewed in Refs. [27, 28]). The use of this panel of inhibitors has greatly accelerated the understanding of MAPK pathways. The second, more mundane reason for SP600125's popularity is its commercial availability. Other compounds including CEP-1347 have been reported [29, 30] but has yet to be commercially released [31].

More than 1300 reports citing the use of SP600125 as a JNK inhibitor in cell cultures and animal models have been published. We have outlined a number of representative *in vitro* and *in vivo* studies below and summarized them in Table **1**. The use of SP600125 has revealed much new knowledge about JNK functions that was not exposed by gene-disruption or over-expression studies. However, this work has also made it clear that, because of specificity concerns, SP600125 should be used only to demonstrate JNK involvement in a process. This issue is addressed in detail in section IV.

i. SP600125 and Arthritis

Several studies have reported the effects of the JNK inhibitor administration in animal models of arthritis. SP600125 blocks IL-1-induced phosphorylation of JNK and c-Jun in cultured synoviocytes from rheumatoid arthritis patients, and impairs the production of matrix metalloproteinase-13 (MMP-13), an enzyme associated with cartilage destruction [24]. SP600125 administration also inhibits JNK activation and collagenase expression in the joints of rats with adjuvant arthritis. These animals showed significant reductions in paw swelling and damage to bone and cartilage [24]. These studies were extended by using JNK2 knockout mice in a model of passive murine collagen-induced arthritis. This work showed that JNK2 is a key determinant of matrix degradation, but is less important for inflammation and paw swelling [32]. In light of these findings, Manning and Davis have proposed that the inhibition of JNK should be considered as a potential therapy for rheumatoid arthritis [9].

ii. SP600125 and Abdominal Aortic Aneurysm

In humans, examination of tissue samples from abdominal aortic aneurysms has shown the presence of upregulated MMP-9 and activated JNK [33]. Treatment of these samples *ex vivo* with SP600125 repressed the release into the culture supernatants of MMP-9, implicating JNK in tissue damage associated with abdominal aortic aneurysms. This hypothesis has also been confirmed by the work of Yoshimura *et al.*, who have used SP600125 and mouse model to show that JNK is critical for the development of abdominal aortic aneurysms. Treatment with SP600125 prevented the development of abdominal aortic aneurysms in $CaCl_2$-induced mouse model. In particular, SP600125 treatment almost completely prevented aortic dilatation and medial thinning while preserving the integrity of the elastic lamellae [33].

iii. SP600125 and Ischemia

Okuno *et al.* showed that JNK inhibition by SP600125 was potently effective in decreasing neuronal apoptosis in the ischemic core after *in vivo* transient focal cerebral ischemia (tFCI) [34]. During ischemia, BimL (Bim long) is induced and phosphorylated in parallel with induction of JNK activity. Co-immunoprecipitation studies have consistently revealed increased interaction of JNK with BimL, as well as BimL with the cell death effector Bax, after tFCI. SP600125 blocked these interactions at a dose that significantly inhibited JNK-induced neuronal apoptosis. That neurons were protected from ischemia-induced apoptosis was determined by TUNEL staining and an apoptosis-related DNA fragmentation assay. Biochemically, SP600125 blocked translocation

Table 1. JNK Functions Revealed by the Use of SP600125 in Mammalian Cells and Animal Models. Representative Studies are Cited

In Vitro Study		
Cell Type	**Function**	**Refs.**
rat cardiomyocyte cell line H9c2	apoptosis	Uetani *et al. J. Biol. Chem.* **2009**
rat neonatal cardiomyocytes	apoptosis	Xie *et al. J. Biol. Chem.* **2009**
human EBV-transfomed B cells	apoptosis	Kim *et al. J. Immunol.* **2008**
human umbilical vein endothelial cells	apoptosis	Ho *et al. J. Biol. Chem.* **2008**
human cholangiocarcinoma cell line KMCH expressing Mcl-1 siRNA	apoptosis	Werneburg *et al. J. Biol. Chem.* **2007**
multidrug-resistant MCF7/ adriamycin-resistant (ADR) human breast carcinoma cells	apoptosis	Lee *et al. J. Biol. Chem.* **2007**
rat primary microglia	apoptosis	Yang *et al. J. Immunol.* **2006**
human bladder cancer cell line RT112, 253J, J82 human prostate cancer cell line LNCap	apoptosis	watanabe *et al. Oncogene* **2006**
rat neural stem cells	apoptosis	kanzawa *et al. Oncogene* **2006**
mouse primary cultures of striatal neurons	apoptosis	Charvin *et al. Proc. Natl. Acad. Sci. U S A* **2005**
human foreskin-derived keratinocyte	gene expression	Wehkamp *et al. J. Invest. Dermatol.* **2006**
human hepatocellular carcinoma cell line HuH-7	gene expression	Higuchi *et al. J. Biol. Chem.* **2004**
primary cultures of human hepatocytes	gene expression	Holt *et al. Genes. Dev.* **2003**
mouse primary splenocytes	gene expression	Brint *et al. J. Biol. Chem.* **2002**
human lung epithelial fibroblast cell line WI38	autophagy	Oh *et al. J. Pharmacol. Exp. Ther.* **2009**
mouse NF-1(+/-) microglia	proliferation, motility, tumor	Daginakatte *et al. Cancer Res.* **2008**
mouse sertori cell line TM4 cells	mRNA degradation	Sze *et al. Biochem. J.* **2008**
human hepatoma cell line Hep3B	inflammation	Nishikawa *et al. J. Immunol.* **2008**
rat fibloblast SCN, pineal gland lung explants	circadian rhythms	Chansard *et al. Neuroscience* **2007**
humam epidermal keratinocytes	differentiation	Gazel *et al. J. Biol. Chem.* **2006**
In Vivo Study		
Model Type	**Function**	**Refs.**
mouse corneal neovascularization model	apoptosis	Ho *et al. J. Biol. Chem.* **2008**
mouse acetaminophen-induced liver injury model	liver injury	Hanawa *et al. J. Biol. Chem.* **2008**
mouse kappa opioid receptor-mediated analgestic model	analgestic response	Bruchas *et al. J. Biol. Chem.* **2007**
mouse Group B streptococcus induced septic shock model	sepsis	kenzel *et al. J. Immunol.* **2006**
mouse LPS-induced lung inflammation model	lung inflammation	Arndt *et al. J. Immunol.* **2005**
mouse antigen-induced airway inflammation model	asthma	Nath *et al. Eur. J. Pharmacol.* **2005**
mouse abdominal aortic aneurysm model	Abdominal aortic aneurysm	Yoshimura *et al. Nat. Med.* **2005**
rat focal cerebral ischemia model	ischemia	Okuno *et al. J. Neurosci.* **2004**
rat adjuvanti-induced arthritis model	inflammatory arthriris	Han *et al. J. Clin. Invest.* **2001**

of Bax from the cytosol to the mitochondria after tFCI. These results suggest that the JNK signaling pathway is involved in ischemia-induced neuronal apoptosis because it stimulates Bax translocation to the mitochondria. In this context, BimL is likely regulated by JNK as a downstream substrate for the transmission of apoptotic signals to Bax. Thus, this use of SP600125 has provided evidence for a role of the JNK signaling pathway in ischemia-induced neuronal apoptosis.

iv. SP600125 and Asthma

The lung inflammation associated with asthma is induced by cytokine and chemical mediators produced by activated mast cells and basophils. These cell types express high levels of surface IgE receptors (FcεRI) that can bind to a range of IgE antibodies. When these IgE antibodies are engaged by multivalent antigen, the FcεRI molecules are crosslinked and initiate intracellular signaling that leads to cellular activation

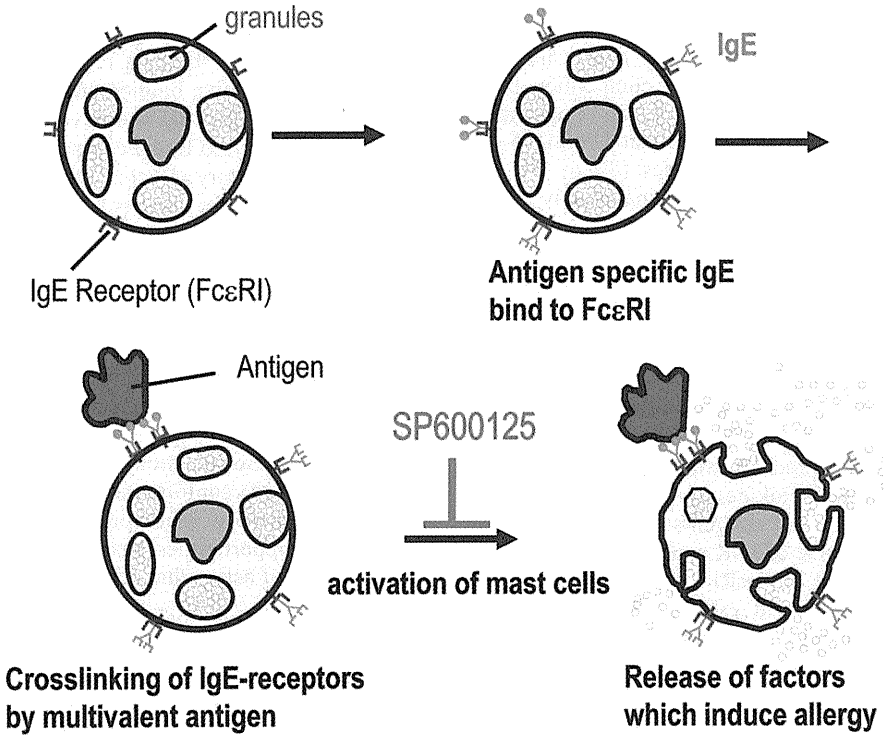

Fig. (2). Triggering of mast cell degranulation.

Mast cells express high levels of FcεRI molecules that can bind to a wide variety of IgE antibodies. The binding of specific multivalent antigen to certain of these fixed IgE antibodies crosslinks the FcεRI molecules and triggers mast cell activation. Activated mast cells release biogenic amines such as histamine and serotonin from their granules, as well as inflammatory cytokines (TNFα, IL-6, IL-13 and others) and lipid mediators (PGD2, LTB4 and others). These factors induce the early-phase allergic reaction and contribute to the inflammatory cell infiltration characteristic of asthma.

(Fig. **2**). Areas of asthmatic inflammation are also characterized by an accumulation of eosinophils, which play an important role in the exacerbation of asthma [35].

Nath *et al.* have used SP600125 to evaluate the function of JNK in a murine model of allergic asthma [36]. Sensitized Balb/C mice subjected to chronic allergen exposure show an accumulation of inflammatory cells, increased numbers of airway smooth muscle cells and goblet cells, and heightened bronchial responsiveness. Pretreatment with SP600125 attenuated allergen-induced bronchial hyperresponsiveness and significantly inhibited eosinophil and lymphocyte accumulation in bronchoalveolar lavage fluid. This amelioration coincided with a decrease in the peroxidase-positive eosinophils within the bronchial submucosa. SP600125 also inhibited allergen-induced increases in airway goblet cells and smooth muscle cells. These observations indicate that JNK activity may be important for the chronic inflammation, airway remodelling and bronchial hyperresponsiveness induced by chronic allergen exposure in the mouse. A decrease in the concentrations of IL-4, IL-13, RANTES (Regulated on Activation, Normal T Expressed and Secreted) and TNFα in the lung was also observed in this model following SP600125 administration [36]. These results suggest that SP600125 might be useful for reducing asthma-associated inflammation, particularly that dominated by mast cells, basophils, Th2 cells and eosinophils.

Taken together, all these reports indicate that SP600125 is a very promising drug with potential therapeutic applicability to a broad range of diseases. Future studies of this sort should add to the list of disorders for which SP600125 treatment may be beneficial.

IV. SPECIFICITY OF SP600125

Despite the favorable data presented above, recent work by Bain *et al.* has raised concerns about SP600125's specificity as an inhibitor of JNK [37, 38]. Fresh examination of IC_{50} values has shown that SP600125 is actually a rather weak inhibitor of JNK isoforms, and that the IC_{50} values reported previously in [23] are likely explained by the lower ATP concentrations used in these assays. The original studies of SP600125 selectivity showed little or no inhibition of the 17 protein kinases and 18 inflammatory enzymes tested [23, 24]. However, Bain *et al.* found that SP600125 was non-specific and inhibited 13 of the 28 protein kinases tested with similar or greater potency than JNK [37]. Notably, serum and glucocorticoid-regulated kinase, p70 ribosomal S6 kinase, AMP-dependent protein kinase, cyclin-dependent kinase 3, casein kinase 1δ and dual-specificity tyrosine–regulated kinase 1A were all inhibited by 10 μM SP600125 to a greater extent than was JNK.

Fabian *et al.* have also re-examined the specificity of SP600125 [39]. These authors developed a new, more sensitive and versatile method for determining kinase inhibitor specificity based on ATP-site-dependent competition binding assays and a T7 phage that serves as a "tag" for kinases. Data obtained using this method showed that SP600125 can bind to a broad range of protein kinases (39 of the 119 tested), confirming that many additional kinases may be targets of SP600125.

The specificity of SP600125 has also arisen as an issue in our work. Our objective has been to examine the physiological role of JNK in mast cells, which play a central role in inflammatory and immediate allergic responses. Engagement of FcεRI of mast cells induces their degranulation and triggers the expression of the genes encoding the inflammatory cytokines IL-6, TNFα and IL-13 (Fig. **3**). We found that the use of SP600125 could almost completely inhibit FcεRI-induced degranulation and cytokine production by mast cells [40]. However, examination of antigen-stimulated mast cells revealed that the time course of JNK activation in these cells did not correlate with that of FcεRI-induced degranulation. Furthermore, degranulation and cytokine gene expression induced by FcεRI engagement were not impaired in MKK7-deficient mast cells, which cannot activate JNK. These results suggested that the altered phenotypes observed in our SP600125-treated mast cells were not due to effects on the JNK signaling pathway. We eventually determined that, rather than inhibiting JNK, SP600125 markedly inhibits the FcεRI-induced activation of phosphatidylinositol 3-kinase (PI3K) pathway in mast cells (Fig. **3**). PI3K is known to be important for both degranulation and cytokine gene expression in mast cells [41]. Our results indicate that SP600125 has non-JNK targets in mast cells, and that these targets may be important for antigen-induced mast cell activation. Cytokines, proteases, biogenic amines and lipid mediators secreted by mast cells induce basophils and eosinophils to congregate in inflamed tissues, so that FcεRI-activated mast cells can be said to make a major contribution to leukocyte infiltration. Therefore, the effects of SP600125 in animal models of allergy (as described above) may be explained in part by SP600125-mediated impairment of PI3K signaling in mast cells. In any case, our work implies that SP600125 may be a potent new drug for the treatment of allergy.

V. FUTURE PERSPECTIVE ON THE NOVEL STRATEGY TO DEVELOP MORE SPECIFIC INHIBITOR FOR JNK

SP600125 has been extensively used as a purported JNK inhibitor both to study the role of JNK in a cell physiology context, and to evaluate the usefulness of JNK inhibition in a therapeutic context. Indeed, SP600125 has shown striking efficacy in some disease models. Ordinarily, such results would mean that JNK might become an attractive new mechanistic target, and that SP600125 would be an effective new drug to treat human disease. However, we and others have raised concerns about the specificity of SP600125 as a JNK inhibitor. The efficacy of SP600125 in some disease models may be derived from its inhibitory effects on targets other than JNK.

Kinase inhibitors are currently under intensive study as a promising new class of targeting drugs. Most of these inhibitors target the ATP-binding region of a kinase protein, setting up a competitive binding situation. However, the structure of the ATP-binding site is very similar among kinases, making it difficult to find a competitive inhibitor of this type that has an inhibitory effect specific to the kinase of interest. Moreover, one of the reasons that kinase inhibitor treatments often generate side effects is that the inhibitor non-specifically shuts down a variety of kinases. At the time of writing, there are eight kinase inhibitor drugs on the market that are approved for use only in oncology. The treatment of chronic diseases with kinase inhibitors may require that these agents show greater selectivity for the target kinase. Kinase inhibitors that attack an allosteric domain rather than the ATP-binding site of a kinase of interest may be the answer to improving kinase specificity, since allosteric inhibitors typically alter kinase conformation and prevent protein substrate binding. Allosteric inhibitors thus may provide exciting therapeutic opportunities based on the exploitation of new mechanisms of action that decrease off-target side effects.

A number of pharmaceutical companies are using *in vitro* and *in vivo* studies to explore the therapeutic potential of JNK inhibitors. Some such inhibitors, including Celgene developing compound CC-401, are already being tested in clinical trials [42]. Other JNK inhibitors, such as CEP-1347 and the small peptide inhibitor I-JIP (Inhibitor of JIP-1), are currently more useful for studying JNK functions than for therapy. CEP-1347 inhibits members of the mixed lineage kinase (MLK) family, with IC50 values of 23-51 nM for MLL1, 2 and 3. MLK1, 2 and 3 are upstream activators of the JNK pathway [29, 30]. Thus, CEP-1347 provides a means of targeting the JNK pathway *via* inhibition of select upstream MEKKs. In contrast, I-JIP is a 21-amino acid peptide inhibitor that directly targets activated JNK enzymes. The sequence of I-JIP was derived from amino acids 143-163 of the JNK-binding domain (JBD) of JIP-1. *In vitro*, I-JIP inhibited JNK-mediated phosphorylation of recombinant c-Jun, Elk and ATF2 by up to 90% [43].

One of the most specific ways to inhibit JNKs may be to block their expression through use of antisense techniques or RNA-mediated interference approaches. This mode of inhibition has already accelerated the discovery of cellular events mediated by JNKs. Whether these types of approaches can be used therapeutically are matters for future exploration.

VI. CONCLUDING REMARKS

We believe that SP600125 can be truly effective as a therapeutic, as has been demonstrated in various animal disease models. However, this efficacy may be attributable to SP600125's inhibition of kinases other than JNK. Future studies employing SP600125 should endeavor to ascertain its true target, so that the production of an inhibitor specific for the kinase responsible for the observed effect can be made. Such an approach should lead to the development of medicines that have greater drug efficacy and fewer adverse effects.

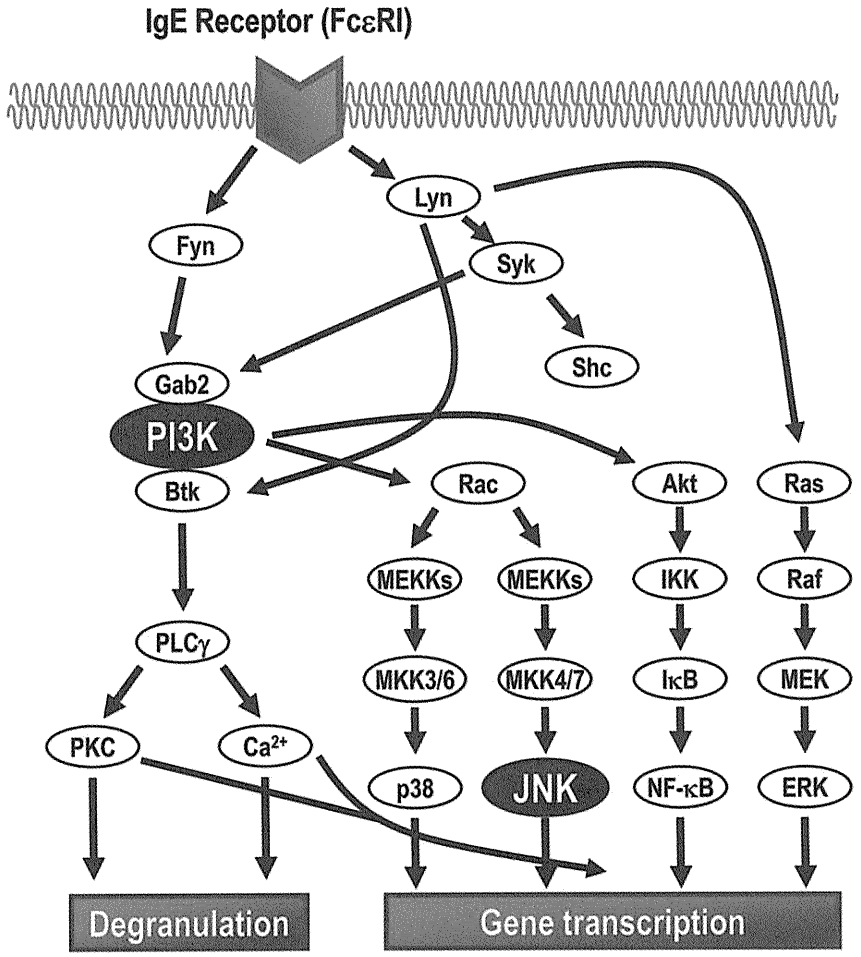

Fig. (3). Signal transduction pathways triggered by FcεRI aggregation.

Aggregation of FcεRI molecules on a mast cell triggers the activation of protein tyrosine kinases (PTKs) such as Syk, Fyn and Lyn. Activated Fyn acts on the scaffolding adaptor protein Grb-2 associated binder (Gab2) to relay the signal from FcεRI to PI3K. PI3K then triggers downstream activation of PKC and calcium signaling, which are essential for mast cell degranulation. Aggregation of the FcεRI also activates MAPKs, particularly ERK, JNK and p38 MAPK. Activation of these kinases is important for the transcription of cytokine genes. Activation of PI3K leads to stimulation of the Akt pathway, which also triggers gene transcription. SP600125 inhibits both PI3K and JNK in mast cells.

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Antiobesity Effect of Eicosapentaenoic Acid in High-Fat/High-Sucrose Diet–Induced Obesity

Importance of Hepatic Lipogenesis

Ayumi Sato,[1] Hiroyuki Kawano,[1] Tatsuto Notsu,[1] Masahiko Ohta,[1] Masanori Nakakuki,[1] Kiyoshi Mizuguchi,[1] Michiko Itoh,[2] Takayoshi Suganami,[2] and Yoshihiro Ogawa[2,3]

OBJECTIVE—Given the pleiotropic effect of eicosapentaenoic acid (EPA), it is interesting to know whether EPA is capable of improving obesity. Here we examined the anti-obesity effect of EPA in mice with two distinct models of obesity.

RESEARCH DESIGN AND METHODS—Male C57BL/6J mice were fed a high-fat/high-sucrose diet (25.0% [w/w] fat, 32.5% [w/w] sucrose) (HF/HS group) or a high-fat diet (38.1% [w/w] fat, 8.5% [w/w] sucrose) (HF group) for 4–20 weeks. A total of 5% EPA was administered by partially substituting EPA for fat in the HF/HS + EPA and HF + EPA groups.

RESULTS—Both the HF/HS and HF groups similarly developed obesity. EPA treatment strongly suppresses body weight gain and obesity-related hyperglycemia and hyperinsulinemia in HF/HS-fed mice (HF/HS + EPA group), where hepatic triglyceride content and lipogenic enzymes are increased. There is no appreciable effect of EPA on body weight in HF-fed mice (HF + EPA group) without enhanced expression of hepatic lipogenic enzymes. Moreover, EPA is capable of reducing hepatic triglyceride secretion and changing VLDL fatty acid composition in the HF/HS group. By indirect calorimetry analysis, we also found that EPA is capable of increasing energy consumption in the HF/HS + EPA group.

CONCLUSIONS—This study is the first demonstration that the anti-obesity effect of EPA in HF/HS-induced obesity is associated with the suppression of hepatic lipogenesis and steatosis. Because the metabolic syndrome is often associated with hepatic lipogenesis and steatosis, the data suggest that EPA is suited for treatment of the metabolic syndrome. ***Diabetes* 59:2495–2504, 2010**

The metabolic syndrome has been defined as a cluster of visceral fat obesity, impaired glucose metabolism, atherogenic dyslipidemia (high plasma triglyceride and low HDL cholesterol), and hypertension (1). There is considerable evidence that visceral fat obesity is a key etiological factor in the metabolic syndrome (2). Enhanced hepatic lipogenesis and hepatic steatosis also appear to play an important role in the pathogenesis of the metabolic syndrome (3). Indeed, nonalcoholic fatty liver disease may constitute the common features of the metabolic syndrome.

Numerous epidemiological studies and clinical trials have revealed that fish oil and n-3 polyunsaturated fatty acids (PUFAs) reduce the risk of coronary heart disease (4). Eicosapentaenoic acid (EPA), one of the major n-3 PUFAs contained in fish oil, has a variety of pharmacological effects such as lipid-lowering (5), anti-platelet (6), anti-inflammatory (7), and anti-atherogenic effects (8,9). Recently, the Japan EPA Lipid Intervention Study (JELIS), a large-scale prospective randomized clinical trial, demonstrated that EPA delays the onset of cardiovascular events via cholesterol-independent mechanisms (10,11), but the molecular mechanisms remain to be elucidated. In a recent sub-analysis of the JELIS, EPA had a great risk reduction of coronary artery events of 53% in patients with high triglycerides and low HDL cholesterol (11), suggesting that EPA may be effective to reduce the incidence of atherosclerosis in the metabolic syndrome. These findings are supported by our recent observations that EPA administration results in decreases in remnant-like particle-triglyceride, small dense LDL, and C-reactive protein and an increase in adiponectin in patients with the metabolic syndrome (12,13).

Given the pleiotropic effect of EPA, it is interesting to know whether highly purified EPA is capable of improving obesity. There is currently a controversy as to the anti-obesity effect of EPA; it has been effective (13,14), has been ineffective (15), or has even increased visceral fat accumulation (16). On the other hand, it is noteworthy that EPA suppresses hepatic lipogenesis and steatosis by reducing mRNA and active protein of sterol regulatory element binding protein-1c (SREBP-1c) (17–19). We, therefore, examined the impact of hepatic lipogenesis on the anti-obesity effect of highly purified EPA.

Here, we demonstrate that EPA strongly suppresses body weight gain and obesity-related hyperglycemia and hyperinsulinemia in high-fat (HF)/high-sucrose (HS)-induced obese mice with enhanced hepatic lipogenesis but not in HF-induced obese mice without enhanced hepatic lipogenesis. This study is the first demonstration that the anti-obesity effect of EPA is related to the suppression of hepatic lipogenesis. Given that the metabolic syndrome is often associated with hepatic lipogenesis and steatosis, the data of this study suggest that EPA is suited for the treatment of the metabolic syndrome.

From [1]Development Research, Pharmaceutical Research Center, Mochida Pharmaceutical, Shizuoka, Japan; the [2]Department of Molecular Medicine and Metabolism, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan; and the [3]Global Center of Excellence Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, Tokyo, Japan.

Corresponding author: Hiroyuki Kawano, h-kawano@mochida.co.jp.

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RESEARCH DESIGN AND METHODS

Highly purified EPA ethyl ester (purity >98%) was obtained from Nippon Suisan Kaisha (Tokyo, Japan). Ethyl palmitate (purity >95%) was purchased from Wako (Tokyo). Nine-week-old male C57BL/6J mice were obtained from CLEA Japan (Tokyo) and acclimated for 1 week before the experiment. Mice were housed under controlled temperature and lighting (0730–1930 light, 1930–0730 dark cycle) with free access to water and a fish meal-free diet (fish meal-free F1: 4.4% fat; Funabashi Farm, Funabashi, Japan). All experiments were carried out in accordance with the guidelines for the use and care of laboratory animals of Mochida Pharmaceutical (numbers 1523, 1524, 2354, and 2389).

Diets. The outline of experiments and composition of diets are in supplementary Tables 1 and 2, respectively (available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1554/DC1).

Plasma analysis. Blood samples were collected via the retro-orbital sinus of nonfasted mice under light anesthesia every 2 weeks. Plasma glucose, total cholesterol, triglycerides, free fatty acid, and insulin were determined by commercially available kits. Plasma concentrations of leptin and adiponectin were measured with the respective enzyme-linked immunosorbent assay kits (leptin: Morinaga, Yokohama, Japan; adiponectin: R&D Systems, Minneapolis, MN).

Hepatic triglyceride content. Liver lipids were extracted by the method of Folch et al. (20). Hepatic triglyceride content was measured with a reagent from Wako.

Gene expression analysis. Expression levels of some genes were determined by quantitative real-time PCR using primers and probes shown in supplementary Table 3. Expression of other genes was determined using TaqMan gene expression assays (Applied Biosystems, Foster City, CA). The assay IDs of TaqMan (R) gene expression assays are shown in supplementary Table 4. 18s rRNA was measured using TaqMan (R) rRNA control reagents (Applied Biosystems) as a control.

Enzymatic activities. Acyl-CoA oxidase, fatty acid synthase (FAS), and hydroxyacyl-CoA dehydrogenase activities were measured spectrophotometrically (21–23).

Cytoplasmic and nuclear protein extracts and Western blot analysis. Cytoplasmic and nuclear proteins were extracted as described (24,25) with slight modifications. The samples were equally pooled from all the mice of each group (n = 7–10), and then 30 μg protein per lane was separated by SDS-PAGE. Western blot analysis was performed using anti–SREBP-1 antibody (H160; Santa Cruz Biotechnology, Santa Cruz, CA). We used anti–β-actin (Cell Signaling Technology, Beverly, MA) and anti-TATA binding protein (TBP) (Abcam, Cambridge, U.K.) antibodies as cytoplasmic and nuclear controls, respectively. The blots were visualized with the ECL Western blotting analysis system (GE Healthcare, Buckinghamshire, U.K.).

Indirect calorimetry analysis. The volumes of consumed O_2 (VO_2) and produced CO_2 (VCO_2) were measured by indirect calorimetry using an Oxymax system (Columbus Instruments, Columbus, OH). The O_2 and CO_2 contents were recorded every 10 min from 1130 to 0900. Mice had unrestricted access to food and water. The respiratory exchange ratio (RER) was calculated by VCO_2/VO_2.

Glycerol release from white adipose tissue. Glycerol release from the epididymal fat ex vivo was measured according to the method of Schweiger et al. (26) with slight modifications.

Hepatic triglyceride secretion rate. A dose of 500 mg/kg tyloxapol (also known as Triton WR-1339; Sigma-Aldrich, St. Louis, MO) in saline was injected intravenously. Mice were fasted overnight before the injection. Immediately before and 1 h after the injection, blood samples were collected and plasma triglyceride concentrations were determined as described above. The hepatic triglyceride secretion rate was calculated as described by Steiner et al. (27).

Fatty acid composition of VLDL. Blood samples were collected via the inferior vena cava of mice 4 h after the tyloxapol injection. Plasma was isolated, and VLDL fraction was obtained by ultracentrifugation, according to the method of Werner et al. (28). Fatty acid composition was analyzed by gas chromatography.

Statistical analysis. Data are presented as means ± SE. ANOVA using a split-plot model was used for the line plots of indirect calorimetry analysis (Fig. 6*C* and *E*). Other data were assessed by a t test. $P < 0.05$ was considered a significant difference.

RESULTS

Effect of EPA on HF/HS- and HF-induced obesity. Both HF/HS and HF groups gained weight with increases in the mesenteric, epididymal, retroperitoneal, and subcutaneous white adipose tissue (WAT) weights relative to the control group after a 20-week feeding (experiments 1 and 2) (Fig. 1*A–D*). There were no appreciable differences between HF/HS and HF groups. The HF/HS-induced increases in body weight and WAT weights were markedly suppressed by treatment with EPA (HF/HS + EPA group). By contrast, EPA treatment did not affect an HF-induced increase in body weight and WAT weights (HF + EPA group). In the HF/HS group, obesity-induced WAT inflammation was evident, as revealed by elevated expression of mRNAs for F4/80, monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α. EPA treatment markedly reduced F4/80 and MCP-1 mRNA expression in WAT (Fig. 1*E*). In WAT from the HF group, expression of F4/80, MCP-1, and tumor necrosis factor-α mRNAs was elevated, which was not affected by EPA (Fig. 1*F*). In this study, caloric intake in the HF/HS group was roughly equivalent to that in the HF/HS + EPA group (Table 1).

Effect of EPA on HF/HS- and HF-induced metabolic abnormalities. In experiment 1, the HF/HS group developed obvious hyperinsulinemia, hyperglycemia, and hepatic steatosis, which were significantly improved by EPA (HF/HS + EPA group) (Fig. 2*A*, *C*, and *E*). The liver weights after a 20-week feeding were 1.33 ± 0.03, 3.10 ± 0.30, and 1.42 ± 0.06 g in the control, HF/HS, and HF/HS + EPA groups, respectively (n = 10 for the control and HF/HS groups, n = 7 for the HF/HS + EPA group; $P < 0.01$ for the control group versus HF/HS group and the HF/HS group versus HF/HS + EPA group). Similarly, the HF group developed hyperglycemia and hyperinsulinemia; however, EPA had a marginal impact on HF-induced hyperglycemia and hyperinsulinemia (Fig. 2*B* and *D*). Although EPA treatment ameliorated hepatic steatosis in the HF group (Fig. 2*F*), liver weight was not decreased by EPA (data not shown). Plasma leptin concentrations were markedly elevated in the HF/HS group relative to the control group and were significantly reduced by EPA ($P <$ 0.01) (Table 1). Although HF/HS did not affect plasma adiponectin concentration, EPA treatment resulted in an ~1.5-fold elevation of plasma adiponectin (Table 1), which is consistent with our previous report (13).

Effect of EPA on gene expression in the HF/HS group. Enhanced expression of genes related to β-oxidation (29), uncoupling proteins (UCPs) (30), and glucose oxidation (31) are known to suppress obesity. However, they were not reduced or rather elevated in the liver and WAT from the HF/HS group relative to the control group (Fig. 3*A* and *E*). Moreover, in the skeletal muscle, except UCP-3, gene expression was not increased by EPA treatment (HF/HS + EPA group) relative to the HF/HS group (Fig. 3*C*). These observations suggest that enhancement of β-oxidation, UCPs, and glucose oxidation does not play a major role in the anti-obesity effect of EPA.

Enhanced lipolysis may promote the degradation of triglycerides accumulated in WAT, thereby leading to the suppression of obesity. Expression of mRNAs for hormone-sensitive lipase and adipose triglyceride lipase in WAT from the HF/HS group did not differ significantly from that in the control group. On the other hand, expression of mRNAs for the β3-adrenergic receptor (β3-AR), triglyceride hydrolase (TGH)-1, and TGH-2 in the HF/HS group was significantly lower than in the control group ($P < 0.01$), which was reversed in the HF/HS + EPA group (Fig. 3*E*).

It is also possible that enhanced lipogenesis promotes energy accumulation through triglyceride synthesis from diet-derived carbohydrates and fatty acids, thereby stimulating obesity and WAT accumulation. In WAT, there was

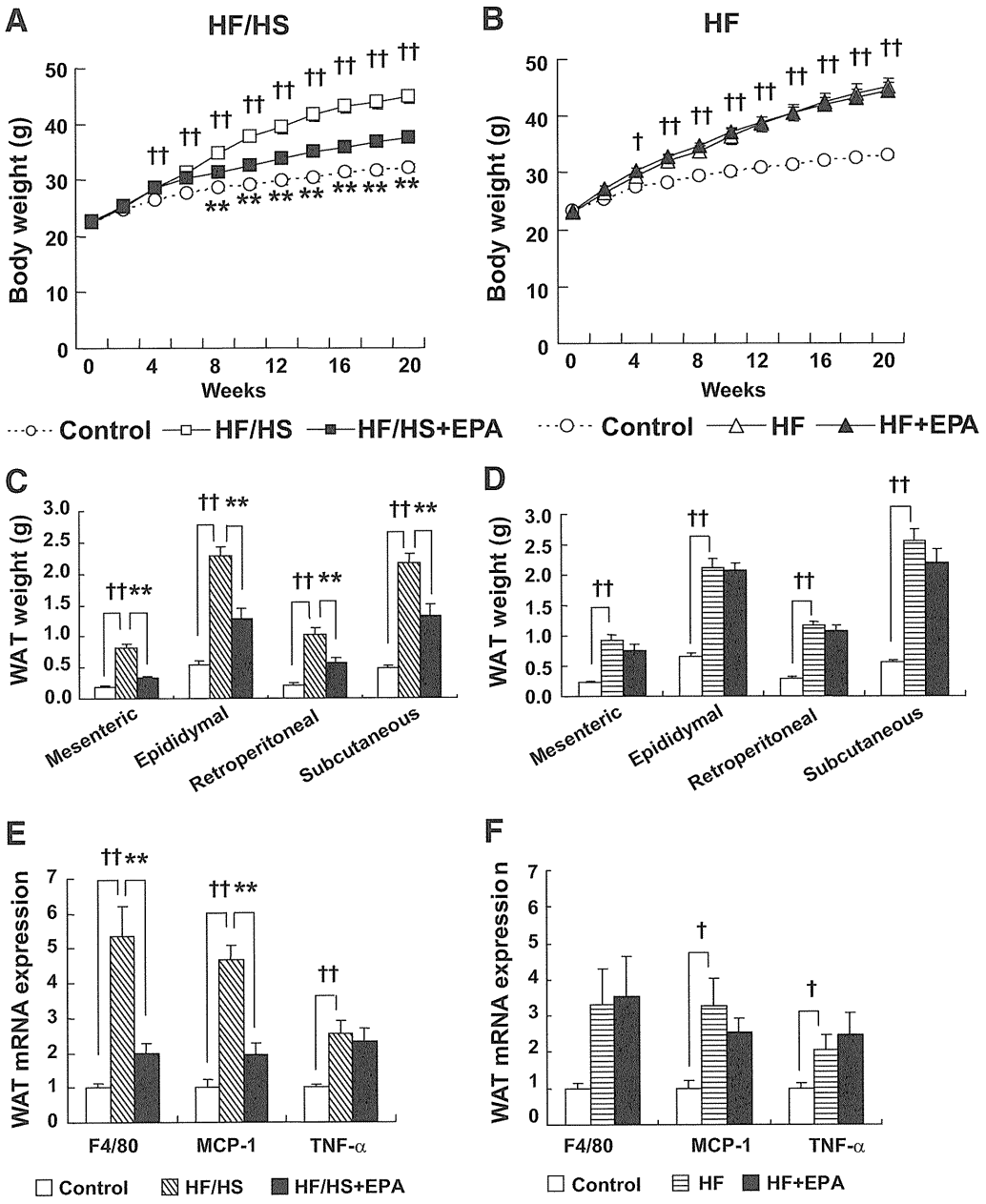

FIG. 1. Effect of EPA on HF/HS- and HF-induced obesity. *A* and *B*: Body weight change. *C* and *D*: WAT weights. *E* and *F*: Proinflammatory gene expression in the epididymal WAT. n = 7–10. †$P < 0.05$; ††$P < 0.01$ vs. control group. **$P < 0.01$ vs. HF (/HS) group.

no appreciable difference in acetyl-CoA carboxylase (ACC)-1 and FAS mRNA expression between the HF/HS and control groups. Expression of ACC-1 and FAS mRNAs was not increased in the HF/HS + EPA group relative to the HF/HS group. Stearoyl-CoA desaturase (SCD)-1 mRNA expression in the HF/HS group was elevated relative to the control group, which was unaffected by EPA treatment (Fig. 3*E*). In the liver, ACC-1, FAS, and SCD-1 mRNA expression in the HF/HS group was elevated relative to the control group, which was reduced by EPA treatment (HF/HS + EPA group) (Fig. 3*A*). In this study, hepatic SREBP-1 protein, a master regulator of lipogenic enzymes, was also increased in the HF/HS group, which was reduced by EPA treatment (Fig. 3*F*).

Effect of EPA on enzymatic activity in the HF/HS group. In this study, elevated enzymatic activities of hepatic FAS and acyl-CoA oxidase in the HF/HS group were reduced by EPA treatment (Fig. 3*B*). Elevated activities of hydroxyacyl-CoA dehydrogenase in the skeletal muscle from the HF/HS group was also reduced by EPA treatment (Fig. 3*D*).

Effect of EPA on gene expression and enzymatic activity in the HF group. We next examined mRNA levels and activities of lipogenic enzymes in the HF group, where EPA did not improve obesity and WAT accumulation (Fig. 1*B* and *D*). Notably, expression of mRNA for lipogenic enzymes and FAS activity in the liver were not elevated in the HF group relative to the control group (Fig.

TABLE 1
Food intake and plasma parameters in experiment 1 and 2 at 18 weeks of feeding

	Experiment 1			Experiment 2		
	Control	HF/HS	HF/HS + EPA	Control	HF	HF + EPA
Food intake (kcal/day/mouse)	8.56	11.15	10.58	9.84	12.72	13.96
Leptin (ng/ml)	4.60 ± 0.82	44.60 ± 5.43††	15.46 ± 2.74**	6.46 ± 0.67	47.02 ± 5.39††	36.24 ± 5.55
Adiponectin (μg/ml)	10.05 ± 0.59	10.78 ± 0.51	15.00 ± 1.45*	10.85 ± 0.92	14.51 ± 0.76††	15.33 ± 1.03
Total cholesterol (mg/dl)	96.8 ± 5.8	236.4 ± 9.6††	104.6 ± 5.5**	101.6 ± 3.7	193.6 ± 7.3††	109.2 ± 7.2**
Triglyceride (mg/dl)	157.2 ± 9.2	105.6 ± 8.1††	80.6 ± 3.7*	143.6 ± 11.6	113.2 ± 12.4	88.0 ± 4.6
Free fatty acid (mEq/l)	0.695 ± 0.060	0.771 ± 0.053	0.593 ± 0.038*	0.670 ± 0.022	0.681 ± 0.059	0.663 ± 0.025

Data are means ± SE (n = 7–10), except for food intake. The results of the food intake are presented as means of two cages. ††$P < 0.01$ vs. the control group; *$P < 0.05$; **$P < 0.01$ vs. the HF (/HS) group.

4*A* and *B*). Moreover, the hepatic nuclear SREBP-1 protein was reduced by a HF diet (Fig. 4*E*), suggesting that HF diet induces obesity without enhancement of hepatic lipogenesis. Although EPA reduced FAS activity, mRNA of lipogenic enzymes was not affected by EPA (Fig. 4*A* and *B*). Treatment with EPA did not affect the activities of β-oxi-

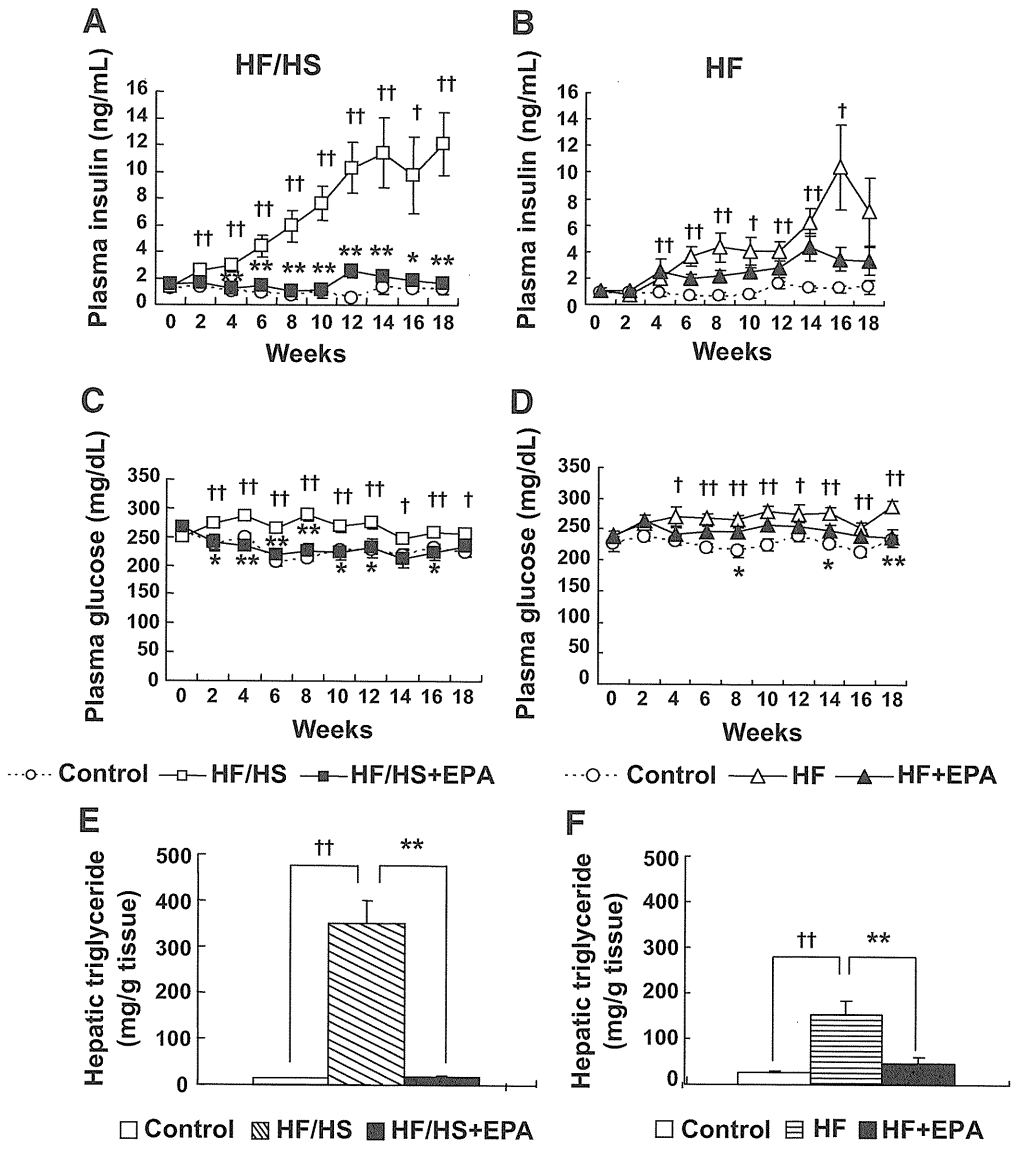

FIG. 2. Effect of EPA on HF/HS- and HF-induced metabolic abnormalities. ***A*** **and** ***B*****: Plasma insulin.** ***C*** **and** ***D*****: Plasma glucose.** ***E*** **and** ***F*****: Hepatic triglyceride content.** †$P < 0.05$; ††$P < 0.01$ **vs. control group.** *$P < 0.05$; **$P < 0.01$ **vs. HF (/HS) group.**

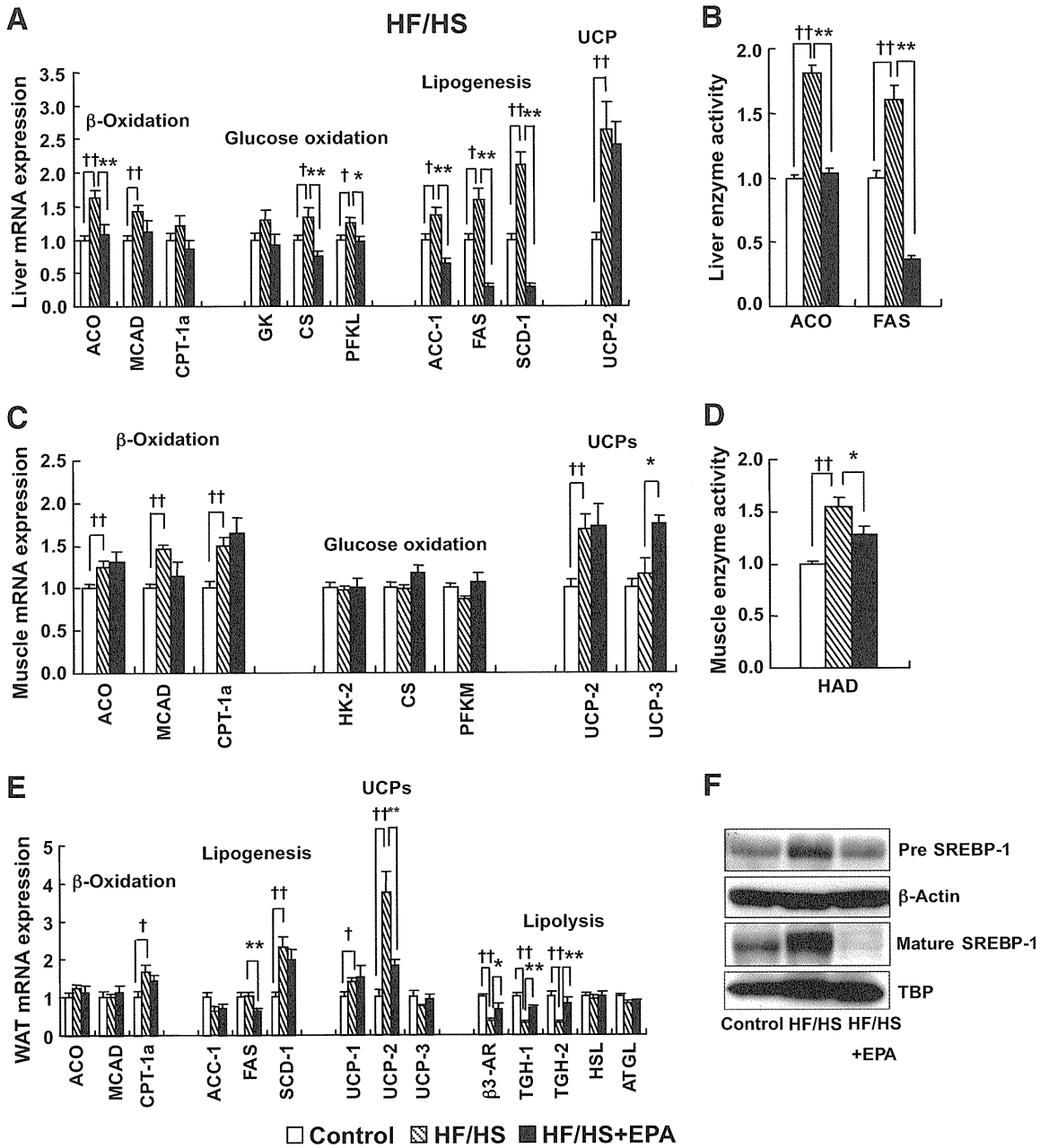

FIG. 3. Effect of EPA on energy metabolism–related genes in the HF/HS groups. *A* and *B*: Liver. *C* and *D*: Skeletal muscle. *E*: Epididymal WAT. *F*: Hepatic SREBP-1 protein in the HF/HS group. n = 7–10. †$P < 0.05$; ††$P < 0.01$ vs. control group. *$P < 0.05$; **$P < 0.01$ vs. HF/HS group. ACO, acyl-CoA oxidase; ATGL, adipose triglyceride lipase; CPT-1a, carnitine palmitoyltransferase-1a; CS, citrate synthase; GK, glucokinase; HAD, hydroxyacyl-CoA dehydrogenase; HK-2, hexokinase-2; HSL, hormone-sensitive lipase; MCAD, acetyl-CoA dehydrogenase, medium chain; PFKL, phosphofructokinase, liver; PFKM, phosphofructokinase, muscle, B-type.

dation enzymes in the liver and muscle or the mRNA expression of lipogenesis or lipolysis-related proteins in WAT from the HF group (Fig. 4*B–D*). Expression of mRNAs for β3-AR and TGH-1 in WAT from the HF group was lower than in the control group. TGH-2 mRNA expression tended to be low relative to the control group, but the difference was not statistically significant (Fig. 4*D*).

Effect of EPA on triglyceride secretion and VLDL fatty acid composition in the HF/HS group. In the liver, triglycerides are synthesized via lipogenesis and secreted as VLDL, which in turn delivered to peripheral tissues such as WAT. Evidence has suggested that some species of fatty acids affect triglyceride accumulation in adipocytes (32). We therefore examined hepatic triglyceride secretion and VLDL fatty acid composition in the HF/HS group (experiment 3). We found the enhanced secretion of triglycerides in the HF/HS group relative to the control group (Fig. 5*A*). Analysis of VLDL fatty acid composition revealed that palmitic (C16:0), palmitoleic (C16:1 n-7), and oleic acids (C18:1 n-9) are increased and stearic acid (C18:0) is

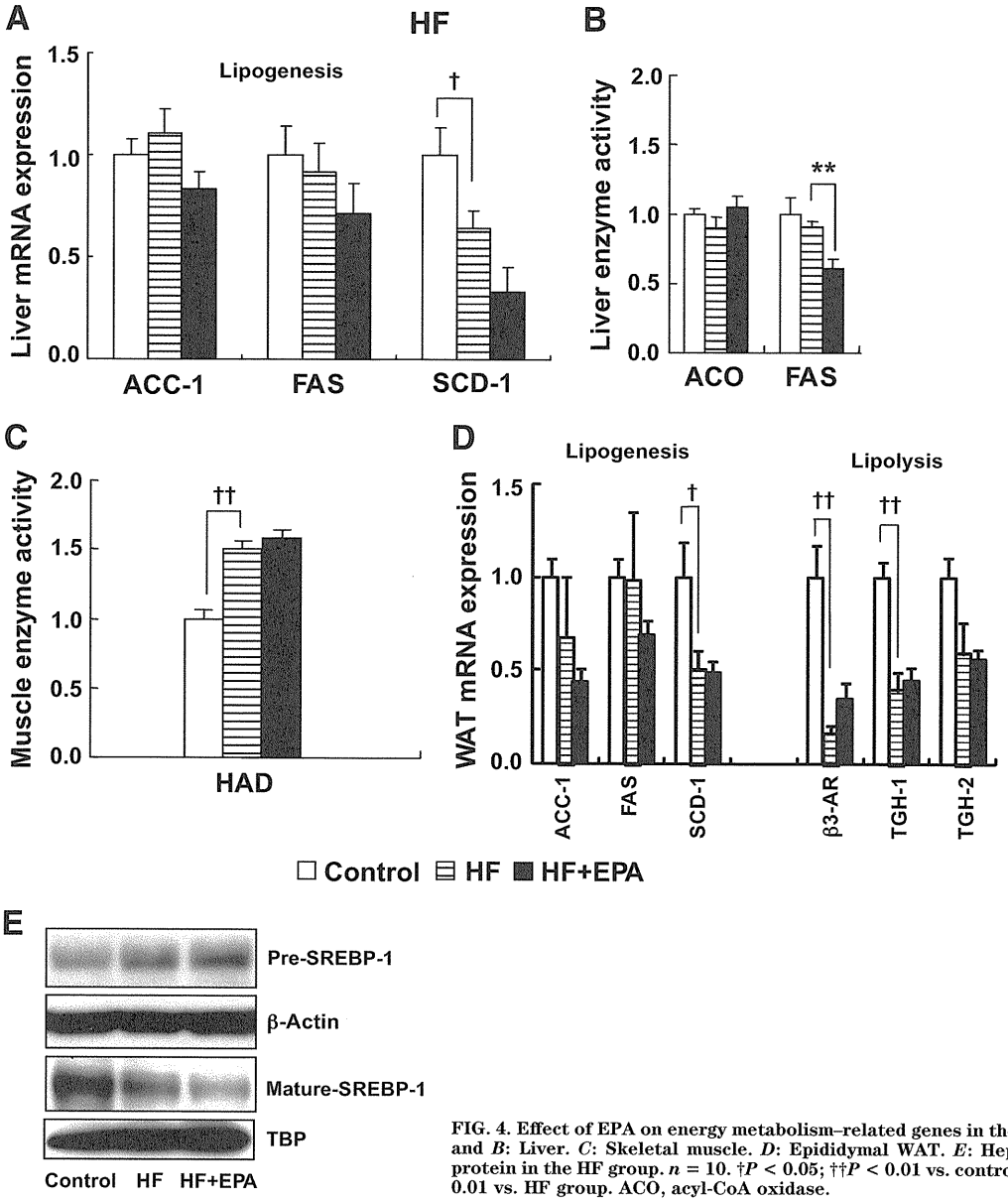

FIG. 4. Effect of EPA on energy metabolism–related genes in the HF groups. *A* and *B*: Liver. *C*: Skeletal muscle. *D*: Epididymal WAT. *E*: Hepatic SREBP-1 protein in the HF group. $n = 10$. †$P < 0.05$; ††$P < 0.01$ vs. control group. **$P < 0.01$ vs. HF group. ACO, acyl-CoA oxidase.

decreased in the HF/HS group relative to the control group (Fig. 5*B*). In this study, EPA (C20:5 n-3) was markedly increased in VLDL obtained from the HF/HS + EPA group relative to the HF/HS and control groups. Increased hepatic triglyceride secretion and the aberrant VLDL fatty acid composition in the HF/HS group were all reversed by EPA treatment (Fig. 5*A* and *B*). Linoleic acid (C18:2 n-6) was markedly decreased in both HF/HS and HF/HS + EPA groups relative to the control group.

Effect of EPA on WAT lipolysis in the HF/HS group. Because reduced expression of β3-AR, TGH-1, and TGH-2 mRNAs in WAT from the HF/HS group was restored by EPA treatment (Fig. 3*E*), we examined the effect of EPA on WAT lipolysis in the HF/HS group (experiment 4). There was no significant difference in glycerol release ex vivo between HF/HS and control groups (Fig. 6*A*). Treatment with isoproterenol, a β-AR agonist, stimulated glycerol release in the control group, which was markedly reduced in the HF/HS group. Interestingly, glycerol release with or without isoproterenol stimulation was higher in the HF/HS + EPA group than in the HF/HS group (Fig. 6*A*).

Effect of EPA on energy consumption in the HF/HS group. There was a marked difference in energy accumulation between HF/HS and HF/HS + EPA groups, despite similar caloric intake (Fig. 1 and Table 1). However, except UCP-3 in the skeletal muscle, gene expression of β-oxidation enzymes and UCPs were unchanged by EPA treatment (Fig. 3*A*, *C*, and *E*). Furthermore, the activities of β-oxidation–related enzymes were reduced by EPA (Fig. 3*B* and *D*). We, therefore, examined the effect of EPA on O_2 consumption and RER using indirect calorimetry (experiment 4). HF/HS feeding increased O_2 consumption and significantly reduced RER 6 weeks after the experiment (Fig. 6*C–F*). In this study, EPA treatment significantly increased the HF/HS-induced O_2 consumption and inhibited the HF/HS-induced decrease in RER throughout

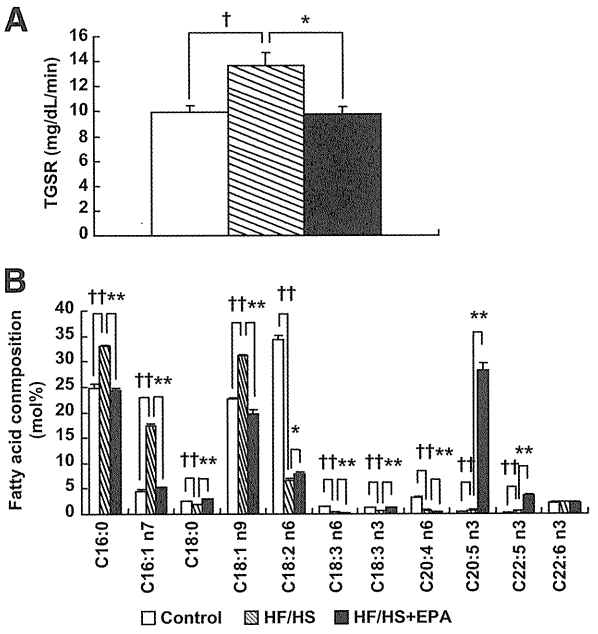

FIG. 5. Effect of EPA on triglyceride secretion rate (TGSR) and VLDL fatty acid composition in the HF/HS group. *A*: TGSR. *B*: VLDL fatty acid composition. $n = 6$. †$P < 0.05$; ††$P < 0.01$ vs. control group. *$P < 0.05$; **$P < 0.01$ vs. HF/HS group.

the experimental period ($P < 0.01$; 130 data points per mouse) (Fig. 6*C* and *E*). The mean values of RER for the light and dark cycles in the HF/HS + EPA group were significantly higher than those of the HF/HS group ($P < 0.01$) (Fig. 6*F*), whereas there was no significant difference in mean values of VO_2 between HF/HS and HF/HS + EPA groups (Fig. 6*D*). EPA also significantly increased O_2 consumption 2 weeks after the experiment ($P < 0.01$) (data not shown).

After 8 weeks of feeding, expression levels of mRNA of UCP-1 in brown adipose tissue (BAT) were unchanged by the HF/HS and HF/HS + EPA groups (Fig. 6*B*). There was no significant difference in UCP-3 mRNA expression in the skeletal muscle among control, HF/HS, and HF/HS + EPA groups (data not shown). EPA also suppressed body weight gain (control group, 28.47 ± 0.64 g; HF/HS group, 35.73 ± 0.99 g; HF/HS + EPA group, 30.78 ± 0.59 g) and epididymal WAT accumulation (control group, 0.36 ± 0.04 g; HF/HS group, 1.51 ± 0.14 g; HF/HS + EPA group, 0.80 ± 0.07 g) (experiment 4). There was no appreciable difference in caloric intake among control, HF/HS, and HF/HS + EPA groups (control group, 14.48 ± 2.03 kcal/day/mouse; HF/HS group, 13.76 ± 0.24 kcal/day/mouse; HF/HS + EPA group, 12.36 ± 0.67 kcal/day/mouse).

DISCUSSION

This study demonstrates that hepatic steatosis is more severe in the HF/HS group than in the HF group. Hyperinsulinemia also develops more rapidly in the HF/HS group than in the HF group, although both HF/HS and HF groups similarly develop obesity and WAT accumulation. In this study, we found that EPA ameliorates HF/HS-induced obesity, WAT inflammation, fatty liver, hyperinsulinemia, and hyperglycemia. By contrast, there is no effect of EPA on obesity in the HF group. These observations indicate the differential effect of EPA on metabolic parameters between HF/HS and HF groups. Expression and activities of hepatic lipogenic enzymes are increased in the HF/HS group, which are abolished by EPA treatment. By contrast, expression of hepatic lipogenic enzymes is not increased in the HF group, where EPA is ineffective against visceral fat accumulation and obesity. It seems that the lower expression of SREBP-1 in the HF/HS + EPA group results in the reduction of hepatic lipogenic enzymes. These observations, taken together, suggest that suppression of enhanced hepatic lipogenesis contributes to the anti-obesity effect of EPA. It is noteworthy that mice with liver-specific disruption of SCD-1 are resistant to high-carbohydrate diet–induced obesity but are sensitive to HF diet–induced obesity (33). In this study, we confirmed that EPA markedly reduces hepatic lipogenic enzymes including SCD-1. The phenotypic effect of liver-specific deficiency of SCD-1 is similar to that observed in this study, thereby supporting the concept that EPA exerts the anti-obesity effect at least in part through the suppression of hepatic lipogenesis.

Deficit of lipogenic enzymes or administration of lipogenic enzyme inhibitors has been reported to inhibit obesity. For instance, administration of FAS inhibitors lowers body weight by reducing food intake (34). Global deficiency of ACC-2 (35) or SCD-1 (36) or administration of antisense oligonucleotide against SCD-1 (37) also shows resistance to obesity through enhanced expression of β-oxidation enzymes or UCPs. On the other hand, neither anorectic effect nor upregulation of β-oxidation enzymes or UCPs has been reported with EPA. Then, how does reduced hepatic lipogenesis inhibit obesity or triglyceride accumulation in WAT? Here, we demonstrated that EPA markedly suppresses the HF/HS-induced hepatic triglyceride secretion and palmitic, palmitoleic, and oleic acids in VLDL. In this study, despite the enhanced hepatic lipogenesis and triglyceride secretion, plasma triglyceride concentrations are reduced in the HF/HS group relative to the control group. This may be because of HF/HS-induced activation of lipoprotein lipase in WAT, as suggested elsewhere (38). Previous studies with mice lacking VLDL receptor or apolipoprotein E demonstrated that VLDL metabolism is closely related to obesity (39,40). There are also several previous reports showing the relationship between triglycerides delivered from the liver and fat accumulation in the adipose tissue. For instance, obese subjects have exhibited enhanced VLDL-triglyceride secretion from the liver (41). Moreover, adenoviral overexpression of diacylglycerol-acyl transferase-1, which catalyzes the final step of hepatic triglyceride synthesis, has resulted in enhanced VLDL-triglyceride secretion and thus obesity (42). On the other hand, there is a report that triglyceride accumulation in WAT is enhanced by a certain species of fatty acids such as oleic and palmitic acids, among which, oleic acid tends to induce triglyceride accumulation in 3T3-L1 adipocytes (32). Moreover, plasma content of palmitoleic acid is positively correlated with obesity in humans (43,44). In this study, we showed that hepatic expression of SCD-1 is enhanced in the HF/HS group and is markedly suppressed by EPA. SCD-1 is known to catalyze the conversion from stearic and palmitic acids to oleic and palmitoleic acids, respectively. Increased oleic and palmitoleic acids in VLDL and hepatic gene expression of SCD-1 in the HF/HS group are both suppressed by EPA. It is, therefore, conceivable that increases in the quantity (i.e., secretion rate) and quality (i.e., fatty acid composition) of triglycerides delivered from the liver contribute to

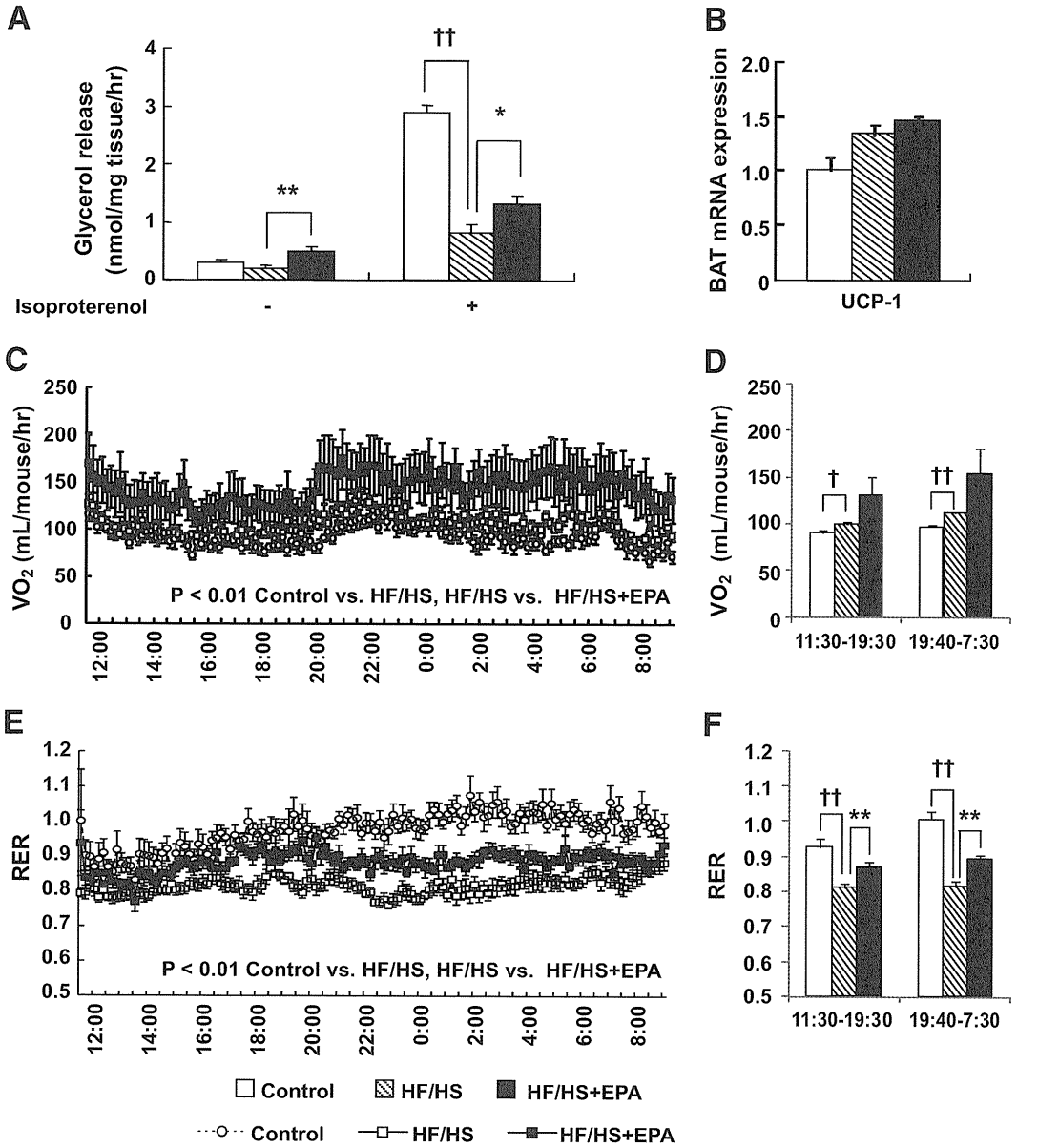

FIG. 6. Effect of EPA on WAT lipolysis and energy consumption in the HS/HF group. *A*: Glycerol release from epididymal WAT with or without 10 μmol/l isoproterenol. *B*: UCP-1 mRNA expression in BAT. Indirect calorimetry analysis of VO_2 (*C* and *D*) and RER (*E* and *F*). $n = 9$. ††$P < 0.01$ vs. control group. *$P < 0.05$; **$P < 0.01$ vs. HF/HS group.

triglyceride accumulation and thus obesity in the HF/HS group. Collectively, we speculate that the anti-obesity effect of EPA is due at least in part to its impact on the quantity and quality of triglycerides through the suppression of hepatic lipogenesis.

Lipogenesis is an efficient means to transform the energy accumulated in the body but not to consume energy. Because EPA results in marked reduction of body weight and fat accumulation in the HF/HS group, it is obligatory that EPA consumes energy. Here, we demonstrated that EPA enhances energy consumption and reverses the decreased RER in the HF/HS group. In this regard, Rustan et al. (45) reported that treatment with docosahexaenoic acid + EPA increases RER, but they did not show increased energy consumption. These observations suggest that energy accumulated not as triglycerides is consumed inside and/or outside the liver. Enhanced energy consumption accompanied by enhanced expression of β-oxidation enzymes and UCPs was reported in SCD-1 knockout mice (36) and mice treated with antisense oligonucleotides of SCD-1 (37). In this study, although EPA strongly suppresses hepatic SCD-1 mRNA expression, it does not enhance β-oxidation enzymes and UCPs. There are appreciable changes in gene expression, which suggests the EPA-induced increase in energy consumption. Expression of β-oxidation and glucose oxidation genes in the liver, skeletal muscle, and WAT is not increased by EPA treatment. Moreover, activities of acyl-CoA oxidase and hydroxyacyl-CoA dehydrogenase in the liver and skeletal muscle, respectively, were reduced by EPA, suggesting a minor contribution of

β-oxidation to enhanced energy consumption. This discussion is also supported by increased RER by EPA.

Expression of mRNAs for UCPs except UCP-3 in the skeletal muscle is not also increased when treated with EPA. Although UCP-3 mRNA expression in the skeletal muscle is only slightly increased by EPA (experiment 1), EPA fails to enhance expression of UCP-3 mRNA in the skeletal muscle as well as UCP-1 mRNA in BAT in experiment 4, suggesting a minor contribution of UCPs to EPA-induced energy consumption. In this regard, our preliminary data show no significant increase in O_2 consumption in the HF + EPA group relative to the HF group (data not shown). These observations, taken together, suggest that the suppressed hepatic lipogenesis is related to enhanced energy consumption in the HF/HS + EPA group. The molecular mechanisms by which EPA enhances energy consumption remain to be elucidated. We also found that EPA only partly restores the otherwise reduced WAT lipolysis in the HF/HS group. Similar to the HF/HS group, β3-AR, TGH-1, and TGH-2 mRNA expression is reduced in the HF group, but EPA fails in the restoration of the reduced gene expression. These observations suggest that EPA restoration of lipolysis is the consequence of the anti-obesity effect of EPA.

The mechanism underlying the anti-obesity effect of EPA has not been extensively studied, and the anti-obesity effect of EPA reported so far is somewhat controversial. Indeed, there is a report showing that EPA does not prevent visceral fat accumulation in rats with HF/HS-induced obesity, where EPA was administered for a much shorter period of time (15). On the other hand, Oh-i et al. (16) reported that EPA promotes body weight gain in rats fed an HF diet through the reduction of brain leptin transport or leptin resistance. The authors used diet without carbohydrate such as sucrose and fructose, when EPA may not exert the anti-obesity effect. In this study, the fatty acid composition was different between the HF/HS and HF diets used. Although the difference might modify the phenotype of our models, this would not invalidate our conclusion on the association between the anti-obesity effect of EPA and suppression of hepatic lipogenesis. In analyzing the anti-obesity effect of EPA, we should be careful of the doses of EPA administered, period of administration, species of animals used, etc., in addition to the proportion of sucrose versus fat in the diet.

Given that hepatic lipogenesis in the HF group was roughly comparable to that in control group, it is likely that hepatic lipogenesis is less associated with HF-induced obesity. This may be related to no appreciable anti-obesity effect of EPA in the HF group, even with a downregulation of hepatic lipogenic genes. In this regard, Kuda et al. (46) reported that treatment with fish oil tends to prevent HF-induced body weight gain, although not with statistical significance. This may be because of the potential difference in the anti-obesity effect between EPA used in this study and fish oil or the doses of administration. On the other hand, EPA reduced hepatic triglyceride in both the HF/HS and HF groups. Rustan et al. (47) reported that EPA inhibits incorporation of other fatty acids into triglyceride, which may explain the EPA-induced reduction of hepatic triglyceride accumulation in the HF group.

In conclusion, this study is the first demonstration that EPA prevents visceral fat accumulation and obesity, possibly through the suppression of hepatic lipogenesis and enhancement of energy consumption (Fig. 7). Because the metabolic syndrome is often associated with enhanced lipogenesis and steatosis or nonalcoholic fatty liver disease, this study suggests that EPA may be effective to improve visceral fat accumulation and hepatic steatosis in patients with the metabolic syndrome. Our data also suggest that EPA is suited for the treatment of the metabolic syndrome.

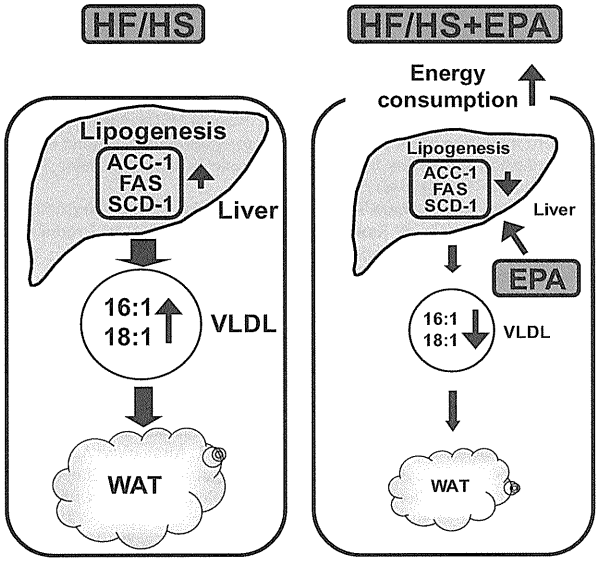

FIG. 7. Possible mechanism underlying the anti-obesity effect of EPA. EPA prevents WAT accumulation in HF/HS-induced obese mice possibly through the suppression of hepatic lipogenesis and enhancement of energy consumption.

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No other potential conflicts of interest relevant to this article were reported.

A.S. researched data, wrote the manuscript, and contributed to discussion. H.K. and Y.O. wrote the manuscript, contributed to discussion, and reviewed/edited the manuscript. T.N., M.O., M.N., K.M., M.I., and T.S. contributed to discussion and reviewed/edited the manuscript.

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Review

Adipose tissue macrophages: their role in adipose tissue remodeling

Takayoshi Suganami and Yoshihiro Ogawa*,†,1*

*Department of Molecular Medicine and Metabolism, Medical Research Institute, and †Global Center of Excellence Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, Tokyo, Japan

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ABSTRACT

The adipose tissue secretes a large number of bioactive substances, adipocytokines, which may be involved in a variety of physiologic and pathologic processes. Unbalanced production of pro- and anti-inflammatory adipocytokines seen in visceral fat obesity contributes critically to the development of the metabolic syndrome. Evidence has accumulated indicating that obesity is associated with a state of chronic, low-grade inflammation, suggesting that inflammation may be a potential mechanism, whereby obesity leads to insulin resistance. Indeed, obese adipose tissue is characterized by adipocyte hypertrophy, followed by increased angiogenesis, immune cell infiltration, extracellular matrix overproduction, and thus, increased production of proinflammatory adipocytokines during the progression of chronic inflammation. The dynamic change found in the adipose tissue can be referred to as "adipose tissue remodeling," in which stromal cells change dramatically in number and cell type during the course of obesity. Among stromal cells, infiltration of macrophages in the adipose tissue precedes the development of insulin resistance in animal models, suggesting that they are crucial for obesity-related adipose tissue inflammation. We have demonstrated that a paracrine loop involving saturated fatty acids and TNF-α derived from adipocytes and macrophages, respectively, aggravates obesity-induced adipose tissue inflammation. Notably, saturated fatty acids, which are released from hypertrophied adipocytes via the macrophage-induced lipolysis, serve as a naturally occurring ligand for TLR4 complex, thereby activating macrophages. Understanding the molecular mechanism underlying adipose tissue remodeling may lead to the identification of novel, therapeutic strategies to prevent or treat obesity-induced adipose tissue inflammation. *J. Leukoc. Biol.* 88: 33–39; 2010.

Abbreviations: ATF3=activating transcription factor 3, CLS=crown-like structure, DAMP=damage-associated molecular pattern, EPA=eicosapentaenoic acid, ER=endoplasmic reticulum, HMGB1=high-mobility group box-1, MKP-1=MAPK phosphatase-1, PAMP=pathogen-associated molecular pattern , PPARγ/δ=peroxisome proliferator-activated receptor γ/δ, PRR=pattern-recognition receptor, S100A8/A9=S100 calcium-binding protein A8/A9, SVF=stromal vascular fraction

Introduction

The metabolic syndrome is a constellation of visceral fat obesity, impaired glucose metabolism, atherogenic dyslipidemia, and blood pressure elevation, which all increase independently the risk of atherosclerotic diseases, such as ischemic heart disease and cerebral stroke [1–5]. The molecular basis for the clustering of such independent risks of atherosclerosis has not been fully elucidated, and visceral fat obesity is considered most important [1–5]. Evidence has accumulated indicating that obesity is associated with a state of chronic, low-grade inflammation, suggesting that inflammation may be a potential mechanism, whereby obesity leads to insulin resistance [1–4].

Adipose tissue secretes a large number of adipocytokines such as leptin, MCP-1, and adiponectin, which may be involved in a variety of physiologic and pathologic processes [1–3, 5, 6]. Unbalanced production of pro- and anti-inflammatory adipocytokines seen in visceral fat obesity critically contributes to the development of many aspects of the metabolic syndrome [1–5]. There is considerable evidence that obese adipose tissue is markedly infiltrated by macrophages; they may participate in the inflammatory pathways that are activated in the adipose tissue [7–9]. Notably, macrophage infiltration and inflammation-related gene expression in the adipose tissue precede the development of insulin resistance in animal models [7, 8], suggesting that infiltrated macrophages are an important source of inflammation in the adipose tissue. This review summarizes the role of macrophages in adipose tissue inflammation.

ADIPOSE TISSUE REMODELING

In addition to lipid-laden, mature adipocytes, the adipose tissue is composed of various cell types; the remaining SVF includes preadipocytes, endothelial cells, fibroblasts, and immune cells [10]. In contrast to "acute inflammation," which resolves by an active termination program [11], "chronic inflammation" is characterized by sustained interaction between

1. Correspondence: Department of Molecular Medicine and Metabolism, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan. E-mail: ogawa.mmm@mri.tmd.ac.jp