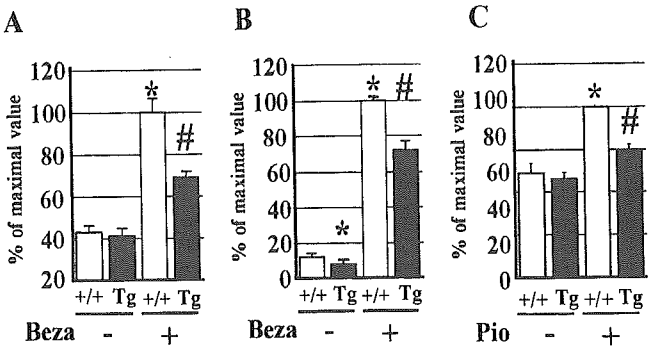

Fig. 4. Northern blot analysis of hepatic mRNA levels for PPARα and PPARγ target genes. The levels of mRNA expression for AOX (A) and CPT-I (B) in bezafibrate (Beza)–treated and untreated Liver mt PPARγ Tg (Tg) and nontransgenic littermates (+/+). The level of mRNA expression for ACC (C) in pioglitazone (Pio)–treated and untreated mice. Quantified data are normalized by the 18S rRNA expression level. Open bars, +/+; dark bars, Liver mt PPARγ Tg (n = 5 for each group), $*P < .05$ vs control +/+, $\#P < .05$ vs Beza +/+ (A and B) or Pio +/+ (C).

3.3. Generation of Liver mt PPARγ Tg

L468A/E471A mutant PPARγ1 was cloned into the downstream of human serum amyloid P component promoter so that transgene expression might be targeted specifically to the liver [37,38], and *Hind*III-*Xho*I fragment was used for microinjection (Fig. 3A). Liver expression of the transgene was confirmed by a 1.95 ± 0.30–fold increase in hepatic PPARγ1 expression by Northern blot analysis (Fig. 3B) (n = 5) and by the expression of transgene-specific mRNA in an RNase protection assay (data not shown) (n = 4). The liver-specific mutant PPARγ transgenic mice were named Liver mt PPARγ Tg.

To confirm the dominant-negative effect in vivo, we examined the mRNA levels for reported downstream target genes of PPARα and PPARγ. In the absence of a PPARα agonist, the liver mRNA level for acyl-CoA oxidase (AOX), a representative PPARα target gene in the liver [21], was not altered in the transgenic mice (Fig. 4A). However, when Liver mt PPARγ Tg were treated with a PPARα agonist, agonist-induced enhancement of AOX expression was significantly suppressed (Fig. 4A). Disturbed up-regulation of AOX by peroxisome proliferator in Liver mt PPARγ Tg is in line with data from PPARα-null mice, which exhibit unaltered basal AOX expression in the liver with an abolished response to PPARα agonist [21]. In contrast, carnitine palmitoyl transferase I (CPT-I), another PPARα target gene [14], was partially but significantly suppressed in Liver mt PPARγ Tg in both the presence and absence of PPARα agonist (Fig. 4B). Augmentation of the expression of acetyl-CoA carboxylase (ACC), a reported PPARγ target gene in the liver [15], in the presence of a PPARγ agonist, was significantly suppressed in Liver mt PPARγ Tg (Fig. 4C). These data provide supporting evidence for the dominant-negative effect of the transgene in vivo.

At the age of 10 weeks, Liver mt PPARγ Tg showed a mean body weight comparable to their nontransgenic littermates (non-Tg). The plasma parameters for glucose and lipid metabolism were comparable between the genotypes (Table 1). The liver weight, histology, and triglyceride content were not altered in the transgenics (Table 1, Fig. 5). Because our goal was to elucidate the impact of mutant PPARγ in steatosis, we next subjected mice to 2 distinct steatogenic stimuli, prolonged fasting and dietary lipid overload.

3.4. Liver mt PPARγ Tg are susceptible to fasting-induced steatosis

Both Liver mt PPARγ Tg and non-Tg developed steatosis as a result of prolonged food deprivation for 72 hours (Fig. 5A). Of note, in the fasted state, the steatosis was drastically aggravated in Liver mt PPARγ Tg in comparison with non-Tg. In non-Tg, microvesicular steatosis was found, localized around the central venule. In contrast, macrovesicular, as well as microvesicular, steatosis was prominent and far-reaching toward the portal area in Liver mt PPARγ Tg (Fig. 5A) (n = 5, representative data). The vacuoles visualized upon H-E staining were positive for lipid by oil red O staining (data not shown). In addition to these histological findings, the liver triglyceride content was also significantly higher in the transgenics (Fig. 5B) (n = 5). Worsening of fasting-induced steatosis in Liver mt PPARγ Tg is reminiscent of PPARα knockout mice, which exhibit increased susceptibility to fasting-induced steatosis [14]. It can be presumed that the worsening of fasting-induced steatosis in Liver mt PPARγ Tg is attributable to PPARα suppression in the liver. Augmented AOX and CPT-I expression in the fasted liver was significantly suppressed in Liver mt PPARγ Tg (Fig. 5C), further supporting a role for PPARα suppression in the severe steatosis in the fasted Liver mt PPARγ Tg.

3.5. Liver mt PPARγ Tg are protected against high-fat diet–induced steatosis

Hepatic expressions of PPARα and PPARγ were substantially increased in HFD-induced steatosis (Fig. 1). To

Table 1
Body weight, liver weight, and plasma parameters of Liver mt PPARγ Tg mice

Genotype	Body weight (g)	Liver weight (g)	ALT (IU/L)	Glucose (mmol/L)	Insulin (ng/mL)	TG (mg/dL)	NEFA (mEq/L)
Wild type	21.4 ± 1.2	1.49 ± 0.03	28.0 ± 4.7	8.0 ± 1.2	1.03 ± 0.13	106.9 ± 23.7	767.5 ± 82.4
Liver mt PPARγ Tg	21.8 ± 2.4	1.48 ± 0.01	26.5 ± 8.0	7.7 ± 1.1	0.98 ± 0.15	97.98 ± 30.4	709.0 ± 117

Ten-week-old male wild-type (n = 5) and Liver mt PPARγ Tg mice (n = 5) maintained on a chow diet were used. Blood was extracted 9:00 to 11:00 AM from mice fed ad libitum. Data are mean ± SEM. No significant difference was observed between the genotypes. ALT indicates alanine aminotransferase; TG, triglyceride; NEFA, nonesterified fatty acids.

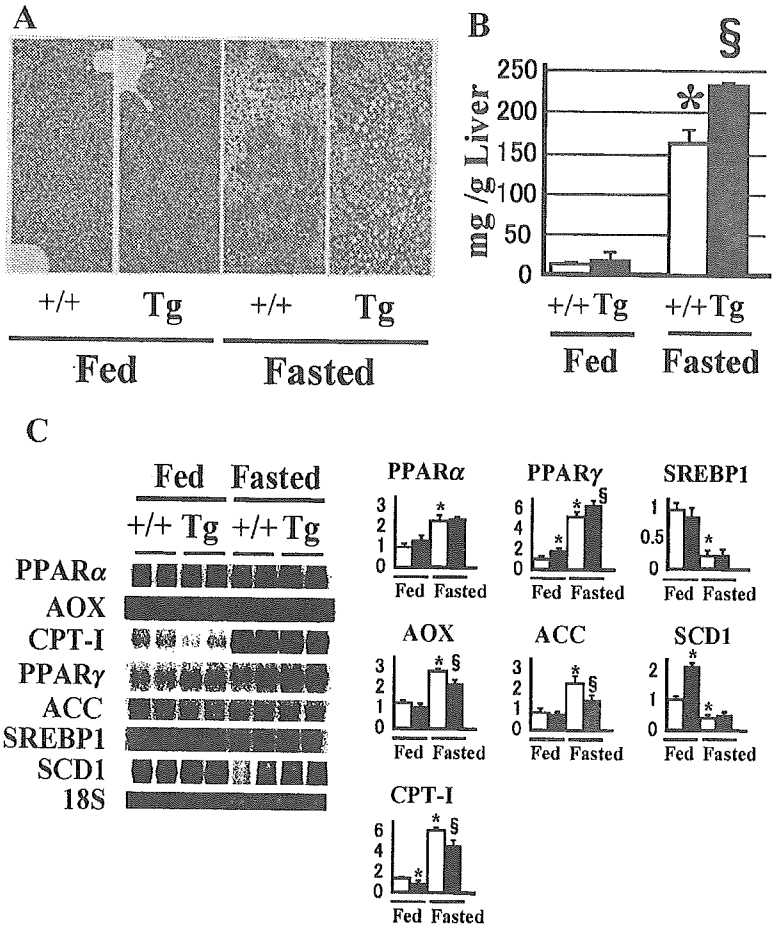

Fig. 5. Liver histology (A) (H-E, magnification ×200; representative data), liver triglyceride content (B), and mRNA levels for a series of PPAR target genes (C) in fasted Liver mt PPARγ Tg (Tg) and nontransgenic littermates (+/+). C, Representative blots and quantified data normalized by the 18S rRNA expression level (in arbitrary units) are shown. Open bars, +/+; dark bars, Liver mt PPARγ Tg (n = 5 for each group), *P < .05 vs Fed +/+, §P < .05 vs Fasted +/+.

elucidate the functional relevance of increased PPARα and PPARγ expressions in HFD-induced steatosis, the liver histology and triglyceride content were analyzed in HFD-fed Liver mt PPARγ Tg. In striking contrast to fasting-induced steatosis, HFD-induced steatosis was markedly ameliorated in Liver mt PPARγ Tg compared with non-Tg (Fig. 6A) (n = 5, representative data). In HFD-fed non-Tg, diffuse and severe macrovesicular steatosis was observed. On the other hand, high-fat feeding resulted only in limited numbers of lipid droplets in perivenular regions in the liver of Liver mt PPARγ Tg (Fig. 6A). In parallel with the histological findings, hepatic triglyceride content was also significantly lowered in Liver mt PPARγ Tg (Fig. 6B) (n = 5). In the transgenics, hepatic expression of both of the PPARα targets, AOX and CPT-I, was suppressed on HFD (Fig. 6C). AOX and CPT-I are critical factors in fatty acid oxidation in the liver, and down-regulation of AOX and CPT-I in PPARα knockout mice is associated with aggravated HFD-induced steatosis [14,21,24]. Liver mt PPARγ Tg are different from PPARα knockout mice in that PPARγ is also suppressed. The mRNA level for ACC, a PPARγ target gene in the liver, was also suppressed in Liver mt PPARγ Tg even on HFD (Fig. 6C). ACC is a representative lipogenic enzyme, and its suppression in the transgenics may partly explain the ameliorated steatosis in HFD-fed Liver mt PPARγ Tg.

3.6. Concerted up-regulation of PPARα and PPARγ target genes in steatosis is abrogated in Liver mt PPARγ Tg

In the wild-type mice, both the PPARα and PPARγ mRNA levels were augmented in both fasting- and HFD-induced steatosis (Fig. 1). Accordingly, the AOX, CPT-I, and ACC mRNA levels were coordinately elevated as a result of fasting and HFD (Figs. 5C and 6C). In contrast, hepatic expression of both sterol regulatory element binding protein 1 (SREBP1) and stearoyl-CoA desaturase 1 (SCD1) was decreased in fasting-induced steatosis, whereas it was augmented in HFD-induced steatosis (Figs. 5C and 6C). These findings suggest distinct molecular mechanisms underlying fasting- and HFD-induced steatosis as far as SREBP1 and SCD1 are concerned.

Expression of SCD1, a potent lipogenic enzyme [41], was augmented in chow-fed Liver mt PPARγ Tg without histological or biochemical signs of steatosis (Fig. 5A and C). SCD1 is a target of PPARα [42], PPARγ [15], and SREBP1 [43] in the liver. The mechanisms whereby

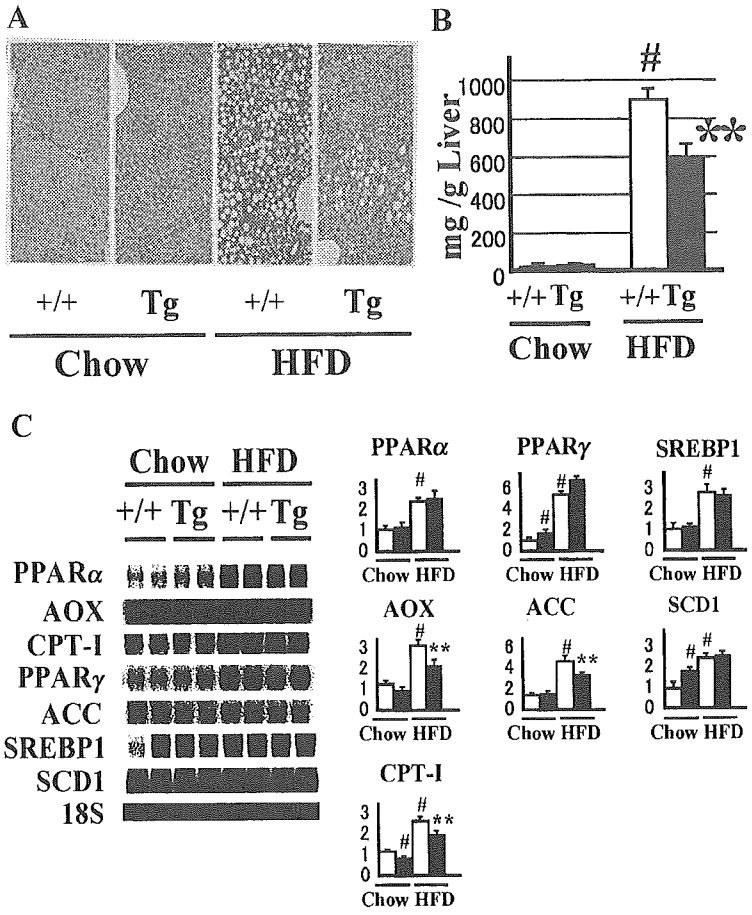

Fig. 6. Liver histology (A) (H-E, magnification ×200; representative data), liver triglyceride content (B), and mRNA levels for a series of PPAR target genes (C) in high-fat diet (HFD)–fed mice. C, Representative blots and quantified data normalized by the 18S rRNA expression level (in arbitrary units) are shown. Open bars, +/+; dark bars, Liver mt PPARγ Tg (n = 5 for each group), #$P < .05$ vs Chow-fed +/+, **$P < .05$ vs HFD-fed +/+.

SCD1 is up-regulated in Liver mt PPARγ Tg must await further investigation.

In Liver mt PPARγ Tg, the AOX, CPT-I, and ACC mRNA levels were all suppressed by fasting as well as HFD (Figs. 5C and 6C). Similar suppression of these genes in Liver mt PPARγ Tg by fasting and HFD does not provide a mechanistic explanation for the different hepatic outcomes as a result of fasting and HFD. On the other hand, suppression of the PPARα and PPARγ target genes in the transgenics in the fasted and HFD-fed states supports the dominant-negative effect of the transgene even in the case of dietary modification. Although the molecular mechanisms still need to be investigated, the distinct responses of Liver mt PPARγ Tg to the 2 types of steatogenic stimuli indicate that PPARα and PPARγ are differently involved in the pathophysiology of fasting- and HFD-induced steatosis.

4. Discussion

To explore the impact of PPARα and PPARγ cosuppression in the liver, we generated liver-specific mutant PPARγ transgenic mice (Liver mt PPARγ Tg), in which the transcriptional activities of liver PPARα and PPARγ are substantially suppressed. Liver mt PPARγ Tg was susceptible to fasting-induced steatosis, whereas HFD-induced steatosis was alleviated in the transgenics.

PPARα is highly expressed in the liver and brown adipose tissue in mice [14]. PPARγ is preferentially expressed in adipocytes but is also present in the liver, and its expression is augmented in murine steatosis [27-30]. It has been reported that, in liver tissue, PPARα and PPARγ are expressed at higher levels in hepatocytes than in endothelial and Kupffer cells [44]. Our data show that PPARα and PPARγ expressions are augmented in both fasting- and HFD-induced steatotic liver, leading us to speculate that cosuppression of PPARα and PPARγ in the liver might have some effects on the development of steatosis.

L468A/E471A mutant PPARγ1 has been reported to serve as a dominant-negative on PPARγ1 and γ2 activities, by recruiting and binding transcriptional corepressors [32]. In addition, it has also been shown that, in JEG-3 trophoblastic cells, L468A/E471A mutant PPARγ1 exerts an inhibitory effect on PPARα transcriptional activity [32]. In our experiment using HEK293 cells, L468A/E471A mutant PPARγ1 exhibited a striking dominant-negative effect on both PPARα and PPARγ1.

It is hard to assess exactly a dominant-negative effect in vivo because numerous factors such as putative endogenous ligands or coregulators are involved in the regulation of PPARα and PPARγ activities [45]. In Liver mt PPARγ Tg, the augmentation of the mRNA levels for AOX and ACC by respective treatment with agonists for PPARα and PPARγ was attenuated. CPT-I expression was significantly decreased in both the presence and absence of the PPARα agonist. Decreased expression of such target genes provides in vivo evidence that transgene-derived L468A/E471A mutant PPARγ1 exerts its dominant-negative effect on both PPARα and PPARγ. Suppression of the target genes in Liver mt PPARγ Tg was also evident in the fasted and HFD-fed conditions. The extent of target gene suppression in vivo was small compared with the reduction in PPRE-luciferase activity in vitro. The difference may partly be explained by regulatory mechanisms for AOX, CPT-I, and ACC independent of PPARα and PPARγ.

A growing body of evidence indicates that lipid accumulation in the liver is associated with insulin resistance [8,9]. The hepatic lipid content is determined by the balance between tissue uptake/synthesis and release/degradation of lipids [13]. PPARα is a key regulator of lipid metabolism in the liver, playing a crucial role in fatty acid oxidation and degradation [14,21-24]. Notably, mainly through enhancement of fatty acid oxidation, treatment with PPARα agonists improved steatosis in lipoatrophic A-ZIP/F-1 mice [46] and ethanol-fed mice [47]. Liver-specific PPARγ disruption has been shown to improve severe fatty liver in *ob/ob* mice [15], with reduced expression of a set of PPARγ target genes involved in lipid synthesis and uptake. Adenoviral overexpression of wild-type PPARγ1 in the liver augments expression of lipogenic enzymes and results in steatosis [31]. These reports indicate that liver PPARα is antisteatotic, whereas liver PPARγ is prosteatotic.

Liver mt PPARγ Tg exhibit opposite responses to fasting and HFD in terms of their susceptibility to steatosis. Enhancement of the mRNA levels for AOX and CPT-I due to fasting was attenuated in the transgenics, raising the possibility that suppression of PPARα-dependent fatty acid oxidation may account for the aggravation of steatosis in Liver mt PPARγ Tg (Fig. 7). In contrast, the augmented ACC expression on HFD was attenuated in the transgenics, indicating that suppression of PPARγ-induced lipogenesis may contribute to the amelioration of steatosis in Liver mt PPARγ Tg (Fig. 7). However, in fasted Liver mt PPARγ Tg, ACC expression was also suppressed compared with fasted non-Tg. Conversely, expression of antisteatotic AOX and CPT-I was reduced in HFD-fed transgenics. Further studies are necessary to elucidate the molecular mechanisms underlying the opposite responses of Liver mt PPARγ Tg to fasting and HFD.

In summary, we demonstrate that liver-specific expression of mutant PPARγ leads to suppression of PPARα and PPARγ target gene expression in vivo. Liver mt PPARγ Tg are susceptible to fasting-induced steatosis, but they are protected against HFD-induced steatosis, suggesting a possible interplay of PPARα and PPARγ in the pathophysiology of steatosis.

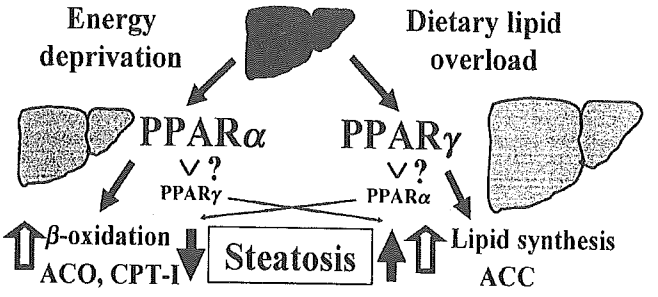

Fig. 7. Hypothetical scheme: possible distinct roles of PPARα and PPARγ in fasting-induced and high-fat diet–induced steatosis in mice. The induction of AOX and CPT-I mRNA levels due to fasting is attenuated in the Liver mt PPARγ Tg, suggesting the possibility that suppression of PPARα-dependent fatty acid oxidation may account for the aggravation of fasting-induced steatosis in the transgenics. In contrast, the augmented ACC expression on HFD is attenuated in the transgenics, indicating that the suppression of PPARγ-dependent lipogenesis may contribute to the amelioration of HFD-induced steatosis. The opposite hepatic outcomes in Liver mt PPARγ Tg as a result of fasting and high-fat feeding may indicate distinct roles of PPARα and PPARγ in 2 different types of nutritionally provoked steatosis.

Acknowledgment

The authors thank Ms Mayumi Nagamoto for technical assistance and Ms Itoe Kitahata and Ms Sayuri Shinhara for secretarial assistance. The authors also thank Dr Yoshihito Tsuji for suggestions during manuscript preparation. The authors are grateful to Prof Iichiro Shimomura and Dr Morihiro Matsuda for generously providing probes for acyl-CoA oxidase, carnitine palmitoyl transferase I, and acetyl-CoA carboxylase. This work was supported by grant-in-aid for Scientific Research (B2) (16390267); grant-in-aid for Scientific Research (S2) (16109007); grant-in-aid for Exploratory Research (16659243); grant-in-aid for Scientific Research on Priority Areas (15081101), the Ministry of Education, Culture, Sports, Science, and Technology of Japan, Research Grant from Special Coordination Funds for Promoting Science and Technology; Research award from Japan Foundation for Applied Enzymology; Tanita Healthy Weight Community Trust; Daiwa Securities Health Foundation; ONO Medical Research Foundation; Yamaguchi Endocrine Research Association; The Ichiro Kanehara Foundation; Yamanouchi Foundation for Research on Metabolic Disorders; The Cell Science Research Foundation; Takeda Medical Research Foundation; and The Study Grant for Japan Insulin Study Group, Smoking Research Foundation, and Study Grant from Metabolic Syndrome Foundation.

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GPR40 gene expression in human pancreas and insulinoma

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Received 17 October 2005
Available online 2 November 2005

Abstract

To assess gene expression of a membrane-bound G-protein-coupled fatty acid receptor, GPR40, in the human pancreas and islet cell tumors obtained at surgery were analyzed. The mRNA level of the GPR40 gene in isolated pancreatic islets was approximately 20-fold higher than that in the pancreas, and the level was comparable to or rather higher than that of the sulfonylurea receptor 1 gene, which is known to be expressed abundantly in human pancreatic β cells. A large amount of GPR40 mRNA was detected in tissue extracts from two cases of insulinoma, whereas the expression was undetectable in glucagonoma or gastrinoma. The present study demonstrates that GPR40 mRNA is expressed predominantly in pancreatic islets in humans and that GPR40 mRNA is expressed solely in human insulinoma among islet cell tumors. These results indicate that GPR40 is probably expressed in pancreatic β cells in the human pancreas.

Keywords: G-protein-coupled receptor; GPR40; Islet of Langerhans; Pancreatic β cell; Insulinoma; Fatty acid; Insulin secretion

Fatty acids (FAs), especially long chain FAs, augment glucose-stimulated insulin secretion (GSIS) from the pancreas [1]. To date, however, the entire mechanism whereby FAs acutely induce GSIS augmentation has not been fully elucidated [2]. In addition, chronic FA exposure causes marked deterioration of β cell function, which is referred to as lipotoxicity [3,4].

GPR40, a membrane-bound G-protein-coupled receptor, is reported to be preferentially expressed in pancreatic β cells in rodents and is involved in the regulation of GSIS after acute exposure to mid- or long-chain FAs in in vitro experiments [5]. A recent study regarding GPR40 knockout mice and β cell-specific GPR40 transgenic mice suggests the possible involvement of GPR40 in diabetes mellitus [6]. Although these findings suggest the implication of GPR40 in the regulation of insulin secretion and glucose homeostasis, gene expression of GPR40 in humans has not been fully elucidated [2,7]. The present study was designed to investigate GPR40 mRNA expression in the pancreas and islet cell tumors in humans.

Materials and methods

Participants, tissue sampling, and pancreatic islet isolation. The present study was performed according to the Declaration of Helsinki and approved by the Ethical Committee on Human Research of Kyoto University Graduate School of Medicine (No. 508, 2003). Signed informed consent was obtained from all patients in the present study. Normal pancreata were obtained from four patients with pancreatic cancer and one patient with insulinoma at the time of surgery. Sample margins contained no signs of tumor invasion, thus these pancreatic samples were thought to be free of tumor. In addition, insulinoma ($n = 2$), glucagonoma ($n = 1$), and gastrinoma ($n = 1$) were collected at surgery. Pancreatic islet tissues were promptly isolated from the pancreas through the mince method [8]. After dithizone staining, pancreatic islets were manually collected using a stereomicroscope (SZ-STB1; Olympus, Tokyo, Japan).

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doi:10.1016/j.bbrc.2005.10.161

Table 1
Sequences of TaqMan primers and probes

Gene	GenBank Accession No.	Probe (FAM-5′ → 3′-TAMRA)	Primers[a] (5′ → 3′)
GPR40	NM005303	TCTGCCCTTGGCCATCACAGCCT	*f* GCCCGCTTCAGCCTCTCT
			r GAGGCAGCCCACGTAGCA
SUR1	NM000352	CCTCACCAACTACCAACGGCTCTGCG	*f* GCTGCCCATCGTTATGAGGG
			r GAATGTCCTTCCGCACCTGG
GAPDH	NM002046	CCTCAAGGGCATCCTGGGCTACACTG	*f* TGAAGCAGGCGTCGGAGG
			r GCTGTTGAAGTCAGAGGAGACC

Abbreviations: FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.
[a] Forward primers are designated by *f* and reverse primers by *r*.

Quantification of mRNA expression of GPR40 and SUR1. We measured mRNA expression of genes encoding GPR40 and sulfonylurea receptor 1 (SUR1) [9]. Total RNA was extracted using the QIAGEN RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) [10] and treated with DNase I to avoid contamination of genomic DNA, because GPR40 gene is a single exon one. First strand cDNA was synthesized by random hexamer primed reverse transcription using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) [11]. The mRNA level was quantified by the TaqMan PCR method using an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) as described [12]. RT-minus samples served as a control to exclude the amplification of potential genomic DNA contamination. To calculate the copy number of each mRNA, standard curves were generated using synthesized oligo DNA fragments (Proligo Japan, Kyoto, Japan) containing the PCR amplicon region. The mRNA level of each gene was normalized to that of GAPDH and expressed in arbitrary units as relative to the GPR40 mRNA level in the normal pancreas from case 1. Table 1 summarizes the sequences of primers and probes used in the present study.

Results

Expression of GPR40 mRNA in human pancreas and isolated pancreatic islets

By use of total RNA samples from patients who underwent pancreatectomy, GPR40 mRNA expression in the human pancreas was examined. GPR40 mRNA was detected in normal pancreata obtained from four patients with pancreatic cancer and one patient with insulinoma, where the relative expression values were from 0.70 to 2.08 in arbitrary unit (1.16 ± 0.39, means ± SEM). The GPR40 mRNA level in freshly prepared isolated pancreatic islets was approximately 20-fold higher than that in the pancreas from the same individuals (Fig. 1).

To evaluate the expression of GPR40 in human pancreatic islets, we measured gene expression of sulfonylurea

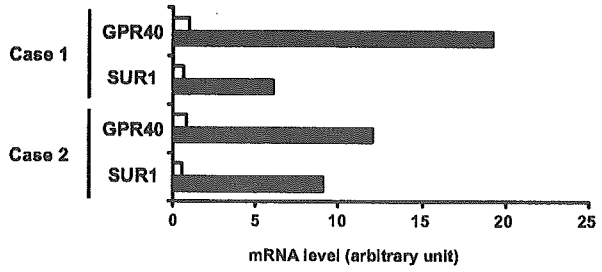

Fig. 1. GPR40 and SUR1 mRNA expression in human pancreas and pancreatic islet. Total RNA extracted from human pancreata (open bars) and pancreatic islets (closed bars) was analyzed in two patients. The mRNA level for each gene was normalized to that of GAPDH.

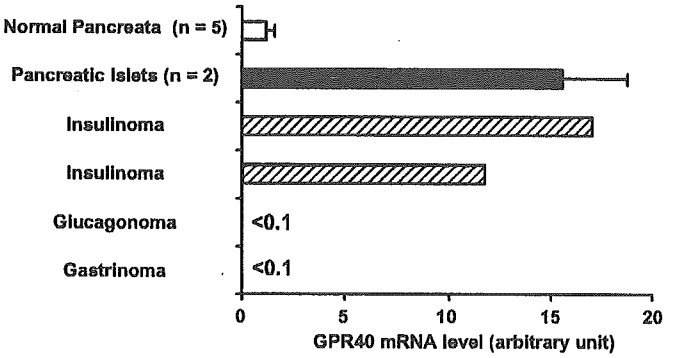

Fig. 2. GPR40 mRNA expression in human pancreas, pancreatic islet, and islet cell tumors. Total RNA extracted from the normal human pancreas (the open bar, $n = 5$), pancreatic islets (the closed bar, $n = 2$), and islet cell tumors (hatched bars). The GPR40 mRNA level was normalized to that of GAPDH. Data are expressed as means ± SEM in the pancreas and pancreatic islets.

receptor, which is known to be expressed abundantly in pancreatic β cells. The estimated mRNA copy number of the GPR40 gene in isolated pancreatic islets was comparable to or rather higher than that of the sulfonylurea receptor 1 (SUR1) gene (Fig. 1).

A large amount of GPR40 mRNA expression in insulinoma tissues

We next explored the expression of GPR40 mRNA in islet cell tumors including insulinoma, glucagonoma, and gastrinoma. GPR40 mRNA was detected in tissue extracts from two cases of insulinoma, which was comparable to that in human pancreatic islets (Fig. 2). In contrast, GPR40 mRNA was undetectable in tissue extracts from glucagonoma or gastrinoma (Fig. 2).

Discussion

As pancreatic tissues are extremely vulnerable to postmortem autolysis, freshly prepared specimens obtained at operation have a great advantage for the precise analysis of the GPR40 mRNA level. Using expeditiously isolated pancreatic islets from the pancreatic tissue obtained during surgery, the present study demonstrates that a large amount of GPR40 mRNA is expressed in pancreatic islets in humans. Levels of GPR40 mRNA expression in

pancreatic islets are 20-fold higher than those of the whole pancreas in the same individuals. The present study also demonstrates that a large amount of GPR40 mRNA is expressed in the insulinoma tissue. In contrast, the mRNA was not detected in glucagonoma or gastrinoma. This is the first demonstration of GPR40 mRNA expression in human insulinoma. These findings indicate that GPR40 is probably expressed mainly in β cells in the human pancreas.

It is important to note that the mRNA level of GPR40 is comparable to that of SUR1 in human pancreatic islets, which is abundantly expressed in human pancreatic β cells and works as a target of anti-diabetic sulfonylurea agents [13]. Thus, the present study indicates that GPR40 is expressed in β cells in the human pancreas, suggesting its involvement in the regulation of insulin secretion.

Acknowledgments

We are grateful to Ms. M. Nagamoto for her excellent technical assistance. This study was supported in part by a research grant from Special Coordination Funds for Promoting Science and Technology (JST, Japan), a Grant-in-Aid for Scientific Research (S2) (16109007), a Grant-in-Aid for Scientific Research (B2) (16390267), a Grant-in-Aid for Exploratory Research (16659243), a Grant-in-Aid for Scientific Research on Priority Areas (15081101) (The Japanese Ministry of Education, Culture, Sports, Science and Technology), a Grant-in-Aid for Research (The Japanese Ministry of Health, Labor and Welfare), and a research award from the Japan Foundation for Applied Enzymology, the Yamaguchi Endocrine Research Association, The Cell Science Research Foundation, the Takeda Medical Research Foundation, the Smoking Research Foundation, and the Metabolic Syndrome Research Foundation.

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Expression of the adrenomedullin gene in adipose tissue

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Received 9 December 2004; received in revised form 16 February 2005; accepted 29 July 2005
Available online 8 September 2005

Abstract

Adrenomedullin (AM) is a potent vasodilating peptide originally isolated from human pheochromocytoma cells. This report concerns the expression and secretion of AM from adipose tissue. Northern blot analysis demonstrated marked expression of AM mRNA in mouse adipose tissue. Expression levels in adipose tissues were 2.5–3.2 times higher than in the kidney. AM mRNA level in mature adipocytes was 7.3 times higher than in the stroma–vascular fraction of adipose tissue. In mature adipocyte culture, time-dependent increase of AM peptide concentration in the culture medium was detected. AM expression was also detected in human subcutaneous adipose tissue. Adipose AM expression significantly increased in obesity mouse model, high-fat diet fed mice and ob/ob mice. These results suggest that adipose tissue, especially mature adipocytes, is major source of AM in the body, and that adipocyte-derived AM plays a pathophysiological role in obesity.

Keywords: Adrenomedullin; Adiopocyte; Fat; Obesity; ob/ob mice

1. Introduction

It has recently been suggested that adipose tissue is an endocrine organ. A wide variety of factors secreted from adipocytes, including leptin, TNF alpha, adiponectin, resistin and free fatty acid, are playing crucial roles in energy expenditure and glucose metabolism [1]. Adipocytes produce a host of vasoactive substances including angiotensinII [2], endothelin-1 [3], nitric oxide [4], prostacyclin [5] and natriuretic peptide [6]. Recent studies have suggested the paracrine/autocrine involvement of these molecules in the regulation of adipocyte growth and differentiation.

Adrenomedullin (AM) is a potent vasodilating peptide that was originally isolated from human pheochromocytoma cells [7]. Structural analysis indicates that AM belongs to the calcitonin gene-related peptide (CGRP) superfamily. AM and CGRP share a common receptor known as the calcitonin-receptor-like-receptor (CRLR). The ligand specificity of CRLR is regulated by the receptor-activity-modifying proteins (RAMPs), which is a family of proteins with a single transmembrane domain [8]. CRLR associated with RAMP1 acts as a CGRP receptor, while it binds to AM when coexpressed with RAMP2/3. It has been reported that AM-producing cells are distributed widely throughout the body, including the adrenal glands, lungs, heart and kidneys [9]. In vitro studies have demonstrated that vascular smooth muscle cells and endothelial cells secrete AM, and have suggested that the major source of AM in the body is the vascular wall [10].

We have previously reported that RAMPs are highly expressed in rat adipose tissue [11], and have posited the existence of an AM system in adipose tissue. The study reported here found massive expression of the AM gene in mouse and human adipose tissue, and its upregulation in obesity mouse models.

2. Materials and methods

2.1. Ethics

This study conforms to the policy of the Ethics Committee on Human Research of the Kyoto University Graduate School of Medicine, and written informed consent was obtained from all subjects.

Abbreviations: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; RAMPs, receptor-activity-modifying protein; sv-f, stroma–vascular fraction; BAT, intrascapular brown adipose tissue; VEC, vascular endothelial cells; VSMC, vascular smooth muscle cells.

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doi:10.1016/j.regpep.2005.07.006

2.2. Mouse adipose tissue

Male C57BL/6 and ob/ob mice aged 14 weeks provided by Shionogi Research Laboratories (Osaka, Japan) were used in this study. They were treated in accordance with our institutional guidelines for animal research, housed in an animal room maintained at 24 °C with a 12:12-h light-dark cycle, fed a standard laboratory diet and given water ad libitum. The retroperitoneal, subcutaneous, omental, epididymal white adipose tissue, intrascapular brown adipose tissue (BAT), kidney, lung and heart were removed and stored at −80 °C until total RNA preparation.

2.3. Mature adipocytes

Murine mature adipocytes were isolated from subcutaneous adipose tissue of C3H/He mice (female, 8 months old) with a modificied version of the method of Rodbell [12]. In brief, the adipose tissue was minced and digested in a 0.2% collagenase solution at 37 °C for 1 h with constant shaking. The digested fluid was filtered through 100 μm nylon mesh and separated by centrifugation performed three times at 180 g for 5 min. Mature adipocytes appeared and were collected as a floating layer, while the sediment consisted of stroma–vascular fraction (sv-f).

2.4. Human adipose tissue

Human abdominal subcutaneous adipose tissue was obtained during plastic surgery after written permission had been obtained. Human kidney and lung mRNA were commercially available (Clontech Laboratories, Inc., Palo Alto, CA, USA).

2.5. RNA extraction and Northern blot analysis

Total RNA was extracted with TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA). Northern blot analysis was performed as previously described using mouse and human AM cDNA as probes [11].

2.6. Measurements of AM released from isolated adipocytes

500 μl (2.5×10^5 cells) of isolated adipocytes from subcutaneous adipose tissue of ICR mouse (male, 12 weeks old) were incubated together with DMEM/H-12 (total volume: 500 μl) containing 10% fetal bovine serum in a CO_2 incubator for 24 h at 37 °C [13]. Aliquots of the incubation medium were removed at 6 and 24 h and stored at −20 °C for measurement of AM. The AM content in 100 μl of the incubation medium was determined by means of a radioimmunoassay (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA).

2.7. TaqMan real time PCR

The quantitative real time RT-PCR was employed to examine the murine AM gene expression. Briefly, AM cDNA was synthesized with Superscript II reverse transcriptase (Gibco-BRL, St. Louis, MO, USA) and used as a template. The primers and probes for TaqMan PCR analysis were designed with primer-express software (Applied Biosystems, Foster City, CA, USA) as follows:

AM forward, 5′-CTCGCTGATGAGACGACAGTTC-3′,
AM reverse, 5′-CTCTGGCGGTAGCGTTTGAC-3′,
detection probe: 5′-CAGCAATCAGAGCGAAGCCCA-CATT-3′.

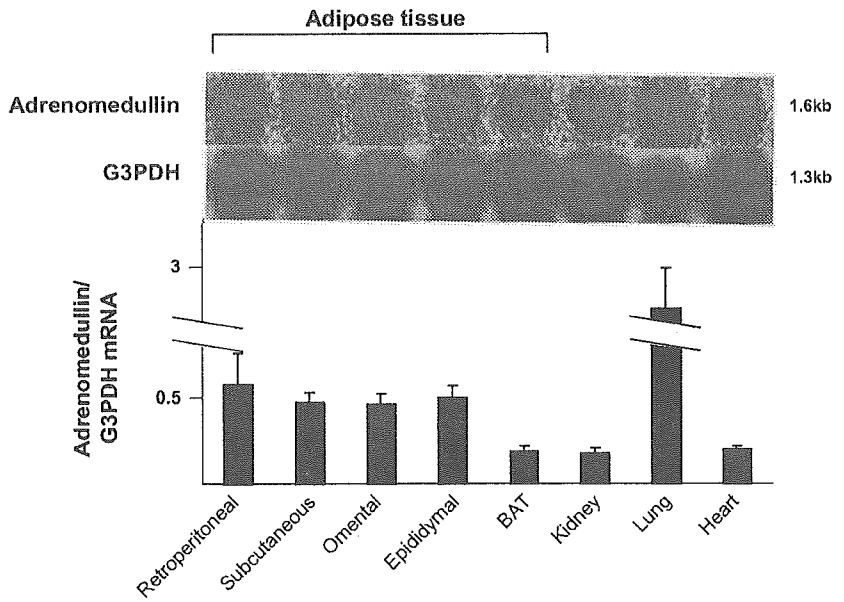

Fig. 1. Northern blot analysis of AM mRNA content of C57BL/6 mice. 10 μg of total RNA in each lane was electrophoresed and hybridized with mouse AM cDNA probes. The lower panel indicates hybridization with a G3PDH probe as an internal control. The ratio of AM to G3PDH mRNA is shown below. Bars represent mean ± S.E.M.

Rodent ribosomal 18S as an internal control was amplified using a commercially available kit (Applied Biosystems) at the same time. Thermal cycling was performed at 40 cycles of 95 °C for 15 s and of 60 °C for 1 min. Reactions were performed in triplicate with the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Input RNA amounts were calculated with a multiplex comparative method for mRNAs of AM and 18S protein.

2.8. High-fat diet (HFD)

Male C57BL/6 mice aged 10 weeks were randomly divided into two groups, one fed a standard fat diet (11% fat by energy) and the other a high-fat diet (60% fat by energy, Research Diets, Inc., New Brunswick, NJ, USA). After 11 weeks, the retroperitoneal, subcutaneous, omental, epididymal white adipose tissue and kidney were removed and stored at −80 °C until total RNA preparation. Mouse plasma AM and leptin concentrations were determined with a radioimmunoassay (Phoenix Pharmaceuticals, Inc.) and an enzyme immunoassay (Immune Biological Laboratory, Gunma, Japan), respectively.

2.9. Statistical analysis

All data are expressed as the mean±S.E.M. Statistical analysis was performed with Student's *t*-test. Values of $P<0.05$ were considered to be statistically significant.

3. Results

3.1. AM gene expression in adipose tissue and mature adipocytes

The expression of AM gene was examined by means of Northern blotting (Fig. 1). A marked expression of AM mRNA was detected in all white adipose tissues from C57BL/6. The expression levels in the retroperitoneal, subcutaneous, omental, and epididymal adipose tissue were 3.2, 2.6, 2.5 and 2.7 times higher than the corresponding levels in the kidney. In addition to mature adipocytes, adipose tissue contains blood vessels, fibroblasts and preadipocytes, among which especially the vascular wall has been regarded as a site of AM production. We therefore separated the adipose tissue into a mature adipocytes fraction and sv-f, and examined AM expression in each fraction (Fig. 2). The AM mRNA level in the mature adipocyte

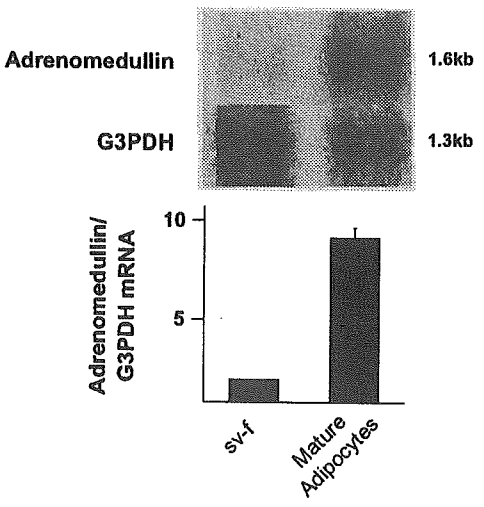

Fig. 2. Northern blot analysis of AM mRNA content in stroma–vascular fraction and isolated mature adipocytes of C3H/He mice. 10 μg of total RNA in each lane were electrophoresed and hybridized with mouse AM cDNA probes. The lower panel indicates hybridization with a G3PDH probe as an internal control. The ratio of AM to G3PDH mRNA is shown below. Bars represent the mean±S.E.M.

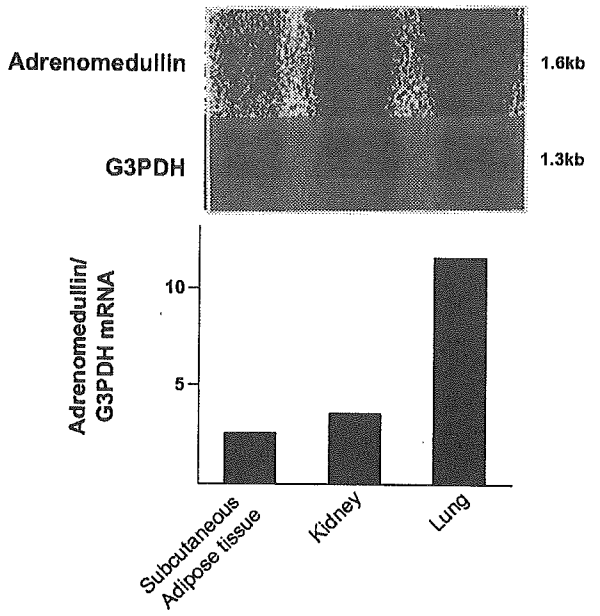

Fig. 3. Northern blot analysis of human AM mRNA content. 10 μg of total RNA in each lane was electrophoresed and hybridized with human AM cDNA probes. The lower panel indicates hybridization with a G3PDH probe as an internal control. The ratio of the AM to G3PDH mRNA is shown below.

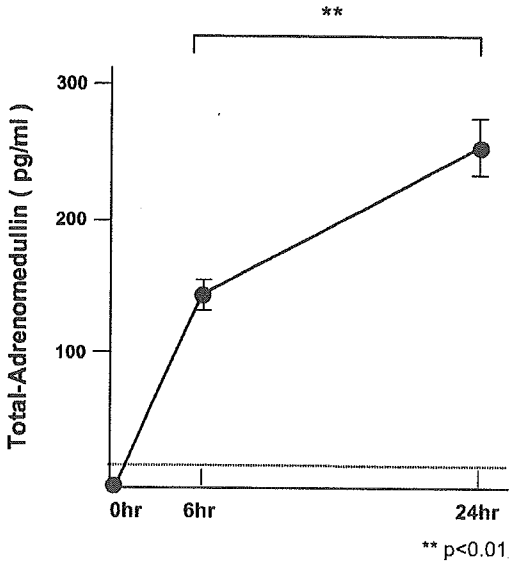

Fig. 4. Time course of AM levels in culture medium of mature adipocytes isolated from ICR mice (12 wk old, male, $n=5$). Mature adipocytes released AM, which significantly increased in a time-dependent manner. Bars represent the mean±S.E.M. Statistical analysis was performed with the *t* test. $^{**}P<0.01$.

Table 1
Body weight and plasma parameters in mice fed with high-fat diet or standard chow

	Standard chow	High-fat diet
Body weight(g)	31.3±1.2	48.6±2.1**
BS (mg/dl)	87.0±8.5	141.5±12.7**
FFA (mEq/ml)	0.51±0.08	0.94±0.18*
Leptin (ng/ml)	0.23±0.02	18.5±3.7***
Total AM (pg/ml)	56.3±17.5	84.9±6.2*

*$P<0.05$; **$P<0.01$, ***$P<0.001$ vs. standard chow group.

fraction was 7.3 times higher than that in the sv-f. In view of the tissue volume, this suggests that the major source of AM mRNA in the adipose tissue is mature adipocytes.

In the human subcutaneous adipose tissues obtained during plastic surgery, AM expression comparable to that in the kidney was detected (Fig. 3).

3.2. AM secretion from mature adipocytes

In addition to AM mRNA expression in the adipose tissue and mature adipocytes, we cultured mature adipocytes and measured AM peptide secretion in the medium. As shown in Fig. 4, AM peptide detected in the cultured medium increased in a time-dependent manner, indicating that AM is secreted from mature adipocytes.

3.3. Changes in AM gene expression in obesity

In order to assess the relationship between obesity and adipose AM production, we examined the AM mRNA expression after administration of the high-fat diet. As shown in Table 1, the HFD group weighed 1.5 times more than the standard chow group, and plasma leptin concentration was significantly higher as well as concentrations of plasma free fatty acid (FFA) and glucose. Plasma AM concentration in the HFD group was significantly higher than that in the standard chow group. After the high-fat diet, AM expression level was elevated in all adipose tissue compared with that in the control group, especially in retroperitoneal, subcutaneous and epididymal white adipose tissue (Fig. 5). In contrast, AM expression levels in the kidney of the HFD group and the control group were comparable.

In addition to HFD, we also examined AM mRNA expression in the obesity mouse model, ob/ob. Fig. 6 shows the AM mRNA level in the adipose tissues of ob/ob and the control, namely, C57BL/6 mice. In ob/ob mice, AM expression level was elevated in all adipose tissues compared with that in C57BL/6, especially in subcutaneous, omental, epididymal white adipose tissue and BAT.

4. Discussion

AM has been implicated in the regulation of circulation and the development of vasculature. Although AM was originally isolated from pheochromocytoma cells, the major site of production in the physiological state is believed to be vascular endothelial cells. Our findings demonstrate that adipose tissue strongly expresses the AM gene. In C57BL/6 mice, AM mRNA expression in all white adipose tissues examined was found to be higher than that detected in kidney. Adipose tissue is capillary-rich, containing blood vessels, fibroblasts and preadipocytes in addition to mature adipocytes. In order to exclude the possibility that AM mRNA in the adipose tissue is of vascular endothelial cell origin, we examined the AM expression in the mature adipocyte fraction and sv-f separately and found that the AM mRNA level in the mature adipocyte fraction was much higher than that in the sv-f, which consists of small vessels, preadipocytes and connective tissue. Moreover, we determined AM peptide secretion from isolated mature adipocytes in the medium. These findings indicate that the major source of AM in the adipose tissue consists of mature adipocytes. A few reports about adipose tissue and AM have

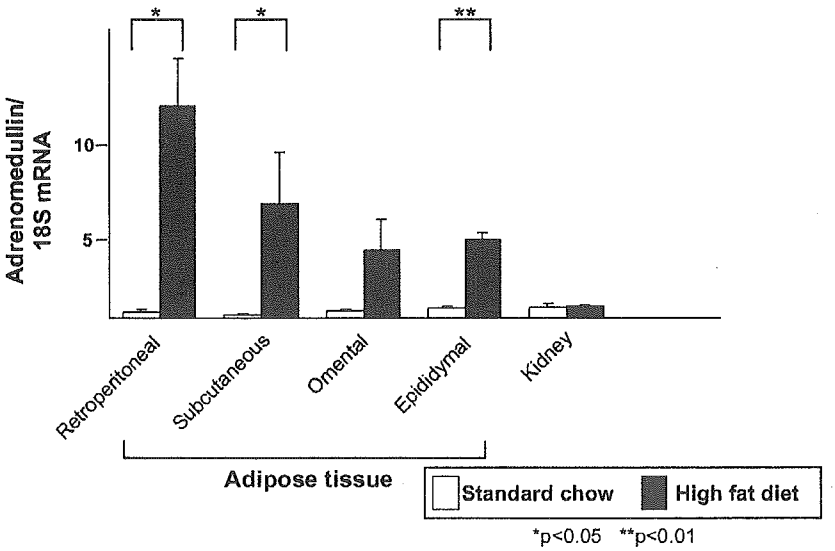

Fig. 5. AM mRNA levels from C57BL/6 mice (21 wk old, male, $n=5$) after 11 weeks of standard chow diet (open bars) or high-fat diet (closed bars). Bars represent the mean±S.E.M. Statistical analysis was performed with the t test. *$P<0.05$; **$P<0.01$.

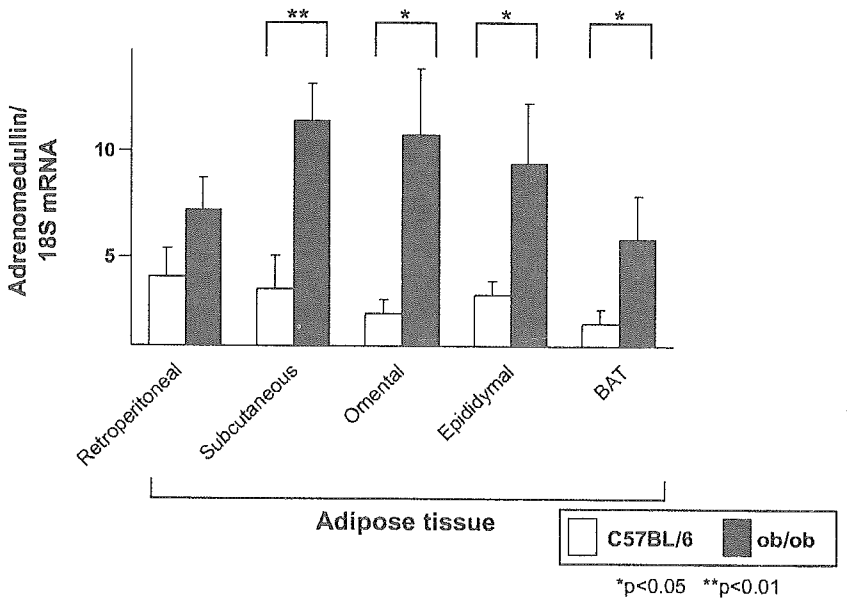

Fig. 6. AM mRNA levels in C57BL/6 (open bars) and ob/ob mice (closed bars) (14 wk old, male, $n=5$). Bars represent the mean±S.E.M. Statistical analysis was performed with the t test. $^{*}P<0.05$; $^{**}P<0.01$.

been published. Using NIH 3T3-L1 cells, Li et al. reported observing AM mRNA expression and secretion in mature adipocytes [14,15]. Recently Fukai et al. found AM mRNA expression in rat and human adipose tissue [16], which agrees with the results of our study.

Human subcutaneous adipose tissue obtained from healthy subjects during plastic surgery exhibited marked AM expression, too. In view of the tissue volume, these results suggest that adipose tissue is one of the major sites of AM production in the body.

The physiological significance of adipocyte-derived AM remains to be clarified. Since it has been reported that the plasma concentration of AM in the physiological state is not high enough to exert a vasodilatory effect [17], it stands to reason that AM secreted from adipocytes functions in an autocrine/paracrine manner. Adipose tissue is distributed throughout the body and works as supportive tissue. AM is a potent vasodilator and a growth factor for vascular endothelial cells (VEC) and vascular smooth muscle cells (VSMC) [18,19]. Many blood vessels are surrounded by fat and AM produced by the adipocytes and the vascular wall itself may make the local concentration of AM high enough to work as a vasodilator or a growth factor for VEC and VSMC. In reconstruction culture of the skin, the epidermal layer is known to grow better on the subcutaneous adipose layer [20]. It has also been reported that AM has a mitogenic effect on human keratinocytes [21]. Along with its enhancing effect on the proliferation and migration of VEC and VSMC, AM secreted from subcutaneous adipose tissue may be crucial for the maintenance and regeneration of the skin. Bone marrow is another organ which is rich in adipose tissue, while it has been reported that AM is expressed in cord blood hematopoietic cells and stimulates their clonal growth [22]. It thus seems reasonable to speculate that bone marrow fat may contribute to haematopoiesis.

It is extremely interesting that AM expression in adipose tissue and plasma AM concentration were significantly augmented with the increase in body weight after the high-fat diet. The same results were observed in ob/ob, leptin-deficient obesity model mice [23]. Furthermore, the massive increase in adipose tissue volume in obesity makes the total AM production in adipose tissue much greater. It has also been reported that the expression of the AM receptor components CRLR and RAMP2 was heightened in HFD rat adipose tissue [16]. Shimosawa et al. reported that heterozygous AM-deficient mice showed obesity, higher blood pressure and insulin resistance in their old age [24]. Taken together, these findings suggest that adipose tissue-derived AM protects against obesity.

The relationship between human plasma AM levels and obesity is not clear yet, although several studies have shown that plasma human AM levels are elevated in obesity [25,26].

In conclusion, our study presented here demonstrated that adipocytes produce AM in human and mouse and the AM production in adipose tissue is enhanced in obesity. Adipose AM may thus have a pathophysiological function in obesity.

Acknowledgements

This study was supported in part by the Smoking Research Foundation.

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Endocrinology. First published December 29, 2005 as doi:10.1210/en.2005-1038

The Neuro-Protective and Vasculo-Neuro-Regenerative Roles of Adrenomedullin in Ischemic Brain and its Therapeutic Potential

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Abbreviated title: Therapeutic effects of AM on ischemic brain

Key words: Adrenomedullin, Stroke, Ischemic brain, Neuro-protection, Vasculo-neuro-regeneration

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Grants: This work was supported by grants from Japanese ministry of Education, Culture, Sports, Science and Technology; ministry of Health, Labor and Welfare; and University of Kyoto 21st century COE (Centers of excellence) program.

Abstract

Adrenomedullin (AM) is a vasodilating hormone secreted mainly from vascular wall and its expression is markedly enhanced after stroke. We have revealed that AM promotes not only vasodilation but also vascular regeneration. In this study, we focused on the roles of AM in the ischemic brain and examined its therapeutic potential. We developed novel AM-transgenic (AM-Tg) mice that overproduce AM in the liver and performed middle cerebral artery occlusion for 20 minutes (20m-MCAO) to examine the effects of AM on degenerative or regenerative processes in ischemic brain. The infarct area and gliosis after 20m-MCAO was reduced in AM-Tg mice in association with suppression of leukocyte infiltration, oxidative stress and apoptosis in the ischemic core. In addition, vascular regeneration and subsequent neurogenesis were enhanced in AM-Tg mice, preceded by increase in mobilization of $CD34^+$ mononuclear cells, which can differentiate into endothelial cells. The vasculo-neuro-regenerative

actions observed in AM-Tg mice in combination with neuro-protection resulted in improved recovery of motor function. Brain edema was also significantly reduced in AM-Tg mice via suppression of vascular permeability. In vitro, AM exerted direct anti-apoptotic and neurogenic actions on neuronal cells. Exogenous administration of AM in mice after 20m-MCAO also reduced the infarct area, and promoted vascular regeneration and functional recovery. In summary, this study suggests the neuro-protective and vasculo-neuro-regenerative roles of AM and provides basis for a new strategy to rescue ischemic brain through its multiple hormonal actions.

Introduction

Adrenomedullin (AM) is a potent vasodilating peptide comprising 52 amino acids, which was originally isolated from human pheochromocytoma tissues in 1993 as a substance to elevate cAMP concentration in platelets (1). It is secreted mainly from the vascular wall into circulating blood to reduce pre- and post-load on the heart via vasodilation, natriuresis, and suppression of aldosterone release. Intravenous administration of AM to patients with heart failure or pulmonary hypertension has already been initiated and beneficial hemodynamic effects have been reported (2).

Along with its vasodilating effect, a number of studies have demonstrated various and significant effects of AM on the regulation of vascular structure, including its development, remodeling and regeneration. Mice lacking the AM gene did not survive their embryonic stage and showed abnormal vasculature with subcutaneous hemorrhage (3, 4). Mice over-expressing AM in endothelial