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Yagi M, Lee T, Awano H, Tsuji M, Tajima G, Kobayashi H, Hasegawa Y, Yamaguchi S, Takeshima Y, Matsuo M	A patient with mitochondrial trifunctional protein deficiency due to the mutations in the HADHB gene showed recurrent myalgia since early childhood and was diagnosed in adolescence	Molecular Genetics and Metabolism	104(4)	556-559	2011
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久保田一生, 深尾敏幸, 堀 友博, 小林弘典, 船戸道徳, 長谷川有紀, 山口清次, 近藤直実	カルニチンパルミトイルトランスフェラーゼ2欠損症のろ紙血血清のアシルカルニチンプロファイルの経時的変化	日本小児科学会雑誌	115(5)	956-960	2011

IV. 研究成果の刊行物・別刷

Apoptosis inhibitor of macrophage (AIM) diminishes lipid droplet-coating proteins leading to lipolysis in adipocytes

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ABSTRACT

Under fasting conditions, triacylglycerol in adipose tissue undergoes lipolysis to supply fatty acids as energy substrates. Such lipolysis is regulated by hormones, which activate lipases via stimulation of specific signalling cascades. We previously showed that macrophage-derived soluble protein, AIM induces obesity-associated lipolysis, triggering chronic inflammation in fat tissue which causes insulin resistance. However, the mechanism of how AIM mediates lipolysis remains unknown. Here we show that AIM induces lipolysis in a manner distinct from that of hormone-dependent lipolysis, without activation or augmentation of lipases. In vivo and in vitro, AIM did not enhance phosphorylation of hormone-sensitive lipase (HSL) in adipocytes, a hallmark of hormone-dependent lipolysis activation. Similarly, adipose tissue from obese AIM-deficient and wild-type mice showed comparable HSL phosphorylation. Consistent with the suppressive effect of AIM on fatty acid synthase activity, the amount of saturated and unsaturated fatty acids was reduced in adipocytes treated with AIM. This response ablated transcriptional activity of peroxisome proliferator-activated receptor (PPARγ), leading to diminished gene expression of lipid-droplet coating proteins including fat-specific protein 27 (FSP27) and Perilipin, which are indispensable for triacylglycerol storage in adipocytes. Accordingly, the lipolytic effect of AIM was overcome by a PPARγ-agonist or forced expression of FSP27, while it was synergized by a PPARγ-antagonist. Overall, distinct modes of lipolysis appear to take place in different physiological situations; one is a supportive response against nutritional deprivation achieved by enhancing lipase activity, and the other is a pathological consequence of obesity, causing subclinical inflammation and metabolic disorders, mediated by abolishing droplet-coating proteins.

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1. Introduction

The homeostasis of adipose tissue is maintained by a metabolic equilibrium between synthesis and degradation of triacylglycerol via types of neural and hormonal signals. The former is termed lipogenesis, while the latter is lipolysis. Although triacylglycerols in white adipose tissues are constitutively turned over through both processes, prominent lipolysis occurs under fasting conditions or during periods of increased energy demand, leading to the release of free fatty acids into the circulation, which are transported to other tissues. Such mobilization of triacylglycerol stores is strictly regulated by catecholamines and other lipolytic hormones, and requires activation and/or an increase in expression of lipases, including hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). This response is dependent on phosphorylative activation of the cAMP-dependent protein kinase A (PKA), which results in phosphorylation of HSL and its lipotransine-mediated translocation from the cytoplasm to lipid droplets, as well as upregulation of the transcription of ATGL mRNA [1–8].

We recently reported that the apoptosis inhibitor of macrophage (AIM) protein induces lipolysis [9]. AIM is a member of the scavenger receptor cysteine-rich superfamily and was initially identified as an apoptosis inhibitor that supports the survival of macrophages against different types of apoptosis-inducing stimuli [10]. AIM is a direct target for regulation by nuclear receptor liver X receptor/retinoid X receptor (LXR/RXR) heterodimers and is solely produced by tissue macrophages [10–13]. As a secreted molecule, AIM is detected in both human and mouse blood at various levels [9,14–18], increasing with the progression of obesity in mice fed a high fat diet (HFD) [9]. Under obese conditions, augmentation of blood AIM levels induces vigorous lipolysis in adipose tissues, increasing local extracellular fatty acid concentration to a level sufficient for the stimulation of toll-like receptor (TLR) 4 expressed

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Y. Iwamura et al./Biochemical and Biophysical Research Communications xxx (2012) xxx–xxx

in adipocytes. This triggers chemokine production by adipocytes, thereby inducing macrophage recruitment [19,20]. This response causes chronic, low-grade inflammation in adipose tissues, which is associated with insulin-resistance, and thus contributes to the development of multiple obesity-induced metabolic and cardio-vascular diseases [19–25]. In agreement with these results, due to reduced lipolysis, although adipocyte hypertrophy was more advanced and the overall mass of visceral adipose tissues was greater in AIM-deficient ($AIM^{-/-}$) than in wild-type ($AIM^{+/+}$) mice fed a HFD, obese $AIM^{-/-}$ mice showed a marked prevention of inflammatory macrophage infiltration into adipose tissue, result-ing in decreased inflammation both locally and systemically, there-by being protected from insulin resistance and glucose intolerance [9,19,20].

Interestingly, unlike many cytokines and growth factors, exoge-nous AIM secreted by macrophages is incorporated into adipocytes and directly functions intracellularly in the absence of signaling. AIM is endocytosed via a scavenger receptor CD36, and binds to cytoplasmic fatty acid synthase (FAS), resulting in decreased FAS enzymatic activity [9,19,20]. We showed that the suppression of FAS activity is responsible for AIM-induced lipolysis, based on the observation that the specific FAS inhibitor C75 [26] also induced lipolysis [9]. Interestingly, however, despite the lipolytic consequences, treatment with AIM or C75 did not upregulate PKA phosphorylation in adipocytes [9]. In addition, the levels of ATGL mRNA did not increase in response to AIM [9]. These results suggest that unlike hormone-dependent lipolysis, inhibition of FAS via AIM does not stimulate the cAMP/PKA signalling cascade. Over-all, the mechanism of how AIM/Fas-inhibition mediates lipolysis remains unclear. In this report, we precisely define the difference between hormone-dependent lipolysis and AIM-induced lipolysis, and elucidate the mechanism of how FAS-suppression via AIM induces lipolysis.

2. Material and methods

2.1. Lipolysis analysis

Lipolysis was assessed by measurement of the amount of glyc-erol released in the medium. Overnight serum-deprived adipocytes (day 6) were incubated in serum-free DMEM containing 2% fatty acid-free BSA in the presence or absence of rAIM or other com-pounds for indicated time. After the incubation, supernatant was collected, and glycerol and FFA contents were measured using a glycerol assay kit, and a fatty acid assay kit (Bio Vision Inc.).

2.2. Luciferase reporter analysis for PPRA γ activity

A ~0.2 kb genomic DNA fragment containing mouse FSP27 reg-ulatory element (−1 to −236) that includes PPRE (TGCCCT CTTGCCT) was subcloned into pGL3-enhancer vector (Promega). The plasmid was linearized by SalI-digestion, and transfected in combination with XhoI-linealized pMC1-neo-polyA into 3T3-L1 preadipocytes. After a G418-selection (800 g/ml), a G418-resistant clone was selected, and used for experiments. Luciferase activities were measured with the Luciferase assay system (Promega).

2.3. Statistical analysis

A two-tailed Mann–Whitney test was used to calculate P-values. (***) $P < 0.001$, (**) $P < 0.01$, (*) $P < 0.05$. Error bars: SEM. Reagents for histological analysis, Purification of rAIM, in vitro adipogenesis, siRNA and Chromatin immuno-precipitation assay, Metabolomics analysis, in vivo starvation study, quantitative PCR assay and primer sequences appear in Supplementary data.

3. Results

3.1. AIM-induced lipolysis is distinctive from hormone-dependent lipolysis

We previously reported that treatment of 3T3-L1 adipocytes with AIM did not upregulate PKA phosphorylation or increase levels of ATGL mRNA, which are both characteristics of hormone-dependent lipolysis observed in fasting situations. To further determine the differences between AIM-induced and hormone-dependent lipolysis, we kinetically assessed various outputs of lipolysis in 3T3-L1 adipocytes. When cells were challenged with the β-adrenergic receptor agonist, isoproterenol (10 M), efflux of glycerol and free fatty acids (FFAs) was observed within 2 h, reach-ing a maximum level in 6 h (Fig. 1A). In contrast, the same efflux in response to recombinant AIM (rAIM) was apparent 24 h after the challenge (Fig. 1A). The effluxed fatty acids stimulate TLR4 expressed on adipocytes and induce inflammatory responses [19,20]. This was also acutely detected in response to isoproterenol (at a maximum level in 2 h) as assessed by quantitative RT-PCR (QPCR) for interleukin-6 (IL-6), monocyte chemotactic protein 1 (MCP-1), and Serum amyloid A-3 (Saa3) mRNA levels, but not appar-ent before 24 h in response to rAIM (Supplementary Fig. S1). At 72 h, levels of FFA efflux and mRNA for MCP-1 and Saa3 induced in response to rAIM were even higher compared to the maximum levels induced by isoproterenol. Thus, AIM-induced lipolysis exhib-ited slow but robust progression.

Activation of the β-adrenergic receptor degrades triacylglycerol by the phosphorylative activation of HSL. In 3T3-L1 adipocytes, HSL phosphorylation occurs rapidly (within 10 min) in response to isoproterenol, followed by degradation of the HSL protein (Fig. 1B). In contrast, despite lipolytic consequences, AIM did not induce HSL phosphorylation even after 72 h (Fig. 1B). Parallel results were also obtained in vivo. Phosphorylation of HSL, or its upstream PKA, in epididymal adipose tissue was not enhanced in obese wild-type mice compared to that in lean mice (Supplemen-tary Fig. S2), though lipolysis was apparently enhanced as deter-mined by elevation of FFAs and glycerol in blood [9]. Similarly, $AIM^{-/-}$ mice fed with HFD also did not show an increase in HSL phosphorylation (Supplementary Fig. S2). In addition, forced induction of lipolysis in obese $AIM^{-/-}$ mice by the intra-venous injection of rAIM did not activate HSL or PKA phosphorylation in epididymal adipose tissue (Supplementary Fig. S3).

An additional observation implicating a distinctive mechanism for AIM-induced lipolysis and hormone-dependent lipolysis was that mRNA levels for FSP27 (also termed cidec) and Perilipin (or Perilipin 1, also termed Plin1), the droplet-coating elements, were profoundly decreased after treatment of 3T3-L1 adipocytes with AIM, whereas this did not occur in response to isoproterenol (Fig. 1C). Previous reports showed that abrogation of expression for either FSP27 or Perilipin in cells or mice reduced the size of lipid droplets in adipocytes and increased the efflux of fatty acids [27–33]. Therefore, it is possible that AIM decreases lipid droplet-coat-ing proteins, leading to the efficient access of constitutive lipases to triacylglycerols without their activation or augmentation. In agree-ment with this idea, overexpression of FSP27 in 3T3-L1 adipocytes using a Lentivirus system overcame the lipolytic effect of AIM (Fig. 1D).

3.2. AIM negatively regulates transcriptional activity of nuclear receptor PPARγ

Although several putative binding sites for transcription factors including peroxisome proliferator-activated receptor (PPARγ), hepatocyte nuclear factor-3 (HNF-3), GATA-binding protein 3
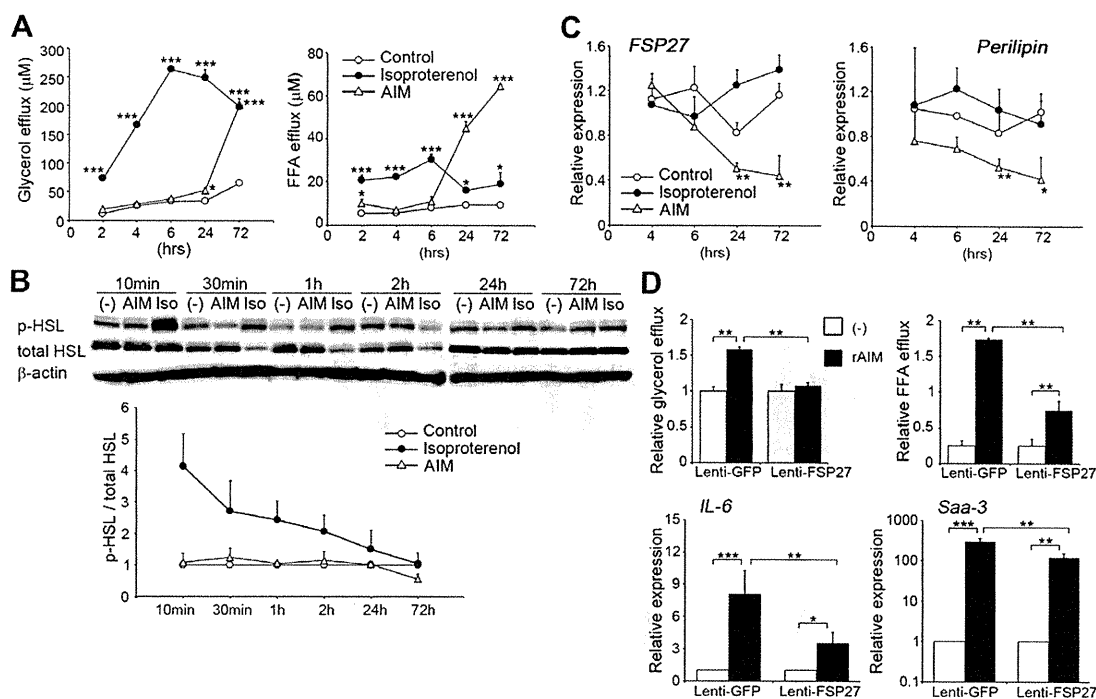
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Fig. 1. AIM-induced lipolysis is distinctive from hormone-dependent lipolysis. 3T3-L1 adipocytes were treated with rAIM (100 μg/ml) or isoproterenol (10 μM), and analysed for (A) the efflux of glycerol and FFAs, (B) HSL (phosphorylated and total) levels by immunoblotting, at indicated time points. The density of the signal was calculated using image analysis software NIH Image J, and the phosphorylative state of HSL normalized by the amount of total HSL is presented. $n = 3$ for each. (C) 3T3-L1 adipocytes incubated with rAIM (100 μg/ml) or isoproterenol (10 μM) were analyzed for mRNA levels of FSP27, and Perilipin by QPCR at indicated time points. Values were normalized to those of β-actin and presented as relative expression to that in cells before the treatment (time 0). $n = 3$ for each. (D) 3T3-L1 adipocytes were infected with a Lentivirus to express FSP27, or GFP as a control, and treated with rAIM for 72 h after the infection. Thereafter, glycerol and FFA efflux as well as induction of IL-6 and Saa-3 expression were analysed to evaluate lipolysis. Three independent experiments were performed.

197 (GATA3), sterol regulatory element-binding protein-1 (SREBP-1),
198 cAMP response element-binding protein (CREBP), and CCAAT-en-
199 hancer-binding protein (C/EBP) are present in the 5-upstream
200 region of the FSP27 gene, recent studies have demonstrated that
201 expression of FSP27 in adipocytes is directly and crucially regulated
202 by PPARγ, a master transcription factor for the differentiation of
203 adipocytes, which is expressed at its highest level in adipose tissue
204 [34]. Indeed, PPARγ expression well correlates with that of FSP27 in
205 adipocytes [35]. It is also known that expression of Perilipin is con-
206 trolled by PPARγ [36]. We previously showed that the progression
207 of lipolysis in 3T3-L1 adipocytes in response to AIM was not
208 accompanied by significant downregulation of PPARγ expression
209 levels [9]. Hence, to test whether AIM influences functional activity
210 of PPARγ in adipocytes, we first assessed whether the presence of
211 rosiglitazone, a selective agonist of PPARγ, or T0070907, a selective
212 PPARγ antagonist, influenced the lipolytic effect of AIM in 3T3-L1
213 adipocytes. As demonstrated in Fig. 2A and Supplementary
214 Fig. S4, a set of parameters with remarkable involvement in AIM-
215 induced lipolysis (i.e. increased glycerol efflux, downregulation of
216 FSP27 and Perilipin mRNA levels, and increased inflammatory gene
217 expression) were inhibited by the presence of rosiglitazone in a
218 dose-dependent fashion. In contrast, a synergistic effect of these
219 lipolytic consequences was detected following the combination
220 of rAIM and T0070907 (Fig. 2 and Supplementary Fig. S5B). To
221 assess the effect of AIM on transcriptional activity of PPARγ more
222 directly, we stably transfected 3T3-L1 cells with a luciferase repor-
223 ter gene conjugated with a PPARγ-binding element (PPRE) at the
224 5′-end [34]. As shown in Fig. 2C (left panel), luciferase activity
225 was upregulated when cells differentiated in response to insulin,
226 dexamethasone (DEX), and isobutylmethylxanthine (IBMX), and
227 this response was markedly enhanced by the presence of rosiglit-
228 azone. The challenge of cells with rAIM significantly decreased

229 the luciferase activity in a dose dependent fashion, at a comparable
230 level to that of T0070907 (Fig. 2C, right panel). In addition, the
231 luciferase activity induced by rosiglitazone was significantly
232 suppressed by AIM (Fig. 2D). Together, these results suggest that
233 treatment of adipocytes with AIM reduces PPARγ activity, resulting
234 in downregulation of mRNA levels for coating proteins, leading to
235 lipolysis.
236 Furthermore, to determine whether AIM enhances the binding
237 efficiency of PPARγ to its binding site, a chromatin immuno-pre-
238 cipitation (ChIP) was carried out. A chromatin fraction was isolated
239 from 3T3-L1 adipocytes incubated in the presence of rAIM or
240 rosiglitazone, digested with DNaseI, then immune-precipitated
241 using a PPARγ antibody. DNA purified from the precipitates was
242 PCR-amplified to detect the presence of PPRE1 or PPRE2 sequences
243 within the 5′ sequence of FSP27 gene (illustrated in Supplementary
244 Fig. S6). No difference in PCR signal level was detected following
245 treatment of cells with rAIM, suggesting that AIM does not sup-
246 press recruitment of PPARγ to DNA, but may modulate PPARγ
247 activity by acting as a co-receptor in FSP27 gene (Supplementary
248 Fig. S6).

249 3.3. Reduction of endogenous fatty acid synthesis decreases PPARΓγ
250 activity

251 Although the identity of the biological ligand(s) for PPARγ
252 remains unknown, extensive studies have suggested that polyun-
253 saturated fatty acids and related molecules can activate PPARγ
254 [37–39]. Because AIM is incorporated into adipocytes and de-
255 creases the enzymatic activity of FAS, this response might reduce
256 production of such PPARγ biological ligand(s). To this end, we first
257 tested whether suppression of FAS activity reduces PPARγ activity
258 using the 3T3-L1 cells transfected with the luciferase reporter gene

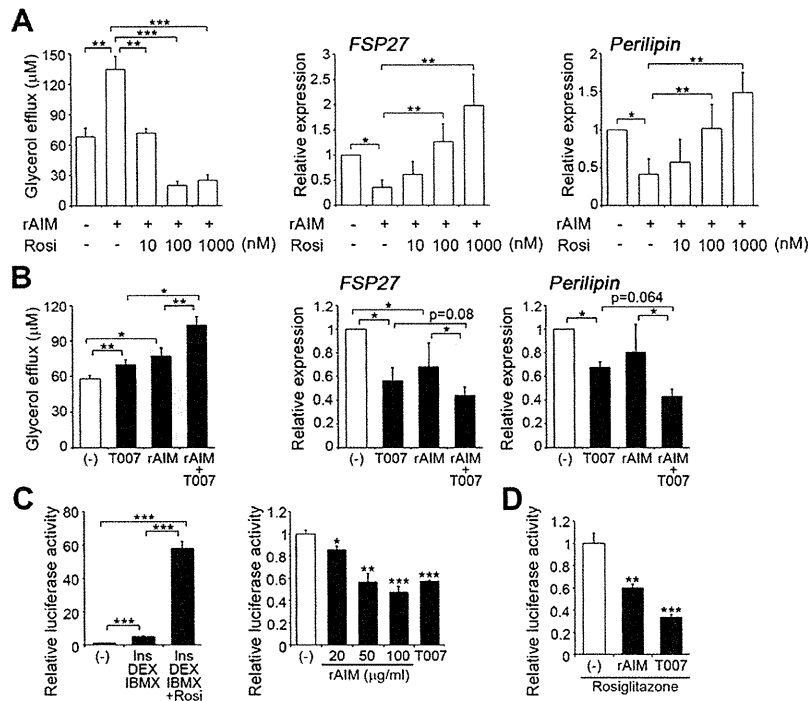4   Y. Iwamura et al./Biochemical and Biophysical Research Communications xxx (2012) xxx–xxx

Fig. 2. AIM decreases transcriptional activity of PPARγ. Glycerol efflux, mRNA levels of FSP27, and Perilipin were assessed in 3T3-L1 adipocytes treated with rAIM (100 μg/ml), and indicated concentration of rosiglitazone (A) or T0070907 (B). n = 3 for each. (C) (Left panel) 3T3-L1 cells stably transfected with a luciferase reporter gene conjugated with a PPARγ-binding element (PPRE) at the 5′-end, were stimulated with insulin, DEX, and IBMX in the absence or presence of rosiglitazone (1 μM). At day 2 after the induction, cells were harvested and the luciferase activity was analyzed. Luciferase activity was increased in response to maturation induction, and this was markedly enhanced in the presence of rosiglitazone, confirming that the reporter construct is useful to evaluate PPARγ activity. (Right panel) Differentiated reporter 3T3-L1 adipocytes were incubated with rAIM (20, 50, or 100 μg/ml) or T0070907 (1 μM) for 24 h, and the luciferase activity was analysed. n = 3 for each. Error bar: SEM. (D). Same cells were incubated with rosiglitazone (μM) alone (−), or in the presence of rAIM (100 μg/ml) or T0070907 (1 μM) for 24 h, and the luciferase activity was analysed. n = 3 for each. Error bar: SEM.

259 (presented in Fig. 2C). As demonstrated in Fig. 3A, like rAIM, the
260 FAS inhibitor cerulenin [40] or abrogation of FAS expression by siR-
261 NA significantly diminished luciferase activity. It therefore appears
262 that cerulenin or FAS siRNA established conditions similar to AIM-
263 induced lipolysis in 3T3-L1 adipocytes. In agreement, cerulenin or
264 the siRNA provided lipolytic consequences in a comparable

manner to that of AIM, including increase in efflux of glycerol 265
(Fig. 3B), downregulation of mRNA for FSP27 and Perilipin, and 266
increases in expression of inflammatory genes (Fig. 3C and Supple- 267
mentary Fig. S7). 268

Changes in intracellular levels of fatty acids caused by AIM via 269
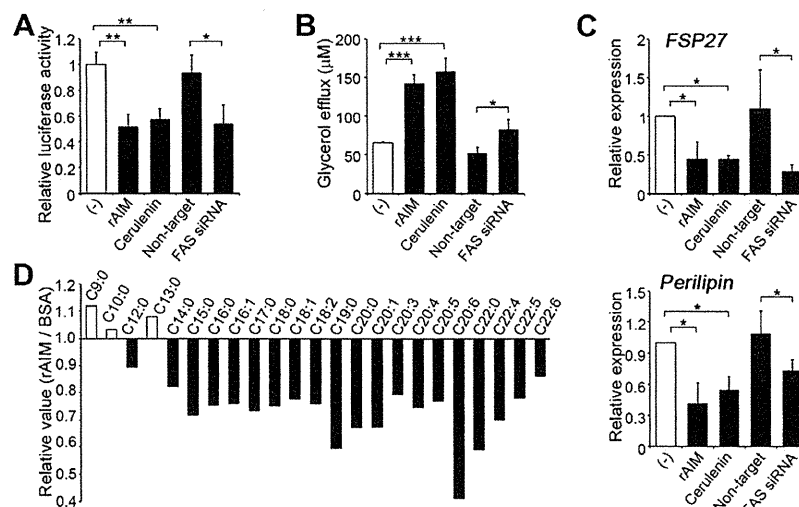the suppression of FAS activity were directly assessed by meta- 270

Fig. 3. AIM reduces endogenous fatty acid synthesis and PPARγ activity. (A) Luciferase activity in the reporter 3T3-L1 adipocytes 24 h after the challenge with rAIM (100 μg/ml), cerulenin (10 μM), or no stimulants (control), or transfection of siRNA for FAS or non-target sequence. n = 3 for each. (B and C) Lipolytic parameters assessed in 3T3-L1 adipocytes 24 h after the challenge with rAIM (100 μg/ml), cerulenin (10 μM), or transfection of siRNA targeting FAS or non-target sequence. n = 3 for each. (D) 3T3-L1 adipocytes were treated with rAIM (100 μg/ml) or control BSA (100 μg/ml) for 24 h, and metabolomics analysis was performed to analyze fatty acid profile in cells. Data are presented as relative values to those from cells treated with BSA. Three independent experiments were performed.
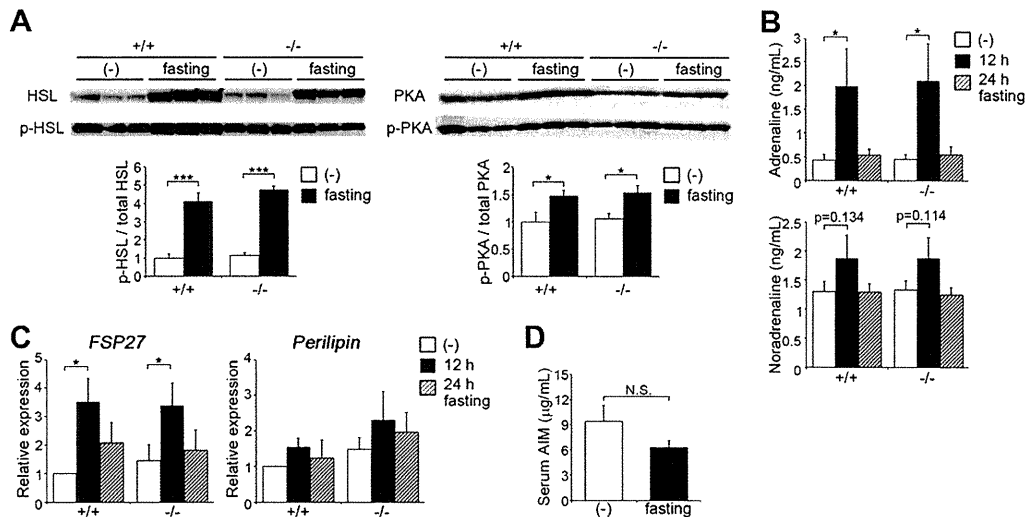
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Fig. 4. Comparative hormone-dependent lipolysis in $AIM^{-/-}$ and $AIM^{+/+}$ mice. (A) Immunoblotting for HSL and PKA (phosphorylated and total) in epididymal fat at 12 h. Data are shown as relative phosphorylative state to that in $AIM^{+/+}$ mice without fasting. $n = 3$ for each. (B) Blood catecholamines levels, (C) relative mRNA level (to that in $AIM^{+/+}$ mice without fasting) for FSP27 and Perilipin in epididymal fat, and (D) blood AIM levels, before and after the 24 h-fasting. $n = 3$ for each.

bolomics analysis using 3T3-L1 adipocytes treated with rAIM for 24 h (Table S2). Consistent with the FAS-suppressing function of AIM, the proportion of palmitic acid (C16:0), the primary product synthesized by FAS, was significantly reduced in cells treated with rAIM. Similarly, proportions of many saturated fatty acids harboring longer chains including stearic acid (C18:0), and related unsaturated fatty acids, also reduced in response to rAIM (Fig. 3D). Since we did not observe a prominent decrease in any specific fatty acid(s) in response to rAIM (Fig. 3D and Supplementary Table S2), it was difficult to define bona fide biological ligand(s) for PPARγ from these results. Indeed, it is possible that not a single fatty acid but a set of different fatty acids generated through FAS might activate PPARγ. This issue also warrants further assessment.

3.4. In vivo analysis

All presented results strongly implicated distinct mechanism between AIM-induced lipolysis and hormone-dependent lipolysis. Consistently, increase in HSL and PKA phosphorylation levels was comparably detected in the epididymal adipose tissue in response to 24-h-fasting in $AIM^{-/-}$ and $AIM^{+/+}$ mice (Fig. 4A). In addition, the increase in blood catecholamine levels in response to fasting was comparable in $AIM^{+/+}$ and $AIM^{-/-}$ mice (Fig. 4B). Also, changes in FSP27 and Perilipin mRNA levels secondary to the acute lipolytic progression were equivalent in $AIM^{+/+}$ and $AIM^{-/-}$ mice (Fig. 4C). Furthermore, blood AIM levels did not significantly change after starvation in $AIM^{+/+}$ mice (Fig. 4D).

4. Discussion

Our current study revealed two distinct modes of lipolysis that occur in different physiological situations: hormone-dependent lipolysis and AIM-induced lipolysis. The former occurs in a starved condition, and is mediated by activation of the hormone-dependent signalling cascade, resulting in phosphorylative activation of HSL and upregulation of ATGL expression levels. Since starvation can be fatal, and thus requires urgent complementation of energy sources, this lipolytic process progresses rapidly. In contrast, AIM-induced lipolysis occurs with progression of obesity, which is accompanied by increases in blood AIM levels. This lipolysis proceeds in a lack-of signalling fashion, and progresses more slowly: AIM is endocytosed into adipocytes, associates with FAS and

suppresses its enzymatic activity, which reduces endogenous generation of fatty acids, resulting in decreased PPARγ activity leading to downregulation of FSP27 and Perilipin mRNA levels. Hence, two characteristics are most contrastive between the two mechanisms for lipolysis: (i) the former forcibly decomposes lipid droplets via activating and augmenting lipases, while the latter targets coating proteins without influencing the activity or expression level of lipases, and (ii) the former is a beneficial response supportive to survival, while the latter is highlighted during obesity progression, a pathological situation that causes subclinical inflammation followed by metabolic disorders.

Previously, we and others [9,41] showed that inhibition of FAS also suppresses preadipocyte differentiation. This may be in part explained by our present findings that FAS-suppression decreases expression levels of droplet-coating proteins. This response should abolish the development of lipid droplets in response to adipocyte-maturation stimuli, even though the biosynthesis pathway of triacylglycerol is activated. Supporting this, $FSP27^{-/-}$ mice were protected from diet-induced obesity, harboring small-size lipid droplets multilocularlly in white adipose tissue [30]. In addition, it is strongly possible that the ablation of PPARγ activity caused by the decrease in fatty acid synthesis may disrupt cross-stimulation by C/EBPα and PPARγ protein to maintain expression of these master genes for adipogenesis, resulting in diminishment of adipocyte maturation [42,43].

To summarize, we have identified a new mode of lipolysis that is AIM-induced and is associated with the progression of obesity. Selective regulation of this type of lipolysis via AIM modulation might be a promising target for next-generation therapy against obesity and obesity-associated metabolic disorders.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.018.

6   *Y. Iwamura et al. / Biochemical and Biophysical Research Communications xxx (2012) xxx–xxx*

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REVIEW

AIMing at Metabolic Syndrome

– Towards the Development of Novel Therapies for Metabolic Diseases via Apoptosis Inhibitor of Macrophage (AIM) –

Toru Miyazaki, MD, PhD; Jun Kurokawa; Satoko Arai, PhD

Metabolic syndrome (MetS) is a cascade of metabolic diseases, starting with obesity and progressing to atherosclerosis, and is often fatal because of serious cardiovascular problems such as heart/brain infarction and hemorrhage. Accumulating evidence has revealed a critical involvement of inflammatory responses triggered by lesional macrophages in the pathogenesis of MetS. Importantly, we found that macrophages are associated with disease progression, not only in the induction of inflammation but also in the production of apoptosis inhibitor of macrophages (AIM), which we initially identified as a soluble factor expressed by macrophages. In atherosclerotic plaques, AIM is highly expressed by foam macrophages and inhibits apoptosis of these cells, which results in the accumulation of macrophages, causing inflammatory responses within the lesion, and ultimately disease progression. In adipose tissue, macrophage-derived AIM is incorporated into adipocytes through CD36-mediated endocytosis, thereby reducing the activity of cytosolic fatty acid synthase. This unique response stimulates lipolysis, resulting in a decrease in adipocyte size, which is physiologically relevant to the prevention of obesity. The lipolytic response also stimulates inflammation of adipocytes in association with the induction of metabolic disorders subsequent to obesity. Thus, AIM is involved in the progression of MetS in both an advancing and inhibitory fashion. Regulation of AIM could therefore be therapeutically applicable for MetS. (Circ J 2011; 75: 2522–2531)

Key Words: Apoptosis inhibitor of macrophage (AIM); Chronic inflammation; Lipolysis; Macrophages; Metabolic syndrome

Metabolic Syndrome (MetS), Macrophages, and Apoptosis Inhibitor of Macrophage (AIM)

MetS comprises a variety of metabolic disorders such as obesity, type 2 diabetes, fatty liver dysfunction, and atherosclerosis. Importantly, these diseases form a cascade of events, occurring sequentially from obesity and progressing towards atherosclerosis, with the stresses of modern life often acting as a catalyst. Difficulties in understanding the events that bridge obesity and insulin resistance (IR) have recently been overcome to reveal that the chronic, low-grade inflammation observed in obese adipose tissue is responsible for triggering IR. This subclinical inflammatory state of adipose tissue is closely associated with IR both in adipose tissue and systemically, thus contributing to the development of multiple obesity-induced metabolic and cardiovascular diseases.[1-5]

Infiltration of a large number of classically activated inflammatory macrophages (M1 macrophages) into adipose tissue has been shown to be responsible for obesity-associated inflammation.[6-8] Lean adipose tissue contains a resident population of alternative activated macrophages (also known as M2 macrophages) that suppress inflammation of both adipocytes and macrophages themselves, partly via the secretion of interleukin (IL)-10. Hence, obesity induces a switch in the macrophage activation state in adipose tissue towards M1 polarization, leading to inflammation.[9-12] Despite this knowledge, the key factors that initiate macrophage recruitment into adipose tissue remain unknown.

In this review, we address this question by focusing on the AIM protein (also known as Sα, Api6, and CD5L). Though AIM was initially identified as an apoptosis inhibitor that supports the survival of macrophages against various apoptosis-inducing stimuli,[13] our recent studies revealed a new role for AIM in adipocytes in the initiation of macrophage recruitment into adipose tissue leading to subsequent metabolic disorders.
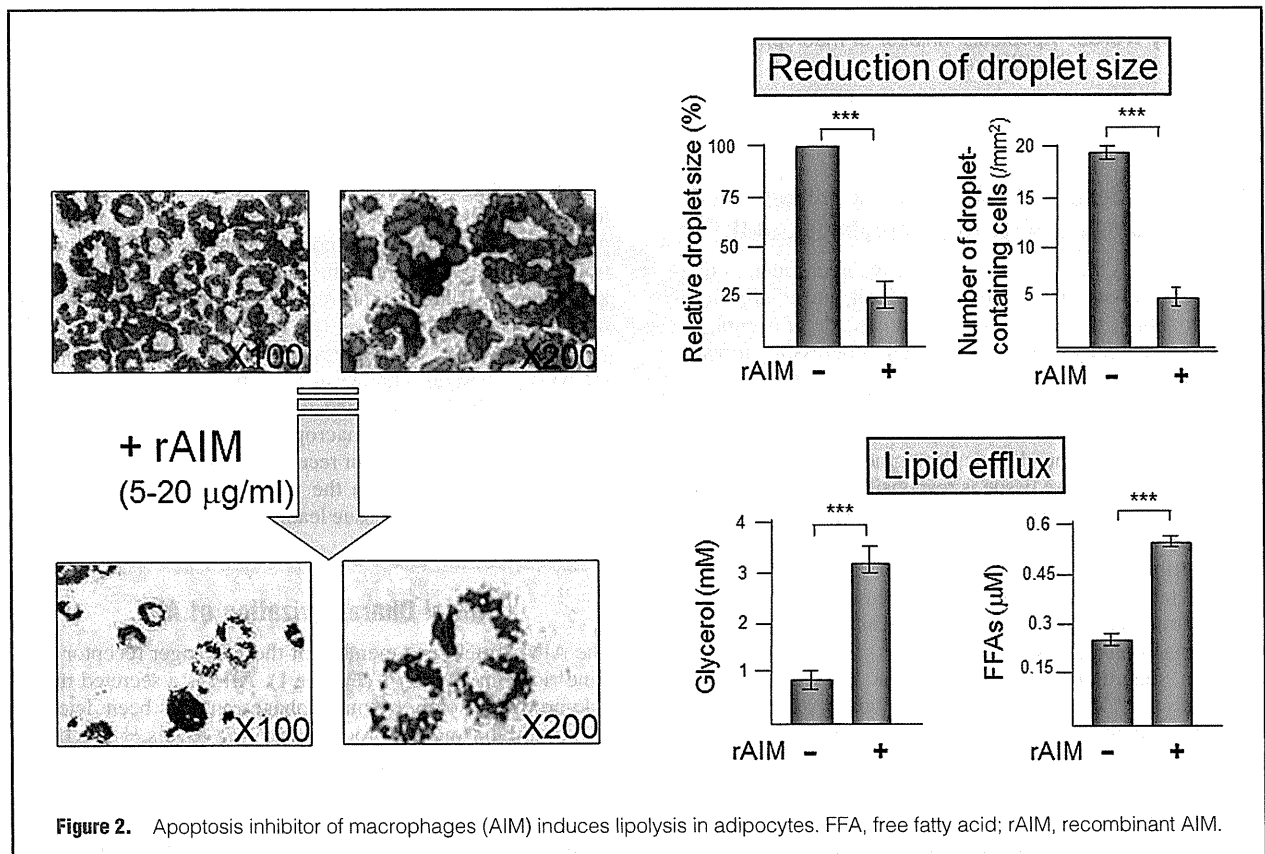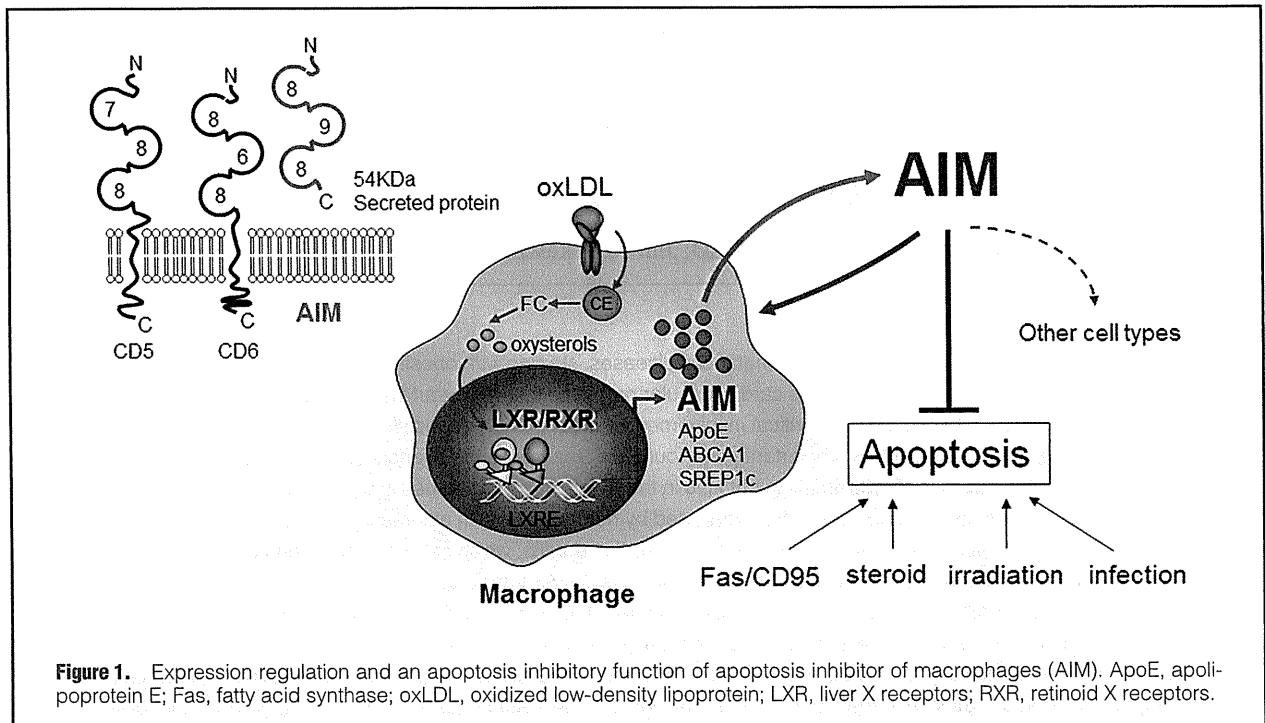
Initial Characterization of AIM

The AIM protein is a member of the scavenger receptor cysteine-rich superfamily[13] (Figure 1). AIM is a secreted molecule produced solely by macrophages and has been detected in human and mouse blood at varying levels.[13-18] AIM is a direct target for regulation by nuclear receptor LXR/RXR

Figure 1. Expression regulation and an apoptosis inhibitory function of apoptosis inhibitor of macrophages (AIM). ApoE, apoli-poprotein E; Fas, fatty acid synthase; oxLDL, oxidized low-density lipoprotein; LXR, liver X receptors; RXR, retinoid X receptors.

Figure 2. Apoptosis inhibitor of macrophages (AIM) induces lipolysis in adipocytes. FFA, free fatty acid; rAIM, recombinant AIM.

Figure 3. (A–C) Apoptosis inhibitor of macrophages (AIM) is incorporated into adipocytes through endocytosis mediated by CD36. (A) Mature adipocytes strongly positive for PPARγ2 (green signals) efficiently incorporated rAIM (red signals) (Left), whereas preadipocytes not stimulated by insulin, DEX, and IBMX did not incorporate rAIM showing no signals (Right).

heterodimers,[19–21] so is produced when macrophages incorporate oxidized low-density lipoprotein (LDL), but not native or acetylated LDL.[22] Based on these findings, we observed that AIM is expressed in lipid-laden macrophages at atherosclerotic lesions, and this induction is associated with atherosclerogenesis by supporting the survival of macrophages within lesions.[22] Indeed, atherosclerotic plaques were markedly reduced in size in mice doubly deficient for AIM and LDL receptor ($AIM^{-/-}LDL^{-/-}$) compared with $AIM^{+/+}LDL^{-/-}$ mice fed a high-cholesterol diet.[22] Other studies have shown that AIM appears to be multifunctional and is effective in cell types other than macrophages, including B and natural killer (NK) T lymphocytes.[23–25]

AIM harbors 3 cysteine-rich domains, resulting in a complex protein structure. The average detected AIM concentration in human and mouse blood varies according to the antibodies used for analysis by ELISA or Western blotting (Miyazaki, unpublished data). In addition, especially in human blood, different types of AIM structure seem to be present in different populations, based on the variable recognition patterns of blood AIM by an AIM-specific antibody (Miyazaki, unpublished data). Therefore, it is essential to evaluate the particular antibody being used for AIM analysis. It is also noteworthy that functional AIM variants exist, because recombinant AIM (rAIM) proteins generated in different host cell types show a wide range of diversity in both structure and function (Miyazaki, unpublished data). In addition to the host cell type, the production efficiency and functional activity of the rAIM protein appear largely dependent on several conditions, including strength of promoter activity of the expression vector, cell culture conditions, including amount and type of fetal bovine

serum (FBS), purification method, including the type of antibody being used, and presence and location of a protein tag. Because of the difficulties experienced in obtaining large quantities of the correct AIM protein, it is also difficult to generate sufficient AIM antibodies; indeed, some rAIM and commercially available antibodies are not fully functional. This makes detailed study of AIM complicated. Although the mechanisms underlying such AIM structural variance are unclear, these could be investigated by analyzing possible associations between AIM structure and disease susceptibility.

AIM Induces Lipolysis in Adipocytes, Suppressing Increased Fat Mass

Besides the apoptosis inhibitory effect, we previously identified a novel AIM function within adipocytes.[26] We initially observed a more accelerated weight increase of both visceral and subcutaneous fat tissue in $AIM^{-/-}$ mice fed a high-fat diet (HFD: fat kcal 60%) for 12 weeks compared with $AIM^{+/+}$ mice. Consistently, visceral fat adipocytes were larger in obese $AIM^{-/-}$ mice than in obese $AIM^{+/+}$ mice, and this was corrected by the intraperitoneal administration of rAIM. Interestingly, both obese $AIM^{-/-}$ and $AIM^{+/+}$ mice showed comparable metabolic rates (such as body temperature, oxygen consumption, and food intake), as well as locomotor activity.[26] Thus, AIM appeared to influence adipose tissue mass by specifically affecting adipocytes. It is also noteworthy that the serum level of AIM was markedly increased in mice receiving a HFD. However, it is unclear whether this increase in the blood AIM levels is brought about by advanced AIM expression in macrophages or other unknown mechanisms.
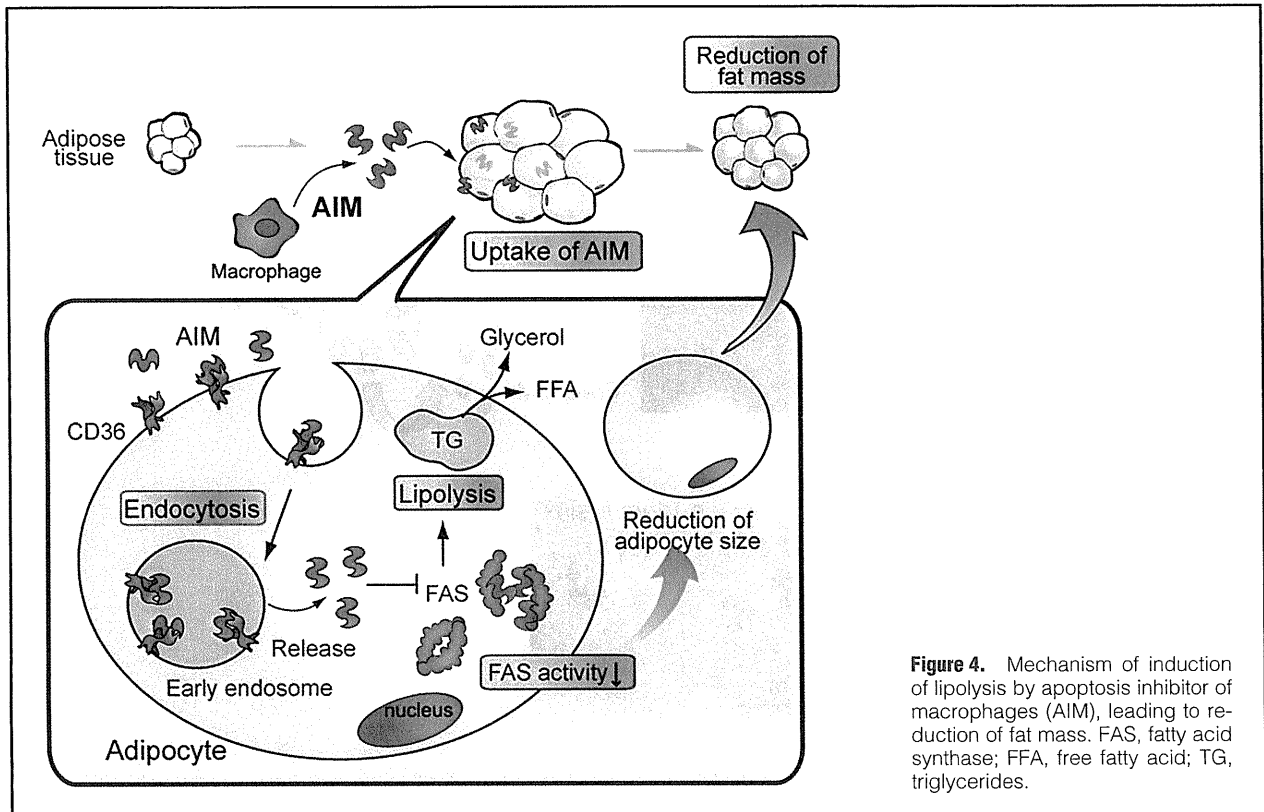
Figure 4. Mechanism of induction of lipolysis by apoptosis inhibitor of macrophages (AIM), leading to reduction of fat mass. FAS, fatty acid synthase; FFA, free fatty acid; TG, triglycerides.

We next assessed the effect of AIM in adipocytes using the 3T3-L1 preadipocyte cell line (**Figure 2**). When differentiated 3T3-L1 adipocytes in culture were challenged with rAIM, the size of the lipid droplets within the cells and the number of cells containing lipid droplets were remarkably decreased.[26] When adipocytes were treated with rAIM, the amount of glycerol and free fatty acids (FFA) in the supernatant increased significantly. Supernatant viscosity was also markedly enhanced by the administration of rAIM, perhaps because of the increased glycerol content. Thus, AIM induces a lipolytic response resulting in the liberation of droplet components such as glycerol and fatty acids from the cells.[27,28] To support these in vitro observations, basal levels of serum FFA and glycerol were lower in obese $AIM^{-/-}$ mice than in obese $AIM^{+/+}$ mice. Therefore, AIM induces lipolysis in adipocytes, resulting in the regulation of fat and body weight.
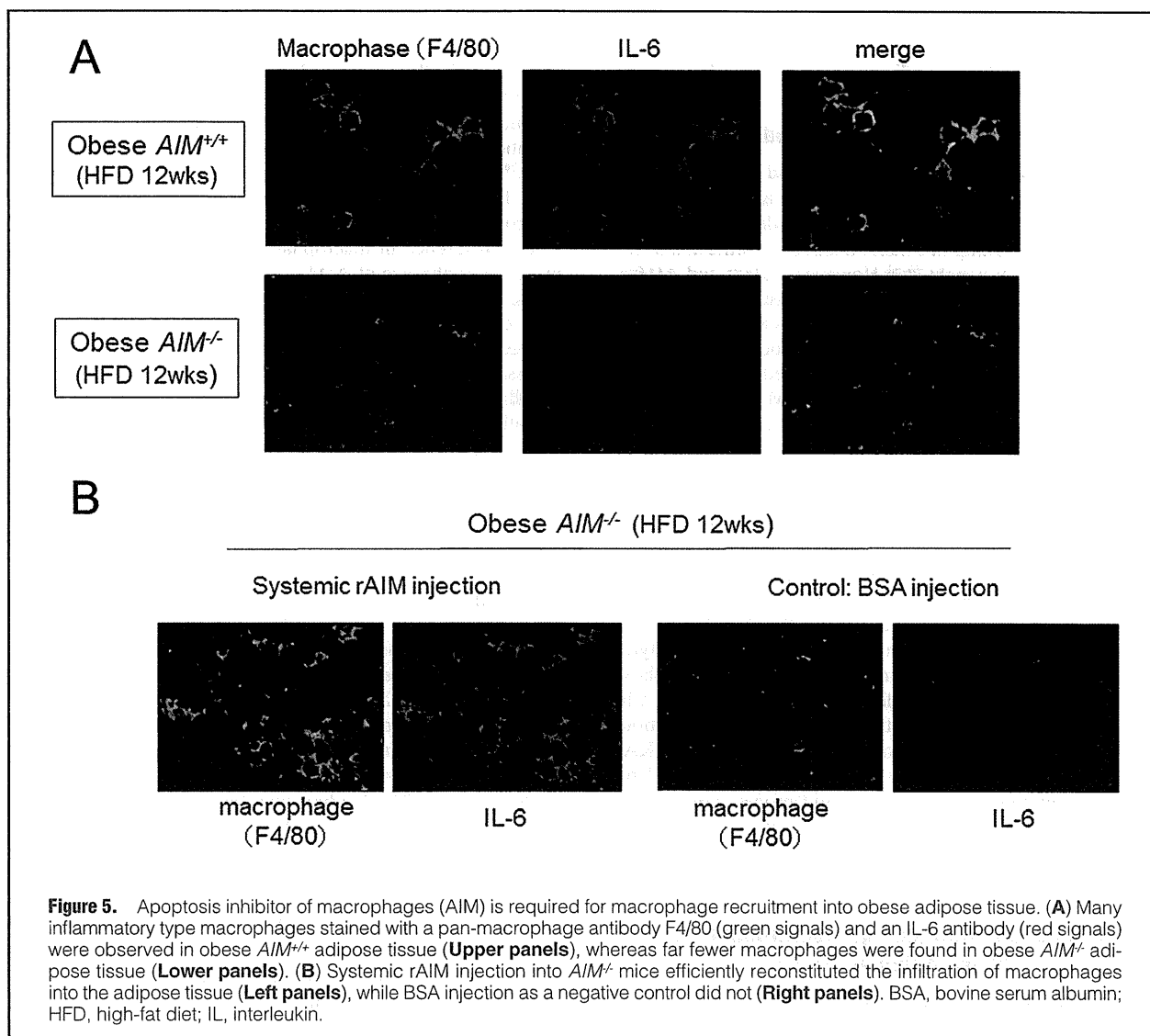
AIM Functions in a Lack-of-Signaling Manner

In most cases, a secreted protein binds to its specific receptor and mediates signal transduction to affect the target cell. Interestingly, however, this is not the case for AIM. Exogenous AIM secreted by macrophages is incorporated into adipocytes where it functions directly. When differentiated 3T3-L1 adipocytes, expressing high levels of peroxisome proliferator-activated receptor γ 2 (PPARγ2),[29] are treated with rAIM, rAIM accumulates within the cytoplasm, forming multiple dots within the intracellular compartment (**Figure 3A Left**). Incorporated rAIM colocalizes with early endosomes, but not with late endosomes or recycling endosomes. Therefore, AIM is endocytosed into adipocytes and is thereafter transported into the cytosol during endosome maturation. Notably, rAIM is not incorporated into immature preadipocytes that are nega-

tive for PPARγ2 expression (**Figure 3A Right**). This specific colocalization of AIM with endosomes was supported by electron microscopy and AIM immunolabeling (**Figure 3B**).

Such direct functioning in the absence of signaling is unusual for a secreted molecule, with only a limited number of previously reported examples, including fibroblast growth factors 1 and 2,[30,31] epidermal growth factor,[32] and some plant and bacterial toxins[33,34] in which the cytosolic delivery of exogenous proteins mediates biological effects in mammalian cells. In addition, in dendritic cells some exogenous antigens can access the cytosol via similar machineries for intracellular transport where they are presented by major histocompatibility complex class I molecules.[35,36] Additional experiments are necessary to clarify the mechanism responsible for AIM translocation from the endosomal compartment to the cytosol.

The internalization of exogenous AIM is mediated by the scavenger receptor CD36, which promotes the internalization of various molecules, including lipoproteins and fatty acids[37,38] and is expressed by adipocytes and macrophages, the target cells for AIM. Indeed, incorporation of rAIM was drastically decreased in the presence of CD36-neutralizing antibody. In addition, when rAIM was intravenously injected into $CD36^{-/-}$ mice, its incorporation into adipocytes in fat tissue was markedly less in $CD36^{-/-}$ mice than in $CD36^{+/+}$ mice.[39] Interestingly, the $CD36^{-/-}$ phenotype is not equivalent to that of $AIM^{-/-}$ mice[39–41] because of the wide-ranging scavenging characteristic of CD36, which allows $CD36^{-/-}$ mice to show a complicated phenotype caused by the deficient incorporation of multiple molecules.

Figure 5. Apoptosis inhibitor of macrophages (AIM) is required for macrophage recruitment into obese adipose tissue. (A) Many inflammatory type macrophages stained with a pan-macrophage antibody F4/80 (green signals) and an IL-6 antibody (red signals) were observed in obese $AIM^{+/+}$ adipose tissue (Upper panels), whereas far fewer macrophages were found in obese $AIM^{-/-}$ adipose tissue (Lower panels). (B) Systemic rAIM injection into $AIM^{-/-}$ mice efficiently reconstituted the infiltration of macrophages into the adipose tissue (Left panels), while BSA injection as a negative control did not (Right panels). BSA, bovine serum albumin; HFD, high-fat diet; IL, interleukin.

AIM Targets Fatty Acid Synthase to Induce Lipolysis

Following the understanding that macrophage-derived AIM enters into adipocytes via CD36-mediated endocytosis (Figure 3C), the next question was how does it induce lipolysis. The first clue was provided by immunoprecipitation–mass spectrometry analysis using lysates from 3T3-L1 adipocytes treated with rAIM. AIM was shown to associate with fatty acid synthase (FAS), which is highly expressed in adipose tissue and catalyzes the synthesis of saturated fatty acids, such as palmitate, from acetyl-CoA and malonyl-CoA precursors. Previous studies have highlighted critical roles for FAS in biological aspects such as early embryogenesis,[42] in addition to its use as a metabolic substrate. The association of AIM and FAS was subsequently confirmed in vitro by HEK293T cell lysates overexpressing FAS and AIM, and in vivo following the coprecipitation of FAS and AIM from fat tissue lysates in obese $AIM^{-/-}$ mice injected with rAIM. Additional in vitro studies revealed that AIM binds to specific domains within FAS, which are involved in the elongation of fatty acid chains, the terminal release of synthesized palmitate, as well as stabi-

lization of FAS dimerization. It is well known that FAS is highly functional as a dimerized form, whereas monomeric FAS possesses little or no activity.[43,44] Apparently owing to AIM binding, the proportion of dimerized FAS was significantly reduced in 3T3-L1 adipocytes treated with rAIM.

AIM association was shown to result in a remarkable reduction in the enzymatic activity of FAS, similar or even greater than that induced by the specific FAS inhibitor, C75,[45] when used at a functional concentration (25 μmol/L). Consistently, FAS activity was significantly increased in the epididymal fat of $AIM^{-/-}$ mice compared with $AIM^{+/+}$ mice, and this activity was subsequently decreased following supplementation of rAIM via direct injection. Thus, through association with multiple regions of FAS, AIM decreases FAS activity both functionally and structurally. Because treatment of 3T3-L1 adipocytes with AIM or C75 has similar consequences, the lipolytic effect of AIM on adipocytes must be through suppression of FAS activity. Indeed, rAIM (5 μg/ml) and C75 (25 μmol/L) were found to induce an increase in the efflux of glycerol and FFA at comparable levels from 3T3-L1 adipocytes. AIM and C75 also similarly prevented preadipocyte morphological differentiation and the suppression of the dif-

ferential upregulation of mRNA levels for "fat genes" such as C/EBPα, PPARγ2, CD36, and GLUT4.[46-49] Figure 4 summarizes the effects of AIM on adipocytes to reduce cell size.

How Does Reduced FAS Activity Lead to Lipolysis?

It is of interest that AIM is the first identified natural inhibitor of FAS. Systemic FAS inhibition via the administration of C75 decreases the production of neuropeptide Y in the mouse hypothalamus, resulting in a marked loss of appetite and overall decreased body weight.[50-58] However, AIM^{-/-} and AIM^{+/+} mice show comparable levels of food intake, suggesting that AIM may not have a neurologic effect. This may be related to a requirement for a specific endocytotic process mediated by CD36, the expression of which is not reported in hypothalamic cells. Obviously, the lipolytic effect of AIM is a direct effect of FAS inhibition on adipocytes, which decreases the size and number of lipid droplets, thereby decreasing adipocyte size.

Lipolysis usually occurs during periods of energy deprivation. Under fasting conditions, adipocytes undergo lipolysis via the hormone-dependent stimulation of a G protein-coupled receptor/cyclic AMP (cAMP)-dependent signaling cascade, followed by phosphorylation of protein kinase A (PKA) which activates hormone-sensitive lipase (HSL). Simultaneously, the level of adipose triglyceride lipase (ATGL) mRNA also increases.[59-65] Interestingly, however, despite the lipolytic consequences, neither rAIM nor C75 upregulates the phosphorylation of PKA or the levels of ATGL and HSL mRNA in 3T3-L1 adipocytes. Thus, unlike conditions of starvation, inhibition of FAS might activate an unknown cAMP/PKA-independent lipolytic pathway. Indeed, lipolysis caused by AIM/FAS inhibition is a slow and mild process contrasting with that observed in starvation, which occurs rapidly and robustly. Further studies are required to identify the mechanisms involved in AIM/FAS-dependent lipolysis.

Lipolysis and Macrophage Recruitment Into Obese Adipose Tissue

Although the mechanism that promotes the infiltration of inflammatory macrophages into obese adipose tissue has been unknown, recent studies have shown that saturated fatty acids released from adipocytes in response to various metabolic consequences of cell hypertrophy, including reduced mitochondrial function, ER stress, and increased rates of basal lipolysis, may contribute to macrophage recruitment.[66-68] In particular, a critical role for lipolysis has been suggested,[69] showing that it promotes macrophage infiltration into adipose tissue during both obesity and weight loss. On the other hand, several reports have emphasized the importance of the chemokine, MCP-1, following the analysis of MCP-1-deficient mice or transgenic mice overexpressing MCP-1 in adipocytes. However, many unanswered questions remain, including "What is the key factor that induces lipolysis along with obesity?", "What promotes MCP-1 expression in obese adipose tissue?", "What links lipolysis and MCP-1?", and "Is lipolysis brought about by AIM involved in this event, and if so, how?"

No Adipose Tissue Macrophage Accumulation in the Absence of AIM

As so far described, adipocyte hypertrophy is more advanced in AIM^{-/-} mice than in AIM^{+/+} mice, and the overall mass of visceral fat and body weight is markedly greater.[26] Interest-

ingly, however, the obesity-associated infiltration of inflammatory macrophages (M1 macrophages) into adipose tissue was dramatically suppressed in AIM^{-/-} mice compared with AIM^{+/+} mice after a 12-week HFD[70] (Figure 5A). In addition, the intraperitoneal administration of rAIM induced the accumulation of M1 macrophages in adipose tissue in obese AIM^{-/-} mice[70] (Figure 5B). Thus, the presence of AIM is indispensable for obesity-associated recruitment of adipose tissue macrophages.

The difference in macrophage accumulation in fat in the presence or absence of AIM is not predominantly because of the anti-apoptotic effect of AIM,[13,22] because the apoptotic state of macrophages (and also of adipocytes) is comparable between obese AIM^{+/+} and AIM^{-/-} epididymal adipose tissue, as assessed by TUNEL staining. Recent reports showed that T cells are also recruited to adipose tissue,[71-75] and that accumulation of a CD8+ T cell population appears to precede macrophage infiltration.[74] However, the number of CD8+ (as well as CD4+) T cells in epididymal fat did not differ significantly between AIM^{+/+} and AIM^{-/-} mice fed a HFD for 6 weeks, which is the early phase of obesity prior to macrophage accumulation.

The lipolytic state of adipose tissue, which progressed along with an increase in blood AIM levels under HFD conditions, was previously shown to be suppressed in AIM^{-/-} mice.[26] Thus, an increase in AIM may induce vigorous lipolysis in obese adipose tissue, thereby stimulating macrophage recruitment. To test this idea, we investigated whether AIM itself attracts macrophages, but found that it showed no chemoattractive activity in a macrophage migration assay using RAW264.1 mouse macrophage cells. By contrast, conditioned medium from 3T3-L1 adipocytes that had been challenged with rAIM for 72h (AIMCM) efficiently attracted macrophage cells.[70] A comparable effect was observed with conditioned medium from cells treated with the specific FAS inhibitor, C75 (C75CM).[26] Furthermore, conditioned medium from 3T3-L1 adipocytes treated with rAIM in the presence of a CD36-neutralizing antibody to inhibit AIM-dependent lipolysis[26] did not efficiently attract macrophages, suggesting that AIM-induced lipolysis in adipocytes appears to be responsible for macrophage recruitment.

Bona Fide Scenario to Attract Macrophages Into Adipose Tissue

Previous work has demonstrated that saturated fatty acids activate Toll-like receptor (TLR) 4, and that this response is tightly associated with obesity-induced inflammation.[76-80] Thus, it is plausible that an increase in blood AIM induces vigorous lipolysis in obese adipose tissue, and that saturated fatty acids effluxed from adipocytes as a result of lipolysis might activate chemokine production in adipocytes via the stimulation of TLR4 in a paracrine/autocrine fashion.[81-83] Indeed, palmitic acid (PA) and stearic acid (SA), the major fatty acids comprising triglyceride droplets[84] and well known stimulators of TLR4,[75,80,85,86] were identified as the components released by adipocytes in response to lipolysis induced by AIM or C75. Consistently, both AIMCM and C75CM efficiently activated the TLR signaling cascade and chemokine production in 3T3-L1 adipocytes, inducing degradation of IkBα and production of chemokines such as MCP-1, CCL5/RANTES, MCP-2, and MCP-3. Similar effects of TLR activation and chemokine production were observed when 3T3-L1 adipocytes were treated with PA and SA.

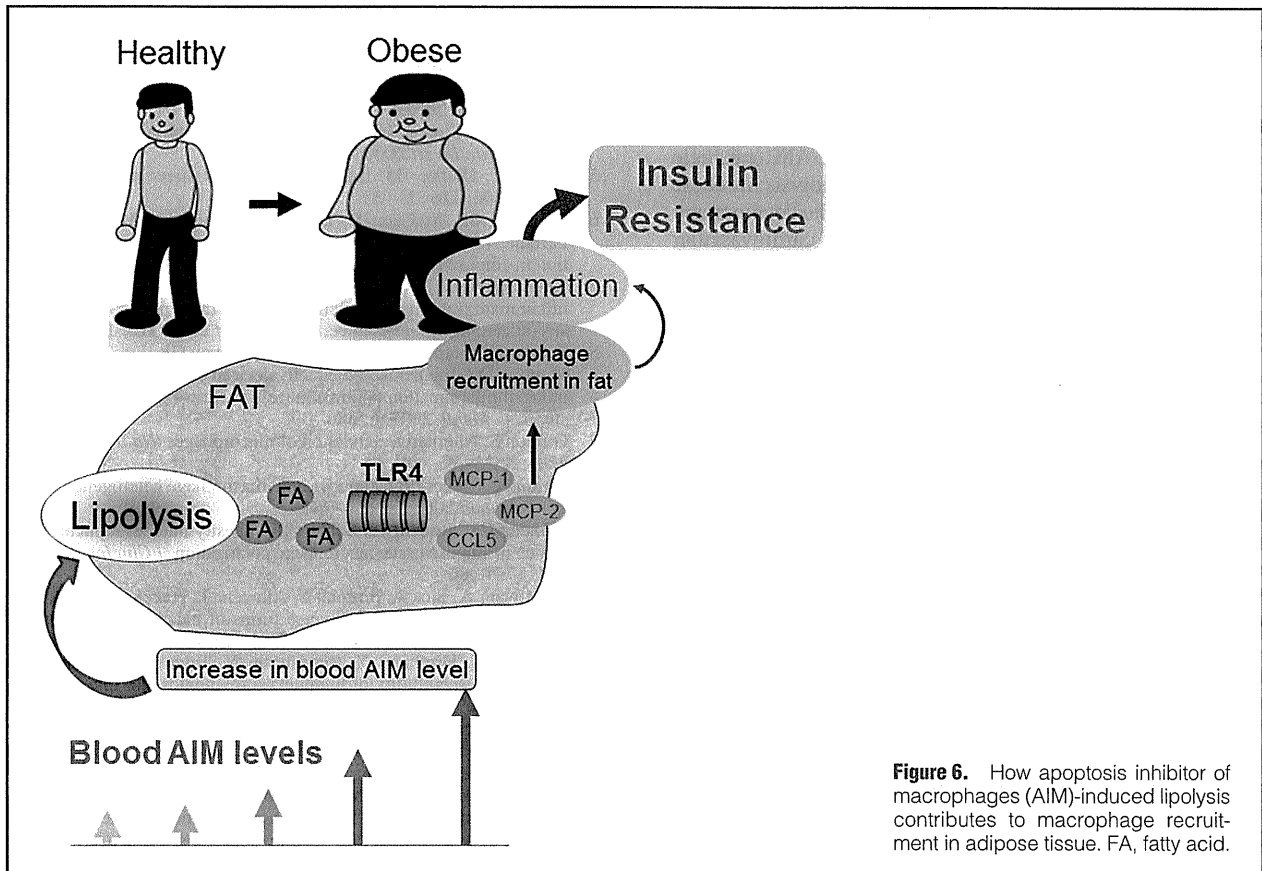The essential role of TLR4 was corroborated in 2 ways.

Figure 6. How apoptosis inhibitor of macrophages (AIM)-induced lipolysis contributes to macrophage recruitment in adipose tissue. FA, fatty acid.

First, suppression of *TLR4* expression by siRNA significantly reduced production of MCP-1 in 3T3-L1 adipocytes. Second, intravenous injection of rAIM into wild-type and *TLR4*−/− mice was assessed according to the state of lipolysis and chemokine production in epididymal adipose tissue. In wild-type and mutant mice, rAIM did induce lipolysis, as shown by increased blood FFA and glycerol levels. By contrast, induction of mRNA for chemokines by rAIM injection was significantly less efficient in *TLR4*−/− than in wild-type mice. Thus, in summary, AIM-induced lipolysis provoked an efflux of saturated fatty acids, including PA and SA, from adipocytes, which stimulated chemokine production in both adipocytes and resident macrophages via TLR4 activation, resulting in M1 macrophage migration (**Figure 6**). Consistent results were obtained in vivo in obese *AIM*+/+and *AIM*−/− mice after 12 weeks on a HFD. In epididymal fat, phosphorylation levels of JNK, representing the state of TLR activation,[87] were decreased in *AIM*−/− mice compared with *AIM*+/+ mice. In addition, chemokine mRNA levels were lower in *AIM*−/− compared with *AIM*+/+ adipose tissue. Overall, these results strongly indicate that AIM-induced lipolysis is the initiating step for macrophage recruitment into obese adipose tissue.

No Inflammation or IR in Obese *AIM*−/− Mice

As a consequence of the abolished infiltration of inflammatory macrophages, the progression of obesity-associated inflammation is prevented both locally and systemically in obese *AIM*−/− mice. In adipose tissue, mRNA levels for proinflammatory cytokines such as tumor necrosis factor α (TNFα), IL-6 and IL-1β, were significantly lower in *AIM*−/− than in *AIM*+/+

mice after a HFD for 12 weeks. Consistent with this finding, serum levels of TNFα and IL-6 were lower in *AIM*−/− mice compared with *AIM*+/+ mice.

Activation of the insulin signaling pathway was studied after the intravenous injection of insulin in *AIM*−/− and *AIM*+/+ mice fed a HFD for 12 weeks. Substantial insulin-stimulated phosphorylation of AKT and GSK3β protein kinases[88] was observed in adipose tissue, skeletal muscle (gastrocnemius), and liver in *AIM*−/− mice in contrast to markedly diminished phosphorylation levels in *AIM*+/+ mice.[70] Thus, insulin sensitivity was maintained in obese *AIM*−/− mice. In line with this, whole-body glucose intolerance and IR observed in obese *AIM*+/+ mice were ameliorated in obese *AIM*−/− mice, as shown by intraperitoneal glucose and insulin tolerance tests. Thus, *AIM*−/− mice showed advanced obesity compared with *AIM*+/+ mice after a 12-week HFD, but still showed normal glucose tolerance.

Conclusion

Is AIM Beneficial or Detrimental for MetS?

AIM is incorporated into adipocytes and induces lipolysis via the reduction of FAS enzymatic activity. This decreases lipid droplet storage within adipocytes, which resists the augmentation of adipose tissue mass on overfeeding. Indeed, the weight increase of visceral fat in mice fed a HFD was accelerated in *AIM*−/− mice, and suppressed by the systemic administration of rAIM. Thus, AIM appears to be a beneficial molecule that impedes the progression of obesity, suggesting that it might be a promising target for next-generation anti-obesity drugs. Intriguingly, however, when this lipolytic effect is excessive

(ie, when an increased level of AIM targets hypertrophic adipocytes), it triggers chronic inflammation via the recruitment of macrophages into adipose tissue, leading to IR. In this regard, AIM is certainly detrimental for metabolic disorders. Thus, during early periods of MetS prior to prominent obesity and with limited lipid storage in adipocytes, AIM can help prevent the progression of obesity through lipolysis; in obese conditions, anti-AIM therapy should prevent the development of metabolic diseases such as diabetes and cardiovascular events, as observed in $AIM^{-/-}$ mice.

One of the criteria for assessing whether AIM or anti-AIM therapy should be administered is the blood AIM level, based on the observation that this increases in line with the progression of obesity in mice fed with HFD. However, 2 things are noteworthy. First, because AIM has a complicated structure, it is possible that a considerable proportion of blood AIM undergoes unsuccessful protein folding, resulting in limited or even no function. In addition, the presence of glycosylation is likely, in particular for murine AIM. Indeed, murine AIM has 3 or 4 regions susceptible to N-glycosylation, and both murine and human AIM proteins have several regions for O-glycosylation. Further, the molecular weights of both murine and human AIM are markedly larger than those estimated from their amino acid sequences, suggesting that they are heavily glycosylated. This modification may also influence the function of AIM. Therefore, not only the amount but also the activity of blood AIM should be evaluated. Second, unlike mice fed with HFD, the body mass index and AIM level are not always parallel in humans (Miyazaki, unpublished data), possibly because of the wide variation in eating habits, as some foods may induce obesity without a remarkable increase in blood AIM, and vice versa. Thus, it is relevant to study which foods increase blood AIM. It is also possible that some foods stimulate AIM production while interfering with its efficient folding and glycosylation. Thus, the evaluation of blood AIM activity together with blood AIM levels is therefore again necessary.

Practically, however, it may be difficult to clearly establish the threshold of blood AIM level or activity, which decides the agonist or antagonist to be used. To this end, large-scale cohort studies for the association of blood AIM and the incidence and levels of disease are certainly required. In the meantime, a set of parameters including blood AIM needs to be defined. In addition, development of a simple method that can maintain the AIM level at a reasonable level via modulating the expression or protein stability of AIM is required.

Perspectives

We have shown that AIM prevents the progression of obesity via lipolysis, and acts as a key factor in the initiation of obesity-associated chronic inflammation leading to IR. Future efforts to establish a diagnosis via the measurement of the blood AIM level and the therapeutic application of both AIM (AIM agonists) and anti-AIM (AIM antagonists) will further progress the development of treatments to prevent the onset of metabolic disorders brought about by the modern lifestyle.

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Apoptosis inhibitor of macrophage (AIM) is required for obesity-associated recruitment of inflammatory macrophages into adipose tissue

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Infiltration of inflammatory macrophages into adipose tissues with the progression of obesity triggers insulin resistance and obesity-related metabolic diseases. We recently reported that macrophage-derived apoptosis inhibitor of macrophage (AIM) protein is increased in blood in line with obesity progression and is incorporated into adipocytes, thereby inducing lipolysis in adipose tissue. Here we show that such a response is required for the recruitment of adipose tissue macrophages. In vitro, AIM-dependent lipolysis induced an efflux of palmitic and stearic acids from 3T3-L1 adipocytes, thereby stimulating chemokine production in adipocytes via activation of toll-like receptor 4 (TLR4). In vivo administration of recombinant AIM to $TLR4$-deficient ($TLR4^{-/-}$) mice resulted in induction of lipolysis without chemokine production in adipose tissues. Consistently, mRNA levels for the chemokines that affect macrophages were far lower in AIM-deficient ($AIM^{-/-}$) than in wild-type ($AIM^{+/+}$) obese adipose tissue. This reduction in chemokine production resulted in a marked prevention of inflammatory macrophage infiltration into adipose tissue in obese $AIM^{-/-}$ mice, although these mice showed more advanced obesity than $AIM^{+/+}$ mice on a high-fat diet. Diminished macrophage infiltration resulted in decreased inflammation locally and systemically in obese $AIM^{-/-}$ mice, thereby protecting them from insulin resistance and glucose intolerance. These results indicate that the increase in blood AIM is a critical event for the initiation of macrophage recruitment into adipose tissue, which is followed by insulin resistance. Thus, AIM suppression might be therapeutically applicable for the prevention of obesity-related metabolic disorders.

diabetes | fatty acid synthase | CD36 | knockout mouse

Chronic, low-grade inflammation observed in adipose tissues is characteristic of obesity. Such a subclinical inflammatory state of adipose tissues is highly associated with insulin resistance both in adipose tissue and systemically and thus contributes to the development of multiple obesity-induced metabolic and cardiovascular diseases (1–4). Evidence has shown that infiltration of a large number of classically activated inflammatory macrophages (M1 macrophages) into adipose tissue is responsible for obesity-associated inflammation (5–7). Lean adipose tissue contains a resident population of alternatively activated macrophages (M2 macrophages), which can suppress the inflammation of both adipocytes and macrophages partly via the secretion of interleukin (IL)-10. Hence, obesity induces a switch in macrophage activation state in adipose tissue toward M1 polarization, which leads to inflammation (8–12). However, the mechanism that promotes infiltration of inflammatory macrophages into obese adipose tissue is as yet unknown.

We recently reported that the apoptosis inhibitor of macrophage (AIM) protein (13) is incorporated into adipocytes via CD36-mediated endocytosis, and induces lipolysis by suppressing the activity of fatty acid synthase (FAS) (14). AIM is a member of the scavenger receptor cysteine-rich superfamily and was initially identified as an apoptosis inhibitor that supports the survival of macrophages against different types of apoptosis-inducing stimuli (13). AIM is a direct target for regulation by nuclear receptor liver X receptor/retinoid X receptor (LXR/RXR) heterodimers (15, 16) and is solely produced by tissue macrophages. As a secreted molecule, AIM is detected in both human and mouse blood at various levels (13, 16–19) and increases in blood with the progression of obesity in mice fed a high-fat diet (HFD) (14). The augmented blood AIM induced lipolysis, as evident by the fact that the increase of free fatty acids (FFAs) and glycerol in blood was suppressed in $AIM^{-/-}$ mice (14). Owing to less lipolysis, adipocyte hypertrophy was more advanced and the overall mass of visceral adipose tissues was greater in $AIM^{-/-}$ than in $AIM^{+/+}$ mice fed a HFD (14). All these observations imply that AIM-induced lipolysis might be responsible for the obesity-associated recruitment of adipose tissue macrophages.

In the present study, we assessed whether AIM affects macrophage accumulation in adipose tissues in obese mice. In addition, we determined the molecular mechanism of how AIM-dependent lipolysis results in the production of chemokines by adipocytes for the effective recruitment of adipose tissue macrophages. Finally, we investigated how the absence of AIM influences the local and systemic inflammatory state and insulin resistance in mice. On the basis of these results, we discuss the putative role of AIM in the initiation of obesity-associated chronic inflammation and subsequent metabolic diseases.

Results and Discussion

Prevention of M1 Macrophage Recruitment into Adipose Tissues in Obese $AIM^{-/-}$ Mice. In $AIM^{-/-}$ mice, adipocyte hypertrophy was more advanced than in $AIM^{+/+}$ mice, and the overall mass of visceral fat as well as body weight was markedly greater compared with that of $AIM^{+/+}$ mice (14). Interestingly, however, far fewer macrophages stained with a pan-macrophage antibody F4/80 were observed in epididymal adipose tissue in $AIM^{-/-}$ mice than in $AIM^{+/+}$ mice fed a HFD for 12 wk (Fig. 1A). The number of IL-6 stained inflammatory type (M1) macrophages in obese $AIM^{-/-}$ mice was markedly lower than in obese $AIM^{+/+}$ mice (Fig. 1A). In addition, almost no M1 macrophage clusters forming crown-like structures (CLS) were observed in obese $AIM^{-/-}$ mice (Fig. 1A). In contrast, the number of M2 adipose tissue macrophages stained for mannose receptor (MR) was not increased in $AIM^{+/+}$ or $AIM^{-/-}$ mice after a 12-wk HFD (Fig. 1B). Furthermore, the stromal-vascular cell fraction (SVF) containing macrophages was isolated from the epididymal fat tissue of lean and obese mice by collagenase treatment and
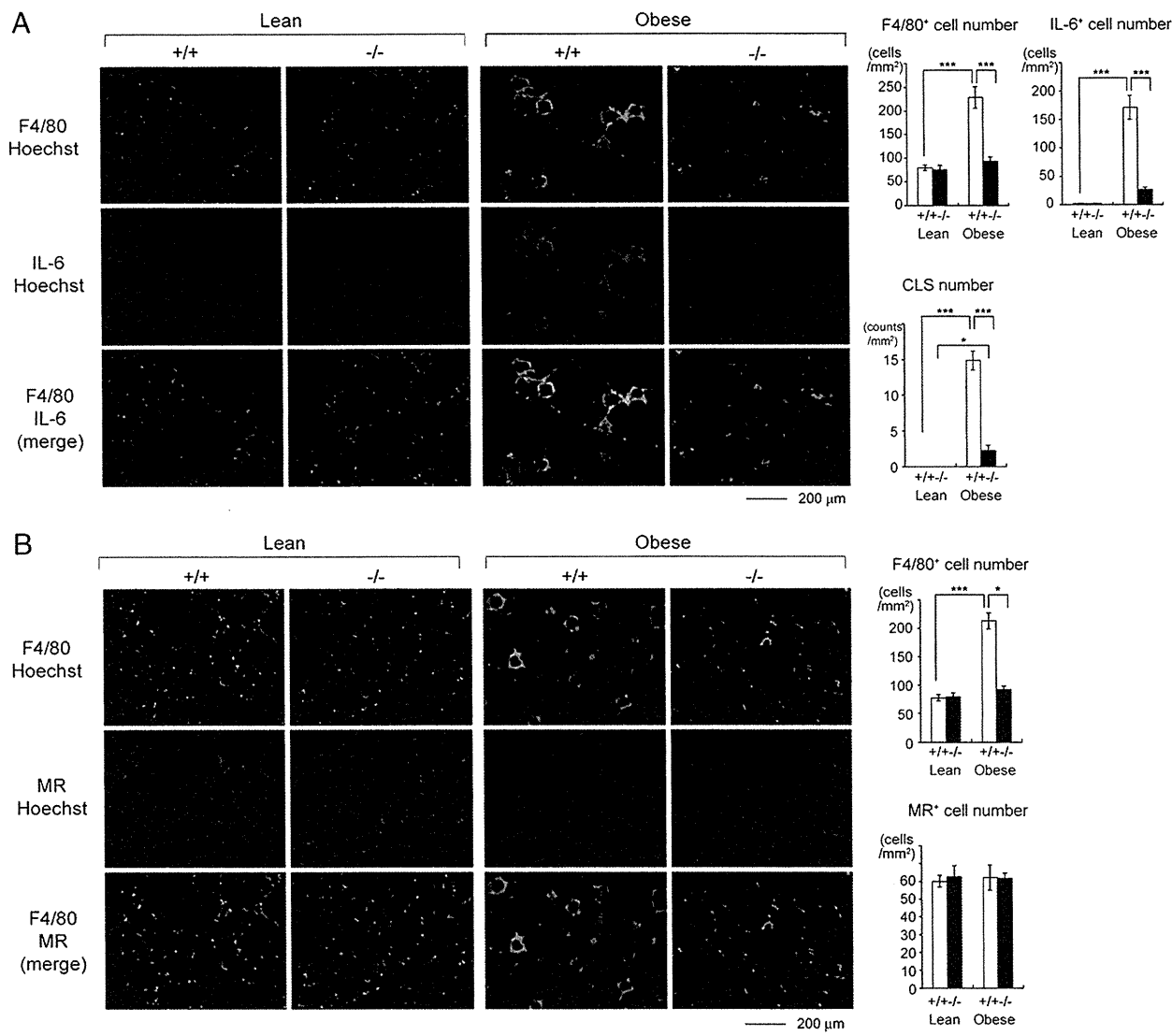
Fig. 1. Requirement of AIM for macrophage recruitment into obese adipose tissue. (*A* and *B*) Specimens of epididymal fat tissue from lean (0 wk) or obese (fed a HFD for 12 wk) $AIM^{+/+}$ and $AIM^{-/-}$ mice were costained for F4/80 (pan-macrophage marker; green), IL-6 (red), and Hoechst (blue) for *A*, and F4/80 (pan-macrophage marker; green), mannose receptor (MR) (red), and Hoechst (blue) for *B*. (Scale bar, 200 μm.) Quantification of F4/80$^+$ cell number, IL-6$^+$ macrophages, and the number of crown-like structures (CLS) are presented for *A*, or F4/80$^+$ cell number and MR$^+$ macrophages for *B* are presented. At least three different areas in three different sections per mouse were analyzed in six to eight mice of each genotype. Results are presented as averages ± SEM.

assessed to determine the number of both types of macrophage by flow cytometry after staining for F4/80 and CD11b (macro-phage), CD11c (M1 marker), and MR. Consistent with the his-tological data, the increase in M1 macrophage number was apparent in obese $AIM^{+/+}$ but not in obese $AIM^{-/-}$ mice (Fig. S1*A*). The M1/M2 ratio of macrophage number was significantly increased in obese $AIM^{+/+}$ than in lean $AIM^{+/+}$ mice, indicating M1 polarization of adipose tissue macrophage (9), whereas this was comparable in lean and obese $AIM^{-/-}$ mice (Fig. S1*B*). Similarly, quantitative RT-PCR (QPCR) analysis with RNA isolated from epididymal fat showed a remarkable increase in mRNA levels for M1 macrophage marker genes, such as *CD11c* and *iNOS*, after a 12-wk HFD in $AIM^{+/+}$ mice, whereas this was not apparent in $AIM^{-/-}$ mice (Fig. S1*C*). In addition, expression levels of antiinflammatory (M2) macrophage marker genes, such as *CD163*, *MR*, and *arginase*, were decreased in epididymal fat of $AIM^{+/+}$ mice fed a HFD, whereas this was not observed in $AIM^{-/-}$ mice (Fig. S1*C*). The reduction in mRNA levels of M2 markers in obese $AIM^{+/+}$ mice is consistent with the increase in

the M1/M2 ratio of macrophage number in obese $AIM^{+/+}$ mice (Fig. 1*D*). The difference in macrophage accumulation in fat in the presence or absence of AIM was not predominantly brought about by the antiapoptotic effect of AIM (13, 20) because the apoptotic state of macrophages (and also of adipocytes) was comparable between obese $AIM^{+/+}$ and $AIM^{-/-}$ epididymal ad-ipose tissues, as assessed by TUNEL staining (Fig. S2). These results implicate an indispensable role of AIM in the obesity-associated recruitment of adipose tissue macrophages.

AIM-Dependent Lipolysis Induces Macrophage Migration. We then tested whether AIM itself attracts macrophages. However, AIM showed no chemoattractive activity in a macrophage migration assay using RAW264.1 mouse macrophage cells (Fig. 2*A*, *Left*). In con-trast, conditioned medium from 3T3-L1 adipocytes that had been challenged with rAIM for 72 h (AIM CM) efficiently attracted macrophage cells (Fig. 2*A*, *Left*). A comparable effect was observed with conditioned medium from cells treated with C75, a specific FAS inhibitor that also induces lipolysis (14). AIM CM also attracted

Kurokawa et al.