II. 研究成果の刊行に関する一覧

研究成果の刊行に関する一覧

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
山口清次	新生児マススクリーニングで異常になったとき	五十嵐隆 高柳正樹	小児臨床ピクシス 23	中山書店	東京	2010	66-68
山口清次	代謝性疾患	白木和夫, 高田哲	ナースとコメディカルのための小児科学	日本小児医事出版社	東京	2010	125-136

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Iwamura, Y., Mori, M., Nakashima, K., Mikami, T., Muratama, K., Arai, S., & Miyazaki, T.	Apoptosis Inhibitor of Macrophage (AIM) diminishes lipid droplet-coating proteins leading to lipolysis in adipocytes.	Biochemical and Biophysical Research Communications	印刷中		2012
Miyazaki, T., Kurokawa, J., & Arai, S.	AIMing at Metabolic Syndrome –Towards the Development of Novel Therapies for Metabolic Diseases via Apoptosis Inhibitor of Macrophage (AIM)-	Circulation Journal	75 (11)	2522-2531	2011
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III. 研究成果の刊行物・別刷

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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.

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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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 cardiovascular 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, resulting in decreased inflammation both locally and systemically, thereby being protected from insulin resistance and glucose intolerance [9,19,20].

Interestingly, unlike many cytokines and growth factors, exogenous 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. Overall, 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 glycerol 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 compounds 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 regulatory element (−1 to −236) that includes PPRE (TGCCCT CTTGCCT) was subcloned into pGL3-enhancer vector (Promega). The plasmid was linearlized 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, reaching 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 apparent 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 exhibited 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 (Supplementary Fig. S2), though lipolysis was apparently enhanced as determined 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-coating proteins, leading to the efficient access of constitutive lipases to triacylglycerols without their activation or augmentation. In agreement 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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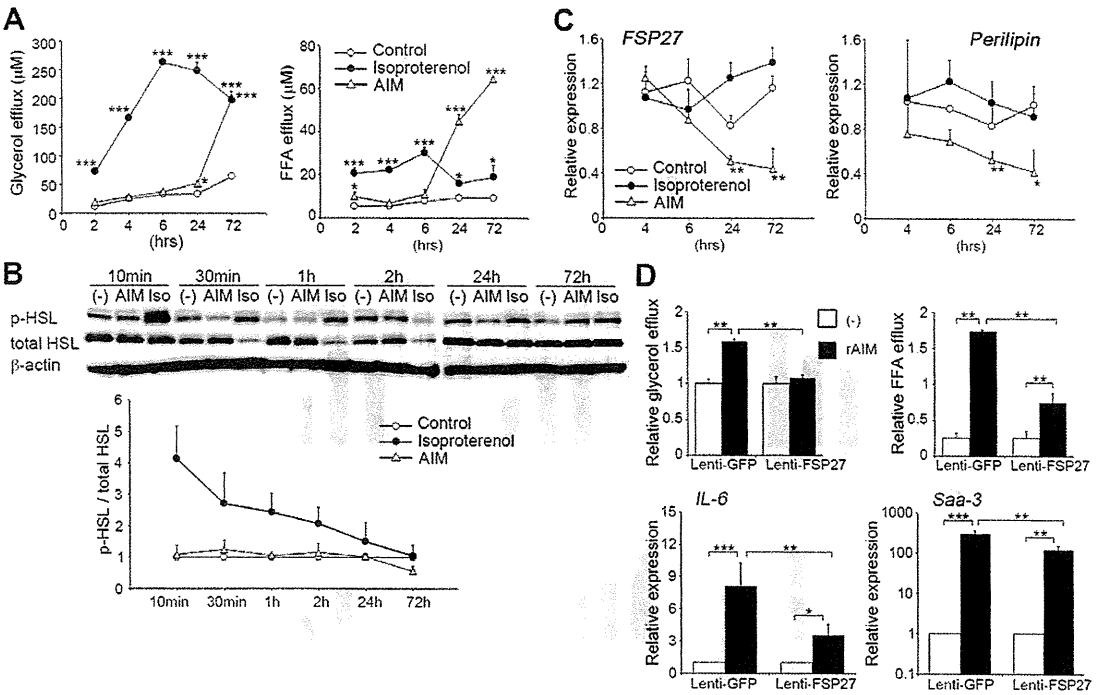

Fig. 1. AIM-induced lipolysis is distinctive from hormone-dependent lipolysis. 3T3-L1 adipocytes were treated with rAIM (100 μg/ml) or isoproterenol (10 μM), and analysed for (A) the efflux of glycerol and FFAs, (B) HSL (phosphorylated and total) levels by immunoblotting, at indicated time points. The 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.

(GATA3), sterol regulatory element-binding protein-1 (SREBP-1), cAMP response element-binding protein (CREBP), and CCAAT-enhancer-binding protein (C/EBP) are present in the 5-upstream region of the *FSP27* gene, recent studies have demonstrated that expression of *FSP27* in adipocytes is directly and crucially regulated by PPARγ, a master transcription factor for the differentiation of adipocytes, which is expressed at its highest level in adipose tissue [34]. Indeed, *PPARγ* expression well correlates with that of *FSP27* in adipocytes [35]. It is also known that expression of *Perilipin* is controlled by PPARγ [36]. We previously showed that the progression of lipolysis in 3T3-L1 adipocytes in response to AIM was not accompanied by significant downregulation of *PPARγ* expression levels [9]. Hence, to test whether AIM influences functional activity of PPARγ in adipocytes, we first assessed whether the presence of rosiglitazone, a selective agonist of PPARγ, or T0070907, a selective PPARγ antagonist, influenced the lipolytic effect of AIM in 3T3-L1 adipocytes. As demonstrated in Fig. 2A and Supplementary Fig. S4, a set of parameters with remarkable involvement in AIM-induced lipolysis (*i.e.* increased glycerol efflux, downregulation of *FSP27* and *Perilipin* mRNA levels, and increased inflammatory gene expression) were inhibited by the presence of rosiglitazone in a dose-dependent fashion. In contrast, a synergistic effect of these lipolytic consequences was detected following the combination of rAIM and T0070907 (Fig. 2 and Supplementary Fig. S5B). To assess the effect of AIM on transcriptional activity of PPARγ more directly, we stably transfected 3T3-L1 cells with a luciferase reporter gene conjugated with a PPARγ-binding element (PPRE) at the 5′-end [34]. As shown in Fig. 2C (left panel), luciferase activity was upregulated when cells differentiated in response to insulin, dexamethasone (DEX), and isobutylmethylxanthine (IBMX), and this response was markedly enhanced by the presence of rosiglitazone. The challenge of cells with rAIM significantly decreased the luciferase activity in a dose dependent fashion, at a comparable level to that of T0070907 (Fig. 2C, right panel). In addition, the luciferase activity induced by rosiglitazone was significantly suppressed by AIM (Fig. 2D). Together, these results suggest that treatment of adipocytes with AIM reduces PPARγ activity, resulting in downregulation of mRNA levels for coating proteins, leading to lipolysis.

Furthermore, to determine whether AIM enhances the binding efficiency of PPARγ to its binding site, a chromatin immuno-precipitation (ChIP) was carried out. A chromatin fraction was isolated from 3T3-L1 adipocytes incubated in the presence of rAIM or rosiglitazone, digested with DNaseI, then immune-precipitated using a PPARγ antibody. DNA purified from the precipitates was PCR-amplified to detect the presence of PPRE1 or PPRE2 sequences within the 5′ sequence of FSP27 gene (illustrated in Supplementary Fig. S6). No difference in PCR signal level was detected following treatment of cells with rAIM, suggesting that AIM does not suppress recruitment of PPARγ to DNA, but may modulate PPARγ activity by acting as a co-receptor in FSP27 gene (Supplementary Fig. S6).

3.3. Reduction of endogenous fatty acid synthesis decreases PPARγ activity

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

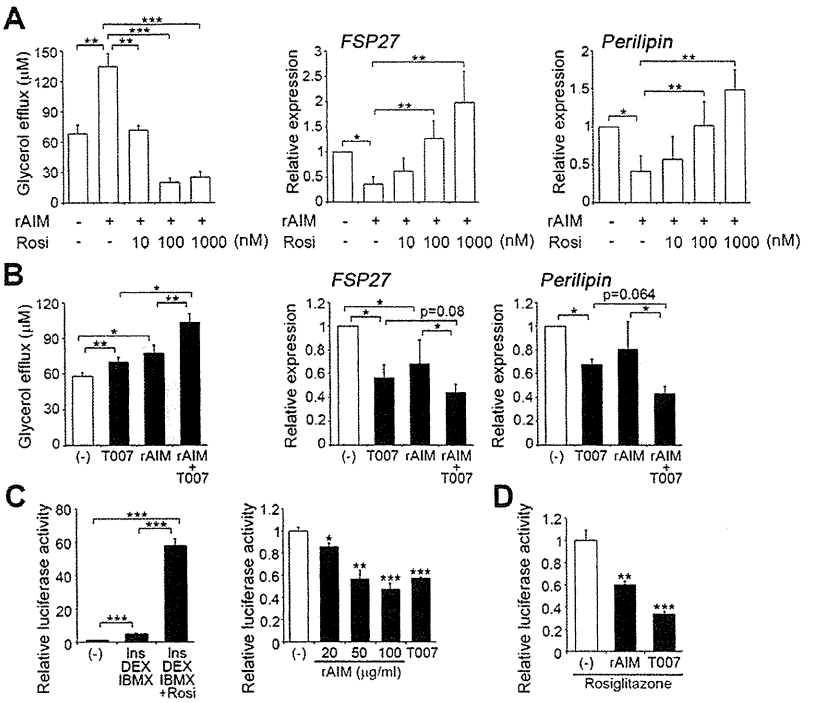

Fig. 2. AIM decreases transcriptional activity of PPARγ. Glycerol efflux, mRNA levels of *FSP27*, and *Perilipin* were assessed in 3T3-L1 adipocytes treated with rAIM (100 μg/ml), and indicated concentration of rosiglitazone (A) or T0070907 (B). $n = 3$ for each. (C) (Left panel) 3T3-L1 cells stably 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.

(presented in Fig. 2C). As demonstrated in Fig. 3A, like rAIM, the FAS inhibitor cerulenin [40] or abrogation of *FAS* expression by siRNA significantly diminished luciferase activity. It therefore appears that cerulenin or FAS siRNA established conditions similar to AIM-induced lipolysis in 3T3-L1 adipocytes. In agreement, cerulenin or the siRNA provided lipolytic consequences in a comparable manner to that of AIM, including increase in efflux of glycerol (Fig. 3B), downregulation of mRNA for *FSP27* and *Perilipin*, and increases in expression of inflammatory genes (Fig. 3C and Supplementary Fig. S7).

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

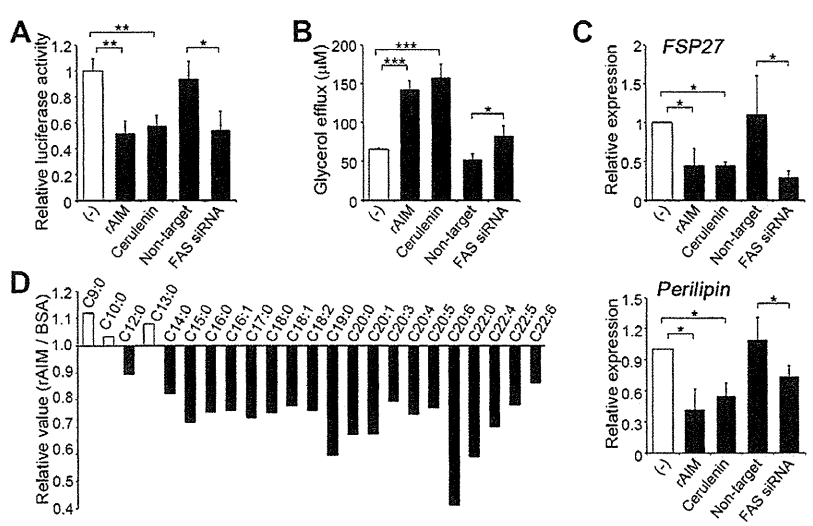

Fig. 3. AIM reduces endogenous fatty acid synthesis and PPARγ activity. (A) Luciferase activity in the reporter 3T3-L1 adipocytes 24 h after the challenge with rAIM (100 μg/ml), cerulenin (10 μM), or no stimulants (control), or transfection of siRNA for *FAS* or non-target sequence. $n = 3$ for each. (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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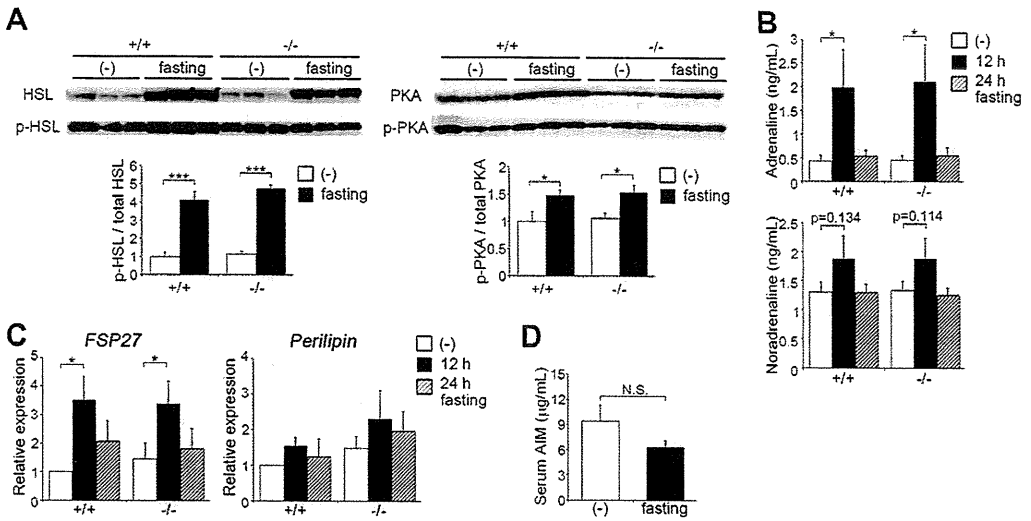

Fig. 4. Comparative hormone-dependent lipolysis in *AIM* $^{-/-}$ and *AIM*$^{+/+}$ mice. (A) Immunoblotting for HSL and PKA (phosphorylated and total) in epididymal fat at 12 h. Data are shown as relative phosphorylative state to that in *AIM*$^{+/+}$ mice without fasting. $n = 3$ for each. (B) Blood 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

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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.

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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

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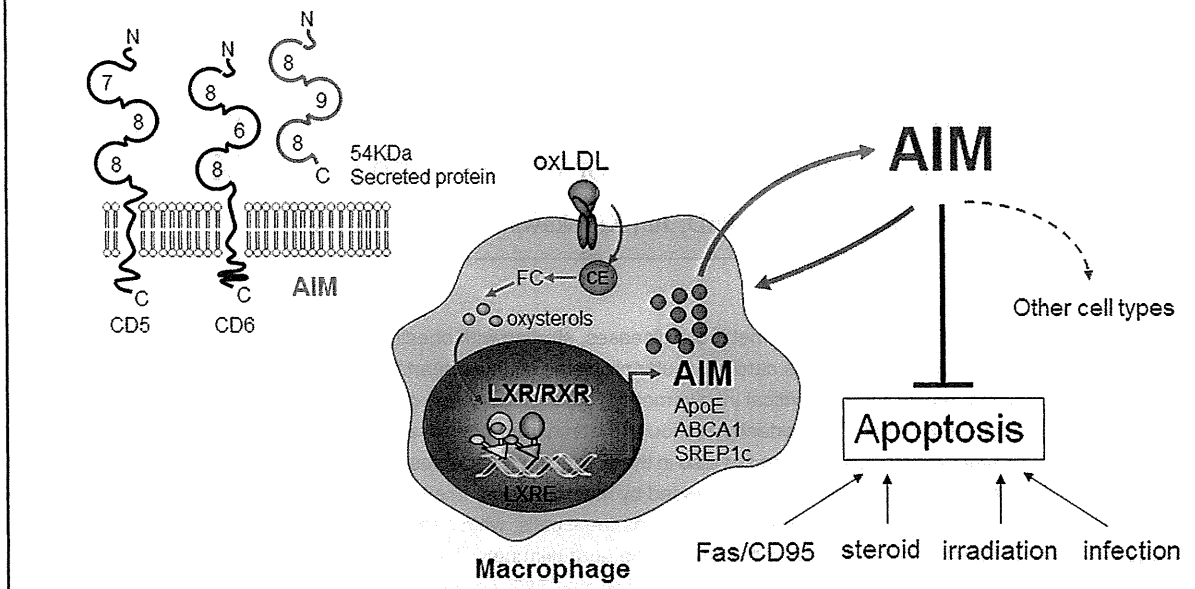

Figure 1. Expression regulation and an apoptosis inhibitory function of apoptosis inhibitor of macrophages (AIM). ApoE, apolipoprotein E; Fas, fatty acid synthase; oxLDL, oxidized low-density lipoprotein; LXR, liver X receptors; RXR, retinoid X receptors.

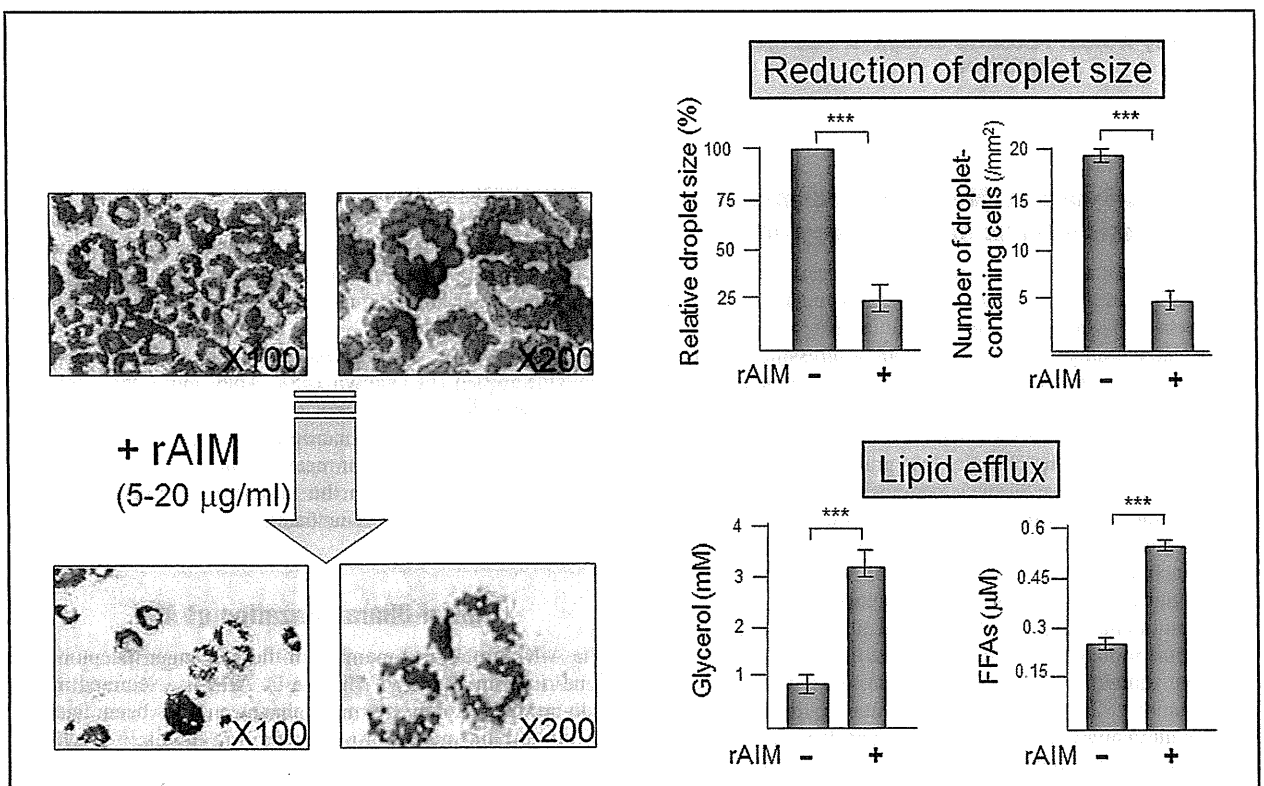

Figure 2. Apoptosis inhibitor of macrophages (AIM) induces lipolysis in adipocytes. FFA, free fatty acid; rAIM, recombinant AIM.

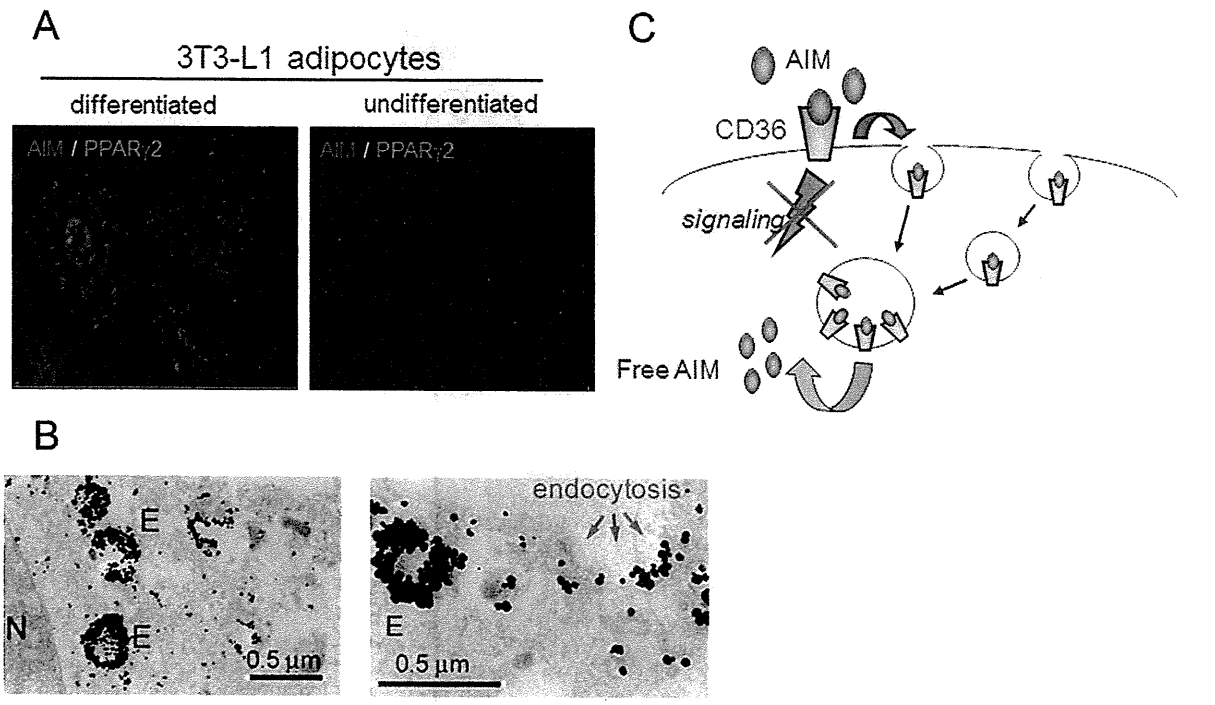

Figure 3. (A–C) Apoptosis inhibitor of macrophages (AIM) is incorporated into adipocytes through endocytosis mediated by CD36. (A) Mature adipocytes strongly positive for PPARγ2 (green signals) efficiently incorporated rAIM (red signals) (**Left**), whereas preadipocytes not stimulated by insulin, DEX, 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.

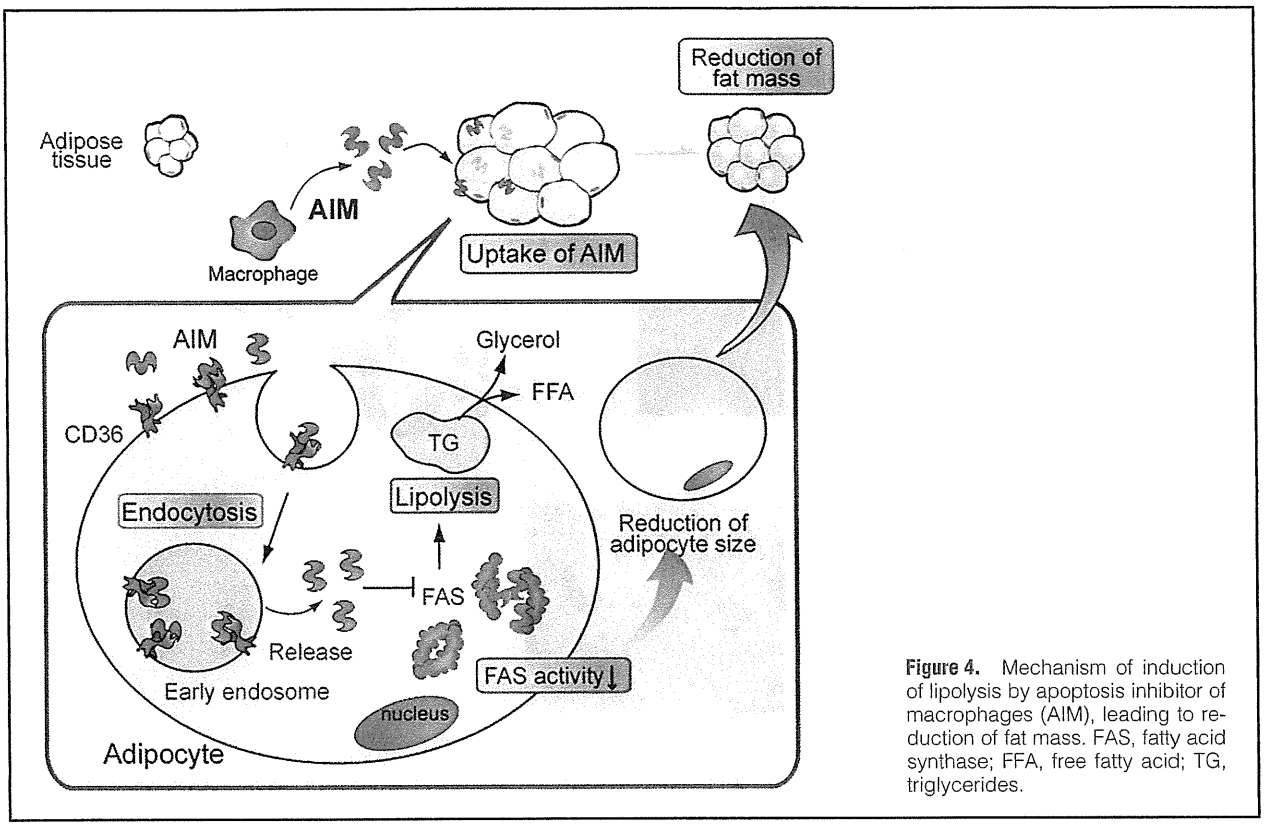

Figure 4. Mechanism of induction of lipolysis by apoptosis inhibitor of macrophages (AIM), leading to reduction of fat mass. FAS, fatty acid synthase; FFA, free fatty acid; TG, triglycerides.

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 negative 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.

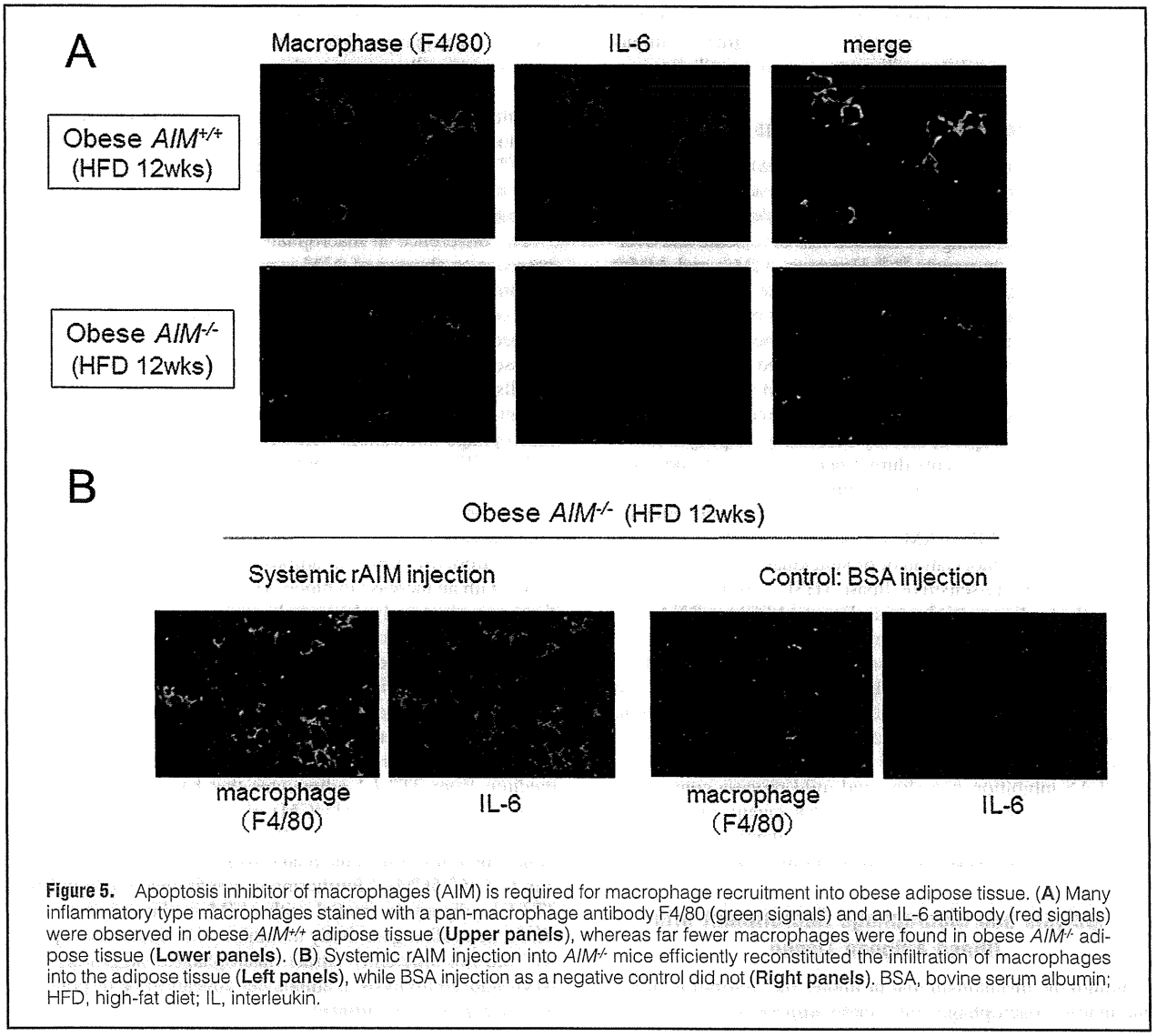

Figure 5. Apoptosis inhibitor of macrophages (AIM) is required for macrophage recruitment into obese adipose tissue. (**A**) Many inflammatory type macrophages stained with a pan-macrophage antibody F4/80 (green signals) and an IL-6 antibody (red signals) were observed in obese *AIM*$^{+/+}$ adipose 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 stabilization 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] Interestingly, 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.