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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

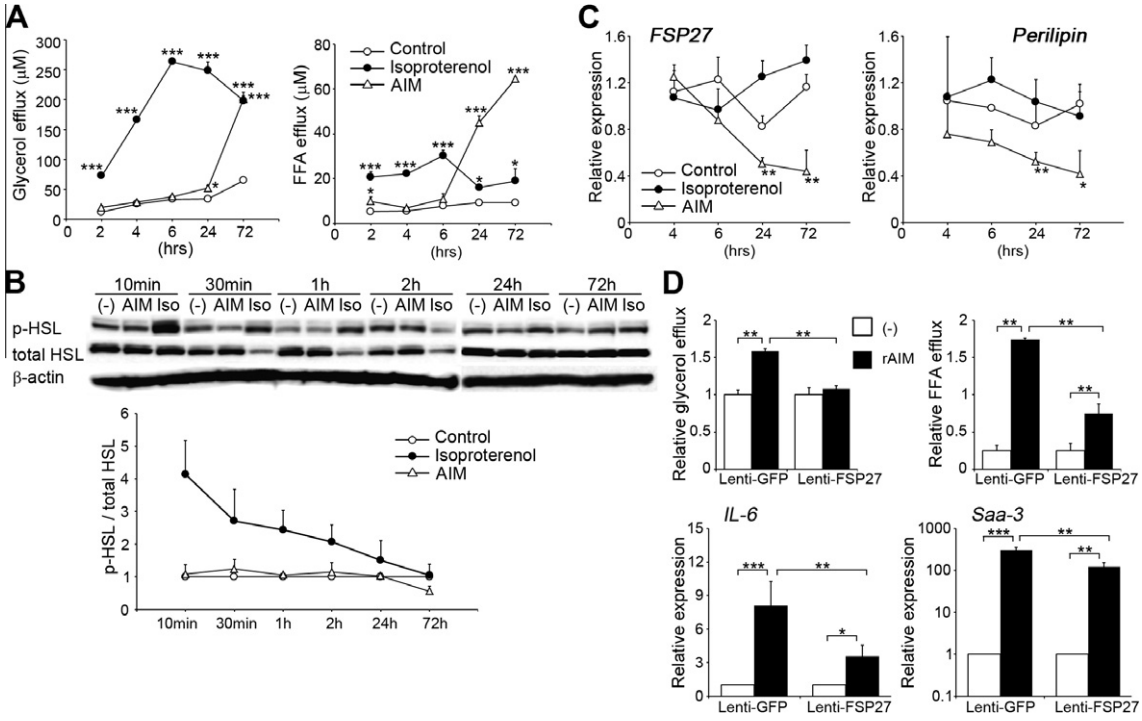

Fig. 1. AIM-induced lipolysis is distinctive from hormone-dependent lipolysis. 3T3-L1 adipocytes were treated with rAIM (100 μg/ml) or isoproterenol (10 μM), and analysed for (A) the efflux of glycerol and FFAs, (B) HSL (phosphorylated and total) levels by immunoblotting, at indicated time points. The 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. S5). 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 PPARΓγ 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

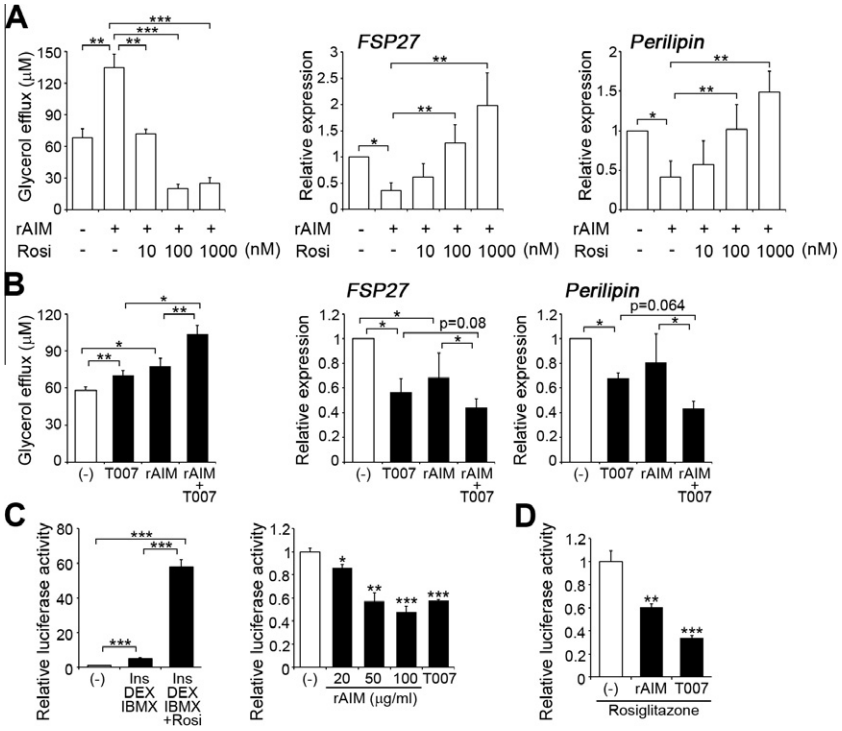

Fig. 2. AIM decreases transcriptional activity of PPARγ. Glycerol efflux, mRNA levels of *FSP27*, and *Perilipin* were assessed in 3T3-L1 adipocytes treated with rAIM (100 μg/ml), and indicated concentration of rosiglitazone (A) or T0070907 (B). $n = 3$ for each. (C) (Left panel) 3T3-L1 cells stably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

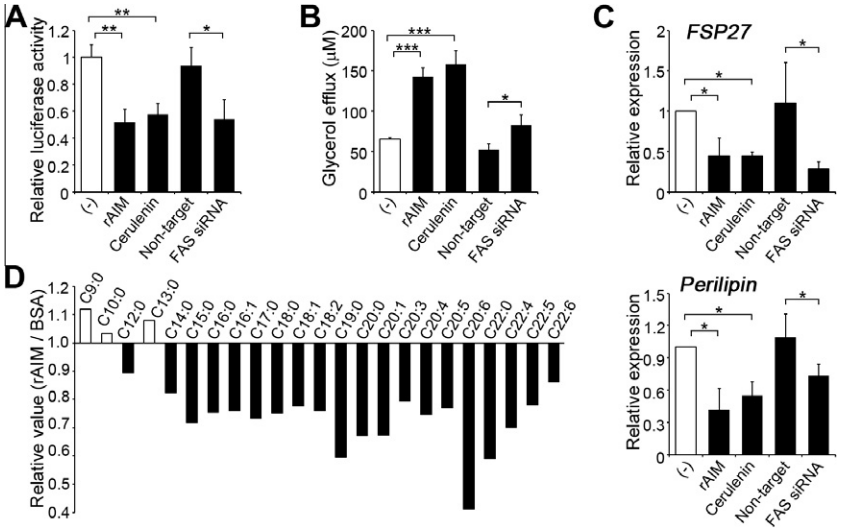

Fig. 3. AIM reduces endogenous fatty acid synthesis and PPARγ activity. (A) Luciferase activity in the reporter 3T3-L1 adipocytes 24 h after the challenge with rAIM (100 μg/ml), cerulenin (10 μM), or no stimulants (control), or transfection of siRNA for *FAS* or non-target sequence. $n = 3$ for each.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

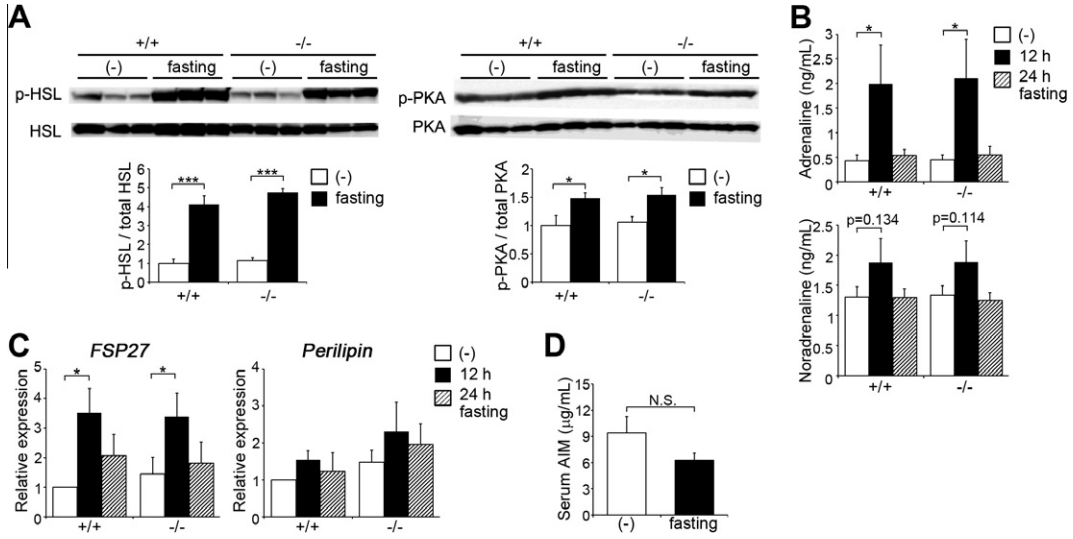

Fig. 4. Comparative hormone-dependent lipolysis in $AIM^{-/-}$ and $AIM^{+/+}$ mice. (A) Immunoblotting for HSL and PKA (phosphorylated and total) in epididymal fat at 12 h. Data are shown as relative phosphorylative state to that in $AIM^{+/+}$ mice without fasting. $n = 3$ for each. (B) Blood catecholamines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

metabolomics analysis using 3T3-L1 adipocytes treated with rAIM for 24 h (Supplementary 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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