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Monoalkylglycerol ether lipids promote adipogenesis

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Abstract

The molecular mechanisms that lead to the generation of adipose tissue (adipogenesis) are of basic and biomedical interest. Cellular models of adipogenesis have proven extremely valuable in defining biomolecules—primarily genes and proteins—that regulate adipogenesis. Here, the analysis of differentiating adipocytes using an untargeted metabolomics approach led to the discovery of the monoalkylglycerol ethers (MAGEs) as a natural class of adipocyte differentiation factors.

Adipogenesis is the process by which undifferentiated fibroblasts undergo morphological changes and accumulate lipid droplets as they become adipose tissue.[1] In an effort to control adipose levels *in vivo* for the treatment of obesity and diabetes, researchers have identified several genes that regulate adipogenesis.[1–8] The 3T3-L1 cell line[1,9,10] has been particularly valuable in studying the molecular mechanisms underlying adipogenesis. The conversion of 3T3-L1 cells into adipocytes occurs through the temporal expression of genes over 6–8 days, and can be initiated *in vitro* by the addition of a hormone cocktail (commonly referred to as DMI) consisting of dexamethasone, 3-isobutyl-1-methylxanthine, and insulin.[1] Importantly, transcription factors such as the nuclear receptors peroxisome proliferator-activated receptor-γ (PPARγ)[5] and COUP transcription factor 2 (COUP-TFII),[4] have been shown to regulate both the differentiation of 3T3-L1 cells *in vitro* and the development of adipose tissue *in vivo*, suggesting the relevance of this cell-line model in the discovery of physiologically relevant adipogenesis pathways.

The observation that adipogenesis can be regulated by nuclear receptors, which in turn are often regulated by endogenous lipids,[11] suggests that lipid metabolism may play an essential role during differentiation. Indeed, previous studies have described an unidentified lipid that is a PPARγ agonist and that is produced during the early stages of adipogenesis (days 1 and 2).[2,12] Moreover, enzymes involved in fatty acid metabolism, including stearoyl CoA desaturase 2 (SCD2),[3] phospholipases,[8] and fatty acid CoA oxidase (FACO),[7] have also been characterized as regulators of adipogenesis. Notably, FACO was identified through a

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Supporting Information Available: Experimental procedures, chemical characterization, full table of targeted ions, cellular production experiments, PPARg binding/activation assays, and the full reference 19 citation are included in the supporting information. This material is available free of charge via the internet at http://pubs.acs.org.

shotgun lipidomics approach, which revealed the emergence of odd chain fatty acids during adipogenesis.[13] These examples support the idea that changes in lipid metabolism, and perhaps lipid signaling, are contributing to the transition of 3T3-L1 cells from preadipocytes to adipocytes. In the context of differentiating embryonic stem cells (ESCs), a metabolomics approach discovered a broad decrease in the levels of unsaturated metabolites in mature neurons and cardiomyocytes, which pointed to the regulation of stem cell redox status during differentiation.[14] In this study we used untargeted metabolomics of the 3T3-L1 cell line (not primary cells) to measure changes in the cellular lipids during adipogenesis. Our approach led to the discovery of the monoalkylglycerol ethers (MAGEs) as a new class of natural adipocyte differentiation promoters.

Lipid extracts from 3T3-L1 cells were analyzed by liquid chromatography-mass spectrometry (LC-MS) and the resulting data were aligned and compared by XCMS[15] to identify changing ions between sets of samples (Figure 1a). We compared 3T3-L1 cells at day 0 and day 2 of the differentiation program due to prior work that demonstrated the presence of a lipophilic PPARγ agonist in day 2 media.[12] A plot of ions that change in abundance during the first two days of differentiation, demonstrates that more ion species were found to be downregulated than upregulated in both positive and negative mode. In this study we focused on a cluster of ions corresponding to a lipid class whose levels were strongly elevated at day 2 of adipogenesis (Figure 1b).

The unique molecular formulae of these elevated ions (e.g., $C_{21}H_{43}O_3^+$ for m/z 343.3207) suggested that these molecules were part of the MAGE lipid class (Figure 2a). This structural assignment was confirmed by comparison of the monooleylglycerol ether isomer (C18:1 MAGE) with a corresponding [^{13}C]-labeled authentic standard in co-elution experiments (Figure 2b). In addition to the MAGEs, monoalkylglycerol vinyl ethers (MAGVEs; e.g., 1-(octadec-1-enyl)glycerol) were elevated (~8-fold) at day 2 but were found to be an order of magnitude lower in abundance (**SI–2**). Lastly, comparison of changes in MAGE levels to other known lipid classes demonstrate that the MAGEs are strongly elevated at day 2, while a majority of the other major lipid classes change in abundance only modestly or not at all (**SI–4**).

Next we determined whether elevated MAGE levels were due to differences in cellular metabolism or were simply a consequence of increased MAGE uptake from the serum-containing media. Cells were cultured with or without DMI in media containing delipidated serum for two days (conditioned media). Consistent with the cellular measurements, we observed a 9-fold increase in the concentration of C18:1 MAGE levels in the conditioned media from DMI-treated cells compared to DMSO-treated control (**SI–6**). The higher MAGE levels from media obtained from differentiating cells imply a net efflux of MAGEs from 3T3-L1 cells into the media. In addition, incubation of 3T3-L1 cells with [^{13}C]-oleyl alcohol, a MAGE precursor, resulted in the production of ^{13}C18:1 MAGE (**SI–6**). Together these data indicate that 3T3-L1 cells are not obtaining MAGE from the media but instead are biosynthesizing and secreting MAGE during differentiation.

Isotope dilution mass spectrometry (IDMS),[16] using the ^{13}C18:1-MAGE standard, was then used to measure the temporal regulation of MAGE levels during adipogenesis. Quantitation

of MAGE levels in 3T3-L1 cells at days 0, 2, 4, and 8 revealed that these lipids are transiently regulated during differentiation (Figure 2c). This type of regulation is reminiscent of many of the key transcription factors involved in the differentiation program, which show transient expression patterns during adipogenesis.[17,18] For example, the transcription factors CCAAT-enhancer binding protein-δ (C/EBPδ) and C/EBPβ are regulated in a transient manner during adipogenesis,[17] as are the nuclear receptors nerve growth factor IB, vitamin D receptor, and COUP-TFII.[18] The finding that MAGE levels are also transiently elevated suggests that these lipids might be connected, directly or indirectly, to the differentiation program.

Given the transient increase in MAGE levels, we decided to investigate what effect, if any, the addition of a MAGE would have on adipogenesis in 3T3-L1 cells. In these experiments the DMI induction cocktail was supplemented with MAGE, MAG, or MAGVE (C18:1 isomers, 20 μM) and administered to 3T3-L1 preadipocytes. Cells treated with DMSO alone were used as a negative control and cells treated with pioglitazone, a PPARγ agonist, were used as a positive control. The addition of C18:1 MAGE to differentiating 3T3-L1 cells caused a significant increase in Oil Red O staining (Figure 3a) compared to the DMSO control. At the cellular level MAGE treatment results in a greater number of differentiated cells with a similar pattern to that seen in the pioglitazone-treated cells. Furthermore, C18:1 MAGE shows a dose dependence between 2–20 μM, as measured by Oil Red O staining (Figure 3b). Attempts to use higher MAGE concentrations (50 μM) lead to cell death, likely due to the ability of MAGE to act as a detergent at high concentrations (data not shown). We tested the ability of MAG and MAGVE to promote differentiation and, in contrast to the results from MAGE addition, we found no significant change in Oil Red O staining compared to the DMI-only control (Figure 3c). We also tested another member of the MAGE family, palmitylglycerol (C16:0 MAGE), in a similar manner and found that it promoted differentiation to a similar extent as treatment with oleylglycerol (**SI–7**). These differentiation experiments suggest that MAGEs are a natural class of adipocyte differentiation factors.

One challenge in these differentiation experiments is that MAGE addition might simply increase intracellular lipid concentrations, which would then lead to an increase in Oil Red O staining. However, we hypothesized that MAGE is regulating adipogenesis through a more complex mechanism because most lipid accumulation occurs after MAGE removal at day 4. To test this idea, we added 20 μM MAGE to differentiating 3T3-L1 cells for three different time periods: days 0–2, 2–4, and 0–4. If MAGE were simply contributing to the lipid content of 3T3-L1 cells, we would expect to see increased Oil Red O staining when the MAGE is added later days (i.e., days 2–4) compared to early time points (i.e., days 0–2); furthermore, the effect of day 0–4 treatment should be additive. We found that treatment of 3T3-L1 cells with MAGE between days 2–4 showed comparable Oil Red O staining to the DMI-only control, while treatment between days 0–2 and 0–4 to show a significant and nearly equivalent increase in Oil Red O staining (Figure 4a). These results suggest that MAGEs are doing more than simply contributing to the lipid pool and may be involved in the early regulation of adipogenesis.

These experiments prompted us to test whether MAGE-promoted adipogenesis was reflected in the induction of genes such as aP2[5] and adiponectin,[19] which are markers of terminal differentiation,[1] through a series of reverse transcription-PCR (RT-PCR) experiments. As in the previous experiment we added MAGE at the same time intervals and measured the expression of aP2 and adiponectin at day 4 using RT-PCR. Similar to the results from Oil Red O staining experiments, MAGE addition between days 2–4 had no impact on gene expression compared to DMI-only control, while addition of MAGE between days 0–2 and 0–4 caused a robust and significant increase in aP2 and adiponectin expression (Figure 4b). In summary, MAGE appears to be an endogenously occurring lipid that promotes adipogenesis and can induce the expression of key genes that are markers of terminal differentiation.

Since aP2 and adiponectin are targets of PPARγ we then asked whether MAGE induction of these genes occurs via direct ligand activation of PPARγ. First, we utilized a fluorescence polarization assay that measures displacement of a fluorogenic ligand from the PPARγ ligand binding domain (LBD) in the presence of candidate binding molecules, such as MAGE. We included pioglitazone as a positive control and MAG as a negative control in these experiments. Pioglitazone gives a robust decrease in fluorescence polarization corresponding to an IC_{50} of 1.5 μM consistent with published results. By contrast, an IC_{50} cannot be calculated for C18:1 MAGE and C18:1 MAG, administered at concentrations up to 200 μM, to indicate that these lipids do not bind to the PPARγ LBD in this assay (**SI–8**). We also tested the influence of MAGE on PPARγ gene expression in a HEK293T cell-based luciferase reporter assay. While pioglitazone strongly activates PPARγ we only observe weak activation with MAGE at 20 μM. However, no activation of PPARγ is detected with 5 μM MAGE, but this concentration still promotes differentiation of 3T3-L1 cells (**SI–8**). Therefore, since MAGE itself does not bind to the PPARγ LBD it is unlikely that direct activation of PPARγ is responsible for the adipogenesis promoting activity of MAGE.

Minimal concentrations of MAGE necessary to promote differentiation are 20–50-fold higher than the natural concentrations of this lipid during differentiation (100–250 nM MAGE in conditioned media compared to 5 uM MAGE to cause differentiation). These levels are consistent with the use of natural lipids as pharmacological reagents in other studies, which typically use these lipids at concentrations ~100-fold higher than their natural concentrations.[20,21] The elevated levels of exogenously added lipids needed to induce cellular effects may reflect difficulty in delivering lipophilic compounds that can aggregate or bind non-specifically to other proteins.

Taken together, these results have led to the discovery that MAGE lipids regulate the differentiation of 3T3-L1 cells from preadipocytes to adipocytes. Previous work has shown that MAGEs protect against leukopenia,[22] the production of white blood cells *in vivo*, which might suggest that these lipids can play a broader role in cellular differentiation. The MAGE pathway has also been linked to the invasiveness of cancer cells *in vitro* and tumor growth in a xenograft model.[23] Thus, this work adds to a growing list of disease relevant biological processes controlled by the MAGE lipids. Lastly, the discovery that MAGEs are adipogenesis promoters highlights the utility of untargeted metabolomics to define bioactive

metabolites and, more generally, demonstrates that the mammalian metabolome likely contains numerous metabolites whose roles require further annotation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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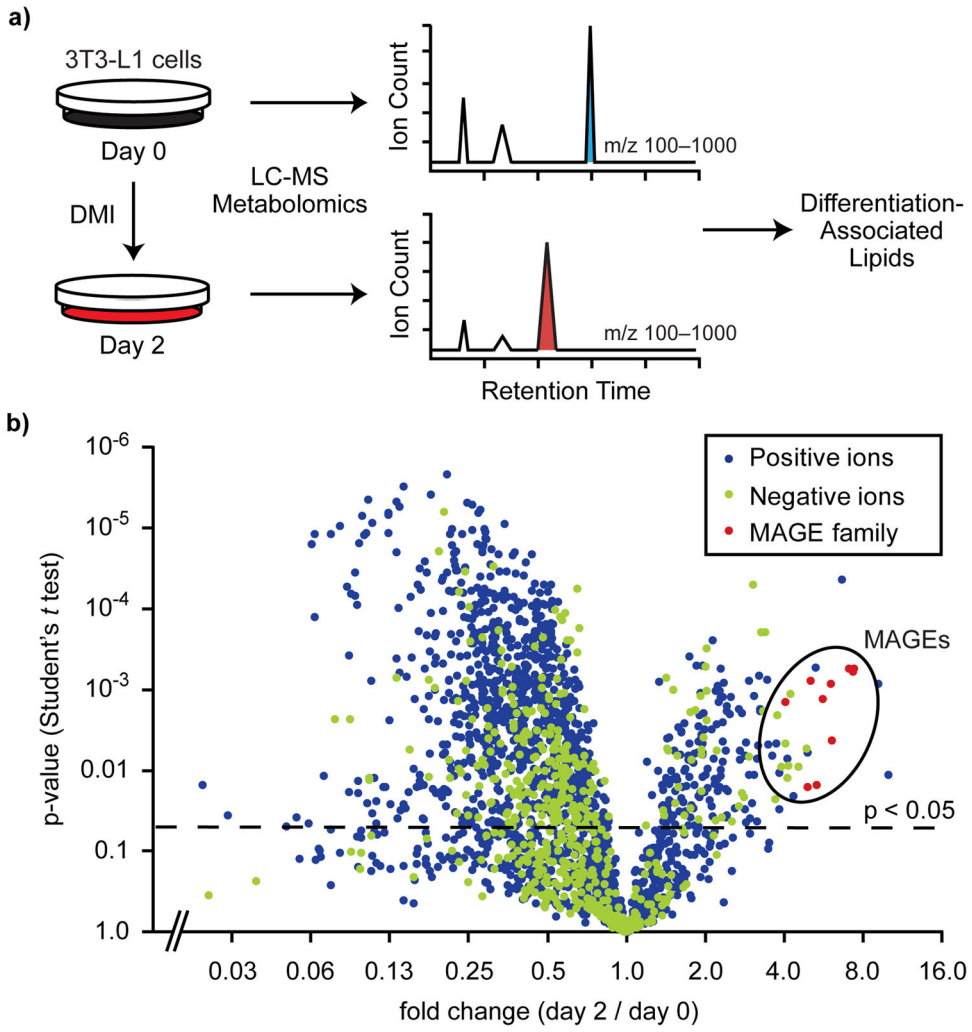

Figure 1.

Untargeted metabolomics of 3T3-L1 adipogenesis. a) Comparison of lipid profiles of 3T3-L1 preadipocytes (day 0) to cells at day 2 of differentiation by LC-MS-based metabolomics. b) Data is visualized by arranging individual metabolite ions along dimensions of biological and statistical significance. Both positive and negative mode MS data are shown, and ions corresponding to members of the MAGE family are highlighted in red.

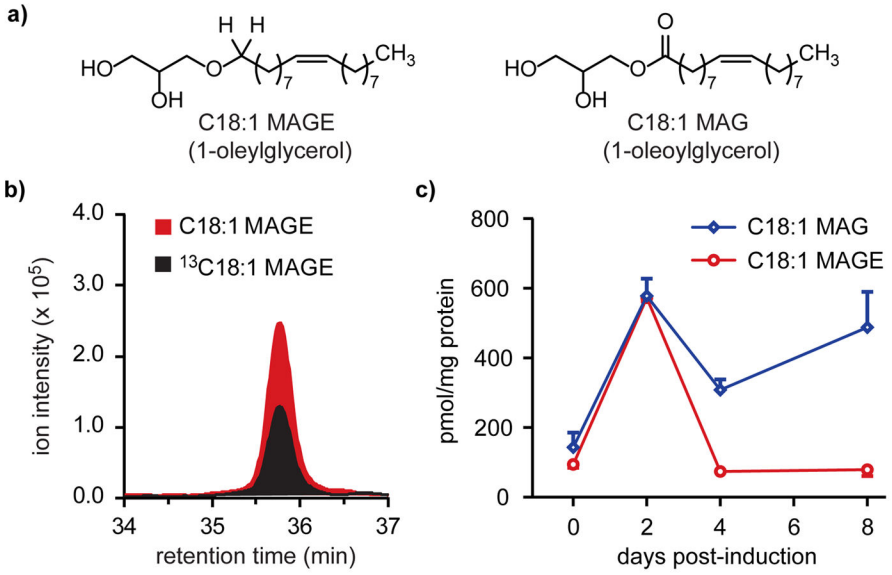

Figure 2.

a) Chemical structures of monooleylglycerol (C18:1 MAGE) and monooleoylglycerol (C18:1 MAG). b) Heavy-labeled C18:1 MAGE was synthesized from all-[^{13}C]-oleic acid and was then co-extracted with 3T3-L1 cells. c) Targeted monitoring of C18:1 MAGE and C18:1 MAG levels over the course of adipocyte differentiation by isotope dilution mass spectrometry (error bars for all graphs: ± s.d.; n = 3).

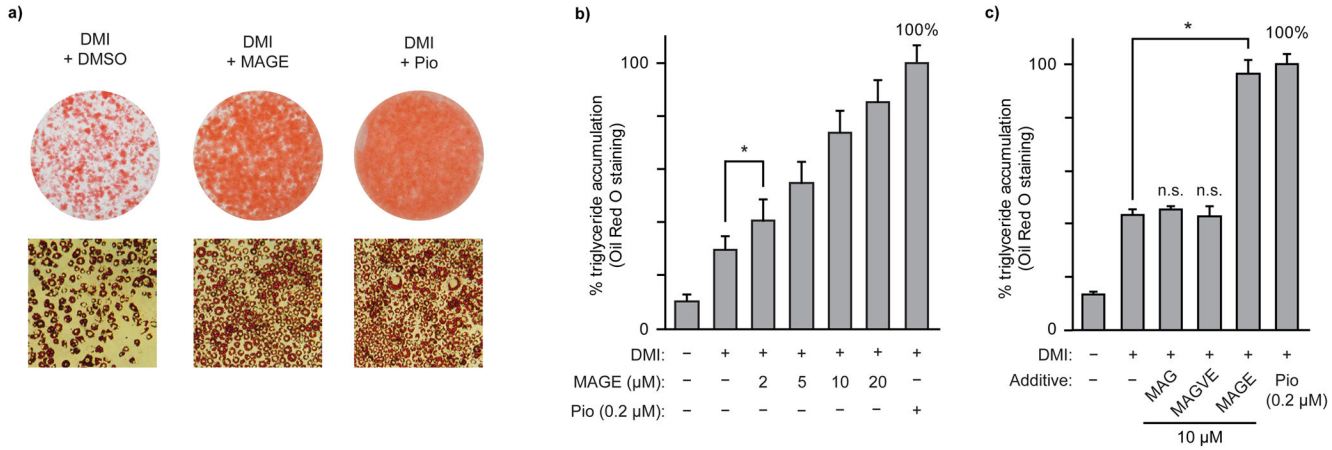

Figure 3.

Addition of MAGE to differentiating 3T3-L1 cells. a) Supplementation of DMI with C18:1 MAGE (20 μM) from day 0 to 4 in 3T3-L1 cells led to increased triglyceride accumulation and number of adipocytes, as assessed by Oil Red O staining of neutral lipid content 8 days post-induction. b) C18:1 MAGE was added to the DMI induction medium at a range of 2–20 μM for days 0 to 4. Cells were fixed and stained on day 7, and the absorbance of extracted Oil Red O stain was monitored at 510 nm (n = 8; *, $p < 0.01$, Student's *t* test). c) 1-Oleoylglycerol (MAG) or 1-(octadec-1-enyl)glycerol (MAGVE) was added to the DMI induction medium at 10 μM for days 0 to 2. Cells were fixed and stained on day 8, and absorbance of extracted Oil Red O stain was measured (n = 3; *, $p < 0.01$).

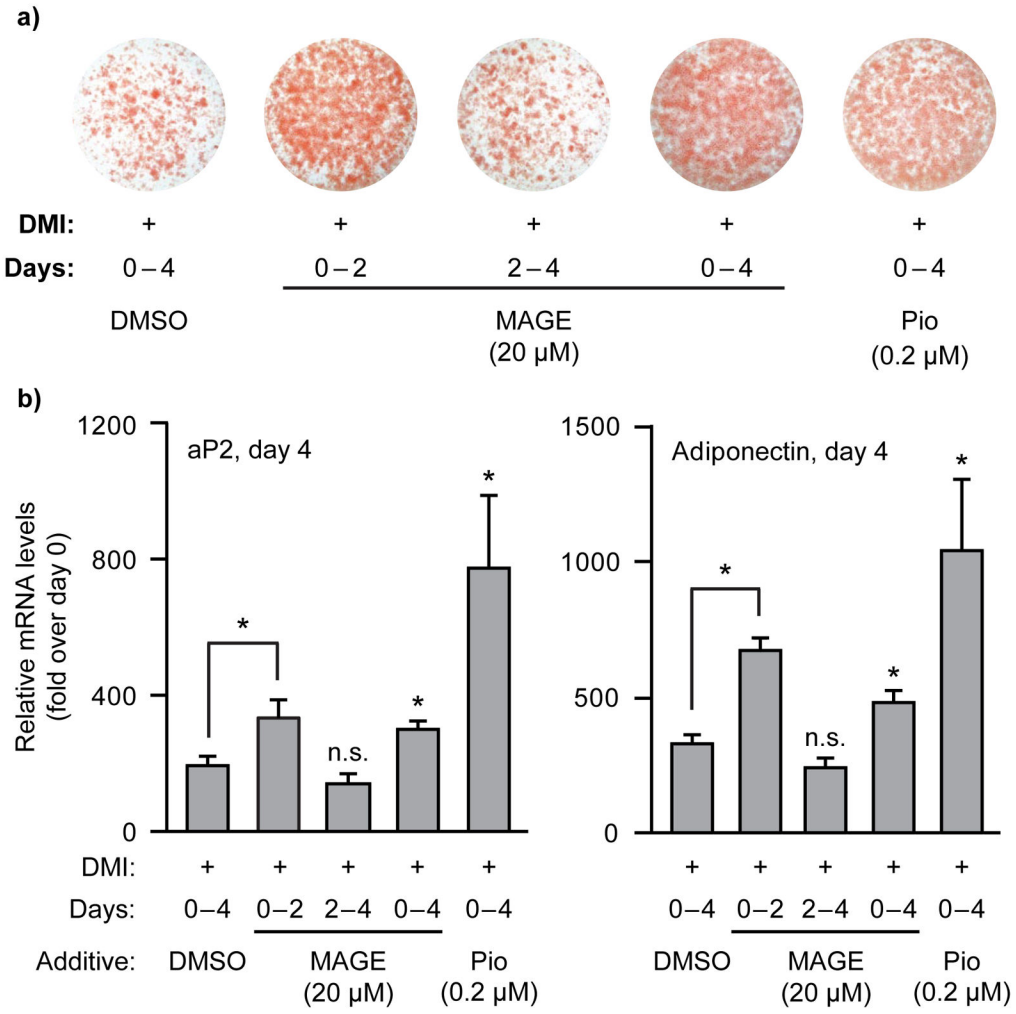

Figure 4.

Effect on gene expression of MAGE addition for different time periods. a) C18:1 MAGE (20 μM) was added to differentiating 3T3-L1 cells during different periods of the DMI treatment: days 0–2, 2–4, and 0–4. b) Relative expression levels of PPARγ target genes, adiponectin and aP2, assessed by RT-PCR using 36B4 as the reference gene. Relative mRNA levels were calculated with respect to the day 0 control using the comparative Ct method. Cells were harvested 96h after induction with DMI supplemented with C18:1 MAGE for the indicated times (n = 3; *, $p < 0.01$).