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Cinnamaldehyde induces fat cell-autonomous thermogenesis and metabolic reprogramming

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Abstract

Objective—Cinnamaldehyde (CA) is a food compound that has previously been observed to be protective against obesity and hyperglycemia in mouse models. In this study, we aimed to elucidate the mechanisms behind this protective effect by assessing the cell-autonomous response of primary adipocytes to CA treatment.

Methods—Primary murine adipocytes were treated with CA and thermogenic and metabolic responses were assessed after both acute and chronic treatments. Human adipose stem cells were differentiated and treated with CA to assess whether the CA-mediated signaling is conserved in humans.

Results—CA significantly activated PKA signaling, increased expression levels of thermogenic genes and induced phosphorylation of HSL and PLIN1 in murine primary adipocytes. Inhibition of PKA or p38 MAPK enzymatic activity markedly inhibited the CA-induced thermogenic response.

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Author Contributions

JJ, MPE, HJ, XQ, JL, and DK performed experiments. JJ, MPE, and JW analyzed data and wrote the manuscript. JW oversaw the study.

Conflict of Interest

The authors declare no conflict of interest.

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In addition, chronic CA treatment regulates metabolic reprogramming, which was partially diminished in FGF21KO adipocytes. Importantly, both acute and chronic effects of CA were observed in human adipose stem cells isolated from multiple donors of different ethnicities and ages and with a variety of body mass indexes (BMI).

Conclusions—CA activates thermogenic and metabolic responses in mouse and human primary subcutaneous adipocytes in a cell-autonomous manner, giving a mechanistic explanation for the anti-obesity effects of CA observed previously and further supporting its potential metabolic benefits on humans. Given the wide usage of cinnamon in the food industry, the notion that this popular food additive, instead of a drug, may activate thermogenesis, could ultimately lead to therapeutic strategies against obesity that are much better adhered to by participants.

Keywords

Cinnamaldehyde; Obesity; Thermogenesis; Subcutaneous adipocytes

1. Introduction¹

The global obesity epidemic calls for effective intervention and treatments. Adipocytes in the subcutaneous fat depots have been reported to provide metabolic benefits, including their capability to be recruited and activated by thermogenic stimuli and thereby potentially improve systemic energy homeostasis [1]. The classical pathway for the activation of thermogenesis in adipocytes is through the activation of PKA and downstream enzymes including p38 MAPK, which in turn leads to the induction of the transcription of key thermogenic genes [2].

Previous studies have shown that cinnamaldehyde (CA), an essential oil found in cinnamon, can reduce diet-induced weight gain and improve glucose homeostasis when given to mice as a supplement in food [3, 4]. Interestingly, cinnamon consumption has also been reported to correlate with lower levels of fasting blood glucose in human studies [5]. However, the molecular mechanisms behind these effects have not yet been elucidated. Here, we show that CA directly acts on subcutaneous adipocytes to activate thermogenesis and PKA signaling and that chronic CA treatment induces thermogenic and metabolic reprogramming in these cells. Importantly, the beneficial effects of CA treatment that we observe in murine adipocytes are conserved in human subcutaneous fat cells, suggesting that CA may potentially be used to counteract obesity and improve systemic metabolism in humans.

2. Materials and Methods

2.1. Cell Culture

Primary preadipocytes were isolated from the inguinal fat depot of mice and differentiated as previously described [6]. Human adipose-derived stem cells (hASCs) were isolated from lipoaspirates from the subcutaneous adipose tissue of donors undergoing voluntary surgery (a generous gift from Dr. Jeffrey M. Gimble, Tulane University, New Orleans, Louisiana, USA).

¹Abbreviations: CA: Cinnamaldehyde, hASC: human adipose stem cell, BMI: Body mass index, ROS: Reactive oxygen species

Donor demographics are included in Supplemental Table 1. All specimens were collected and handled under the protocols reviewed and approved by the Western Institutional Review Board (Puyallup, WA) and the University of Michigan Medical School Institutional Review Board (IRBMED). For further isolation, culture, and differentiation information, see the supplemental materials and methods.

2.2. RNA Isolation and RT-qPCR Analysis

RNA was extracted from cells and tissue using TRI Reagent (Sigma, Saint Louis, MO, USA) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA using M-MLV (Invitrogen, Carlsbad, CA, USA) and qPCR was performed using SYBR Green (Applied Biosystems, Warrington, UK) on a 7900HT Fast Real-Time PCR system (Applied Biosystems). Fold change was determined using the $\Delta\Delta C_t$ method with samples normalized to the reference gene *36B4* (*RPLP0*). A list of qPCR primers used in this study is included in Supplemental Table 2.

2.3. Western Blot Analysis

Cells were lysed with RIPA buffer supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) and phosphatase inhibitors. Protein extracts were run on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Blots were exposed using ECL (Amersham, Pittsburgh, PA, USA). For antibody information, see the supplemental materials and methods.

2.4. Imaging

Oil red O staining was described in the supplemental materials and methods. For immunofluorescence, primary cells were seeded onto collagen-coated coverslips and differentiated as described above. Differentiated cells were probed using p-PLIN1 Ser517 antibody (Vala Sciences, San Diego, CA, USA), anti-rabbit Alexa 594 antibody (Abcam, Cambridge, MA, USA), and BODIPY (Invitrogen), and imaged using an Olympus FV300 confocal laser-scanning microscope (Melville, NY, USA).

2.5. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6. Data are expressed as mean $\pm$ SEM. Statistical significance was determined using an unpaired two-tailed Student's t-test for two-group comparisons ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). A one-way analysis of variance (ANOVA) was applied for multiple group comparisons involving one independent variable. The data presented is representative of 2–4 independent experiments.

3. Results

3.1. CA activates thermogenesis through PKA signaling in mouse subcutaneous fat cells

While the beneficial effects of CA on systemic metabolism have recently been observed [3, 4, 7], the underlying molecular basis of these effects remains largely unknown. To explore the possibility that CA influences whole body metabolism through direct action on adipocytes, we treated fully differentiated adipocytes isolated from the murine inguinal

subcutaneous fat depot with CA. Subcutaneous fat plays a unique role in maintaining energy homeostasis, demonstrating profound “browning” upon cold exposure and mediating protective metabolic functions during obesity [8]. Acute CA treatments induce expression of thermogenic markers including *Fgf21* and *Ucp1* without altering expression of general adipogenic markers such as *Pparg* and *Adipoq* in these primary subcutaneous adipocytes (Fig. 1A, Supplemental Fig. 1 and 2A). The induction of thermogenesis in response to acute stimuli, such as treatment with β -adrenergic agonist isoproterenol (Iso), is mainly mediated through the activation of PKA and subsequent phosphorylation of p38 MAPK [2]. Indeed, we observed a robust phosphorylation of PKA substrates and p38 MAPK by CA at a comparable level to Iso in inguinal adipocytes (Fig. 1B). In addition to p38, PKA dependent phosphorylation of hormone-sensitive lipase (HSL) and lipid droplet-associated protein perilipin 1 (PLIN1) are also activated by CA in inguinal adipocytes (Fig. 1B and C). CA has been reported to be an agonist for transient receptor potential cation channel, member A1 (TRPA1) [4]. Emerging evidence suggests that signaling through the transient receptor potential (TRP) channels may cell-autonomously mediate adipocyte function [6, 9]. Interestingly, this CA-induced thermogenic response is TRPA1-independent since a similar response was observed in TRPA1KO inguinal adipocytes (Fig 1D and E, Supplemental Fig. 3).

Phosphorylation of PKA substrates, p38 MAPK, HSL and PLIN1 by CA treatment were significantly blocked by PKA inhibitor H-89 in primary inguinal adipocytes (Fig. 1F). Consistently, the induction of *Fgf21* and *Ucp1* by CA in inguinal adipocytes was substantially suppressed by H-89 and partially reduced by p38 MAPK inhibitor SB203580 (Fig. 1G and H), suggesting that CA-stimulated thermogenesis is dependent on PKA/p38 MAPK signaling. These results were confirmed in immortalized C3H-10T1/2 cells, indicating that the observed response to CA in primary inguinal fat is adipocyte cell-autonomous (Supplemental Fig. 4). Taken together, these data revealed that CA directly stimulates a thermogenic response and PKA activation in murine subcutaneous fat cells.

3.2. CA activates thermogenesis and promotes metabolic reprogramming in human subcutaneous fat cells

It has been reported that in addition to the supraclavicular area, thermogenic adipocytes also exist in human subcutaneous fat depots [8, 10]. We observed a clear activation of the thermogenic response and PKA signaling in differentiated hASCs isolated from subcutaneous fat depots (Fig. 2A–F, Supplemental Fig. 2B). This phenomenon is consistently observed across multiple human donors of different ethnicities and ages and with varied BMI (Supplemental Table 1 and Supplemental Fig. 5). Previous studies have shown that long-term CA supplementation in food can protect mice from diet-induced obesity and hyperglycemia [3, 7]. *In vitro*, upon chronic treatment with CA, *Fgf21* and *Ucp1* were significantly upregulated in both murine inguinal adipocytes and human subcutaneous fat cultures (Supplemental Fig. 6 and Fig. 2J, L, and M). Thermogenic genes (*Cidea*, *Cyts*, *Tfam*) and genes encoding key enzymes in the citric acid cycle (*Idh3a*, *Cs*) were upregulated after chronic exposure to CA. Cumulative evidence supports that many aspects of lipid metabolism can be altered in response to cold exposure or β -adrenergic agonist treatment [11, 12]. We observed that chronic CA treatment upregulates the expression of lipolytic

genes (*Hsl*, *Atgl*), key regulators in fatty acid oxidation (*Cpt1b*, *Ppara*), lipogenic genes (*Gyck*, *Dgat1*), and *Pdk4*, an enzyme that enhances lipid metabolism and has recently been reported to be responsible for major metabolic adaptations during white-to-beige conversion of human adipocytes (Fig. 2J, L, and M) [13]. Increased oxidative metabolism in mitochondria is known to be accompanied by increased reactive oxygen species (ROS) production. Chronic CA treatment also upregulated the expression of ROS-detoxifying enzymes including *Sod1* and *Sod2*, which may protect activated thermogenic adipocytes from oxidative damage (Fig. 2J, L, and M). Murine brown adipocytes additionally showed a thermogenic response to both acute and chronic CA treatment, including an increase in oxygen consumption in cells treated with chronic CA (Supplemental figure 7).

Acute CA treatment led to considerable induction of *Fgf21*, an important metabolic regulator which can be secreted by adipocytes and function in an autocrine or paracrine manner to induce thermogenesis and affect metabolic pathways [14]. In wild type (WT) inguinal adipocytes, CA treatment induced a clear elevation of *Egr1* and *cFos*, known FGF21 target genes (Fig. 2G) [15]. This induction was completely abolished in adipocytes isolated from FGF21KO mice (Fig. 2H, Supplemental Fig. 8), suggesting that FGF21-dependent signaling is activated by CA. The FGF21 knockout did not affect the PKA-mediated acute response to CA treatment in inguinal adipocytes (Fig. 2I). However, metabolic reprogramming upon chronic CA treatment is blunted in the absence of FGF21, suggesting that FGF21 may contribute to certain aspects of the long-term effects of CA on adipocytes through autocrine or paracrine pathways (Fig. 2K).

4. Discussion

Thermogenic adipocytes in subcutaneous fat depots are highly recruitable under certain conditions such as cold exposure [1]. These cells play an important role in maintaining energy homeostasis and improving overall metabolic health [16]. Herein, we show that CA treatment activates thermogenic and metabolic responses in human subcutaneous fat cells derived from multiple donors (Fig. 2 and Supplemental Fig. 5). Recent studies demonstrate that lipolysis and lipogenesis are coupled in adipose tissue during chronic β 3-adrenergic receptor stimulation [12]. In cold-exposed mice, genes involved in lipid catabolism and anabolism are both upregulated [11]. Cold-stimulated thermogenic fat activation in humans triggers triglyceride-free fatty acid cycling accompanied with altered metabolic gene profiles [17]. Consistent with these observations, we found that chronic CA treatment leads to a futile metabolic cycle in subcutaneous fat cells which may constitute the key mechanism through which CA consumption leads to metabolic protection. Notably, a recent clinical trial with healthy subjects suggests that acute CA ingestion increases energy expenditure and postprandial fat oxidation, in which the CA dose is judged as “sensorially acceptable” by participants [18]. A pharmacokinetic study revealed that circulating levels of CA were detectable 20 h after oral delivery, indicating that consumption of cinnamon may be a feasible way to activate thermogenesis in subcutaneous fat and ultimately protect against obesity and metabolic disorders [19].

This study investigated the thermogenic and metabolic actions of CA on adipocytes and the mechanisms by which they take place. We found that CA acts through a PKA/p38 MAPK-

dependent pathway. Consistently, the phosphorylation of lipolysis- and thermogenesis-related proteins was diminished by treatment with the PKA inhibitor H-89, and thermogenic gene expression was attenuated by treatment with the p38 inhibitor SB203580. We also found that some of the chronic effects of CA are mediated by FGF21, an adipokine that is known to activate thermogenesis in adipocytes [14].

In summary, this study provides evidence that CA induces thermogenesis as well as chronic metabolic remodeling in a fat cell-autonomous manner. Particularly, the thermogenic and metabolic responses of human subcutaneous adipocytes to CA suggest that this may be a promising therapeutic target for obesity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Acute CA treatment upregulates thermogenic gene expression and activates PKA signaling in murine subcutaneous fat cells. **(A)** Real-time qPCR analyses of thermogenic genes (*Fgf21* and *Ucp1*) and adipogenic markers (*Pparg* and *Adipoq*) in differentiated murine inguinal adipocytes (n=4) after 400 μ M CA treatment for 4 h. It has been reported that CA treatment in 3T3-L1 preadipocytes negatively influences adipogenesis [7]. We observed similar levels of adipogenesis and equal pan-fat marker expression (*Pparg* and *Adipoq*) in control and CA treated groups. **(B)** Representative immunoblots of PKA substrate phosphorylation, p38 MAPK phosphorylation, HSL phosphorylation (Ser660) and PLIN1 phosphorylation (Ser517) in differentiated inguinal adipocytes exposed to 400 μ M CA for the indicated time or 10 μ M Iso for 10 min as a positive control. **(C)** Representative immunofluorescence staining of PLIN1 phosphorylation (red) in differentiated inguinal adipocytes treated with 400 μ M CA for 1 h or 10 μ M Iso for 1 h as a positive control. BODIPY was used to stain lipid droplets (green). Scale bar = 20 μ m. **(D)** Real-time qPCR analyses of thermogenic genes (*Fgf21* and *Ucp1*) and adipogenic markers (*Pparg* and *Adipoq*) in differentiated WT or TRPA1KO inguinal adipocytes following vehicle control or 400 μ M CA treatment for 4 h (n=6). **(E)** Representative immunoblots of p38 MAPK phosphorylation, HSL phosphorylation (Ser660) and PLIN1 phosphorylation (Ser517) in differentiated TRPA1KO inguinal adipocytes treated with 400 μ M CA for the indicated time or 10 μ M Iso for 10 min as a control. **(F)** Representative immunoblots of phosphorylated PKA substrates,

phosphorylated p38 MAPK, phosphorylated HSL (Ser660) and phosphorylated PLIN1 (Ser517) in differentiated inguinal adipocytes treated with 50 μ M H-89 for 30 min and then 400 μ M CA for 1 h or 10 μ M Iso for 10 min as a positive control. (**G** and **H**) Real-time qPCR analyses of thermogenic markers (*Fgf21*, *Ucp1*) in differentiated inguinal adipocytes treated with 50 μ M H-89 (**G**, n=3) or 10 μ M SB203580 (SB, **H**, n=3) for 1 h and then 400 μ M CA for 4 h. All data in **A**, **D**, **G**, and **H** are presented as mean $\pm$ SEM. * p <0.05, ** p <0.01, *** p <0.001.

Fig. 2.

CA activates a thermogenic response and regulates lipid metabolic gene profiles in human subcutaneous fat cells. (A) Locations of the human subcutaneous fat biopsy sites used in this study and representative Oil Red O staining of differentiated hASCs. Scale bar = 50 μ m. (B) Real-time qPCR analyses of thermogenic genes (*FGF21* and *UCP1*) and adipogenic markers (*PPARG* and *ADIPOQ*) in differentiated hASCs after 200 μ M CA treatment for 4 h (n=4). (C and D) Representative immunoblots of phosphorylated p38 MAPK, phosphorylated HSL (Ser660) and phosphorylated PLIN1 (Ser522) in differentiated hASCs treated with 200 μ M CA for the indicated time (C) or 50 μ M H-89 for 30 min and then 200 μ M CA for 2 h (D).

(**E** and **F**) Real-time qPCR analyses of thermogenic markers in differentiated hASCs treated with 50 μ M H-89 (**E**, n=3) or 10 μ M SB (**F**, n=3) for 1 h and then 200 μ M CA for 4 h. (**G** and **H**) Real-time qPCR analyses of thermogenic genes (*Fgf21* and *Ucp1*), FGF21-target genes (*Egr1*, *cFos*) and the adipogenic marker *Pparg* in differentiated inguinal adipocytes of WT mice (**G**, n=4) or FGF21KO mice (**H**, n=4) after 400 μ M CA treatment for 4 h. (**I**) Representative immunoblots of phosphorylated p38 MAPK, phosphorylated HSL (Ser660) and phosphorylated PLIN1 (Ser517) in differentiated inguinal adipocytes from FGF21KO mice treated with 400 μ M CA for the indicated time. (**J–M**) Real-time qPCR analyses of thermogenic and metabolic genes in differentiated WT (**J**, n=4) or FGF21KO (**K**, n=4) murine inguinal adipocytes after 200 μ M CA treatment for 48 h, or differentiated hASCs stimulated with 200 μ M CA for 24 h (**L** and **M**, n=4). All data in **B**, **E–H**, and **J–M** are presented as mean $\pm$ SEM. * p <0.05, ** p <0.01, *** p <0.001.