Constitutively Active TRPC Channels of Adipocytes Confer a Mechanism for Sensing Dietary Fatty Acids and Regulating Adiponectin

Piruthivi Sukumar, Alicia Sedo, Jing Li, Lesley A. Wilson, David O’Regan, Jonathan D. Lippiat, Karen E. Porter, Mark T. Kearney, Justin F.X. Ainscough, David J. Beech

Rationale: Calcium entry is pivotal in the heart and blood vessels, but its significance and mechanisms in adipose tissue are largely unknown. An important factor produced by adipocytes is adiponectin, which confers myocardial protection, insulin-sensitization, and antiatherosclerotic effects.

Objective: To investigate the relevance of calcium channels to adipocytes and the production of adiponectin.

Methods and Results: Microarray analysis led to identification of transient receptor potential canonical (TRPC)1 and TRPC5 as channel subunits that are induced when adipocytes mature. Both subunits were found in perivascular fat of patients with atherosclerosis. Intracellular calcium and patch-clamp measurements showed that adipocytes exhibit constitutively active calcium-permeable nonselective cationic channels that depend on TRPC1 and TRPC5. The activity could be enhanced by lanthanum or rosiglitazone, known stimulators of TRPC5 and TRPC5-containing channels. Screening identified lipid modulators of the channels that are relevant to adipose biology. Dietary ω-3 fatty acids (eg, α-linolenic acid) were inhibitory at concentrations that are achieved by ingestion. The adipocyte TRPC1/TRPC5-containing channel was functionally negative for the generation of adiponectin because channel blockade by antibodies, knock-down of TRPC1–TRPC5 in vitro, or conditional disruption of calcium permeability in TRPC5-incorporating channels in vivo increased the generation of adiponectin. The previously recognized capability of α-linolenic acid to stimulate the generation of adiponectin was lost when calcium permeability in the channels was disrupted.

Conclusions: The data suggest that TRPC1 and TRPC5 contribute a constitutively active heteromultimeric channel of adipocytes that negatively regulates adiponectin and through which ω-3 fatty acids enhance the anti-inflammatory adipokine, adiponectin. (Circ Res. 2012;111:191-200.)

Key Words: calcium channel • transient receptor potential • α-linolenic acid • adipocyte • adiponectin

A dipocytes are sites for metabolism, storage, and effects of fatty acids. The cells are also pivotal in generating the endocrine organ of adipose tissue, which impacts on whole body metabolism and inflammation through secretion of adipokines. A key adipokine is adiponectin, which is anti-inflammatory, insulin-sensitizing, and protective against atherosclerosis and myocardial decline. Decreased concentrations of adiponectin occur in obesity-induced insulin resistance and are associated with endothelial dysfunction, diabetes, and hypertension. Diminished adiponectin secretion from adipose tissue of human coronary arteries has been suggested to be an initiator of atherosclerosis.

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The concentration of free cytoplasmic calcium (Ca2+) and the amplitude and rhythmicity of its fluctuations have primary importance in a plethora of cell types. For many cells there has been extensive study of intracellular Ca2+ signals, including investigation of the plasma membrane ion channels that directly permit Ca2+ influx or control Ca2+ influx indirectly. There is, by contrast, relatively little known about Ca2+-signaling in adipocytes, despite its suggested importance.

A major class of Ca2+-permeable channels is formed by transient receptor potential (TRP) proteins, which are en-

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coded by 28 genes in mammals. The proteins span the plasma or intracellular membranes, assembling around central ion pores as homo- or heteromultimers to allow influx of cations such as Ca\(^{2+}\) and Na\(^+\). The proteins are classified into subfamilies based on amino acid sequence; one of these is the canonical subfamily, which contains 6 members in humans (transient receptor potential canonical [TRPC], 1–7). Unlike many other ion channels, they are not voltage- or neurotransmitter-gated. Instead, they couple relatively slow chemical and physical activators to intracellular Ca\(^{2+}\)-signaling. Activator chemicals include dietary factors such as capsaicin, which activates TRPV1, and menthol which activates TRPM8. Several TRP channels are expressed, albeit not exclusively, in sensory neourones, supporting the concept of TRP channels as mechanisms by which animals detect external chemical signals. Although there is potential for importance of chemical-sensing ion channels in adipocyte biology, there are only 2 reports on TRP channel function in this context, both addressing TRPV1: One of the reports suggested function of TRPV1 in preadipocytes, whereas the other suggested no function in preadipocytes or adipocytes but a role in sensory nerves of adipose tissue. Here we sought Ca\(^{2+}\) channels that are important in adipocyte function and have potential relevance to cardiovascular health and disease. The investigation highlights TRPs from the canonical subfamily.

Methods
An expanded Methods section is provided in the online-only Data Supplement.

Human and Mouse Tissues
See the online-only Data Supplement.

Transgenic Mice
Dominant negative TRPC5 mutant (DNT5) cDNA was cloned into the pTRE vector from Clontech (Online Figure I). After Asel restriction digestion transgene was purified and microinjected into the pronucleus of C57BL/6 mouse embryos (MRC Harwell). Double transgenics were generated by breeding with mice carrying transgene transgenics were generated by breeding with mice carrying transgene that activates TRPM8. Several TRP channels are expressed, albeit not exclusively, in sensory neourones, supporting the concept of TRP channels as mechanisms by which animals detect external chemical signals. Although there is potential for importance of chemical-sensing ion channels in adipocyte biology, there are only 2 reports on TRP channel function in this context, both addressing TRPV1: One of the reports suggested function of TRPV1 in preadipocytes, whereas the other suggested no function in preadipocytes or adipocytes but a role in sensory nerves of adipose tissue. Here we sought Ca\(^{2+}\) channels that are important in adipocyte function and have potential relevance to cardiovascular health and disease. The investigation highlights TRPs from the canonical subfamily.

Cell Culture and Transfection
HEK 293 cells stably expressing human TRPC5 under a tetracycline inducible promoter and expression of TRPC1 using FuGene HD (Roche, UK) have been described. The 3T3-L1 cell line was obtained from the American Type Culture Collection and cultured in DMEM-F12 containing 10% fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. To induce differentiation, cells were grown to confluence and 2 days postconfluence, the medium was changed to medium containing 5 µg/mL insulin, 0.25 µmole/L dexamethasone, and 0.5 mmole/L IBMX with 10% fetal calf serum and antibiotics. After 48 hours, medium was changed to medium containing 5 µg/mL insulin, 10% fetal calf serum, and antibiotics. Cells were fed with fresh maintenance medium every 2 days until the day of experiments. Cells were differentiated for 12 to 16 days. Accell short-interfering RNA (siRNA) delivery was according to the manufacturer’s protocol (Dharmacon, UK). siRNA sequences are in Online Table I. For investigation of adipocytes from mice, preadipocytes were isolated using methods adapted from previous studies. Epididymal fat pad was dissected and digested in collagenase II (500 µg/50 mg tissue) for 1 hour at 4°C and 2 hours at 37°C and then centrifuged at 200 g for 10 minutes. The pellet was dissolved in erythrocyte lysis buffer, filtered, and centrifuged again. Preadipocytes were cultured and differentiated as described for 3T3-L1 cells but, in addition, all media contained 5 µg/mL doxycycline. Cells were differentiated for 9 days. For Ca\(^{2+}\) measurement, cells were plated on fibronectin-coated glass bottom dishes (Fluorodish, World Precision Instruments, USA).

Intracellular Ca\(^{2+}\) Measurement and Electrophysiology
3T3-L1 cells were plated in 96-well biocat plates (Corning) to 80% to 90% confluence for 24 hours. Prior to recordings, cells were incubated for 1 hour at 37°C in 4 µmole/L fluo-4AM in standard bath solution containing (mmole/L): 140 NaCl, 5 KCl, 1.2 MgCl\(_2\), 1.5 CaCl\(_2\), 8 glucose, and 10 HEPES titrated to pH 7.4 using NaOH. Cells were washed for 0.5 hours in standard bath solution at 37°C. Except for measurements from mouse adipocytes, recordings used the FlexStation II in 96-well mode (Molecular Devices, USA). Mouse adipocytes were studied using a Nikon Eclipse TE2000 microscope equipped with a 40 × objective and confocal fluorescence system (Thorlabs, Sterling, VA). Images from approximately 20 cells per dish were collected using ThorImageLS (Thorlabs) and analyzed using Image software. Consistent with a previous report, a fluorescence artifact between fura-2 and the lipid droplets of mature adipocytes prevented ratiometric Ca\(^{2+}\) measurements. Therefore, the nonratiometric fluo-4 Ca\(^{2+}\) indicator was used with 3T3-L1 cells or mouse adipocytes. Flu-4 was excited at 485 nm (FlexStation) or by a 488-nm laser (microscope), and emission was collected at 525 nm. Experiments were at room temperature (21 ± 2°C). For HEK 293 cells, the protocol was similar except fluo-4-AM was used with 0.01% pluronic acid and 2.5 mmole/L probenecid, or 2 µmole/L fura-2-AM was used. Fura-2 was excited at 340 and 380 nm and emitted light was collected at 510 nm; intracellular Ca\(^{2+}\) was indicated by the ratio of emission intensities for the excitation wavelengths. For electrophysiology methods, see the online-only Data Supplement.

Adipokine Measurement
3T3-L1 cells were differentiated in 6-well plates. On day 12, cells were serum-starved for 24 hours and then treated with dialyzed anti-TRPC1 (T1E3) and/or anti-TRPC5 (T5E3) antisera for 24 hours. For α-linolenic acid (lino) treatment, cells were incubated with 50 µmole/L lino or its vehicle (0.5% DMSO). After 24 hours, supernatant was collected and centrifuged at 1000 rpm for 10 minutes. Full-length adiponectin and soluble leptin were measured using ELISA kits (R&D Systems, UK). For organ cultures, epididymal fat tissue was harvested from 8- to 12-week-old male C57BL/6 mice and about 0.5 cm\(^3\) pieces were kept in DMEM-F12 containing 10% fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin for 24 hours. The tissues were treated with agents (as in...
3T3-L1 experiments) and the supernatant collected. For tissue from transgenic mice, the medium was supplemented with 5 μg/mL doxycycline. For mouse plasma adiponectin or leptin levels, the mice were terminally bled and anticoagulant (EDTA) containing blood was centrifuged at 7000 rpm for 7 minutes and the supernatant plasma was used.

Immunostaining, Western Blotting, RNA Isolation, RT-PCR, and Microfluidic Cards
See the online-only Data Supplement and Online Table II for PCR primer sequences.

Chemicals and Antibody Reagents
All chemicals were from Sigma (UK) except for fura-2AM and fluo-4AM (Invitrogen) and the fatty acid library (Biomol, Enzo Life Sciences, UK). For functional antibody experiments cells were pretreated with anti-TRPC1 T1E3 (1:500) or anti-TRPC5 T5E3 (1:100) antisera with or without preadsorption to the relevant antigenic peptide (10 μmol/L). T1N1 was custom-made rabbit anti-TRPC1 antibody targeted to intracellular N-terminal sequence (EVMALKDREVKEENTC) of TRPC1. Dialyzed antisera were diluted in DMEM medium and incubated with cells for 2 to 3.5 hours at 37°C prior to recordings. Chemical identity and purity of α-linolenic acid was confirmed by liquid chromatography-mass spectrometry.

Data Analysis
Data were collected in control and test pairs, expressed as mean±SEM and compared statistically using Student’s t-tests; n is the number of independent experiments and N is the number of wells in multi-well assays (when only N is stated, the data are from one 96-well plate). Probability (P) <0.05 (*) indicates statistically significant difference; n.s. indicates no significant difference. All results were from at least 3 independent experiments. Origin software was used for data analysis and presentation.

Results
TRPC1 and TRPC5 Are Expressed When Adipocytes Mature
As a first step toward elucidating ion channel types that are important in adipocytes we performed an unbiased screen to identify ion channel transcript expression that upregulates on maturation of preadipocytes to adipocytes. As a basis for the screen, we chose mouse 3T3-L1 cells that have been extensively characterized as a model of in vivo adipocytes and can be compared in 2 groups: preadipocytes and differentiated mature adipocytes. Appropriate differentiation of the cells was validated by Oil-red O staining and expression of the adipocyte markers PPARγ, aP2, adiponectin, and leptin (Online Figure II). Total RNA was isolated from each group of cells and ion channel expression was investigated in microfluidic PCR array cards representing 185 ion channel genes. Expression of 51 ion channel genes was indicated. Of these, 18 are known to confer Ca2+-permeability and 6 are TRPs; the most highly upregulated in adipocyte maturation was TRPC1. TRPC mRNAs were therefore investigated in independent quantitative RT-PCR reactions. Expression of TRPC1 mRNA was confirmed and TRPC5 mRNA was also detected, whereas mRNAs encoding TRPC3–TRPC4/TRPC6 to TRPC7 were not detected (Figure 1A; Online Figure III). Notable was the marked upregulation of TRPC1 (15.5 times) and TRPC5 (36.9 times) mRNAs as the cells differentiated (Figure 1A and 1B). TRPV4 and TRPP2 mRNAs were also detected on the array card and are potentially relevant, but neither was upregulated on differentiation (Online Figure III).

Western blotting and immunostaining were used to investigate TRPC1 and TRPC5 proteins. Neither protein was detectable in undifferentiated 3T3-L1 cells but both were expressed after differentiation (Figure 1C). Similarly, immunofluorescence experiments showed that TRPC1 and TRPC5 were expressed on differentiation (Figure 1D; Online Figure IV). These TRP proteins were not only expressed in 3T3-L1 cells but also in native mature adipocytes of mice and humans. In mice, TRPC1 and TRPC5 mRNAs were detected in native epididymal fat (Figure 1E). We also investigated perivascular fat because it is considered to be crucial in atherosclerosis.3 TRPC1 and TRPC5 were detected in perivascular fat of the mouse aorta (Online Figure V). To investigate perivascular fat in humans, we obtained internal mammary artery during coronary artery bypass surgery. TRPC1 and TRPC5 mRNAs (Figure 1F) and proteins (Figure 1G) were detected and localized to adipocytes (Figure 1H). The data suggest that expression of TRPC1 and TRPC5 is induced in mature adipocytes and relevant to endogenous fat of mice and humans, including perivascular fat.

TRPC1 and TRPC5 Confer Constitutive Calcium Entry in Adipocytes
To investigate if TRPC1 and TRPC5 are functionally relevant we performed intracellular Ca2+ measurements. Differentiated 3T3-L1 cells showed higher basal fluo-4 signal (Figure 2A), which depended on extracellular Ca2+ (Figure 2B), suggesting the presence of constitutively active Ca2+ entry channels. Moreover, whole-cell patch-clamp recordings revealed larger basal currents in differentiated 3T3-L1 cells (Figure 2C). We tested the effect of extracellular lanthanum ions (La3+) because a distinguishing feature of TRPC5-containing channels is that they may be stimulated by lanthanides such as La3+ or gadolinium (Gd3+). Consistent with the presence of functional TRPC5-containing channels, La3+ stimulated Ca2+-entry in differentiated 3T3-L1 cells (Figure 2A, 2B, and 2D). Another unusual property of TRPC5 is that it is stimulated by the PPARγ agonist rosiglitazone but not by a related thiazolidinedione pioglitazone and only slightly but not significantly by troglitazone.17 In differentiated 3T3-L1 cells, rosiglitazone stimulated Ca2+ entry whereas pioglitazone had no effect, and troglitazone caused a delayed increase in Ca2+ (Figure 2E, F).

To investigate more directly if Ca2+ signals related to TRPC1 and TRPC5, we used antibodies that target extracellular peptides in TRPC1 or TRPC5 and acutely inhibit channel function.14 Antibody to either TRPC1 or TRPC5 suppressed constitutive and La3+- or rosiglitazone-evoked Ca2+ signals in differentiated 3T3-L1 cells (Figure 2G–2J). There was a trend toward anti-TRPC5 antibody having a greater effect, compared with anti-TRPC1 antibody, on the rosiglitazone response (Figure 2J). Control antibody targeted to the N-terminus of TRPC1 (which is intracellular and therefore not accessible to extracellular agents) had no effect (Figure 2H and 2I). The anti-TRPC blocking antibodies had no effects on ATP-evoked Ca2+-release, consistent with them being specific (Figure 2K).
The data suggest that ion channels containing both TRPC1 and TRPC5 generate constitutive Ca\(^{2+}\) entry that is upregulated in differentiated 3T3-L1 cells. The channel activity may be further enhanced by La\(^{3+}\) or rosiglitazone.

**Figure 1. Transient receptor potential canonical (TRPC)1 and TRPC5 in mature adipocytes.**

A, Summary of mRNA detection in undifferentiated (Undiff) and differentiated (Diff) 3T3-L1 cells and mouse brain (+, expression; ++, higher expression; −, undetected). B, Abundances of TRPC1 and TRPC5 mRNAs in 3T3-L1 cells relative to 18S. C, 3T3-L1 proteins labeled with T1E3 anti-TRPC1 and T5E3 anti-TRPC5 antibodies. Expected masses are indicated by arrows. Equal total protein was loaded in each lane. A β-actin blot is shown in Online Figure II. D, Labeling of 3T3-L1 cells with T1E3 or T5E3 (green). Cell nuclei were stained with DAPI (blue). Representative of 3 experiments. The scale bar is 20 μm and for all images. Controls are shown in Online Figure IV. E, Products from RT-PCR on RNA from mouse epididymal fat tissue. F, Analysis of mRNA from human coronary artery disease (CAD) perivascular fat by RT-PCR. G, Lysates showing single protein bands of the expected sizes for TRPC1 and TRPC5. H, As for (G) but protein analysis by immunostaining (brown color in the upper panels indicates channel detection). The control was the antibody (Ab) preadsorbed to its antigenic peptide (+ pep). Scale bar, 100 μm.

**Identification of Negative Impact on Adiponectin**

To investigate whether there is a relationship of TRPC1 and TRPC5 channels to adiponectin, we first incubated differentiated 3T3-L1 cells with blocking antibodies targeted to...
controls showing suppression of rosiglitazone-evoked Ca\(^{2+}\) responses by T1E3 and T5E3 compared with the controls of antibodies preadsorbed to antigenic peptides (+pep) (n/N=3/9, n/N=3/9). K, Mean data for responses to extracellular 100 \(\mu\) mole/L ATP, showing no effects of T1E3 and T5E3 (n=3 for each).

TRPC1 or TRPC5. Anti-TRPC1 or anti-TRPC5 antibody enhanced the generation of adiponectin (Figure 3A). As an independent test, differentiated 3T3-L1 cells were transfected with siRNAs to knock-down TRPC1 and TRPC5 expression. Cellular delivery of siRNAs by standard transfection methods was inefficient but cell-permeable Accell siRNA achieved 70% to 90% knock-down (Online Figure VI). Combined knock-down of TRPC1 and TRPC5 increased adiponectin generation (Figure 3B). There was less effect compared with the blocking antibodies (Figure 3B compared with 3A), possibly because the antibodies inhibited the channels more effectively than the siRNA. To investigate the relevance of the channels to native adipocytes, organ-cultured mouse fat tissue was incubated with anti-TRPC blocking antibodies, and again there was increased adiponectin (Figure 3C). Addition of both antibodies together did not generate a significantly greater effect than either antibody alone (Figure 3C). The antibodies had less effect than in 3T3-L1 cells (Figure 3C compared with 3A), which may reflect inadequate penetration of the tissue by antibodies. Collectively the data suggest that channels comprising TRPC1 and TRPC5 impact negatively on the generation of adiponectin.

**Regulation of Adiponectin In Vivo**

To determine the relevance of the above findings to endogenous channels in vivo we used a dominant negative (DN) ion pore mutant of TRPC5 (DNT5) to engage with and disrupt channel complexes that can accept TRPC5 (Figure 3D). DNT5 was therefore generated as a broad expression across multiple cell types. As predicted, DNT5 expression occurred in adipose tissue of doxycycline-treated double transgenic mice but not doxycycline-treated single transgenics or mice carrying neither transgene (con-
The data suggest that constitutive Ca\(^{2+}\) entry through TRPC1/TRPC5-containing channels suppresses the generation of adiponectin by adipose tissue in vivo.

**TRPC Inhibition by Dietary Fatty Acids**

We hypothesized that TRPC1/TRPC5-containing channels might act as sensors of chemical factors that are important in adipocyte biology and coronary artery disease. We therefore screened for novel activators or inhibitors of the channels, first testing chemicals against signals arising from TRPC5 expressed alone in HEK 293 cells.

Using an intracellular Ca\(^{2+}\) indicator as the read-out of channel function, 66 fatty acids (Online Tables III and IV) were screened against TRPC5. A 2-step addition protocol first delivered the fatty acid and then the TRPC5 stimulator, Gd\(^{3+}\) (Figure 4A). None of the fatty acids stimulated TRPC5 but 19 had inhibitory effects (Figure 4A, Online Table III). A relationship of TRPC5 to anti-inflammatory fatty acids was indicated. Included were dietary \(\omega-3\) fatty acids, lino and docosahexaenoic acid, which are present in oily plants and fish.\(^{20,21}\) Inhibitory action of these fatty acids was confirmed with adversity,\(^8\) we studied mice that were either fed chow diet or high-fat diet for 6 weeks, the latter inducing expression of inflammatory indicators (Online Figure VII) but not obesity. In each litter there was a mixture of genotypes: double transgenics (DNT5+rtTA), single transgenics (DNT5 only or rtTA only), and mice carrying neither transgene. At 8 weeks of age, doxycycline was administered to all of the mice for 1 week. Double transgenic (DNT5, test) and single transgenic and no transgene (controls) mice were compared. No differences in weight or well-being of the mice in each group were observed. However, in chow-fed and fat-fed mice, DNT5 significantly increased the circulating adiponectin concentration without affecting leptin (Figure 3G and 3H). Serum adiponectin and leptin concentrations in mice expressing DNT5 and rtTA transgenes, DNT5 transgene only, rtTA transgene only, or neither transgene (none). The PCR primers were for DNT5 or rtTA transgenes, DNT5 transgene only, or neither transgene.

The data suggest that constitutive Ca\(^{2+}\) entry through TRPC1/TRPC5-containing channels suppresses the generation of adiponectin by adipose tissue in vivo.

Figure 3. Negative coupling to adiponec-
tin. A and B, Adiponectin secreted from differentiated 3T3-L1 cells. C, Adiponectin secreted from organ-cultured mouse fat tissue. A, Effects of T1E3 anti-transient receptor potential canonical (TRPC)1 and T5E3 anti-TRPC5 antibodies on adiponectin (n=5 for each). B, As for A but the effect of TRPC1 and TRPC5 short-interfering RNA (siRNAs) compared with control siRNA (n=3). C, As for A but intact excited fat and including data for both antibodies combined (n=4 for each). D, Diagram of the wild-type TRPC1–TRPC5 channel with Ca\(^{2+}\) and Na\(^{+}\) permeability (top) and the channel after incorporation of DNTRPC5 (DNT5), which inhibits ion permeability (bottom). E, RT-PCR analysis for mRNA from adipose tissue of mice containing the DNT5 and rtTA transgenes, DNT5 transgene only, rtTA transgene only, or neither transgene (none). The PCR primers were for DNT5 or 18S. Expected product sizes are indicated by arrows. F, Confocal fluo-4 Ca\(^{2+}\) measurements for adipocytes from transgenic mice expressing DNT5 (n=4) or controls (n=5). G and H, Serum adiponectin and leptin concentrations in mice expressing the DNT5 transgene (n=6 and 6 chow-fed, n=13 and 9 fat-fed) compared with matched control mice (n=9 and 6 chow-fed, n=11 and 11 fat-fed). I, Adiponectin secreted from fat excised from chow-fed mice, shown for mice expressing DNT5 normalized to controls (n=5 each). Controls were litter mates expressing neither transgene, rtTA only, or DNT5 only. See online-only Data Supplement for more details.

Adiponectin was significantly higher in the DNT5 group (Figure 3I).
other dietary ω-3 fatty acid, eicosapentaenoic acid, was also an inhibitor of TRPC5 (Figure 4F and 4G). Inhibition occurred independently of the type of TRPC5 activator because TRPC5 activity evoked by other, nonlanthanide, agonists was also inhibited (Figure 4H and 4I). Resolvin D1, an endogenous substance that is related to the dietary ω-3 fatty acids, had no effect when applied at the putative physiological concentration of 50 nmole/L (Figure 4J). TRPC1 and TRPC5 mix together to form a heteromultimeric channel that has different electrophysiological characteristics compared with TRPC5 alone, showing an almost linear I-V.\(^\text{14}\) We therefore investigated if lino inhibited the heteromultimeric channel. Figure 4K–4M show that there was strong inhibition of coexpressed TRPC1–TRPC5.

The data suggest that the dietary ω-3 fatty acids lino, docosahexaenoic acid, and eicosapentaenoic acid inhibit the TRPC5 homomeric and TRPC1–TRPC5 heteromeric channels.

Inhibition of Endogenous Adipocyte Channels by Fatty Acids

Whole-cell patch-clamp recording from differentiated 3T3-L1 cells revealed a constitutively active ionic current that averaged about −300 pA at −80 mV (Figure 5A). The I-V of the inhibited current was similar to that of the TRPC1–TRPC5 heteromultimeric channels in HEK 293 cells (Figure 5B compared with Figure 4). The current was inhibited by lino in differentiated but not in undifferentiated 3T3-L1 cells (Figure 5C). Antit-TRPC5 antibody suppressed the constitutive ionic current and no effect of lino was seen in differentiated 3T3-L1 cells (Figure 5C). Anti-TRPC5 antibody suppressed the constitutive ionic current and no effect of lino was seen in differentiated 3T3-L1 cells (Figure 5C). Anti-TRPC5 antibody suppressed the constitutive ionic current and no effect of lino was seen in differentiated 3T3-L1 cells (Figure 5C). Anti-TRPC5 antibody suppressed the constitutive ionic current and no effect of lino was seen in differentiated 3T3-L1 cells (Figure 5C). Anti-TRPC5 antibody suppressed the constitutive ionic current and no effect of lino was seen in differentiated 3T3-L1 cells (Figure 5C).

Because lino inhibited the TRPC channels, we hypothesized that it should stimulate the production of adiponectin, consistent with prior reports.\(^\text{22,23}\) In support of this, lino enhanced the generation of adiponectin by differentiated 3T3-L1 cells (Figure 5G) and adipose tissue excised from wild-type mice (Figure 5H). Strikingly, in excised adipose tissue from transgenic mice, lino failed to enhance the generation of adiponectin if it had already been enhanced by DNT5 (Figure 5I). The data suggest that the capability of lino...
to stimulate adiponectin production depended on its ability to suppress Ca\(^{2+}\) entry through TRPC5-incorporating channels.

**Discussion**

This study gives insight into a Ca\(^{2+}\) entry mechanism of adipocytes. Molecular components, TRPC1 and TRPC5, were upregulated as mature adipocytes formed, leading to constitutively active heteromeric Ca\(^{2+}\)-permeable channels. The arising Ca\(^{2+}\) influx inhibited the generation of adiponectin, without effect on leptin. Most assays showed about 25% increase in the generation of adiponectin when the TRPC channels were inhibited. Although TRP channels in general increase in the generation of adiponectin when the TRPC channels are upregulated as mature adipocytes formed, leading to constitutively active heteromeric Ca\(^{2+}\)-permeable channels. Molecular targets of the channels' activity have not been elucidated but it was possible, although lipid effects on TRPC5 have previously been found to be stimulatory.\(^3\) Intriguingly, the possible, although lipid effects on TRPC5 have previously been found to be stimulatory.\(^3\) TRPV1 modulation by which inhibits TRPC5.\(^3\) Therefore, protein kinase C is a putative transduction mechanism. More direct effects are possible, although lipid effects on TRPC5 have previously been found to be stimulatory.\(^3\) Intriguingly, the Drosophila TRP channel is activated directly by polyunsaturated fatty acids,\(^5\) our data indicate that mammalian orthologues (ie, TRPC1/TRPC5) are also sensitive to such fatty acids but that...
the functional consequence is the opposite (ie, inhibition). Substantial sequence differences between the mammalian and Drosophila channels make it difficult to predict which residues are responsible for the reversal of polarity.

Fatty acid inhibitors of TRPC1–TRPC5 channels are predicted to oppose the adverse effects of TRPC channel activation in inflammation and cardiovascular disease. There may be additional inhibitory factors acting similarly on TRPCs, such as resveratrol, vitamin C, and gallic acid17 (Online Figure IX). These factors are exogenous to the body, suggesting that a general function of TRPC channels may be to enable coupling between external chemicals and the internal biology of the body. Previously studies have focused on TRP channels other than TRPCs as integrators of cells external signals.10 The study used 3T3-L1 cells as a foundation, but data obtained using human tissue and mouse samples and through genetic manipulation in vivo supported the 3T3-L1 findings, and studies of overexpressed TRPCs supported the conclusion that the specified channel is a target of ω-3 fatty acids. There was technical difficulty in measuring intracellular Ca2+ in the mature adipocytes, but independent electrophysiological studies supported the data obtained with the fluo-4 Ca2+ indicator.

This study identified a Ca2+-permeable cationic channel (TRPC1/5) mechanism of adipocytes. Inhibition of the mechanism raised adiponectin levels and would thus be expected to confer cardiovascular protection. Constitutive activity of the channels was significant, suggesting that inhibitors are likely to be important even in the absence of an activator. Novel inhibitors of the channels were identified (ie, ω-3 fatty acids), adding to previously identified TRPC inhibitors that are associated with protection against major cardiovascular diseases.

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Disclosures

None.

References


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**Novelty and Significance**

**What Is Known?**

- Adiponectin secreted by adipocytes confers protection against cardiovascular disease.
- Secretion from adipocytes is calcium regulated.
- Transient receptor potential canonical (TRPC) proteins form calcium-permeable channels that promote cardiac and vascular smooth muscle cell hypertrophy, migration, and proliferation.

**What New Information Does This Article Contribute?**

- TRPC1 and TRPC5 form calcium-permeable channels in adipocytes.
- Inhibition of adipocyte TRPC channels increases the circulating concentration of adiponectin.
- \(\omega-3\) fatty acids inhibit adipocyte TRPC channels.

Adipocytes are vital for storing fats and regulating cardiovascular health, yet little is known about how calcium entry is mediated in these cells. Our study demonstrates that calcium entry into adipocytes modulates the generation of the cardiovascular protector, adiponectin. We show that TRPC channels play a role in this response and that dietary \(\omega-3\) fatty acids inhibit this channel. TRPC was identified through molecular and cell biology experiments in vitro and the results were then extended to the intact animal by generating a transgenic mouse for conditional inhibition of calcium permeation through the channels in vivo. TRPC inhibition led to an increased concentration of adiponectin in the plasma, arising from adipose tissue. Upregulation of adiponectin by \(\omega-3\) fatty acids was prevented by inhibition of the channels. The study reveals a previously unrecognized mechanism in adipocytes that is likely to be relevant for cardiovascular health in which inhibition of adipocyte TRPC channels confers protection, in part, through increased levels of adiponectin.
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Constitutively-active TRPC channels of adipocytes confer a mechanism for sensing dietary fatty acids and regulating adiponectin

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**Supplemental Methods**

**Human and mouse tissues**

Left internal mammary artery segments with fat were obtained anonymously and with informed consent from patients undergoing open heart surgery. Approval was granted by the Local Research Ethics Committee. Eight week old male C57BL/6 mice were killed by CO\textsubscript{2} asphyxiation and cervical dissociation in accordance with Schedule 1 Code of Practice, UK Animals Scientific Procedures Act 1986. Thoracic aorta with fat and epididymal fat were placed in ice-cold Hanks solution.

**Electrophysiology**

Whole cell patch-clamp recordings were performed at room temperature\textsuperscript{1}. Patch pipette solution 1 contained (mmole/L): 135 CsCl, 2 MgCl\textsubscript{2}, 5 Na\textsubscript{2}ATP, 0.1 NaGTP, 1 EGTA and 10 HEPES at pH 7.2 (CsOH). Patch pipette solution 2 contained (mmole/L): 115 CsCl, 2 MgCl\textsubscript{2}, 5 Na\textsubscript{2}ATP, 0.1 NaGTP, 10 EGTA, 5.7 CaCl\textsubscript{2} and 10 HEPES at pH 7.2 (CsOH). Pipette solution 1 was for 3T3-L1 cell recordings. Pipette solution 2 was for HEK 293 cell studies. The extracellular solution was SBS or SBS in which Ca\textsuperscript{2+} was substituted by Ba\textsuperscript{2+} where indicated. Whole cell patch-clamp signals were amplified with an Axopatch 200B patch clamp amplifier (Molecular Devices, USA) and software was Signal 3.05 (CED, UK). A ramp voltage protocol from -100 mV to +100 mV of 1 s duration was applied every 10 s from a holding potential of 0 mV. Current signals were filtered at 1 kHz and digitally sampled at 3 kHz. Patch pipettes had resistances of 3-5 M\Omega.

**Immunostaining**

Cells were fixed with 3 % formaldehyde and then incubated with primary antibodies in 0.5 % donkey serum overnight at 4 °C and then with FITC-conjugated secondary antibodies for 2 hr at room temperature. Coverslips were mounted using DAPI-hardest mounting medium (Vector Labs). Perivascular and epididymal fat tissue and aortic segments were fixed in 10 % formalin and embedded in paraffin. 8 \mu m sections were cut, hot-plated and dried overnight. Dewaxing, rehydration, and antibody
staining were according to standard protocols. Sections were incubated with primary antibodies overnight at 4 °C, labelled using ABC kit and visualised with 3,3’-diaminobenzidine substrate (Vector labs). Sections were counter-stained with eosin.

**Western blotting**

Cells or minced tissue were lysed with 1.25 % SDS containing 1 mmole/L dithiothreitol at 80-100 °C for 15 min. Proteins were separated on 10 % SDS-PAGE gels and transferred to nitrocellulose membrane (Millipore) and probed with primary antibodies diluted in 5 % milk. Secondary antibody was conjugated with horseradish peroxidase. Incubation in primary antibody was overnight at 4 °C and in secondary antibody was 2 hr at room temperature. Membranes were washed with PBST and labelling was detected with ECLplus chemiluminescence reagent (Amersham).

Antibodies were: anti-PPARγ (1:100; Santa Cruz), anti-β actin (1:1000; Santa Cruz), anti-TRPC1 (1:500; custom made T1E3) or anti-TRPC5 (1:100; custom made T5E3).

**RNA isolation, RT-PCR and microfluidic cards**

Total RNA was isolated using a standard TriReagent protocol and treated with DNase I (Ambion); an aliquot was used for cDNA synthesis using a high capacity RT kit (Applied Biosystems, UK) containing Oligo-dT and random primers. PCR primer sequences are in Online Table II. Primers were used at 0.5 μmole/L with 3 mmole/L Mg²⁺. Thermal cycling was 40 cycles of: 94 °C (30 s); 56.6 °C (1 min); 72 °C (1 min). PCR products were electrophoresed on 1.5 % agarose gels containing ethidium bromide. Direct sequencing confirmed product identity. Custom TaqMan Array (MFC_384) microfluidic cards were loaded with primers and Taqman probes (Applied Biosystems) and real-time PCR was performed in an ABI prism 7900HT Sequence Detection System controlled by SDS 2.2 software (Applied Biosystems).

**DNTRPC5 (DNT5) specificity**

cDNA encoding DNT5 was transfected into wild-type HEK 293 cells or HEK 293 cells stably over-expressing TRPM2 or TRPM3 channels. Transfection was achieved using FuGene HD transfection reagent (Roche) according to the manufacturer’s protocol. Briefly, transfection reagent and 2 μg of plasmid DNA was added to 194 μl of Opti-MEM medium (Gibco) and the mixture was incubated at room temperature for 15-20 min and then added to 80 % confluent cells in 10 cm² plate (one well of a 6-well plate) containing 2 ml of culture medium. TRPM2 and TRPM3 were investigated by measuring intracellular Ca²⁺, as described for TRPC5 recordings. For voltage-dependent K⁺ current recordings, 500 ms duration voltage steps were applied starting from -120 mV and incrementing by 10 mV every 10 s to a final voltage of +80 mV. The holding potential was -80 mV.

**Supplemental Results**

More detailed information for the adiponectin measurements in fat-fed mice is as follows: Statistical comparison of the groups using an unpaired t-test not assuming equal variances gave a P value of 0.001, indicating a high probability that our conclusion is correct (i.e. that DNT5 stimulated the generation of adiponectin). Independent analysis of each set of data arising from different litters also indicated statistically significant differences between the test and control groups (data not shown). 15 male mice from 2 litters were investigated in the chow group, and 24 male mice from 3 litters in the fat-fed group.
Supplemental Discussion

There is a report of TRPC1, TRPC4 and TRPC6 mRNAs in human pre-adipocytes\(^2\). These data support our undifferentiated mouse 3T3-L1 data, which suggest that TRPC1 mRNA is detectable in pre-adipocytes. We did not detect TRPC4 or TRPC6 mRNA in the 3T3-L1 cells. The study of human pre-adipocytes did not provide quantitative data or make a comparison with differentiated (mature) adipocytes.

Supplemental Figure Legends

**Online Figure I. DNTRPC5 (DNT5) specificity, transgene construct and detection.**

**A**, Diagram of the membrane topology of a single TRPC5 protein, showing the location of the LFW amino acid sequence that was mutated to AAA in order to generate a dominant negative (DN) TRPC5 (DNT5) that inhibits \(\text{Ca}^{2+}\) flux. **B**, Mean normalised data showing the effects of transfection of HEK 293 cells with vector expressing DNT5 (DNTRPC5) or vector alone (controls). Four sets of experiments are shown for cells: (i) with stable conditional over-expression of wild type TRPC5; (ii) with stable conditional over-expression of wild type TRPM2; (iii) with stable conditional over-expression of wild type TRPM3; or (iv) not transfected but showing endogenous voltage-gated \(K^+\)-current (\(K_V\) current). TRPM2 was activated by 1 mmole/L \(H_2O_2\) and TRPM3 by 10 \(\mu\)mole/L pregnenolone sulphate. Each experimental set had its own control even though only one control bar is shown. The data show that DNT5 inhibited wild type TRPC5 but not TRPM2, TRPM3 or \(K_V\) current. **C**, Diagram of the DNT5 transgene construct containing LacZ and DNT5 either side of a bi-directional promoter complex with a tetracycline response element (TRE), two opposing minimal CMV promoters (CMV\(_{\text{min}}\)), and SV40 and \(\beta\)-globin polyadenylation sequences. Ase1 sites were used for construct linearization and removal of plasmid sequence prior to injection. **D**, Validation of DNT5-specific PCR primers (see Online Table II for sequences) in PCR reactions that used wild-type (WT) TRPC5 or DNT5 cDNAs as templates. DNT5 primers only generated a product (360 bp) in reactions containing DNT5 template. WT primers generated products from both templates as they recognise sequence outside the mutated site of DNT5. ‘M’ indicates the DNA marker ladder.

**Online Figure II. Confirmation of differentiation in mouse 3T3-L1 cells.**

**A**, Oil-Red-O staining of undifferentiated (a) and differentiated (b, c) 3T3-L1 cells (scale bar, 100 \(\mu\)m). **B**, Mean abundances of PPAR\(_\gamma\) and aP2 mRNAs in undifferentiated (Undiff) and differentiated (Diff, adipocytes) 3T3-L1 cells (n=3). **C**, Western blots for lysates from Undiff and Diff 3T3-L1, showing labelling by anti-PPAR\(_\gamma\) (top panel) and anti-\(\beta\)-actin (bottom panel) antibodies. Predicted masses of PPAR\(_\gamma\) and \(\beta\)-actin are 58 and 43 kDa. **D**, PCR products from total RNA with (+) and without (-) reverse transcriptase (RT) reactions. Expected PCR product sizes were 363 bp (adiponectin) and 191 bp (leptin). **E**, Loading controls for the western blots in Figure 1C showing single bands labelled by anti-\(\beta\)-actin antibody.

**Online Figure III. Messenger RNA species encoding additional TRP channels.**

PCR products from total RNA of mouse brain or differentiated (Diff) or undifferentiated (Undiff) 3T3-L1 cells with (+) or without (-) reverse transcriptase
(RT) reaction. Expected PCR product sizes were 222 bp (TRPC3), 349 bp (TRPC4), 272 bp (TRPC6), 193 bp (TRPC7), 232 bp (TRPV4), 186 bp (TRPP2). TRPC3, TRPC4, TRPC6 and TRPC7 mRNA species were detected in brain but not 3T3-L1 cells. TRPV4 and TRPP2 mRNA species were detected similarly in differentiated and undifferentiated 3T3-L1 cells. TRPC2 mRNA was detected on the microfluidic card and was indicated to be slightly up-regulated on adipocyte maturation (~1.4 PCR cycle shift). Although TRPC2 may be functionally relevant in murine adipocytes it was not studied further because it is not relevant to humans, where TRPC2 is a pseudogene.

Online Figure IV. Control data for the detection of TRPC1 and TRPC5 proteins in 3T3-L1 cells by immunofluorescence.
Shown is labelling of undifferentiated (Undiff) and differentiated (Diff) 3T3-L1 cells with T1E3 anti-TRPC1 (A) or T5E3 anti-TRPC5 (B) antibodies. The controls were antibodies preadsorbed to their antigenic peptides (+pep). Positive labelling is indicated by the green colour of the FITC-conjugated secondary antibody. Cell nuclei were stained with DAPI (blue). Images are representative of 3 independent experiments. The scale bar is 20 µm and applies to all images.

Online Figure V. Immunofluorescence data for expression of TRPC1 and TRPC5 in mouse peri-vascular fat.
Immunostaining of mouse thoracic aorta (a) and the surrounding perivascular fat (pvf). Upper panels show positive staining (brown colour) for anti-TRPC1 (T1E3) and anti-TRPC5 (T5E3) antibodies, whereas lower panels show control experiments when antibodies had been boiled to denature the protein content (scale bar, 100 µm).

Online Figure VI. Validation of TRPC1 and TRPC5 knock-down by RNA interference in 3T3-L1 cells.
Mean quantitative real-time RT-PCR data for TRPC1, TRPC5 and TRPP2 mRNAs, showing specific knock-down of TRPC1 and TRPC5 mRNAs by the mixture of siRNAs targeting TRPC1 and TRPC5 (n=3). Each knock-down reaction was normalised to its own control from cells treated with scrambled siRNA.

Online Figure VII. Validation that fat-feeding induced inflammation.
Mean quantitative real-time RT-PCR data for interleukin-6 (IL-6) and tumour necrosis factor α (TNFα) mRNAs from adipose tissue of the mice fed chow (n=7) or high fat (n=7). The mice were from the groups used for analysis of adiponectin, as shown in the main paper. The data from fat-fed mice are normalised to those of the chow-fed mice.

Online Figure VIII. Suppression of mouse adipocyte Ca^{2+} entry by α-linolenic acid. Example confocal fluo-4 Ca^{2+} measurement from all adipocytes within a single field in a dish (control chow-fed mouse). Representative of n=5. Substances bath-applied to the cell were 100 µmole/L rosiglitazone (ros) and 50 µmole/L α-linolenic acid (lino.).

Online Figure IX. Diagram summarising the findings of the study. The grey square symbolises a mature adipocyte and the heterotetrameric cylinders at the top are TRPC 1 and 5 proteins around a central ion pore that is permeable to Ca^{2+} and Na^{+}. The large black cross on the horizontal white arrow indicates that the Ca^{2+} entry suppressed the generation of adiponectin. Adiponectin is anti-inflammatory and so
inhibition of adiponectin is described as leading to inflammatory effects that include atherosclerosis. Constitutive channel activity and enhancers of the TRPC1/5 channels are shown in the top left corner (e.g. stress factors). At the top right corner are examples of dietary factors that suppress the TRPC1/5 channel activity (e.g. ω-3 fatty acids). By inhibiting the channel, these factors reduce Ca\(^{2+}\) entry, reducing the inhibitory effect of the TRPC1/5-mediated Ca\(^{2+}\) entry on adiponectin; that is, the factors inhibit an inhibitor, conferring a double-negative that leads to a positive effect on adiponectin.

**Supplemental Table Legends**

**Online Table I. Sequences of TRPC1 and TRPC5 siRNAs.**

**Online Table II. PCR primers.**

**Online Table III. Summary data for fatty acids that inhibited Gd\(^{3+}\)-evoked TRPC5 activity in HEK 293 cells.** Fatty acids were tested at 50 μmole/L. The number of independent experiments (n) was 3 for each fatty acid.

**Online Table IV. Negative results from screening of the fatty acid library against Gd\(^{3+}\)-evoked TRPC5 activity in HEK 293 cells.** Fatty acids were tested at 50 μmole/L. The fatty acids which gave non-specific Ca\(^{2+}\) signals in non induced HEK 293 cells were omitted from final analysis. The number of independent experiments (n) was 3 for each fatty acid.

**Supplemental References**


Online Figure I
Online Figure II
Online Figure III
Online Figure IV
Online Figure V
Online Figure VI
Online Figure VII
• constitutive activity
• stimulation by stress factors (e.g. oxidized phospholipids)

• inhibition by protective dietary chemicals
  ω-3 fatty acids (e.g. α-linolenic acid)
  stilbenes (e.g. resveratrol)

plasma membrane TRPC channels

Ca^{2+}

Na^{+}

adiponectin

mature adipocyte

inflammation

endothelial dysfunction
diabetes
hypertension
atherosclerosis
heart failure

Online Figure IX
Short-interfering (si) RNA molecules

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Online Table I
## PCR primers

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<td>Mouse tumour necrosis factor α (TNFα)</td>
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## Fatty acids that inhibited TRPC5

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<th>Fatty acid</th>
<th>% inhibition</th>
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<td>9(E)-octadecenoic acid</td>
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<td>undecanoic acid</td>
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<td>10(E)-pentadecenoic acid</td>
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<td>10(Z),13(Z)-nonadecadienoic acid</td>
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<td>4(Z),7(Z),10(Z),13(Z),16(Z),19(Z)-docosahexaenoic acid (DHA)</td>
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**Online Table III**
## Fatty acids that had no effect on TRPC5

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