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# Citrulline induces fatty acid release selectively in visceral adipose tissue from old rats

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**Scope:** During aging, increased visceral adipose tissue (AT) mass may result in impaired metabolic status. A citrulline (CIT)-supplemented diet reduces AT mass in old rats. We hypothesized that CIT could directly affect fatty acid (FA) metabolism in retroperitoneal AT.

**Methods and results:** A 24-h exposure of AT explants from old (25 months) rats to 2.5 mM CIT induced a 50% rise in glycerol and FA release, which was not observed in explants from young (2 months) animals. The phosphorylated form of hormone-sensitive lipase, a key lipolytic enzyme, was 1.5-fold higher in CIT-treated explants from old and young rats, whereas glyc-eroneogenesis, that provides glycerol-3P requested for FA re-esterification, and its key enzyme phosphoenolpyruvate carboxykinase, were down-regulated 40–70%. Specifically in young rats, beta-oxidation capacity and gene expressions of carnitine palmitoyl transferase 1-b and very long chain acyl-CoA dehydrogenase were strongly up-regulated by CIT. In contrast, in old rats, while glyceroneogenesis was lower, beta-oxidation was not affected, enabling increased FA release.

**Conclusion:** Hence, in visceral AT, CIT exerts a specific induction of the beta-oxidation capacity in young rats and a selective stimulation of FA release in old rats, therefore providing a direct mechanism of CIT action to reduce AT mass.

## Keywords:

Adipose tissue / Aging / Citrulline / Fatty acids / Glyceroneogenesis

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**Abbreviations:** ARG, arginine; ASL, argininosuccinate lyase; ASS, argininosuccinate synthase; AT, adipose tissue; ATGL, adipocyte triglyceride lipase; BSA, bovine serum albumin; CIT, citrulline; CPT1-b, carnitine palmitoyl transferase 1-b; 8CPT-cAMP, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt; FA, fatty acid; Gyl, glycerol kinase; HSL, hormone-sensitive lipase; KREBS, Krebs Ringer buffer saline; NEFA, nonesterified fatty acids; NO, nitric oxide; NOS, NO syn-

## 1 Introduction

It is of common knowledge that the distribution of adipose tissue (AT) mass is altered during aging. After middle age, subcutaneous fat mass declines whereas intraabdominal fat tends to be either preserved or enlarged [1–4]. This situation leads to an augmented ratio of visceral to subcutaneous AT which is associated with an increased risk of

thases; PEPCK-C, cytosolic phosphoenolpyruvate carboxykinase; P-HSL, phosphorylated-HSL; PPAR $\alpha$ , peroxisome proliferator-activated receptor-alpha; PPAR $\gamma$ , peroxisome proliferator-activated receptor-gamma 2; RET AT, retroperitoneal AT; VLCAD, very long chain acyl dehydrogenase

atherosclerosis and diabetes [5, 6]. Among the intraabdominal depots, epididymal and retroperitoneal AT (RET AT) are considered as visceral depots and have been largely used for metabolic regulation studies [3, 7–10].

The AT is heterogeneous, encompassing several cell types in addition to adipocytes [11]. Specifically, the amount of resident macrophages increases with AT mass, causing a chronic low-grade inflammatory state [12]. The expansion of AT mass can be the result of either cell proliferation followed by differentiation into adipocytes or size enlargement of preexisting adipocytes because of an increased cell capacity to store triglycerides. The latter situation is reversed when lipolysis, i.e. triglyceride hydrolysis, is stimulated. Adipocyte triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) are the two main lipolytic enzymes [13, 14]. ATGL has no known posttranslational modification affecting its activity whereas, following phosphorylation, phosphorylated-HSL (P-HSL) is translocated at the surface of lipid droplets to hydrolyze triglycerides and diglycerides. Upon lipolysis induction, nonesterified fatty acids (NEFA) and glycerol can be released in the blood. However, under such a situation, plasma NEFA concentration depends also on other metabolic processes in addition to lipolysis. NEFA disposal is finely regulated by re-esterification, a pathway linked to glyceroneogenesis, for which the cytosolic isoform of phosphoenolpyruvate carboxykinase (PEPCK-C) is the key enzyme, and by mitochondrial beta-oxidation [15–17]. Glycerol output is also balanced by its direct phosphorylation when glycerol kinase (Gyk) is induced. Any treatment to fight visceral obesity would be efficient if it reduces re-esterification and increases beta-oxidation, in addition to stimulating lipolysis. Such a treatment would favor adipocyte emptying.

Citrulline (CIT) is an amino acid that plays important roles although it is not incorporated in proteins. CIT is involved in urea production in the liver and is coupled to nitric oxide (NO) production [18]. CIT recycling into arginine (ARG) is well described in macrophages that express argininosuccinate synthase (ASS), argininosuccinate lyase (ASL), and NO synthases (NOS) [19]. This CIT-derived process could participate in the cellular response to inflammation [20]. CIT is poorly present in natural foods, except watermelon, hence dietary supplementation with ARG or CIT was used to treat and prevent human metabolic disorders [21]. However, when administered to humans, ARG is metabolized in the liver whereas CIT is not and therefore becomes all available [20]. CIT was also shown to modulate protein metabolism in muscles of malnourished aged rats [22]. Some of us recently described that a 3-month treatment of old rats with a CIT-supplemented diet resulted in an increased lean mass and a decreased AT mass, particularly the visceral depot and was able to modify lipid raft structure in the brain [23, 24]. Furthermore, a similar effect on body composition was observed by Wu and colleagues in a model of obese ZDF diabetic rats fed with a diet supplemented with watermelon juice which is naturally enriched in CIT [25].

The purpose of the present study was to evaluate whether CIT could directly affect adipocyte metabolism in AT explants from young and old rats. We focused our studies on lipolysis, glyceroneogenesis, and beta-oxidation.

## 2 Materials and methods

### 2.1 Materials

Dulbecco's PBS, DMEM, penicillin, streptomycin, SuperScript<sup>®</sup> III First-Strand Synthesis SuperMix kit were from Invitrogen Life Technologies (Saint Aubin, France). RNeasy lipid tissue mini kit, Qiazol Reagent, and DNase set were purchased from Qiagen (Courtaboeuf, France). [ $1-^{14}\text{C}$ ]-Pyruvate acid, sodium salt (9.5 mCi/mmol), and (9,10- $^3\text{H}$ ) palmitic acid (60 Ci/mmol) were from PerkinElmer (Waltham, MA, USA). Rat insulin enzyme immunoassay kit was from Bertinpharma (SPI-BIO) (Montigny le Bretonneux, France). Glucose Assay Kit was from Abcam (Cambridge, UK). Triacylglycerol assay kit was from Biomérieux (Craponne, France). Hybond-N<sup>+</sup> membranes and Amersham Hyperfilm enhanced chemiluminescent (ECL) were from GE Healthcare Life Sciences (Velizy-Villacoublay, France). Free Fatty Acids Half Micro test and SYBR Green were from Roche (Meylan, France). Glycerol test (glycerol UV-method) was from R-Biopharm (Saint Didier au Mont d'Or, France). For Western blot analyses, SDS-PAGE was carried out using an SDS-MOPS running buffer and a Novex 4–12% Bis-Tris gel from Invitrogen (Villebon sur Yvette, France). Rabbit antiHSL and anti-P-HSL antisera were purchased from Ozyme (Saint Quentin en Yvelines, France). Sheep anti-PEPCK-C antibody was a gift from Professor E. Beale (Lubbock, TX, USA). Goat anti- $\beta$ -actin antibody was from Santa-Cruz Biotechnology (Heidelberg, Germany). Nylon mesh was from Sefar (Switzerland). Fatty acid (FA)-free albumin, sodium pyruvate and Tween 20 and all other products were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). CIT was a gift from Citrage company (<http://www.citrage.com>).

### 2.2 Animals

For in vivo experiments, 22-month-old male Sprague–Dawley rats from Charles River (L'Arbresle, France) were randomized into two groups after 2 weeks of acclimatization. In the CIT group ( $n = 5$ ) rats were fed ad libitum with standard diet supplemented with CIT at  $1 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  during 12 weeks [24]. Rats of the control group ( $n = 5$ ) were fed ad libitum with standard diet.

For in vitro experiments, 25-month-old male Sprague–Dawley rats were a gift from Sainte Anne Hospital (Paris, France). Two-month-old male Sprague–Dawley rats were obtained from Centre d'Élevage de Rats Janvier (Berthevin, France).

All rats were maintained at constant room temperature (24 °C) on a 12-h-light/-dark cycle. Rats were fed a standard balanced diet (60% carbohydrates, 16% proteins, 3% lipids, 5% vitamins and minerals, including retinol (1.98 mg/kg), cholecalciferol (506  $\mu$ g/kg), d- $\alpha$ -tocopherol (20 mg/kg), and micronutrients such as calcium pantothenate, nicotinic acid, calcium iodate, ferrous carbonate, and copper sulfate) from Safe (Augy, France) and tap water ad libitum. Rats were anaesthetized and killed by decapitation.

Blood was taken from the jugular vein. Aliquots were centrifuged and the supernatants were stored at -20 °C for further glucose, insulin, and triacylglycerol determination. Samples of epididymal and RET AT were removed and weighted.

The protocol for the animal studies was conducted according to the French Guidelines for the care and use of experimental animals.

## 2.3 Histology

For in vivo experiments, 5–8 g of RET AT was analyzed to measure adipocyte sizes. RET AT was removed and immediately fixed in 4% paraformaldehyde in PBS, pH 7.4, for 48 h at 4 °C. After fixation, tissues were dehydrated in ethanol, cleared, and finally embedded in paraffin blocks. Paraffin sections (5- $\mu$ m thick) were stained by hematoxylin and eosin and observed under a microscope (Zeiss, Germany). Adipocyte size was analyzed by using an image processing software (ImageJ, <http://rsbweb.nih.gov/ij/index.html>) with the determination for all adipocytes on a tissue section of minimal Feret diameter (corresponding to the lowest length of two parallel lines that just touch each side of the cell).

## 2.4 Rat AT explants

For in vitro experiments, RET AT from rats was removed for analyses. About 200 mg of tissue were cut in about 20 mg fragments and cultured in Krebs Ringer buffer saline (KREBS) medium (0.63% NaCl, 0.32% KCl, 0.014% CaCl<sub>2</sub>, 0.015% KH<sub>2</sub>PO<sub>4</sub>, 0.025% MgSO<sub>4</sub>, 0.2% NaHCO<sub>3</sub>) containing 5 mM glucose at 37 °C in 10% CO<sub>2</sub>. At 6 h, KREBS medium was renewed with 5 mM glucose. At 24 h of incubation, KREBS medium was renewed with 5 mM glucose with or without 2.5 mM CIT (this dose was chosen because it corresponds to the plasma CIT concentration observed after CIT supplementation [22]). Explants were treated by CIT for 8 or 24 h then frozen before further studies [26].

## 2.5 Isolation of adipocytes from AT

Isolated RET adipocytes were prepared as previously described [27]. Once digestion was complete, samples were passed through a sterile 250  $\mu$ m nylon mesh. Then the floating adipocytes-enriched fraction was washed three times

with KREBS medium containing 3% bovine serum albumin (BSA). The supernatant (adipocytes) was placed in Qiazol lysis reagent and kept at -80 °C for later RNA extraction.

## 2.6 Culture and differentiation of 3T3-F442A adipocytes

3T3-F442A preadipocytes were grown to confluence in DMEM containing 25 mM glucose, 200 IU/mL penicillin, 50 mg/L streptomycin, 2 mg/mL biotin, and 4 mg/L pantothenate supplemented with 10% newborn calf serum. Cells were cultured at 37 °C in a 10% CO<sub>2</sub> incubator. At confluence, newborn calf serum was changed to fetal bovine serum and 20 mM insulin was added to the medium named differentiation medium as described previously [28]. Culture medium was changed every 2–3 days. For experiments, medium was replaced by insulin-deprived regular medium 24 h ahead of the treatment with CIT. After 8 or 24 h of treatment with CIT, RNA and protein were frozen for further analyses.

## 2.7 Glycerol and FA release

### 2.7.1 Culture of explants from in vivo CIT-supplemented rats

Two hundred milligrams of tissues from CIT-supplemented or control rats were cut into fragments of about 20 mg and cultured in KREBS medium, glucose 5 mM, as described previously [9]. The medium was changed at 6 h. To study lipolysis, medium was changed with KREBS glucose-free medium containing 2% FA-free BSA and 5 mM pyruvate. Two hours later, the incubation medium was recovered for the estimation of lipolytic NEFA and glycerol.

### 2.7.2 Culture of explants from nonsupplemented rats

Two hundred milligrams of tissue were cut into fragments of about 20 mg and cultured in KREBS medium glucose 5 mM. The medium was changed at 6 h. Two hours later, the medium was replaced with KREBS medium, 5 mM glucose, containing or not 2.5 mM CIT. At 24 h of incubation, the medium was replaced with KREBS, glucose-free medium, containing 2% FA-free BSA and 5 mM pyruvate. Two hours later, the incubation medium was recovered for the estimation of lipolytic NEFA and glycerol.

### 2.7.3 3T3 F442A adipocytes

One million 3T3 F442A adipocytes was treated or not with 2.5 mM CIT for 24 h. Then, the medium was replaced with KREBS glucose-free medium containing 2% FA-free BSA and

5 mM pyruvate. Two hours later, the incubation medium was recovered for the estimation of lipolytic NEFA and glycerol.

## 2.8 Glyceroneogenic flux

To investigate the proportion of re-esterification of NEFA, glyceroneogenic flux was followed by the incorporation of  $^{14}\text{C}$  from  $[1-^{14}\text{C}]$ -pyruvate into neutral lipids, followed by lipid extraction as described previously [9, 26]. AT explants (200 mg) were incubated in KREBS medium 5 mM glucose with or without 2.5 mM CIT for 24 h, then media were changed and explants were incubated at 37°C under 10%  $\text{CO}_2$  for 2 h with KREBS glucose-free medium containing 2% FA-free BSA and 5 mM pyruvate.  $[1-^{14}\text{C}]$ -Pyruvate (9.5 mCi/mmol) was used at an isotopic dilution of 1/250. At 2 h of incubation, each sample was rinsed and frozen in liquid nitrogen before lipid extraction, according to the simplified method of Bligh and Dyer [29]. The subsequent  $[1-^{14}\text{C}]$ -pyruvate incorporation was estimated by counting the radioactivity associated with the fragments [9, 26].

## 2.9 Beta-oxidation

Palmitate oxidation rates from RET AT (100 mg) were assessed after 24-h incubation in the presence or absence of CIT. Tritiated water release experiments were carried out in triplicate as previously described [30]. Cultured explants layers were washed three times with PBS and 200  $\mu\text{L}$  of (9,10- $^3\text{H}$ ) palmitic acid (60 Ci/mmol) bound to FA-free albumin (final concentration: 125  $\mu\text{M}$ , palmitate:albumin 2:1) were added per well. Incubation was carried out for 2 h at 37°C. After incubation, the mixture was removed and added to a tube containing 200  $\mu\text{L}$  of cold 10% trichloroacetic acid. The tubes were centrifuged 10 min at 3300  $\times g$  at 4°C and aliquots of supernatants (350  $\mu\text{L}$ ) were removed, mixed with 55  $\mu\text{L}$  of 6N NaOH, and applied to ion-exchange resin. The columns were washed with 1.7 mL of water and the eluates were counted.

## 2.10 RNA extraction, cDNA synthesis, and real-time RT-qPCR

Total RNA was extracted from RET AT and isolated adipocytes. RNA was quantified by spectrophotometric Nanodrop (ND-1000; Nyxor Biotech). Total RNA (500 ng) was reverse transcribed using SuperScript<sup>®</sup> III First-Strand Synthesis SuperMix kit. Samples of cDNA were diluted 1:40, amplified, and used for RT-qPCR measurements using SYBR Green. RT-qPCR was performed in the LightCycler1536 instrument (Roche) as follows: 75°C for 2 min, 95°C for 10 min, 40 cycles of denaturation (95°C for 15 s to the denaturation, 60°C for 30 s to the annealing, 72°C for 30 s to the extension). Results were analyzed with the LightCycler1536 software from Roche. Ribosomal RPL13 RNA was

**Table 1.** Primer sequences of RNA

RNA	Forward (5' 3')	Reverse (5' 3')
(A) Rat primer sequences of RNA tested in RT-qPCR.		
<i>Cd68</i>	Agagcaggtcaaggtgaacag	Agggtggaagaaggtgaagc
<i>Cpt1b</i>	Acaatgggacattccaggag	Aaagactggcgctgctca
<i>Emr1</i>	Gtggaggatggggatggac	Acaacaggaaggtggctatgg
<i>IL6</i>	Ttggatgggtcttggtccttagcc	Tcctacccaactccaatgctc
<i>Nos2</i>	Cccagagcctctagacctcaa	Catgtgtaacacgttcttgg
<i>Ppara</i>	Aagccatcttcacgatgctg	Tcagaggtcctgaacagtg
<i>Pparg</i>	Ttatgctgttatgggtgaaa	Caaaggaatgggagtgtc
<i>Rpl13</i>	Tggcaggggcttcag	Tgggcatcacaggtcc
<i>Tnf</i>	Gcttgggtgttggtctacgac	Actgaacttcggggtgattg
<i>Acadvl</i>	Tgaatgacctgccaag	Ccaaatctctgccaagc
(B) Murin primer sequences of RNA tested in RT-qPCR.		
<i>Cpt1b</i>	Ttccgacaaacctgaagct	Taagatctgggctatctgtgtcc
<i>Ppara</i>	Aagccatcttcacgatgctg	Tcagaggtcctgaacagtg
<i>Pparg</i>	Ttatgctgttatgggtgaaa	Caaaggaatgcgagtgttc
<i>Rpl13</i>	Ggatccctccaccctatgaca	Ctggacttccaccgacctc
<i>Acadvl</i>	Tgaatgacctgccaag	Ccaaatctctgccaagc

The abbreviations of the genes, their 5'–3' nucleotide sequences of the forward and reverse primers are presented.

used to normalize cDNA. Quantification of mRNA was carried out by comparing the number of cycles required to reach reference and target threshold values ( $2^{-(C_t - C_{\text{ref}})}$  method) as described previously [31]. Sequences of the rat sense and anti-sense nucleotides corresponding to the different tested genes are presented in Table 1.

## 2.11 Western blot

Protein fractions were prepared from AT explants in a buffer containing 10 mM HEPES pH 7.9, then separated by SDS-PAGE, transferred to nitrocellulose filters, and blocked with 3% BSA in PBS containing 0.05% Tween 20. Blots were incubated with 1:1000, 1:1000, 1:2500, and 1:500 dilutions for the HSL, P-HSL, PEPCK-C, and  $\beta$ -actin antisera, respectively. For PEPCK-C detection, bound antibodies were visualized using ECL according to the manufacture's instruction. Blots were exposed to Hyperfilm and signals were quantified by scanning densitometry. For  $\beta$ -actin, HSL, and P-HSL, the Odyssey method was used for detection, following the procedure described by the manufacturer (Li-COR). Quantitative results of Western blotting were obtained by densitometry using ImageJ software.

## 2.12 Statistical analysis

Data are presented as means  $\pm$  SEM. Each independent experiment was carried out in triplicate. Statistical analyses were carried out using the nonparametric Mann–Whitney  $U$ -test for pairwise comparisons which was applied due to the small number of experiments ( $n < 10$ ).  $*p < 0.05$  versus Ctrl;



**Table 2.** Anthropometric and biological parameters of young and old rats

	Young <i>N</i> = 5	Old <i>N</i> = 5
Body weight (g)	256 ± 5	651 ± 15**
Epididymal AT weight (g)	4.40 ± 0.45	9.00 ± 0.43**
Retroperitoneal AT weight (g)	4.5 ± 0.80	19.08 ± 2.70**
Glycemia (mM)	5.42 ± 0.10	5.73 ± 0.28
Insulinemia (ng/mL)	0.249 ± 0.022	0.286 ± 0.023
Triacylglycerol (g/L)	1.52 ± 0.09	1.55 ± 0.21

Two-month-old male young rats (*n* = 5) and 25-month-old male rats (*n* = 7) were under a standard balanced diet. Blood samples and adipose tissue were collected for analyses. Data are means ± SEM.

\*\**p* < 0.01 versus young.

\*\**p* < 0.01 versus Ctrl; \*\*\**p* < 0.001 versus Ctrl. A value of *p* < 0.05 was considered as significant.

### 3 Results

#### 3.1 Body and metabolic parameters

Old rats presented a significant higher body weight than young animals (Table 2). The weight of both epididymal and RET AT increased in old rats in comparison with young animals. Furthermore, the ratio of RET AT to body weight increased with age from 1.8 to 2.9% whereas that of epididymal AT remained similar (1.7 versus 1.4%). Thus, we decided to focus our studies on RET AT. Interestingly, young and old rats used in this study had similar blood glucose, insulin, and triglyceride levels (Table 2).

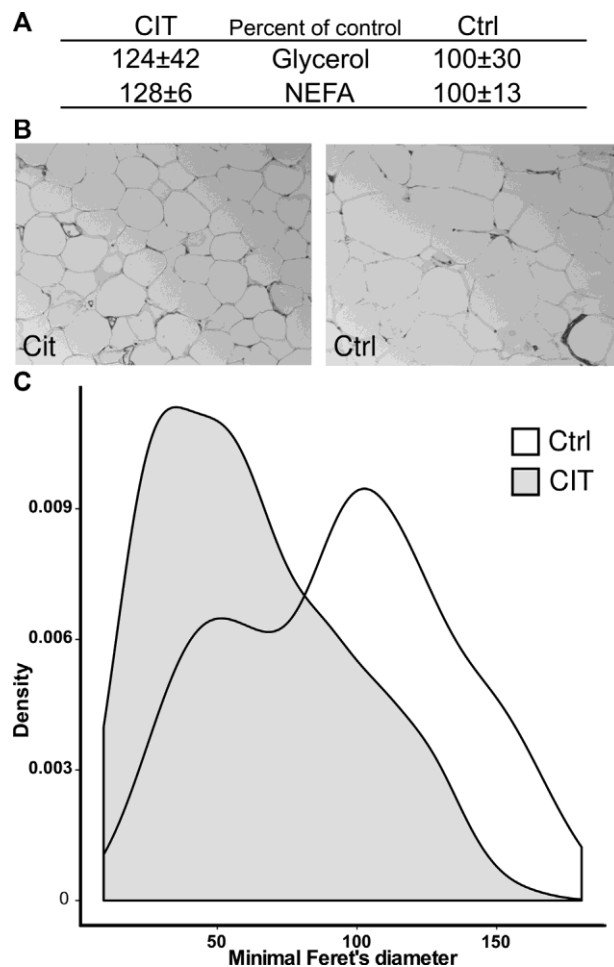
#### 3.2 In vivo effect of CIT in AT from old rats

We studied the lipolytic effects of CIT in RET AT explants from old rats maintained for a long-term (3 months) under the CIT-supplemented diet or the control diet.

As described in Fig. 1, the release in the incubation medium of glycerol and NEFA from explants of CIT-treated rats remained unchanged. However, adipocyte size was significantly lower in CIT-supplemented rats than in controls.

#### 3.3 In vitro effect of CIT in AT from young and old rats

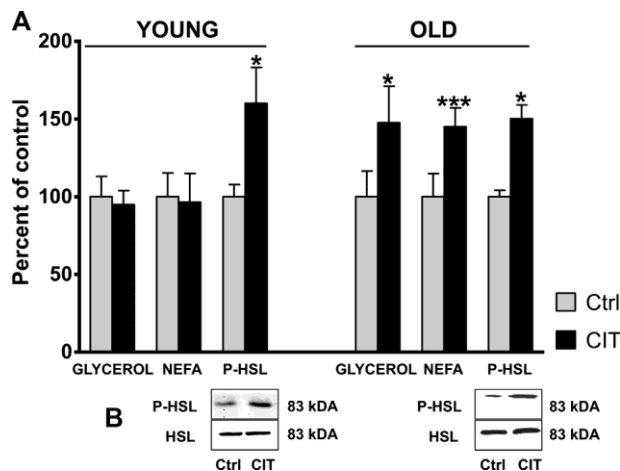
We investigated the effect of CIT on RET AT explants from young (2 months) and old rats (25 months). A concentration of 2.5 mM CIT was chosen to mimic the plasma concentration of this amino acid in rats supplemented with CIT [22]. We determined the glycerol and NEFA concentrations in the culture medium of explants maintained for 24 h in the presence of CIT. As shown in Fig. 2A, a significant increase in glycerol



**Figure 1.** Effect of CIT in RET AT explants from old rats maintained for 3 months under the CIT-supplemented diet. (A) Glycerol and NEFA release from RET AT explants of old rats. Twenty-two-month-old rats were fed either a chow supplemented with CIT, 1 g·kg<sup>-1</sup>·day<sup>-1</sup>, or a control diet for 12 weeks. Explants of RET AT were prepared and incubated for 24 h as described in Materials and Methods for the determination of glycerol and NEFA concentrations. Results are expressed in percent of control and each value represents the mean ± SEM from five independent experiments, each carried out in triplicate. In control rats, absolute values were 4.51 ± 1.35 nmol·2 h<sup>-1</sup>·mg<sup>-1</sup> tissue for glycerol, and 2.94 ± 0.38 nmol·2 h<sup>-1</sup>·mg<sup>-1</sup> tissue for NEFA. (B) Histological hematoxylin staining of RET AT from 25-month-old rats fed either a chow supplemented with CIT, 1 g·kg<sup>-1</sup>·day<sup>-1</sup> or a control diet for 12 weeks. (C) Frequency distribution of adipocyte size (minimal Feret's diameter) from RET AT of the same rats as in B. CIT treatment induced a significant (\*\*\**p* = 2.2 × 10<sup>-16</sup>, Kolmogorov–Smirnov test) shift toward smaller size in adipocyte distribution.

(48%) and NEFA (45%) release in the incubation medium occurred selectively in old rats. The protein concentrations per gram of tissue were identical whether explants were treated or not with CIT (data not shown).

In addition to the total ATGL and HSL amounts, we determined the proportion of P-HSL. We observed that a 24-h

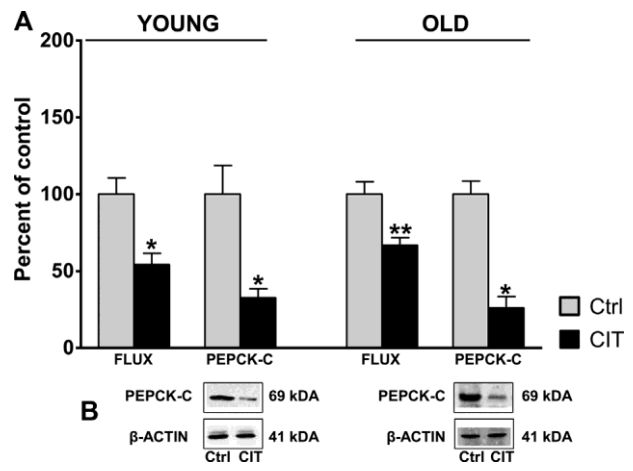


**Figure 2.** Effect of CIT on glycerol and NEFA release and on phosphorylated-HSL (P-HSL) of RET AT explants from young and old rats. (A) Glycerol and NEFA concentrations in culture medium of RET AT explants, from young (2-month-old) and old (25-month-old) rats, treated or not for 24 h with CIT 2.5 mM. In young rats, absolute values were  $4.54 \pm 0.60 \text{ nmol} \cdot 2 \text{ h}^{-1} \cdot \text{mg}^{-1}$  tissue for glycerol and  $3.13 \pm 0.48 \text{ nmol} \cdot 2 \text{ h}^{-1} \cdot \text{mg}^{-1}$  tissue for NEFA. In old rats, absolute values were  $4.61 \pm 0.44 \text{ nmol} \cdot 2 \text{ h}^{-1} \cdot \text{mg}^{-1}$  tissue for glycerol and  $2.90 \pm 0.89 \text{ nmol} \cdot 2 \text{ h}^{-1} \cdot \text{mg}^{-1}$  tissue for NEFA. Results were expressed in percent of control (Ctrl) and each value represents the mean  $\pm$  SEM from five to seven independent experiments, each carried out in triplicate. Histograms of P-HSL represent the mean  $\pm$  SEM of the quantification of four Western blots carried out from different explants. (B) Representative autoradiogram of four Western blots of total HSL and P-HSL in explants from young and old rats, at 24 h of exposure or not (Ctrl) to CIT. \* $p < 0.05$  versus Ctrl; \*\*\* $p < 0.001$  versus Ctrl.

exposure to CIT did not modify ATGL (data not shown), while this treatment induced P-HSL in explants from both young (58%) and old (50%) rats (Fig. 2B).

The fact that neither glycerol nor NEFA output was modified by CIT in AT from young rats, while P-HSL was increased, suggesting that lipolysis occurred, was puzzling. Glycerol could be reused if Glyk was induced. Using RT-qPCR, we monitored Glyk mRNA in CIT-treated explants versus controls. GK gene expression presented a twofold induction in CIT-treated explants from young rats whereas it remained unchanged when old rats were used (data not shown).

The release of NEFA from AT strongly depends on their intracellular utilization which is modulated by re-esterification and beta-oxidation. Accordingly, we first monitored glyceroneogenesis as the first step in FA re-esterification, evaluated by the incorporation of  $[1-^{14}\text{C}]$ -pyruvate into neutral lipids. In the presence of CIT, glyceroneogenesis was found significantly attenuated (46 and 34%) in AT from young and old animals, respectively, when compared with untreated explants (Fig. 3A). Since PEPCK-C is the key enzyme of glyceroneogenesis, the amount of this protein was determined by Western blot analysis. As expected, a 24-h exposure to CIT reduced PEPCK-C amount in explants from both young



**Figure 3.** Effect of CIT on glyceroneogenesis and on PEPCK-C amount in RET AT explants from young and old rats. (A) Explants from young (2-month-old) and old (25-month-old) rats were incubated or not (Ctrl) with CIT 2.5 mM for 24 h before measuring the incorporation of  $[1-^{14}\text{C}]$ -pyruvate into neutral lipids, as described in Materials and Methods. Control (Ctrl) values were  $14.47 \pm 1.53 \text{ nmol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$  for young rats and  $17.05 \pm 1.4 \text{ nmol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$  for old rats. Results were expressed in percent of control (Ctrl), and each value represents the mean  $\pm$  SEM from five to seven independent experiments, each carried out in triplicate. Histograms of cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C) represent the mean  $\pm$  SEM of the quantification of four Western blots carried out from different explants. (B) Representative autoradiogram of four Western blots of PEPCK-C in explants from young and old rats, at 24 h of exposure or not (Ctrl) to CIT. \* $p < 0.05$  versus Ctrl; \*\* $p < 0.01$  versus Ctrl.

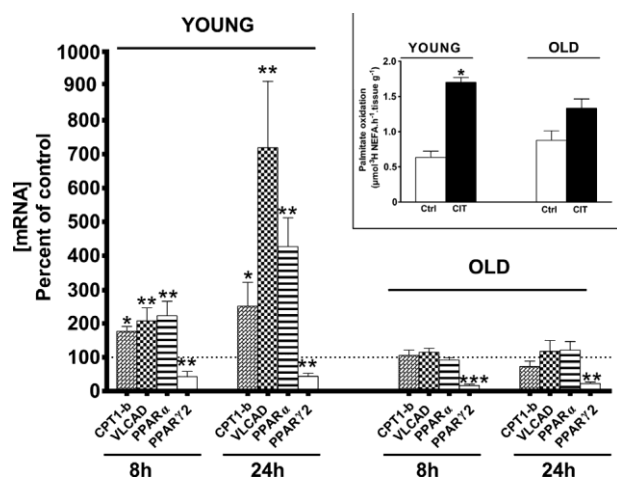
(68%) and old (74%) rats (Fig. 3B). Thus, CIT reduced NEFA re-esterification in RET AT from both ages.

The above results can explain the increased NEFA release in the medium of explants from old rats but cannot account for the absence of NEFA output in young rats. Thus, we investigated the beta-oxidation capacity of the explants under the same conditions of exposure to CIT. Results show that CIT induced palmitate oxidation rates about threefold in AT from young rats (Fig. 4, insert). In contrast, beta-oxidation did not change in response to CIT in AT from old rats.

Therefore, in young rats, the glyceroneogenic flux is reduced and the beta-oxidation capacity is augmented, opening an explanation for the apparent absence of CIT effect on net NEFA output. In contrast, in old rats, while glyceroneogenesis is reduced, beta-oxidation is not affected, thus resulting in an increased NEFA release in the medium (Fig. 2A).

### 3.4 Effects of CIT on metabolic protein-encoded genes

We first evaluated the stability of the expression of the series of genes involved in the metabolic processes under study. Although large variations occurred in some of these genes



**Figure 4.** Effect of CIT on beta-oxidation and on the expression of genes coding for proteins involved in beta-oxidation and in mitochondrial biogenesis. Explants from young (2-month-old) and old (25-month-old) rats were incubated or not (Ctrl) with CIT 2.5 mM for 8 or 24 h. Carnitine palmitoyl transferase 1-b (CPT1-b), very long chain acyl-CoA dehydrogenase (VLCAD), peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ), peroxisome proliferator-activated receptor- $\gamma$  2 (PPAR $\gamma$ 2) transcript levels were evaluated by RT-qPCR. Results were normalized using RPL13 mRNA. Results are expressed in percent of control (Ctrl) and each value represents the mean  $\pm$  SEM of five to seven independent experiments carried out in triplicate. Insert: Explants were incubated or not (Ctrl) with CIT for 24 h before following the incorporation of  $^3\text{H}_2\text{O}$  from (9,10- $^3\text{H}$ )-palmitate as described in Materials and Methods. Each value represents the mean  $\pm$  SEM of four independent experiments carried out in triplicate. \* $p < 0.05$  versus Ctrl; \*\* $p < 0.01$  versus Ctrl; \*\*\* $p < 0.001$  versus Ctrl.

between their expression in freshly isolated tissues and at 24 h of incubation, no significant modifications were detected later on (data not shown). Hence, the expression of these genes remains satisfactorily stable in control explants whether obtained from young or old rats.

Then, we monitored the expression levels of genes coding for proteins involved in beta-oxidation in AT explants from young and old rats treated for 8 or 24 h with 2.5 mM CIT. Selectively in young rats, CIT induced the expression of carnitine palmitoyl transferase 1-b and very long chain acyl dehydrogenase mRNA to reach, respectively, 177 and 251% at 8 h and 208 and 719% at 24 h (Fig. 4).

We then wondered whether the expression of peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ) and peroxisome proliferator-activated receptor- $\gamma$  2 (PPAR $\gamma$ 2) were affected by CIT. These two transcription factors play important roles on metabolic and oxidative genes in adipocytes. We observed a large CIT-induced increase in PPAR $\alpha$  mRNA selectively in explants from young rats (Fig. 4). In contrast, the *Pparg* gene was down-regulated by CIT whatever the age of the animals and the time of treatment, with a stronger effect in old rats (Fig. 4).

### 3.5 Effects of aging and CIT on inflammatory protein-encoded genes

The impact of aging and of CIT on the expression of genes involved in inflammation was analyzed in RET AT explants from young and old rats. The expression of the genes coding for two macrophage markers—F4/80 and CD68—was both raised about eightfold (Fig. 5A) in explants from old rats compared to young rats. The mRNAs encoding the inflammatory markers—interleukin-6 and tumor necrosis factor  $\alpha$ —were also increased, respectively, about fourfold and threefold with aging. In addition, *Nos2* gene expression was stimulated about twofold in old versus young rats (Fig. 5A). A 24-h treatment of explants with CIT affected neither the expression of macrophage markers nor that of inflammatory markers whatever the age of the rats (Fig. 5B). In contrast, CIT up-regulated NOS II mRNA (270%) selectively in AT explants from old rats (Fig. 5B).

### 3.6 Effects of CIT in cultured adipocytes

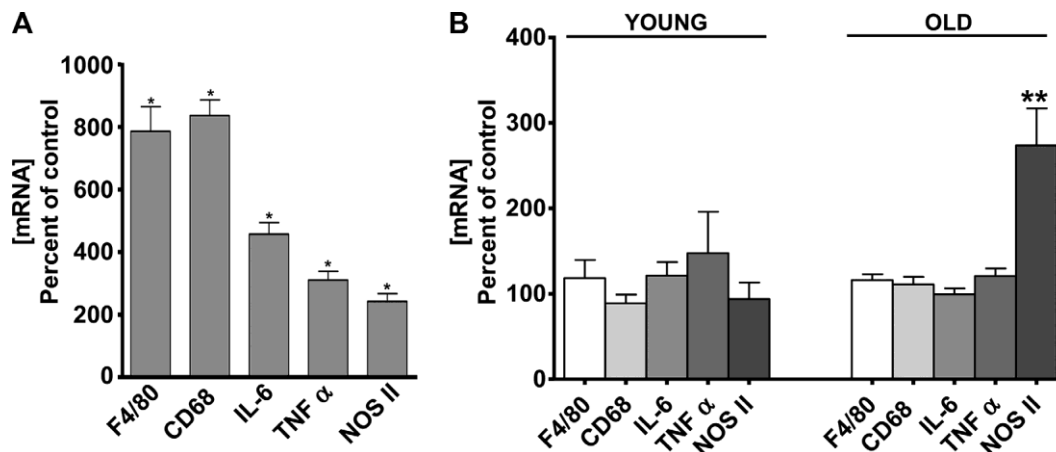
AT is heterogeneous, comprising fibroblasts, endothelial cells of capillaries, resident macrophages, and lymphocytes, in addition to adipocytes. To evaluate the effect of CIT on adipocytes, we used the adipocyte cell line 3T3-F442A. Twelve days after confluence, cells were differentiated into adipose-like cells. When treated with 2.5 mM CIT for 8 and 24 h, these cells did not release additional glycerol and NEFA in the culture medium, when compared to controls (Fig. 6A). In contrast, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt, a well-known lipolytic agent, induced glycerol and NEFA output about twofold, as expected [32]. However, as in RET AT (Fig. 2), CIT increased HSL phosphorylation and down-regulated PEPCK-C amount (Fig. 6A).

3T3-F442A adipocytes, such as RET AT from young rats, responded to CIT by a large induction of CPT-1b mRNA both at 8 and 24 h (Fig. 6B). *Acadol*, gene expression was up-regulated about twofold only at 24 h whereas PPAR $\alpha$  increased already at 8 h. In contrast, PPAR $\gamma$ 2 transcript was strongly down-regulated both at 8 and 24 h (Fig. 6B).

## 4 Discussion

Our data show that a CIT-enriched diet administered to old rats for 3 months induces a decrease in adipocyte diameter of RET AT, in line with the original demonstration that the CIT-rich watermelon juice reduces RET AT mass in ZDF rat [25]. However, watermelon juice contains other nutrients (e.g. lycopene) with pharmacological properties. Thus, our study clearly shows the specific effect of CIT. The action of CIT in old (25-month-old) and young (2-month-old) rats is somewhat different. Selectively in AT explants from old rats, CIT induces FA and glycerol release in the culture medium. FA and glycerol output are considered as the hallmarks of

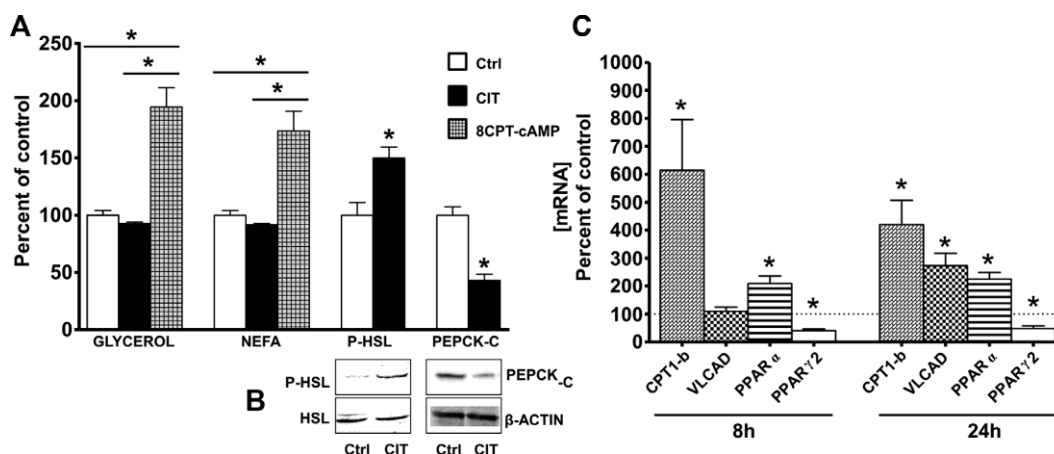




**Figure 5.** Effect of aging and of CIT on the expression of genes coding for proteins involved in macrophage markers and in inflammation F4/80, CD68, interleukin-6 (IL-6), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), nitric oxide synthase 2 (NOS II) mRNA levels were evaluated by RT-qPCR. Results were normalized using RPL13 mRNA. (A) Effect of aging. Explants from young (2-month-old) and old (25-month-old) rats were incubated in the absence of CIT for 24 h. Results represent the ratio of the values obtained in old rats versus the values obtained in young rats, taken as controls. (B) Explants from young (2-month-old,  $n = 5$ ) and old (25-month-old,  $n = 7$ ) rats were incubated or not (Ctrl) with CIT 2.5 mM for 24 h. Results are expressed as percent of control and represent the mean  $\pm$  SEM of five to seven independent experiments carried out in triplicate. \* $p < 0.05$  versus Ctrl; \*\* $p < 0.01$  versus Ctrl; \*\*\* $p < 0.001$  versus Ctrl.

lipolysis. However, FA and glycerol that originate from triglyceride breakdown can be immediately metabolized in intracellular pathways, therefore preventing their release. Such a situation appears to occur here. Whatever the age of the rat, CIT is lipolytic since it significantly increases the amount of P-HSL, the active form of this key lipolytic enzyme. The total ATGL amount did not vary in response to CIT but the

proportion of lipid droplet-linked ATGL could be increased, although such a hypothesis requires further work. In addition, Gyl mRNA is also up-regulated by CIT specifically in explants from young rats (unpublished results), thus giving a clue for the lack of increase in glycerol output in CIT-treated explants. The demonstration that glyceroneogenesis is decreased together with the CIT-induced down-regulation of



**Figure 6.** Effect of CIT on differentiated 3T3-F442A adipocytes. (A) Glycerol and NEFA concentrations in the culture medium of 3T3-F442A cells, incubated or not (Ctrl) with CIT 2.5 mM for 24 h. Values are expressed in percent of control (Ctrl). In Ctrl, absolute values were  $4.42 \pm 0.19$  nmol/2 h/ $\mu$ g protein for glycerol, and  $3.02 \pm 0.12$  nmol/2 h/ $\mu$ g protein for NEFA. (B) Representative autoradiogram of four Western blots of total hormone-sensitive lipase (HSL) and phosphorylated-HSL (P-HSL) expression and of cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C) protein levels in 3T3-F442A adipocytes, at 24 h of exposure or not (Ctrl) to CIT. (C) Effects of CIT on the expression of metabolic genes coding for proteins involved in beta-oxidation and mitochondrial biogenesis in 3T3-F442A adipocytes, treated or not (Ctrl) for 8 or 24 h with 2.5 mM CIT. Carnitine palmitoyl transferase 1-b (CPT1-b), very long chain acyl-CoA dehydrogenase (VLCAD), peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ), peroxisome proliferator-activated receptor- $\gamma$ 2 (PPAR $\gamma$ 2) mRNA levels were evaluated by RT-qPCR. Results were normalized using RPL13 mRNA. Results are expressed as percent of control (Ctrl) and represent the mean  $\pm$  SEM of four independent experiments carried out in triplicate. \* $p < 0.05$  versus Ctrl.

its key enzyme, PEPCK-C, strongly suggests that this amino acid reduces re-esterification of lipolytic FA into triglycerides [26]. CIT-induced reduction of PEPCK-C level is concomitant with a decrease in the expression of its mRNA (data not shown). This is consistent with the fact that the expression of PPAR $\gamma_2$ , a key transcription factor controlling *Pck1* expression, was found to be strongly down-regulated in response to CIT in both young and old rats.

While an increased FA output occurs in AT from old rats, this effect was not observed in young animals. Although the basal beta-oxidation capacity in RET AT is not statistically different between young and old rats, its induction by CIT occurs selectively in AT from young rats. This process probably compensates the observed reduction in FA re-esterification, thus potentially explaining the absence of induction of FA release in young rats. Altogether, these results suggest that CIT augments FA beta-oxidation, in line with the up-regulation of *Cpt1b*, *Acadvl*, and *Ppara* expressions in AT from young rats. Increased levels of acylcarnitine and carnitine were reported to follow elevations in CIT in neonates with intrahepatic cholestasis caused by citrin deficiency [33]. Since a carnitine-supplemented diet increases beta-oxidation in muscle, due to acetylcarnitine efflux from mitochondria to cytosol, such a mechanism could be one of the causes of CIT effect in AT [34]. If it were so, the combined supplementation with CIT and carnitine could be an original approach to treat metabolic disorders. Further works are required to clarify this point.

We made the hypothesis that the age-dependent CIT effect on beta-oxidation could be the result of a differential CIT-induced NO production. For NO production with CIT as a substrate, ASS and ASL are required [19]. Although *Ass* and *Asl* are expressed in RET AT, these mRNAs are below the level of detection by RT-qPCR in isolated adipocytes from this AT (unpublished results from our group). Hence, adipocytes are not NO producers from CIT as a substrate. However, AT is made of several cell types in addition to adipocytes, including endothelial cells of vessels and resident macrophages in which ASS and ASL are present in large amount [19,20]. Therefore, both cell types can synthesize ARG from CIT, which in turn can produce NO from NOS III and NOS II, respectively [27]. It is well known that the number of macrophages increases in AT with aging and gain of fat mass [12, 35]. We show here that macrophage markers are strongly up-regulated with age in RET AT. Therefore, NO production from AT-resident macrophages could indeed be increased with aging. Another observation is that CIT induces *Nos2* expression selectively in AT from old rats. This effect is in line with studies pointing out the ability of CIT to favor NO production. Hence, in vitro, CIT enhanced NOS III expression in endothelial cells [36]. Furthermore, in endotoxemic rats, CIT administration increased phosphorylation of NOS III in enterocytes [37]. Such a synergy could favor a large increase in NO production in this AT, compared with a weaker NO synthesis from NOS III in explants from young animals. NO could then easily diffuse toward adipocytes and affect FA metabolism, with a noteworthy effect on beta-oxidation. Such

a hypothesis would mean that high NO production would be deleterious for beta-oxidation whereas low amounts of NO favor beta-oxidation in agreement with previously published observations [38,39]. A similar opposite effect of low and high productions of NO on leptin action was also demonstrated [9,40,41].

Altogether our results provide a metabolic explanation for the reduction in adipocyte size observed in RET AT from in vivo CIT-supplemented old rats for 3 months. Previously published observations demonstrated that dietary L-ARG supplementation reduced AT mass by down-regulating the expression of genes that encode lipogenic enzymes [25,42]. This down-regulation is concomitant with an increase in skeletal muscle and brown fat mass in obese rats [42, 43]. Other data showed that, in old malnourished rats, a CIT-supplemented diet induced a rise in protein content in muscle [22]. Although ARG and CIT were shown to significantly affect AT mass in vivo, no data of a direct CIT effect on AT was available. Our results demonstrate that CIT is able to act directly on adipose cells.

The increase in NEFA output triggered by CIT appears in line with the study of Faure et al. that demonstrated that CIT supplementation to malnourished old rats stimulated muscle energy metabolism [44]. Accordingly, it can be thought that potential deleterious effects of the rise in circulating glycerol and NEFA could be counteracted by the lipo-oxidative action of CIT in nonadipose cells, particularly in muscle cells. Of particular interest is the observation that in AT from young rats and in 3T3-F442A cells, CIT directly induces the mitochondrial beta-oxidation, and therefore exerts a direct protector role. Altogether, this metabolic action of CIT in the young rat sounds as a favorable mechanism to limit lipid storage and to facilitate fat burning. In advanced age, fat depots are redistributed, leading to an increased ratio of visceral to subcutaneous fat mass [1, 2, 6]. The CIT-induced reduction of fat deposit in RET AT could prevent this increase in ratio of visceral to subcutaneous fat, and hence could limit the associated pathological risks (e.g. diabetes, cardiovascular diseases) related to aging. In obesity, the rise in AT mass also affects predominantly visceral fat, and CIT effects in this pathophysiological situation remain an opened question.

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