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ANTIADIPOGENIC EFFECTS OF SUBTHERMAL ELECTRIC STIMULATION AT 448 kHZ ON DIFFERENTIATING HUMAN MESENCHYMAL STEM CELLS

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INTRODUCTION

Obesity is a risk factor involved in a number of severe diseases, including diabetes, heart conditions and different cancer types (1,2). A variety of physical techniques including radiofrequencies (RF: 100 kHz-3 GHz), have been assayed in antiobesity treatments (3-5). RF have been reported effective in therapies for adipose tissue reduction (6-9). Their antiadipogenic or lipolytic effects, which are dependent on the signal frequency and power, have received support from *in vivo* and *in vitro* studies (10-12). Specifically, in capacitive-resistive electric transfer (CRET) therapy, the target tissues are heated through exposure to 448 kHz, sine wave electric current, the therapy is applied manually, exerting pressure with the electrodes over the skin, so that the underlying target tissues receive two simultaneous stimuli: thermal (electrically-induced) and mechanical (8). Previous studies applying 448 kHz CRET currents that increased the temperature of the culture medium up to 42 ˚C, induced a significant reduction in intracellular lipid depot in OP9 mouse preadipocytes differentiated into mature adipocytes (13). These results supported the hypothesis that the antiadipogenic or lipolytic effects of CRET are due to the cellular or tissular response to the electrically-induced hyperthermia. On the other hand, it has been shown that in vitro exposure to CRET in the 448-570 kHz range significantly affects essential cellular functions, including the control of proliferation in different human cell types, even when the currents are administered at subthermal doses (14-19). These results revealed that, at least at the cellular level, CRET currents can exert an electrostimulatory action that is independent of the electrically-induced hyperthermia. Therefore, the aim of the present study was to determine if, besides the antiadipogenic effects induced by thermal treatment with 448 kHz CRET (13), electric stimulation with the same signal can also interfere, by itself and under conditions of normothermia, with the adipocytic differentiation. The present study investigated the effect of *in vitro* exposure to CRET current at 448 kHz, administered at a subthermal density, on the early adipogenic differentiation of human adipose-derived stem cells (ADSC). The study subsequently delved into the molecular basis of the observed response. Therefore, the action of CRET on kinases 1 and 2 of the enzyme mitogen-activated protein kinase (MEK1/2), and on the transcription factor, nuclear peroxisome proliferator-activated receptor (PPAR)γ, both of which serve important roles in the regulation of adipogenesis (20-22). Additionally, since PPARγ directly activates a number of genes involved in lipid synthesis and/or storage in adipocytes, the action of CRET on the expression levels of selected genes was assessed in ADSC at the early stages of adipogenic differentiation. Throughout, PPARγ refers to the expression of PPAR protein, whereas PPARG refers to PPAR gene expression

MATERIALS AND METHODS

Cell culture. uman ADSC were used for two fundamental reasons: i) ADSC are responsive to subthermal treatment with CRET (15); ii) ADSC are an optimal model for investigating phenomena involved in adipogenic processes. All experiments were performed with cells between passages 3 and 7.

Adipogenic differentiation of ADSC. After 4 days of growing in Petri dishes, cultures were incubated in adipogenic differentiation medium and maintained in this medium for periods of 2-9 days, with the medium being renewed every 3-4 days. Samples were CRET or sham exposed during the last 48 h of the adipogenic treatment. Therefore, the electric stimulation was applied to cells at early or intermediate stages of the adipogenic differentiation.

CRET exposure. It was performed by means of pairs of sterile stainless steel electrodes which were fitted inside all Petri dishes, CRET-exposed and sham-exposed. Only cells grown on the rectangular area located within the electrode gap were used. The electrode pairs were connected in series to a signal generator (INDIBA® device, INDIBA SA, Barcelona, Spain). For sham-exposure, the electrode pairs inserted in control dishes were also connected to the generator, but not energized.

Oil red O staining and quantification of lipid content. To assess the adipogenic differentiation of ADSC, the quantity of fatty acids synthesized by cultures grown for 2 or 9 days in differentiating medium were quantified and compared with those in samples maintained in basal medium for the same intervals.

Immunoblotting. Proteins from cultured cells were separated by SDS-polyacrylamide gel electrophoresis and transferred to Odyssey® nitrocellulose membranes. Membranes were incubated with antibodies against monoclonal anti-human anti-PPARγ, monoclonal anti-human anti-phosphorylated (p-)MEK1/2 and monoclonal anti-human anti-β-actin in blocking buffer. Following, membranes were subsequently incubated with LI-COR Odyssey secondary antibodies and the fluorescent intensity of the blots was measured and evaluated. PPARγ antibody enables detection of the two PPARγ isoforms of interest for the present study: PPARγ1 and PPARγ2.

Immunofluorescence. Cells from cultures between passages 3 and 5

were incubated with monoclonal anti-human anti-PPARγ antibody and fluorescently stained with anti IgG conjugated to Alexa Fluor 488. Cell nuclei were counterstained with bisBenzimide.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from ADSC was extracted to generate cDNA. RT-qPCR amplification was performed. Melting curves were evaluated and PCR reaction products were separated and stained to confirm the presence of a single product. The efficiency of the reaction was evaluated by amplifying serial dilutions of cDNA.

RESULTS

CRET effect on lipid content. Exposure to CRET during the last 48 h of incubation significantly reduced cell lipid content with respect to sham-exposed controls, in samples grown in differentiating medium for 2 days (61.32±15.05% below controls; P<0.05) or 9 days (8.48±2.37% below controls; P<0.05).

CRET effects on the expression of PPARγ. From the above results, and since the ligand-activated transcription factor PPARγ is crucial in lipid metabolism and in adipocyte differentiation, the potential effects of CRET on the expression of PPARγ was assessed by immunoblotting analysis. Samples incubated in basal medium expressed no PPARγ at either at 2 or 9 days of incubation (Fig. 1). The increased expression of PPARγ induced by the adipogenic medium was unaffected by CRET when applied during the first two days of differentiation. By contrast, when applied from incubation day 7 to 9, CRET significantly reduced the expression of PPARγ, 17% below the sham-exposed, differentiated controls.

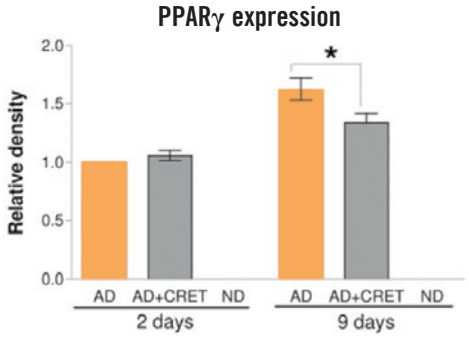

Fig. 1. Densitometric assessment of the immunoblot analysis for PPARγ (PPARγ/β-actin ratio) corresponding to the bands for PPARγ1 and PPARγ2. The data are presented as the mean ± standard deviation over controls grown in differentiating medium for 2 or 9 days (*: P<0.05). PPARγ expression levels in the ND controls were vestigial and non-quantifiable, therefore, were assigned a value of 0. PPAR, peroxisome proliferator-activated receptor; CRET, capacitive-resistive electric transfer; AD, control samples incubated in adipogenic medium for 2 or 9 days and sham-exposed to CRET; AD + CRET, samples grown in adipogenic medium and exposed to CRET during the final 48 h; ND: samples grown in basal, non-differentiating medium.

Effects of CRET on the nuclear expression and intracellular location of PPARγ. The number of cells exhibiting nuclear expression of PPARγ in CRET-exposed samples was significantly reduced with respect to sham exposed, differentiated controls, both in samples with 2 or 9 days of incubation, (42 and 67% reduction, respectively; Fig. 2). Samples with 9 days of differentiation and treated with CRET, immunostaining revealed a predominantly cytoplasmic location of PPARγ, which is in contrast with the preferential nuclear location of PPARγ in sham-exposed differentiated controls and in undifferentiated cells. The electric stimulation induced no such response in samples with 2 days of adipogenic differentiation.

Effect of CRET on the expression of p-MEK. Mitogenic stimuli can induce increased nuclear expression of MEK1, whose interaction with PPARγ results in the inactivation and translocation of PPARγ to the cytoplasm (20-22). The possibility that CRET exposure can induce changes in the expression of p-MEK that are associated with the observed cytoplasmic expression of PPARγ was investigated by immunoblotting of the expression of p-MEK. In the absence of electrical stimulation, incubation in adipogenic medium for 2 or 9 days induced a statistically significant decrease in the expression of p-MEK (42 and 35%, respectively, below non-differentiated controls; Fig. 3). In differentiated cultures, CRET stimulation induced overexpression of p-MEK with respect to the sham-exposed controls (12 and 21% at 2 and 9 days, respectively), the effect being statistically significant in samples differentiated for 9 days.

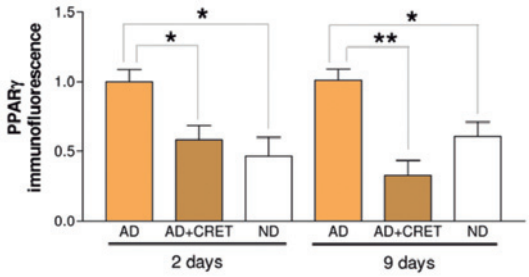

Fig. 2. Immunofluorescence analysis of the expression of PPARγ. Only cells showing nuclear PPARγ were scored as positive. The data are means ± standard error over controls differentiated for 2 or 9 days and sham-exposed to CRET (*: P<0.05; **: P<0.01). Same notations as in Fig. 1.

Effects of CRET on the regulation of genes intervening in adipocyte differentiation. Incubation for 2 or 9 days in adipogenic medium significantly increased mRNA expression of PPARG and of the other selected genes intervening in lipid storage and PPARγ activation. The results in Fig. 4 demonstrated that when applied during the two initial days of differentiation, CRET exposure failed to induce significant changes in the mRNA expression levels of the studied genes. By contrast, when the electric stimulus was administered from day 7-9 of adipogenesis, mRNA expression of PPARG1 was significantly reduced by 9%, with respect to sham-exposed controls. CRET also induced significant decreases in the mRNA expression of genes located downstream of PPARG, including perilipin (18%), angiopoietin-like (ANGPTL)4 (20%) and fatty acid synthase (FASN; 11%)

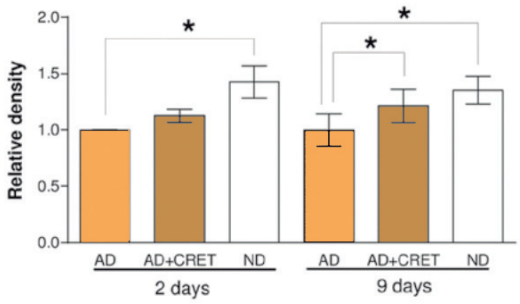

Fig. 3. Results of the densitometric analysis of the immunoblots for p-MEK1/2 (p-MEK/β-actin ratio). The data are means ± standard deviation, normalized over controls grown in differentiating medium for 2 or 9 days and sham-exposed to CRET (*P<0.05). Same notations as in Fig. 1.

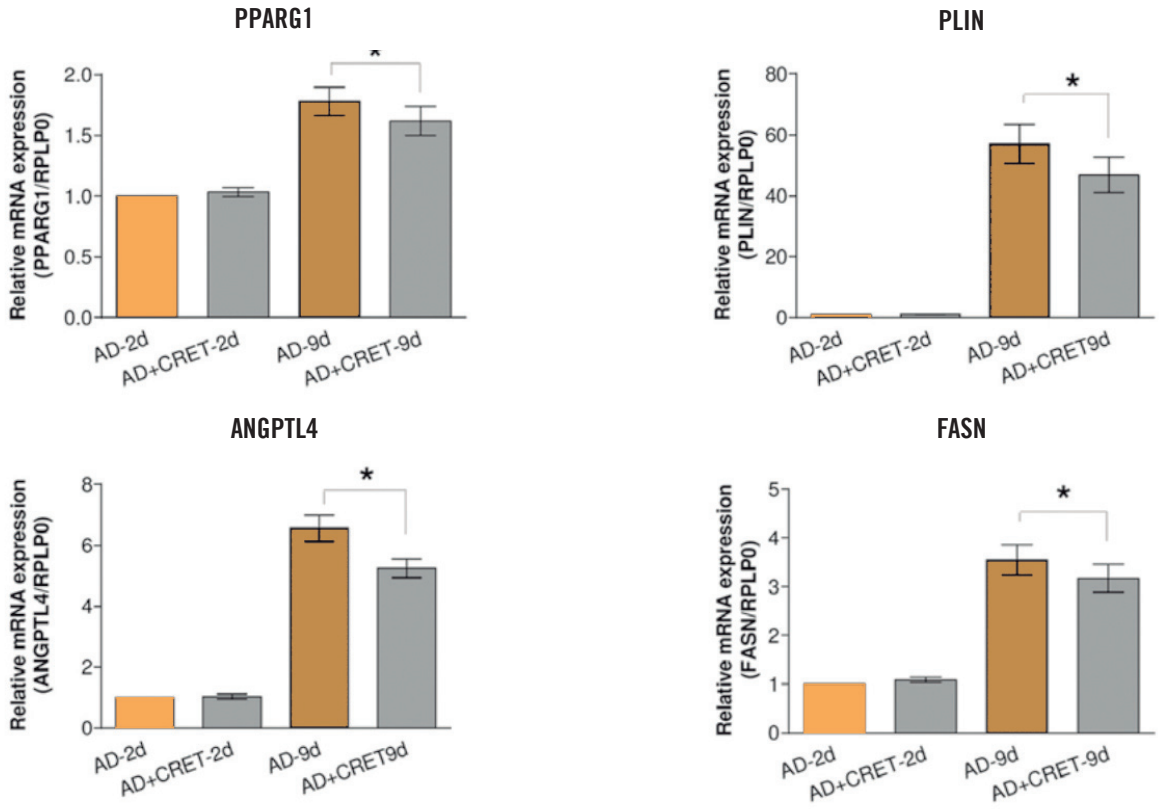

Fig. 4. Reverse transcription-quantitative polymerase chain reaction analysis of the effects of CRET stimulation on the expression levels of PPARG1, PPARG2, FABP4, PLIN, ANGPTL4, SREBP1c, SCD and FASN. The data are means ± standard deviation, normalized over samples differentiated for 2 or 9 days and sham-exposed to CRET (*P<0.05). PPARG, peroxisome proliferator-activated receptor gamma; PLIN, perilipin; ANGPTL, angiopoietin-like; FASN, fatty acid synthase. The rest notations are the same as in Fig. 1.

DISCUSSION

ADSC can differentiate to form mature adipocytes. Since restrictive dieting is known to induce a reduction in size, but not in number, of adipocytes in adipose tissue, it has been postulated that cytoplasmic accumulation of fat during adipogenic differentiation is crucial in human obesity (27). Therefore, the experimental study of the effects of antiobesity therapies on the regulation of the adipogenic differentiation can provide relevant information for therapy optimization. For instance, at thermal doses, stimulation with 448 kHz CRET has been reported to cause a significant decrease in the quantity of fatty deposits in OP9 mouse stromal cells in advanced stages of differentiation (13). In the present study exposure to 448 kHz CRET currents resulted in significant and consistent reduction of lipid content in human ADSC at the early stages of the adipogenic differentiation. These results suggested that, when administered simultaneously with the thermal stimulus and mechanical pressure applied in the CRET therapy, the electric stimulus could significantly reduce lipids present in advanced stages of adipogenesis. Additionally, subthermal electric stimulation can induce an antiadipogenic action in the earlier stages of adipogenesis.

The present study hypothesizes that the early antiadipogenic action of the subthermal stimulus is exerted through a chain of responses at the molecular level, rather than through a more direct effect of emptying of the lipid vesicles. To investigate the processes potentially involved in the subthermal effects, the protein and mRNA expression levels of PPARγ, the key nuclear transcription factor in adipogenesis control, were determined. Immunoblotting assay failed to detect significant changes with respect to the controls in the expression of PPARγ in the samples at the earlier stages. By contrast, in samples differentiated for 9 days, it induced a significant decrease in PPARγ with respect to sham exposed controls. On the other hand, immunofluorescence assay revealed that CRET treatment significantly decreased the number of cells displaying nuclear PPARγ, both in samples differentiated for 2 or 9 days, the latter showing a predominantly cytoplasmic location of PPARγ.

Although little is known about its intracellular distribution, experimental evidence locates PPARγ in the nucleus of the quiescent cells. The apparent discordance between the responses obtained at 2 days in the immunoblot vs immunofluorescence assays, may be attributable to the fact that immunoblotting quantifies total PPARγ protein in the sample, regardless of its cellular location. By contrast, immunofluorescence exclusively quantifies PPARγ that, being present in the cell nucleus, acts as an adipogenic transcription factor. Therefore, the mentioned discordance would not occur if only the nuclear, active PPARγ protein was susceptible to the electric stimulus. As a whole, these results are coherent with the observed reduced lipid content in CRET-treated ADSC and indicate that such an effect can be mediated by electrically-induced changes in the expression of PPARγ.

It has been reported that when the cell receives a mitogenic stimulus, p-MEK, the kinase enzyme of the MAPK-ERK1/2 pathway, is overexpressed and binds physically to PPARγ, causing its translocation from the nucleus to the cytoplasm (20-22) and preventing PPARγ from activating adipogenic effect on genes. To elucidate whether this is a potential mechanism involved in the observed translocation of PPARγ to the cytoplasm of the samples differentiated for 9 days and treated with CRET, the expression of p-MEK was analyzed. While in undifferentiated samples immunoblotting revealed a high expression of p-MEK, typical of proliferating cultures, the differentiation medium induced significant low expression of p-MEK (Fig. 3). Compared with differen-

tiated, sham-exposed controls, CRET treatment induced overexpression of p-MEK, although this effect only reached statistical significance in cultures with 9 days of differentiation. These results suggested that this overexpression of p-MEK may result in the downregulation of the genomic activity of PPARG. Therefore, the 448 kHz electric signal may be interpreted by the cell as a mitogenic stimulus, which induces the overexpression of p-MEK. In cultures undergoing adipogenic differentiation, early activation of this pathway may not result in changes in cell proliferation, however, only in the translocation of PPARγ towards the cytoplasm, and subsequent hindering of the adipogenic differentiation. CRET stimulation may result in at least one non-genomic interaction of PPARγ with different protein partners, leading to alternative cytoplasmic signaling, which would result in partial inhibition of early adipogenic differentiation.

Regarding the influence of CRET on the expression of genes involved in the adipogenic metabolism and differentiation, no significant changes were detected in ADSC differentiated for 2 days and treated with CRET. By contrast, in samples differentiated for 9 days, CRET caused decreased expression of PPARG1, ANGPTL4, perilipin and FASN, without significantly affecting the other genes analyzed (Fig. 4). These results revealed that CRET stimulation not only induces decreased protein expression of PPARγ, but also results in the downregulation of PPARG, which leads to the reduced expression of a number of genes whose transcription requires PPARγ protein directly, like ANGPTL4 and perilipin, or indirectly, like FASN (28). ANGPTL4 is known to irreversibly inactivate lipoprotein lipase (LPL) (29). Therefore, it was hypothesized that the decreased expression of this gene may result in increased lipolytic activity of LPL. Perilipin is a protein covering lipid vesicles, protecting them against the action of cytosolic lipases (30), therefore, decreased gene expression of perilipin may promote mobilization of intracellular reservoirs of triacylglycerol. Finally, FASN is a multi-enzyme protein that, in the presence of NADPH, catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA to long chain saturated fatty acids (31). Therefore, the CRET-induced decrease in FASN expression may also contribute to reduced quantities of intracellular lipids. These results indicated that the hindering effects of CRET on early adipogenic stages would be mediated, at least in part, by electrically-induced underexpression of genes involved in the control of the synthesis and mobilization of fatty acids during adipogenesis.

In conclusion, 448 kHz CRET therapy simultaneously applies three stimuli: electrical, thermal and mechanical, which may act in synergy in antiobesity treatments. RF at 448 kHz can, by itself and in the absence of the accompanying thermal and mechanical components, interfere with the synthesis and mobilization of fat at early adipogenic stages by, at least, the activation of MEK1/2, reduced expression of PPARγ protein and downregulation of the gene expression levels of PPARG1, perilipin, ANGPTL4 and fatty acid synthase. The possibility cannot be disregarded that these effects also contributed to the antiadipogenic action exerted on mature adipocytes by thermal treatment with CRET (13), even if the extent of such potential contribution remains to be determined.

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