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RAPID COMMUNICATION

Effects of type 5-phosphodiesterase inhibition on energy metabolism and mitochondrial biogenesis in human adipose tissue *ex vivo*

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ABSTRACT. ***Objective:*** **An excess of adipose tissue (AT) in obese individuals is linked to increased cardiovascular risk and mitochondria have been shown to be defective in the muscle and AT of patients with metabolic disorders such as obesity and Type 2 diabetes. Nitric oxide (NO) generated by endothelial NO synthase (eNOS) plays a role in mitochondrial biogenesis through cyclic-GMP (cGMP). AT harbors the whole molecular signaling pathway of NO, together with type 5-phosphodiesterase (PDE-5), the main cGMP catabolising enzyme.** ***Aim:*** **Our aim was to evaluate the effect of the modulation of NO pathway, through PDE-5 inhibition, on energy metabolism and mitochondria biogenesis in human omental AT.** ***Methods and measurements:*** **Cultured human omental AT was stimulated with PDE-5 inhibitor, vardenafil, at different concentration for 24 and 72 h. Analysis of the expression of both key-regulator genes of adipocyte metabolism and mitochondria-biogenesis markers was performed.** ***Results:*** **We found an increased gene expression of peroxisome proliferator-activated receptor-γ (*PPAR-γ*), *adiponectin*, and proliferator-activated receptor gamma coactivator-1 α (*PGC-1α*) after a 24-h stimulation with vardenafil at the lowest concentration employed compared to controls ($p<0.05$). After 72 h of stimulation, a significant increase of mitochondrial DNA was found compared to control samples ($p<0.05$).** ***Conclusion:*** **Our data suggest that PDE-5 inhibition could have an impact on mitochondrial content of human AT suggesting a positive effect on energy metabolism and adding new elements in the comprehension of AT pathophysiology.**

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INTRODUCTION

The expansion of body fat, particularly of visceral adipose tissue (AT), has a great influence on insulin sensitivity and both glucose and lipid metabolism. Moreover, an excess of AT in obese individuals, especially of visceral AT, is linked to increased cardiovascular risk (1). Among the different mechanisms implicated in the pathogenesis of insulin resistance, the existence of an impaired mitochondrial function has been recently highlighted. The mitochondria of the main tissues involved in glycemic control, muscle, and AT, are in fact defective or less functional in subjects affected by obesity with insulin resistance or by Type 2 diabetes. This deficit has been hypothesized to contribute to the impairment of signal transduction, and substrate transport and oxidation (2, 3).

Mitochondrial biogenesis describes the generation of more mitochondria in response to increased energy demands (e.g. cell growth and division). Conversely, the copy number of specific mitochondrial proteins and the functional capacity of each mitochondrial pathway may vary greatly between different tissues and different physiological conditions (4). Nisoli et al. suggested that nitric oxide (NO), generated by endothelial NO synthase (eNOS), might play a major role in the regulation of increasing mitochondrial biogenesis in several tissues including white adipocytes (5). A cyclic-GMP (cGMP) mediated pathway represents a way of action of NO, leading to an upregulation of peroxisome proliferator-activated receptor γ (PPAR-γ) coactivator-1 α (PGC-1α), one of the main regulators of mitochondrial biogenesis (6). Engeli et al. showed how AT harbors the whole molecular signaling pathway of NO, including eNOS, guanylate ciclase (GC) and cGMP-dependent-protein kinase (PKG) (7). On the other side, conversion of cGMP to linear GMP is one of the main regulatory event of NO-pathway and is performed by cGMP-specific enzyme called phosphodiesterase type 5 (PDE-5) (8). It is known that adipocytes express also PDE-5 (9). PDE-5 inhibition can affect adipocyte function by modulating adipogenesis and glucose uptake in *in vitro* animal models (10). However, the available data about this pathway in humans are not conclusive (11).

In this study, we investigated the effect of the modulation of the NO pathway in human omental AT (OAT) through PDE-5 inhibition, focusing on the expression of the key genes involved in mitochondria biogenesis.

Key-words: Adipose tissue, nitric oxide, cyclic-guanylyl monophosphate, type 5-phosphodiesterase, mitochondrial biogenesis.

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MATERIALS AND METHODS

Cell cultures

Samples of human OAT were obtained from 5 male patients (mean age 62±4 yr), undergoing elective surgical correction at the Geriatric Surgery Clinics of the University Hospital of Padua of umbilical hernia or laparocele, pathologies classically reducing physical activity. Exclusion criteria were absence of cancer, metabolic disorders, and a therapy with oral anticoagulants, thiazolidinediones, PDE5 inhibitors, NO-donors. The study was approved by the local Ethics Committee. Patients were treated in accordance with the principles of the Declaration of Helsinki.

Surgical samples (4-5 g) of OAT were purged, minced under sterile conditions and placed in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1.5% BSA and antibiotic/antifungal mixture (penicillin G/streptomycin/amphotericin B). Mature adipocytes were the main constituents of OAT (>95%). A solution of PDE-5 inhibitor, vardenafil (Bayer, Milano, Italy) in Dimethyl sulfoxide (DMSO) (Sigma Aldrich, Milano, Italy) was added to a final concentration ranging from 10^{-12}M to 10^{-8}M. The NO donor DETA NONOate (Cayman Chemicals purchased by INALCO, Milano, Italy) was employed as positive control at the final concentration of 10^{-5}M (12). Considered the very small amount of OAT obtainable from each patient, we decided to focus specifically on rapid (24 h) and long-term (72 h) stimulation for the analysis of the variation gene expression profile.

Tissue fragments were stimulated up to 72 h and medium containing vehicle (DMSO, negative control), PDE-5 inhibitor or NO donor was changed every 24 h. Experimets were performed in duplicate. Finally OAT samples were harvested and stored at –80 C until total RNA and DNA extraction.

Quantitative real-time PCR

Total RNA from up to 200 mg of OAT tissue was isolated with the RNeasy Mini kit (Qiagen, Milan, Italy) after separation of aqueous phase from lipid phase with RNA Bee reagent (Duotech, Milan, Italy). The amount of RNA isolated (ranging from 3 to 5 µg of total RNA from each sample) was determined by spectrometry at 260 nm with a Nanodrop 1000 (Euroclone, Milan, Italy). Total RNA was used for first-strand cDNA synthesis using the Superscript III Reverse Transcriptase (Life Technologies, Milan, Italy) and Random Hexamers (Life Technologies) according to the manufacturer's instructions. cDNA were tested by PCR using specific oligonucleotide primers for the housekeeping gene β-actin.

Different sets of primers (intron spanning) were used to quantify mRNA expression level of *PPAR-γ*, *adiponectin*, and *PGC-1α* by quantitative real time PCR with SYBR Green (Life Tecnologies). Melt curves were performed for the Sybr Green analysis. The cDNA was amplified and quantified using a Step One plus Real Time PCR System (Life Tecnologies). Data were normalized to Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as internal housekeeping gene.

To estimate the mitocondrial DNA (mtDNA) copy number we used the previously reported set of primers and probes for *APP* and *CO II* used by Malena et al. (13) on genomic DNA extracted with the QIAamp DNA Blood mini kit (Qiagen). Experiments were performed 3 times in triplicate. Data are shown as the mean±SD of the mean of 3 different experiments performed in triplicate. Data elaboration was performed as relative quantification analysis using the ΔΔcycle-threshold method.

Statistical analysis

All statistics were performed using SPSS software (version 15.1; SPSS Inc. Chicago, Illinois, USA). Student's t-test and analysis of variance were used to compare means of gene expression and mtDNA analysis. The significance level was set to p=0.05. Variables are given as mean±SD.

RESULTS

After 24 h, NO donor induced a significant increase of the expression of *PPARγ* in OAT compared to control samples (p<0.05) (Fig. 1A). Vardenafil stimulation induced a significant increase of this gene at the concentrations of 10^{-12}M and 10^{-10}M compared to controls (p<0.05) (Fig. 1A-I). The same behavior was observed for *adiponectin* gene expression compared to control samples (p<0.05) (Fig. 1A-II). After 72-h treatment, no difference in gene expression was found between control and all treated samples (data not shown).

NO donor induced a significant increase of the expression of *PGC-1α* both after 24 and 72 h in OAT compared to controls (p<0.05) (Fig. 1B-I), whereas vardenafil stimulation induced a significant increase of this gene only after 72 h at 10^{-12}M concentration (p<0.05) (Fig. 1B-I).

mtDNA content showed no significant variation for all conditions tested after 24 h (Fig. 1B-II). On the other hand, a significant increase of mtDNA was observed after a 72-h treatment with NO donor and vardenafil at 10^{-12}M and 10^{-10}M compared to control samples (p<0.05) (Fig. 1B-II).

DISCUSSION

In this study, we have reported the effect of the treatment of whole human OAT with a specific PDE-5 inhibitor, vardenafil, showing an up-regulation of *PPAR-γ*, *adiponectin*, and *PGC-1α* after 24 h and of mtDNA after 72 h at lower dosages. PDE-5 inhibitors, that ultimately increase intracellular cGMP concentration, are worldwide employed for the treatment of erectile dysfunction (ED). Endothelial dysfunction, atherosclerosis, metabolic, and hormonal abnormalities play a central role in the pathogenesis of ED (14). On the other side, ED represents an early marker of several clinical conditions such as hypertension, cardiovascular disease, diabetes mellitus, and mainly metabolic syndrome and obesity. The administration of PDE-5 inhibitors in patients classically presenting a high incidence of metabolic derangements prompted us to investigate on the effect of the modulation of the NO pathway in human OAT.

PPAR-γ is a key transcription factor, up-regulated during adipogenesis, that modulates the expression of several downstream genes typical of mature adipocytes, such as glucose transporter *GLUT-4*, and *adiponectin* (15). On

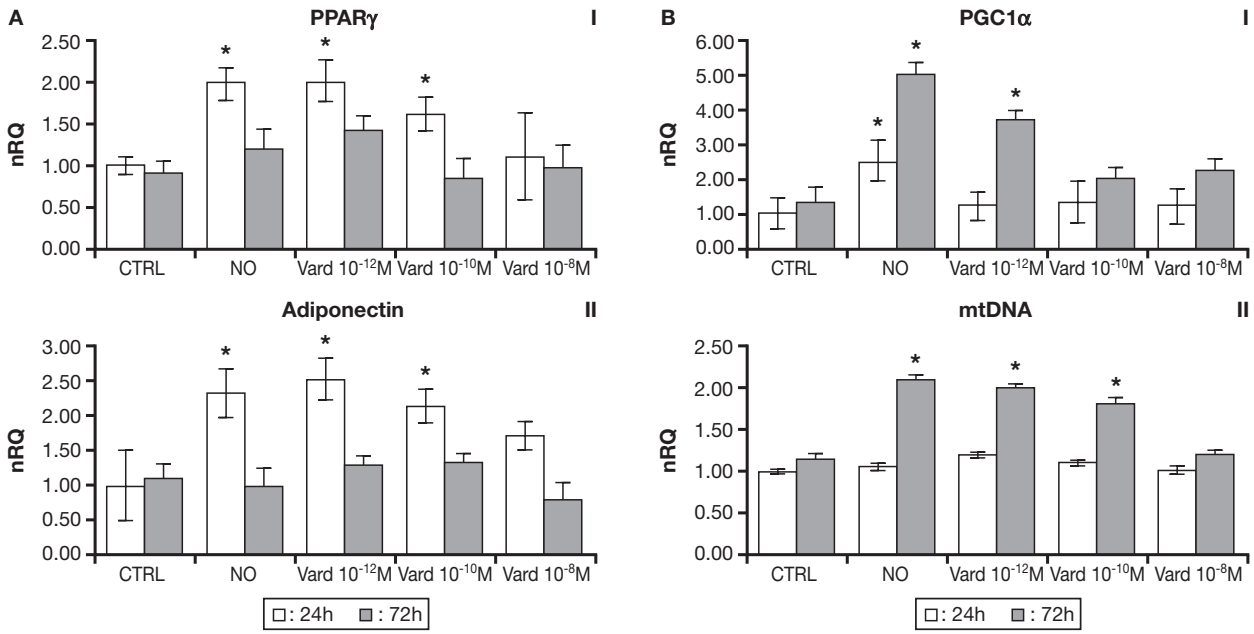

Fig. 1 - *Gene expression analysis and quantification of mitochondrial DNA (mtDNA) in human omental adipose tissue stimulated* in vitro *with vardenafil at concentration ranging from 10^{-12}M to 10^{-8}M. In negative control (CTRL) any stimulation was omitted whereas in the positive control (NO), nitric oxide donor was added at the final concentration of 10^{-5}M. A) Gene expression analysis of peroxisome proliferator-activated receptor γ (*PPARγ*) (I) and* adiponectin *(II) after 24 h (white bars) and 72 h (black bars) of stimulation. Data are expressed as normalized relative quantity (nRQ). Significance* *p<0.05 *compared to time-matched control. B) Gene expression analysis of PPAR-γ coactivator-1 a (*PGC-1α*) (I) and quantification of mitochondrial DNA (II) after 24 h (white bars) and 72 h (black bars) of stimulation. Data are expressed as nRQ. Significance* *p<0.05 *compared to time-matched control.*

the other side, *adiponectin* is an adipokine acting as strong positive modulator of insulin sensitiveness of peripheral tissues (15, 16). *PGC-1α* gene expression and *mtDNA* are, instead, both markers associated with an increased mitochondrial biognenesis. Moreover, mitochondrial function has been linked to adiponectin synthesis in adipocytes and mitochondrial dysfunction in AT may explain decreased plasma adiponectin levels in obesity (17). eNOS system plays, in fact, an important role in adiponectin synthesis in adipocytes by increasing mitochondrial biogenesis and enhancing mitochondrial function (18). Our data suggest that PDE-5 inhibition partially reflects the direct stimulation of OAT with NO, obtained by the use of an NO-donor, that similarly stimulates *PPAR-γ* (10), the master regulator of adipogenesis, *PGC-1α*, the master regulator of mitochondrial biogenesis, and *adiponectin*, the most important insulin-sensitizing adipokine. However, we cannot exclude that PDE-5 inhibition could act through different pathways. In fact, it has been demonstrated in L6E9 myoblasts that reduction of intracellular levels of reactive oxygen intermediates (ROI), such as superoxyde, can act as molecular switch detouring G1-phase cells towards differentiation instead of cell growth and division (19). Enhanced levels of cGMP are known to impair NADPH-oxidase expression, one of the main enzymes involved in superoxyde production (20). It could alternatively be speculated that PDE-5 inhibition could reduce ROI levels in an indirect manner, inducing adipocyte differentiation, featured by increased *PPAR-γ* expression, and improving mitochondrial toxicity exerted by ROI (21). Furthermore, in our study vardenafil, exerted its action at doses much lower (10^{-12}-10^{-10} M) that those showed by Zhang et al. with sildenfil in mouse mesenchymal cell-derived adipocytes (10). This discrepancy could be likely due to the higher affinity of vardenafil for PDE-5 (22) and to the use of 3-isobutyl-1-methyl-xanthine (IBMX) as PDE inhibitor for adipocyte differentiation, forcing to higher doses of sildenafil to evidence any effect on cultured cells. Finally, our findings are also in accordance with previous data from animal experimental studies, where mitochondrial biogenesis was increased in cultured adipocytes from transgenic mice undergoing an hyper-activation of NO pathway through the blockade of cannabinoid receptor (23). The NO acts through the second messanger cGMP and the subsequent increase of oxidative phosphorylation has profound consequences on energy metabolism (6). Recent studies performed on animal models revealed that the hyper-production of cGMP, obtained in transgenic mice through the hyper-expression of NO and the consequent constant activation of GC, leads to protection from weight gain and to an increased oxygen consumption under high fat diet (24), but the significance of these effects is not clearly understood in humans. On these basis, the intriguing hypothesis that the modulation of the NO pathway in human AT could partially modulate energy metabolism remains to be further investigated both *in vitro*, in particular on function of isolated mitochondria, and *in vivo* experiments (25).

Besides, the lack of data on protein-expression and on

the use of a specific cGMP-dependent kinase inibitor, the heterogeneity and reduced number of patients represent the main limitation of this study. Further studies will be necessary to evaluate the proper extensibility of this data. Our results add new elements in the comprehension of AT physiology and open new cues for the pharmacological modulation of AT function.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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