ONLINE APPENDIX

Cannabinoid receptor stimulation impairs mitochondrial biogenesis in mouse white adipose tissue, muscle, and liver: the role of eNOS, p38 MAPK, and AMPK pathways

Laura Tedesco,1,2 Alessandra Valerio,1,3 Marta Dossena,1,3 Annalisa Cardile,1 Maurizio Ragni,1 Claudio Pagano,4 Uberto Pagotto,5 Michele O. Carruba,1,2 Roberto Vettor,4 and Enzo Nisoli1,2

1Integrated Laboratories Network, Center for Study and Research on Obesity, Department of Pharmacology, Chemotherapy and Medical Toxicology, School of Medicine, University of Milan, Milan, Italy; 2Istituto Auxologico Italiano, Milan, Italy; 3Department of Biomedical Sciences and Biotechnologies, University of Brescia, Brescia, Italy; 4Endocrine-Metabolic Laboratory, Internal Medicine, Department of Medical and Surgical Sciences, University of Padova, Padova, Italy; 5Endocrinology Unit, Department of Internal Medicine and Gastroenterology, and Center for Applied Biomedical Research S. Orsola-Malpighi Hospital, Alma Mater Bologna University, Bologna, Italy.
SUPPLEMENTAL DATA

Figure S1.

The endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) decrease mitochondrial biogenesis in cultured mouse white adipocytes. A: mtDNA amount, analyzed by means of quantitative PCR in white adipocytes treated either with vehicle (0.002% DMSO) or different doses of endocannabinoids for 2 days. Values of the vehicle-treated cells were taken as 1.0 (n = 3 experiments). B: PGC-1α, NRF-1, and Tfam mRNA levels were analyzed by means of quantitative RT-PCR. The cycle number at which the various transcripts were detectable was compared to that of β-actin, and expressed as relative expression versus values in vehicle-treated cells taken as 1.0 (n = 3 experiments). C: COX IV and Cyt c protein levels detected by immunoblot with densitometric analysis referred to β-actin, with values from vehicle-treated cells taken as 1.0 (n = 3 experiments). D: Citrate synthase activity (nmol citrate/min/mg protein) and E: oxygen consumption (nmol O₂/min/mg protein) were expressed as fold-change vs. vehicle-treated cells taken as 1.0 (n = 3 experiments). A-E: *P < 0.05 and **P < 0.01 vs. vehicle-treated cells. All data represent mean ± SEM.
Figure S2.

Low concentrations of ACEA do not affect mitochondrial biogenesis in mouse white adipocytes. A: PGC-1α, NRF-1, and Tfam mRNA levels were analyzed by means of quantitative RT-PCR white adipocytes treated either with vehicle (0.002% DMSO, Veh) or ACEA for 2 days. The cycle number at which the various transcripts were detectable was compared to that of β-actin, and expressed as relative expression versus values in vehicle-treated cells taken as 1.0 ($n = 5$ experiments). B: mtDNA amount, analyzed by means of quantitative PCR and expressed as mtDNA copy number per nuclear DNA copy number. All data represent mean ± SEM.
The CB1 receptor agonist ACEA decreases mitochondrial biogenesis in human white adipocytes. 

**Figure S3.**

The CB1 receptor agonist ACEA decreases mitochondrial biogenesis in human white adipocytes. 

**A:** PGC-1α, NRF-1, and Tfam mRNA levels were analyzed by means of quantitative RT-PCR in visceral and subcutaneous white adipocytes treated either with vehicle (0.002% DMSO, Veh) or 0.01 μM ACEA for 2 days. The cycle number at which the various transcripts were detectable was compared to that of β-actin, and expressed as relative expression versus values in vehicle-treated cells taken as 1.0 (n = 5 experiments). *P < 0.05 and **P < 0.01 vs. vehicle-treated cells. 

**B:** mtDNA amount, analyzed by means of quantitative PCR and expressed as mtDNA copy number per nuclear DNA copy number. **P < 0.01 vs. vehicle-treated cells. 

**C:** COX IV and Cyt c proteins were detected by immunoblot analysis. The relative values from the densitometric analysis are referred to β-actin levels; vehicle-treated cell measurement is given a value of 1.0. **P < 0.01 vs. vehicle-treated cells. 

**D**: Citrate synthase activity (nmol citrate/min/mg protein) and **E:** oxygen consumption (nmol O2/min/mg protein) were expressed as fold-change vs. vehicle-treated cells taken as 1.0 (n = 3 experiments). **P < 0.01 vs. vehicle-treated cells. All data represent mean ± SEM.

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Figure S4.
CB1 and CB2 receptor expression in mouse white adipocytes. CB1 and CB2 receptor mRNA levels were analyzed by means of quantitative RT-PCR in white adipocytes treated either with vehicle (0.002% DMSO, Veh) or different doses of ACEA for 2 days. The cycle number at which the various transcripts were detectable was compared to that of β-actin, and expressed as relative expression versus values in vehicle-treated cells taken as 1.0 (n = 5 experiments). **P < 0.01 vs. vehicle-treated cells. All data represent mean ± SEM.
TRPV1 activation is not involved in ACEA-mediated decrease of mitochondrial biogenesis in cultured mouse white adipocytes.  

A: TRPV1 expression in white adipocytes transfected with either TRPV1 siRNA or non-targeting (NT) siRNA. *** $P < 0.001$ vs. vehicle-treated cells.  

B-E: mitochondrial biogenesis parameters in white adipocytes transfected with either TRPV1 siRNA or NT-siRNA and treated with vehicle (0.002 % DMSO, Veh) or with ACEA for 2 days.  

B: eNOS expression and C: PGC-1α, NRF-1, and Tfam expression. Relative expression values of the vehicle-treated cells were taken as 1.0 ($n = 5$ experiments; ** $P < 0.01$ vs. vehicle-treated cells).  

D: mtDNA amount and E: citrate synthase activity. ** $P < 0.01$ vs. vehicle-treated cells. All data represent mean ± SEM.
Figure S6.

ACEA reduces AMPK phosphorylation in cultured mouse white adipocytes. AMPK phosphorylation in primary white adipocytes exposed to vehicle (0.002% DMSO) or ACEA for 48 h (one experiment representative of three reproducible ones. Relative values from the densitometric analysis (phospho-AMPK/total AMPK) are shown below the blots, with vehicle values taken as 1.0.
Figure S7.

CB1 receptor stimulation down-regulates mitochondrial biogenesis in the tibialis muscle of obese mice. A: eNOS mRNA, analyzed by means of quantitative RT-PCR, in tibialis muscle of both ACEA- and vehicle-treated mice on chow regular diet or HFD (n = 8 animals; **P < 0.01 vs. vehicle-treated mice on chow regular diet, and †P < 0.05 vs. vehicle-treated mice on HFD). B-C: PGC-1α, NRF-1, Tfam mRNA levels and mtDNA amount, in muscle of both ACEA- and vehicle-treated mice on chow regular diet or HFD (n = 8 animals; **P < 0.01 vs. vehicle-treated mice on chow regular diet, and †P < 0.05 vs. vehicle-treated mice on HFD). D: COX IV and Cyt c protein levels detected by immunoblot with densitometric analysis (n = 3 experiments; **P < 0.01 vs. vehicle-treated mice on chow regular diet and †P < 0.05 vs. vehicle-treated mice on HFD). E: Citrate synthase activity (n = 3 experiments; **P < 0.01 vs. vehicle-treated mice on chow regular diet and †P < 0.05 vs. vehicle-treated mice on HFD). F: p38 MAPK phosphorylation in muscle of vehicle- and ACEA-treated mice on HFD. G: AMPK phosphorylation in muscle of vehicle- and ACEA-treated mice on HFD. All data represent mean ± SEM.
**Figure S8.**

CB1 receptor stimulation down-regulates mitochondrial biogenesis in the liver of obese mice. 

*Figure S8.* CB1 receptor stimulation down-regulates mitochondrial biogenesis in the liver of obese mice. *A:* eNOS mRNA, analyzed by means of quantitative RT-PCR, in liver of both ACEA- and vehicle-treated mice on chow regular diet or HFD (*n* = 8 animals; **P* < 0.01 vs. vehicle-treated mice on chow regular diet, and †*P* < 0.05 vs. vehicle-treated mice on HFD). *B-C:* PGC-1α, NRF-1, Tfam mRNA levels and mtDNA amount, in liver of both ACEA- and vehicle-treated mice on chow regular diet or HFD (*n* = 8 animals; **P* < 0.01 vs. vehicle-treated mice on chow regular diet, and †*P* < 0.05 vs. vehicle-treated mice on HFD). *D:* COX IV and Cyt c protein levels detected by immunoblot with densitometric analysis (*n* = 3 experiments; **P* < 0.01 vs. vehicle-treated mice on chow regular diet and †*P* < 0.05 vs. vehicle-treated mice on HFD). *E:* Citrate synthase activity (*n* = 3 experiments; **P* < 0.01 vs. vehicle-treated mice on chow regular diet and †*P* < 0.05 vs. vehicle-treated mice on HFD). *F:* p38 MAPK phosphorylation in liver of vehicle- and ACEA-treated mice on HFD. *G:* AMPK phosphorylation in liver of vehicle- and ACEA-treated mice on HFD. All data represent mean ± SEM.