Activation of Cannabinoid Receptors Attenuates Endothelin-1-induced Mitochondrial Dysfunction in Neonatal Rat Ventricular Myocytes

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Supplemental Data File

1. Optimization of working conditions for mitochondrial bioenergetics assays.

Mitochondrial bioenergetics of cardiac myocytes were assessed in the presence of glucose/pyruvate and palmitate as major substrates. It was important to first determine the optimal conditions for mitochondrial bioenergetics assays. Therefore, the glucose/pyruvate model was used to optimize cell seeding density and oligomycin concentration. Furthermore, as the maximal effective concentration of FCCP may vary depending on the different energy substrate provided [1], optimal concentrations of FCCP were ascertained for both glucose and palmitate.

Primary neonatal rat cardiac myocytes were seeded into 24-well X24 microplates with test densities: 100,000, 200,000, 300,000 and 400,000 cells/well. As shown in Figure S1, oxygen consumption rates (OCRs) declined with increases in cell density. In addition, Hill et al. detected a linear increase in OCR in neonatal rat ventricular myocytes within a range of 25,000 to 75,000 cells/well [2]. Thus, OCR peaks at a seeding density of 75,000-100,000 cells/well, whereas underseeding or overseeding impacts the accuracy of OCR measurements. Here, 100,000 cells/well was chosen for future experiments.

Figure S1.

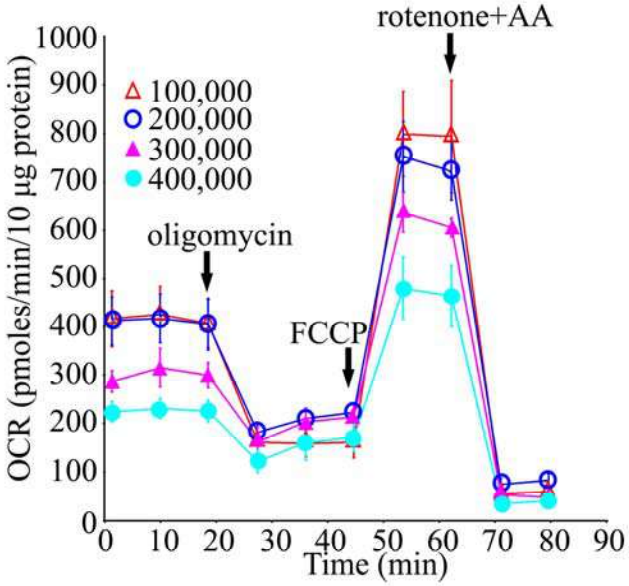

Figure S1. OCR were measured using the Seahorse Bioscience XF24 analyzer in cultured neonatal rat cardiac myocytes at cell densities ranging from 100,000 to 400,000 myocytes per well. Basal OCRs were measured in media containing 10 mM glucose and 1 mM pyruvate, followed by the sequential addition of oligomycin (1 µg/mL), then FCCP (2 µM) and R/AA (rotenone, 1 µM and antimycin A, 1 µM). The results are presented as the OCR per 10 µg protein. A cell density of 100,000 myocytes per well resulted in the maximal protein-normalized OCR. n=5 replicate cultures (5 replicate wells/n).

Increasing concentrations of oligomycin were titrated with glucose/pyruvate as provided substrates. OCR plots were presented as percent values normalized to basal levels (basal OCR was set as a baseline of 100%) (Figure S2). Addition of increasing concentrations of oligomycin (0.5, 1.0, 5.0 and 10 µg/mL) reduced OCRs to similar degrees. A working oligomycin concentration of 1 µg/mL was selected for subsequent experiments.

Figure S2.

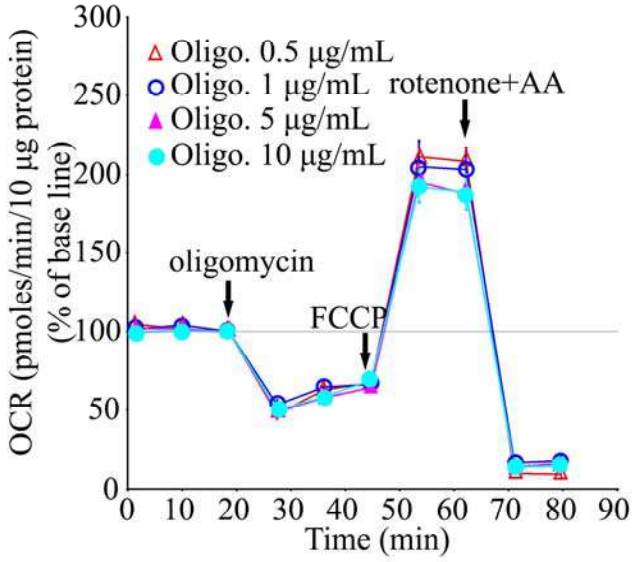

Figure S2. OCRs of cultured myocytes at basal levels were measured using the Seahorse Bioscience XF24 Analyzer in media containing 10 mM glucose and 1 mM pyruvate, followed by the sequential addition of increasing concentrations of oligomycin (0.5-10 µg/mL), then FCCP (2 µM) and R/AA (rotenone, 1 µM and antimycin A, 1 µM). Results are presented as the percent values of basal OCR. There were no concentration-dependent differences in oligomycin-induced OCR reductions between groups. Oligomycin at 1 µg/mL was chosen for subsequent experiments. n=5 replicate cultures (5 replicate wells/n).

Effective concentrations of FCCP vary according to cell type and energy substrate [1-4]. Hence, it is important to determine the optimal concentration applicable to cardiac myocytes in the context of glucose or palmitate as substrate. Increasing concentrations of FCCP were tested in the presence of either palmitate/BSA or glucose/pyruvate. As shown in Figure S3A, during glucose oxidation, FCCP concentrations ranging from 1 µM to 10 µM did not generate significant differences, except perhaps only a minor increase at 2 µM. In contrast, the effect of FCCP on fatty acid oxidation was concentration-dependent, and 10 µM achieved the maximal response (Figure S3 B). Therefore, 2 µM and 10 µM of FCCP were selected for glucose- and fatty acid-dependent OCR respectively.

Figure S3.

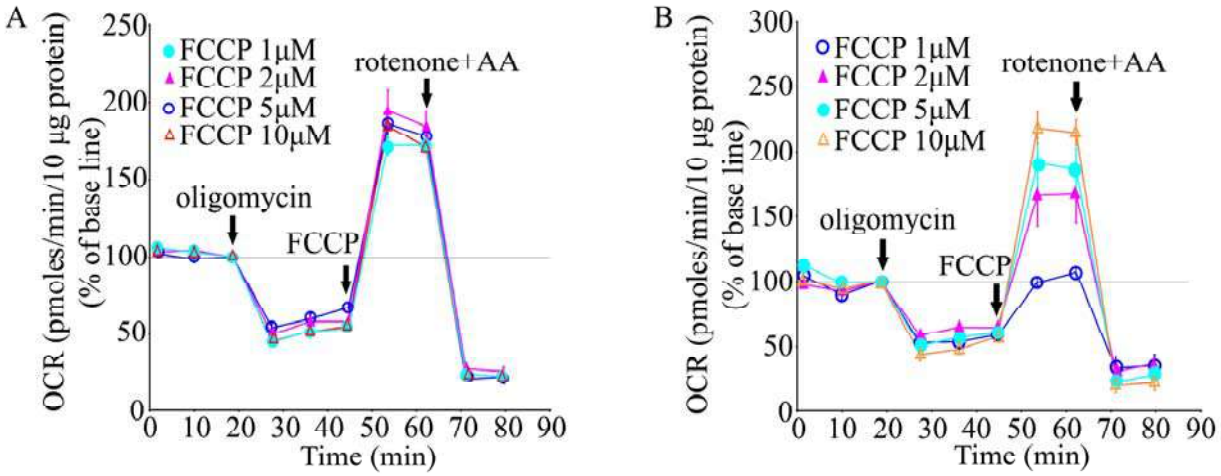

Figure S3. OCRs of cultured myocytes at basal levels were measured using the Seahorse Bioscience XF24 Analyzer in media containing *A)* glucose (10 mM)/pyruvate (1 mM) and *B)* palmitate/BSA (200 µM), followed by the sequential addition of oligomycin (1 µg/mL), FCCP (1-10 µM) and R/AA (rotenone, 1 µM and antimycin A, 1 µM). Results are presented as the percent of basal OCR. When glucose/pyruvate were provided as the primary substrates, differences between groups are subtle; FCCP of 2 µM gives an apparent maximal rate of OCR. Whereas when palmitate/BSA was provided as the major substrate, FCCP at 10 µM gives a maximal response of increased OCR. n=5 replicate cultures (5 replicate wells/n).

2. Determination of fatty acid oxidation-associated OCR in cultured neonatal rat cardiac myocytes.

OCR was measured using the Seahorse Bioscience XF24 analyzer to determine the capacity of mitochondrial respiration. Cultured neonatal rat cardiac myocytes were exposed to palmitate/BSA conjugates to facilitate the study of fatty acid-dependent respiration. To verify that responses were due to utilization of exogenous palmitate by mitochondria, subgroups were pretreated with etomoxir (40 μM) 15 min prior to the assay to inhibit CPT-1. As CPT-1 facilitates fatty acid transport across the mitochondrial membrane [5], etomoxir would inhibit oxidation of exogenous fatty acids. BSA, the carrier of palmitate, was injected to the cells in the presence or absence of etomoxir, and served as negative control. As shown in Figure S4, the addition of etomoxir dramatically inhibited palmitate-related OCR, and BSA only generated negligible OCR. These data confirm that here, oxygen consumption was due to oxidation of exogenous palmitate.

Figure S4.

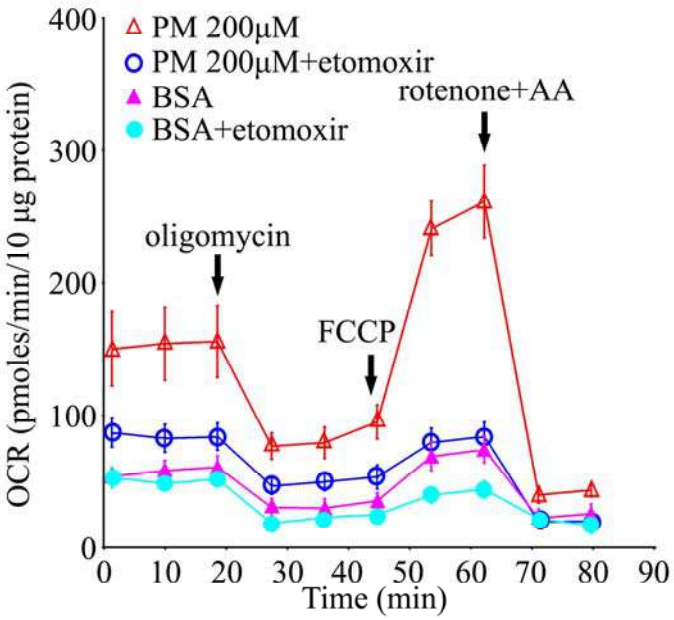

Figure S4. OCRs at basal levels were measured in the Seahorse Bioscience XF24 Analyzer in cultured myocytes (100,000 cells per well) with palmitate/BSA complex (PM, 200 μM) or BSA (vehicle carrier) as the substrate, in the absence or presence of etomoxir (inhibits CPT-1 and therefore mitochondrial fatty acid uptake), followed by the sequential addition of oligomycin (1 μg/mL), FCCP (2 μM) and R/AA (rotenone, 1 μM and antimycin A, 1 μM). The difference between PM (Δ) and PM + etomoxir (○) suggests that exogenous fatty acids were utilized as substrate. n=5 replicate cultures (5 replicate wells/n).

3. Determination of AMPKα1/2 knockdown in cultured neonatal rat cardiac myocytes.

CB-13 activated AMPKα by increasing phosphorylation at Thr172; therefore, we queried the role of AMPK in CB-13-mediated mitochondrial protective effects. To ascertain the role of AMPK, neonatal rat cardiac myocytes were infected for 24 h with lentiviral vectors expressing shRNA against AMPKα1 and α2, followed by 72 h incubation. Degree of knockdown was confirmed by western blotting. As shown in Figure S5, this achieved significant, simultaneous reductions of AMPKα1 and α2.

Figure S5.

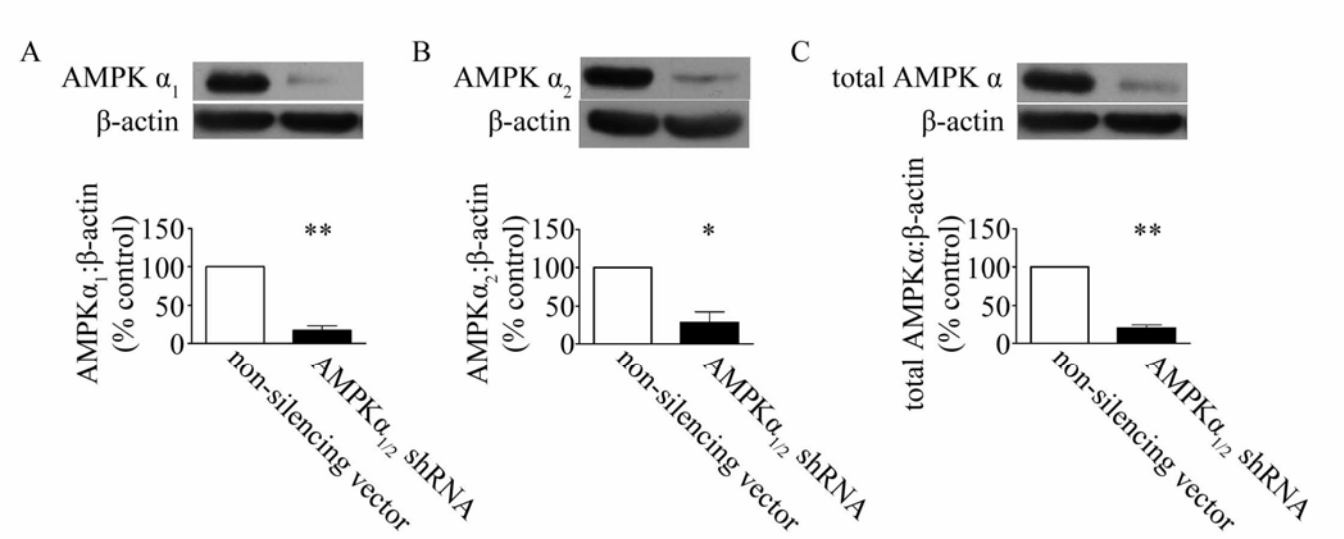

Figure S5. Myocytes were infected with lentivirus carrying shRNA against AMPK α1 and α2 or non-silencing empty vector. 72 h post-infection, *A,* AMPKα1, B, AMPKα2, and C, total AMPKα were measured by conventional western blotting of total cell lysates. Results are presented as percent of normalized protein *vs.* empty vector-treated controls. n=3 (≥3 replicates per n-value). *p<0.05 and **p<0.01 *vs*. non-silencing vector treated control (open bars).

4. Quantification of cannabinoid receptor protein concentration in neonatal rat cardiomyocytes.

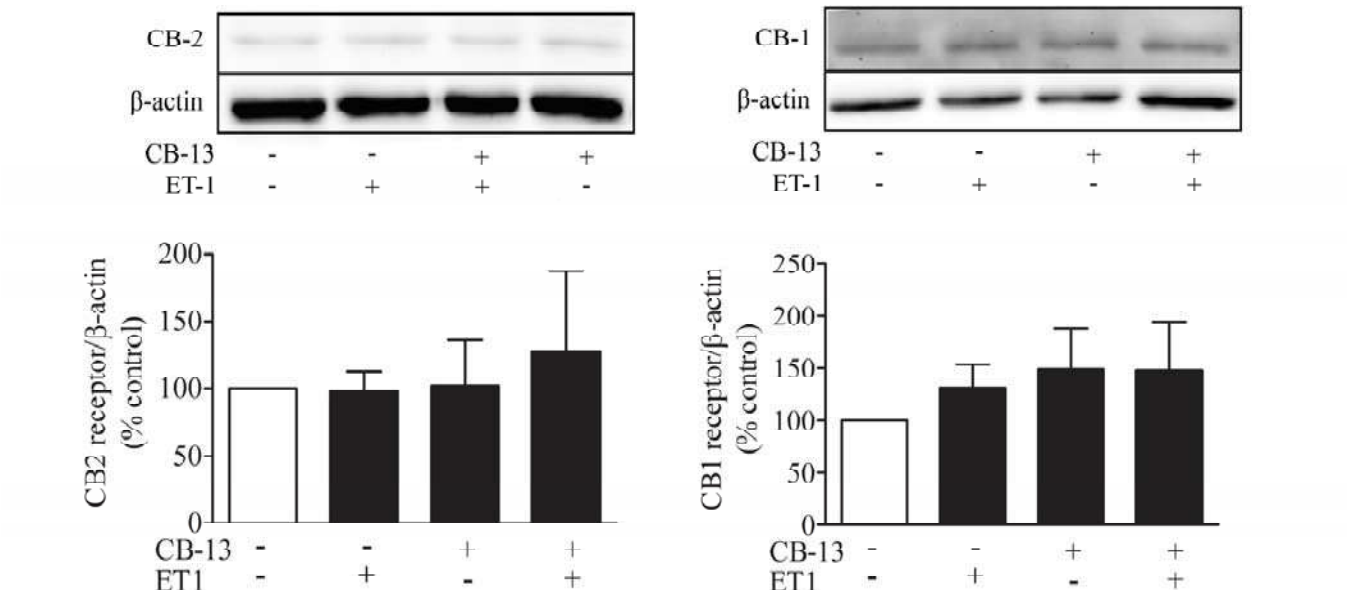

Figure S6. Myocytes were exposed to ET1 (0.1 μM; 4 h) in the presence or absence of vehicle or CB-13 (1 μM). *A*, representative blot, *B*, CB1 and *C*, CB2 receptor expression following CB-13 (1 μM) and ET1 (0.1 μM) treatment in neonatal rat cardiomyocytes. CB1 and CB2 levels were measured by conventional western blotting of total cell lysates and were not significantly different. n=3 (≥3 replicates per n-value).

5. Schematic of events as elucidated from experimental NRVM's results.

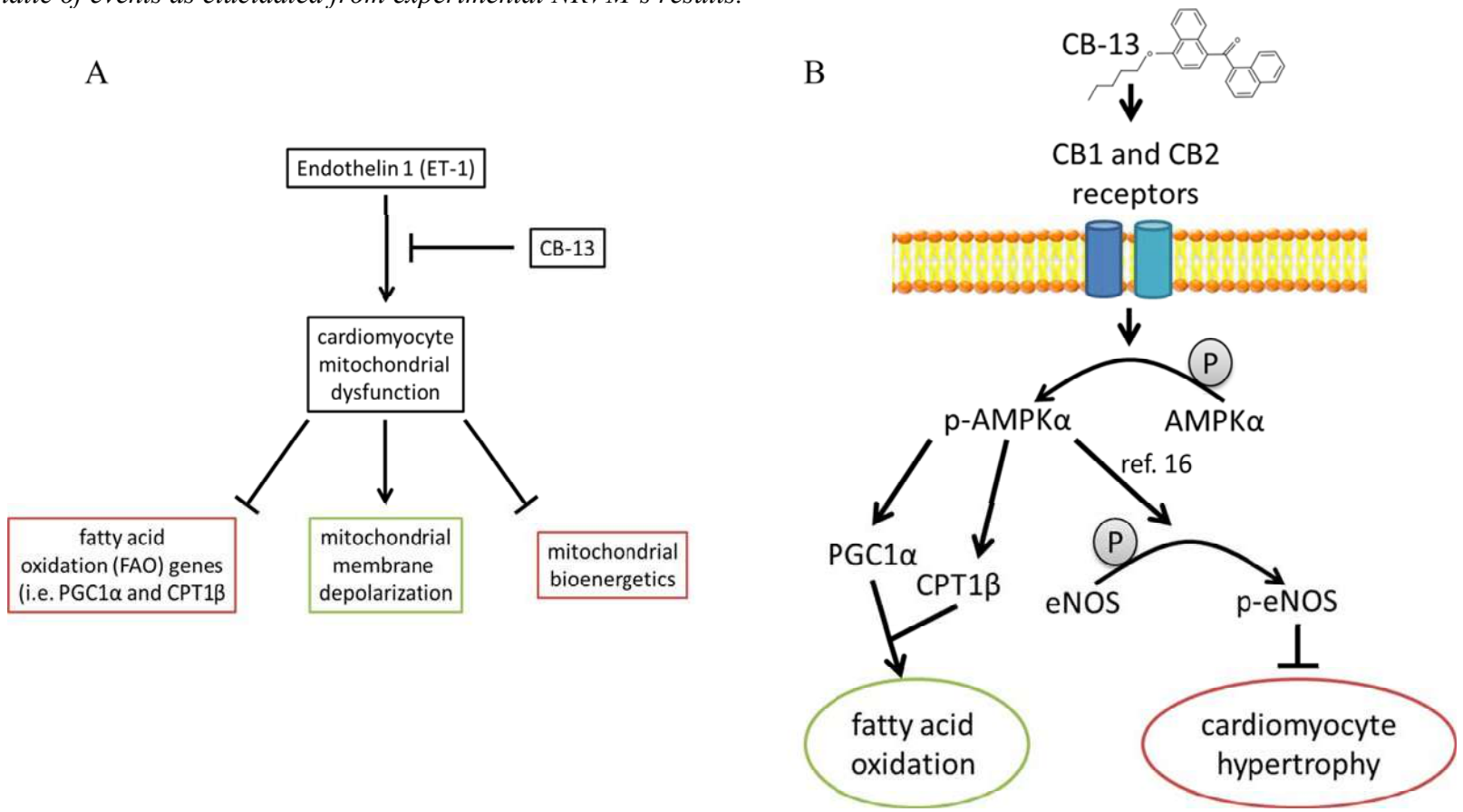

Figure S7. Schematic representation of putative bioenergetic and signaling events triggered by *A,* endothelin-1 (ET-1) and *B*, CB-13 in neonatal rat cardiac myocytes.

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