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Cannabidiol protects C2C12 myotubes against cisplatin-induced atrophy by regulating oxidative stress

Running title: CBD prevents cisplatin-induced muscle atrophy in vitro

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32 **ABSTRACT**

33 Cancer and chemotherapy induce a severe loss of muscle mass (known as cachexia), which
34 negatively impact cancer treatment and patient survival. The aim of the present study was to
35 investigate whether CBD administration may potentially antagonize the effects of cisplatin in
36 inducing muscle atrophy, using a model of myotubes in culture. Cisplatin treatment resulted in
37 a reduction of myotube diameter (15.7 ± 0.3 vs. 22.2 ± 0.5 μm , $p<0.01$) that was restored to control
38 level with $5\mu\text{M}$ CBD (20.1 ± 0.4 μM , $p<0.01$). Protein homeostasis was severely altered with a
39 $\approx 70\%$ reduction in protein synthesis ($p<0.01$) and a 2-fold increase in proteolysis ($p<0.05$) in
40 response to cisplatin. Both parameters were dose dependently restored by CBD co-treatment.
41 Cisplatin treatment was associated with increased TBARS content (0.21 ± 0.03 to 0.48 ± 0.03
42 nmol/mg prot , $p<0.05$), catalase activity (0.24 ± 0.01 vs. 0.13 ± 0.02 $\text{nmol/min}/\mu\text{g prot}$, $p<0.01$),
43 whereas CBD co-treatment normalized TBARS content to control values (0.22 ± 0.01 nmol/mg
44 prot , $p<0.01$) and reduced catalase activity (0.17 ± 0.01 $\text{nmol/min}/\mu\text{g prot}$, $p<0.05$). These
45 changes were associated with increased mRNA expression of GPX1, SOD1, SOD2 and CAT
46 mRNA expression in response to cisplatin ($p<0.01$), which was corrected by CBD co-treatment
47 ($p < 0.05$). Last, cisplatin treatment increased the mitochondrial protein content of NDUFB8,
48 UQCRC2, COX4 and VDAC1 (involved in mitochondrial respiration and apoptosis), and CBD
49 co-treatment restored their expression to control values. Altogether, our results demonstrated that
50 CBD antagonizes the cisplatin-induced C2C12 myotube atrophy and could be used as an
51 adjuvant in the treatment of cancer cachexia to help maintain muscle mass and improve patient
52 quality of life.

53

54 **New and noteworthy:** In an in vitro model, cisplatin treatment led to myotube atrophy
55 associated with dysregulation of protein homeostasis and increased oxidative stress, resulting
56 in increased apoptosis. Co-treatment with cannabidiol was able to prevent this phenotype by
57 promoting protein homeostasis and reducing oxidative stress.

58

59 **KEYWORDS**

60 cachexia, endocannabinoid system, protein homeostasis, oxidative stress, mitochondrial
61 function

62

63 INTRODUCTION

64 Cachexia is a multifactorial wasting syndrome characterized by a severe involuntary loss of
65 body weight (i.e. more than 5% body weight loss in the last 12 months or less), loss of skeletal
66 muscle mass (with or without loss of fat mass), anorexia, and dysregulated energy and protein
67 metabolism (1). Prevalence of cachexia varies with cancer type, from 15% in prostate cancer
68 up to 60% in pancreatic cancer (2). Several studies have clearly demonstrated that survival
69 times are shorter in patients who have experienced weight loss than in those who have not.
70 Weight loss is not only predictive of survival but also of response to chemotherapy (3). One of
71 the mechanisms that has been advanced to explain why patients with cachexia have poorer
72 survival is their higher incidence of complications related to surgical, radiotherapeutic and
73 chemotherapeutic treatments (4). Cachexia is associated with increased fatigue and frailty,
74 reduced physical activity leading to loss of autonomy, and decreased quality of life (5, 6). In
75 some cases, the impact of cachexia is severe enough to necessitate chemotherapy dose
76 reductions, treatment postponements or permanent discontinuation, in which case patients who
77 lose weight do not get the full potential benefit of their cancer therapy (4, 7). Maintaining
78 skeletal muscle mass in cancer patients is therefore crucial to their management, improved
79 response to associated therapies, and improved quality of life. There are no formal guidelines
80 for the management of cancer-related muscle wasting, but an effective strategy should aim to
81 reduce muscle wasting in order to promote survival in patients with advanced cancer (8). One
82 straightforward way to do this is through nutrition (9).

83 Cancer treatment often involves chemotherapy, which itself contributes to the development and
84 progression of muscle atrophy and weakness in treated patients and preclinical models (10-13).
85 Chemotherapy has been found to reduce muscle mass index and strength in lung and breast
86 cancer patients (10), and cisplatin treatment in head and neck cancer patients decreased muscle
87 strength measured by chair-lift and arm flexion tests (11). This same type of muscle dysfunction
88 is also observed in preclinical models of cancer and chemotherapy. For example, in mice,
89 implantation of C26 colonic tumor or lung carcinoma led to muscle atrophy associated with a
90 decrease in mitochondrial respiration (14) and an alteration of the processes regulating
91 mitochondrial biogenesis and dynamics (fusion/fission) (15). Similarly, treatments using
92 cisplatin or doxorubicin, two widely-used chemotherapy agents, increased muscle
93 mitochondrial dysfunction by altering mitochondrial biogenesis and dynamics (16-18). These
94 data are consistent with the mitochondrial dysfunction, decreased mitochondrial content, and
95 alterations in mitochondrial dynamics that are well-documented in cancer patients (19). As a

96 result, damaged mitochondria accumulate in skeletal muscle and, in addition to being less
97 bioenergetically efficient, promote oxidative stress through increased production of reactive
98 oxygen species (ROS). In mice, administration of doxorubicin or cisplatin leads to an increase
99 in ROS production (16-18). Mitochondrial dysfunction and increased oxidative stress disrupt
100 protein turnover pathways, leading to decreased protein synthesis and increased activity of
101 muscle proteolytic systems (proteasome, autophagy) (12, 20), ultimately resulting in muscle
102 fiber atrophy (16, 17).

103 The endocannabinoid system is a major molecular system responsible for controlling
104 metabolism throughout the body, and is becoming an increasingly popular target for
105 pharmacotherapy. Endocannabinoids (EC) and phytocannabinoids are the two main subclasses
106 of cannabinoids. EC are produced by mammals, whereas phytocannabinoids, including
107 cannabidiol (CBD), are produced by plants such as *Cannabis sativa* (21). EC exert their
108 pharmacological effects via the endogenous endocannabinoid system, mainly by interacting
109 with various receptors, primarily CB1 and CB2 (21). CBD is a weak agonist of CB1 and CB2,
110 and can activate multiple cellular targets (e.g. TRPV1, PPAR γ) or inhibit (e.g. GPR55) (22).
111 The EC system is involved in numerous physiological processes, such as memory, appetite, and
112 the regulation of metabolic energy balance (21). EC exert a central effect by stimulating food
113 intake but also by modulating lipid and carbohydrate metabolism in the liver, adipose tissue
114 and skeletal muscle to favor energy accumulation (21). There is also growing evidence that the
115 EC system and CBD plays an important role in regulating mitochondrial biogenesis, membrane
116 integrity and oxidative capacity (23). Our laboratory and other teams have demonstrated that
117 the EC system also controls muscle development (24, 25) and that alterations in the EC system
118 are associated with muscle dysfunction (26-29). The use of CBD could therefore hold benefit
119 for improving the treatment of cancer and cancer-induced cachexia, in particular by protecting
120 skeletal muscle mass.

121 Recent studies have shown that CBD has antineoplastic and anti-inflammatory properties in
122 numerous in vitro models (30). Recent evidence also indicates that CBD regulates oxidative
123 activity and mitochondrial content in the myocardium by modulating the expression of several
124 markers of mitochondrial biogenesis that had been severely reduced by doxorubicin treatment
125 (13). In addition to its effects on mitochondria, CBD also has beneficial effects on skeletal
126 muscle. In mdx mice, i.e. a model of Duchenne muscular dystrophy, Iannotti *et al.* reported that
127 CBD was able to prevent loss of motor activity by promoting myotube formation and reducing

128 inflammation (27). CBD also reduces the production of ceramides (deleterious lipid derivatives
129 responsible for mitochondrial dysfunction) in high-fat diet-induced obesity (31).

130 Taken together, these data show that there is currently no treatment to prevent or reduce
131 cachexia, and that CBD could be a promising candidate compound for use as an adjuvant in
132 cancer treatment, due to its demonstrated positive effects on mitochondrial function, oxidative
133 stress, and skeletal muscle development, metabolism and. Here, we used a model of myotubes
134 in culture to investigate whether CBD treatment was able to counteract the muscle atrophy
135 induced by chemotherapy (cisplatin).

136

137 **MATERIAL AND METHODS**

138 **Chemicals and reagents.** Dulbecco's modified Eagle medium (DMEM) and phosphatase
139 inhibitor cocktail were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Fetal
140 bovine serum, horse serum, trypsin-EDTA, PBS, and penicillin-streptomycin were purchased
141 from PAA (Pasching, Austria). Primary antibodies were obtained from the following sources.
142 Thr389-phosphorylated S6K (#34475, 1/1000) total S6K (#9202, 1/1000), Ser473-
143 phosphorylated Akt (#9271, 1/1000), total Akt (#9272, 1/1000), Ser51- phosphorylated
144 eIF2 α (#3398, 1/1000), total eIF2 α (#5324, 1/1000), Thr172-phosphorylated AMPK (#2535,
145 1/1000), total AMPK (#2532, 1/1000), caspase 3 (#9662, 1/1000), and VDAC (#4866, 1/1000)
146 antibodies were from Cell Signaling Technology (distributed by Ozyme, Saint-Quentin-en-
147 Yvelines, France). Mouse anti-puromycin mAb (clone 12D10) (#MABE343, 1/1000) was from
148 Sigma-Aldrich (Saint-Quentin-Fallavier, France). Horseradish peroxidase-conjugated
149 secondary antibodies were from DAKO (Trappes, France). GAPDH antibody (#9545, 1/5000)
150 was from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Polyubiquitin antibody (#ENZ-
151 ABS840-0100, 1/1000) was purchased from Enzo Life Sciences (Villeurbanne, France). Total
152 OXPHOS antibody (#MS604, 1/1000) was purchased from Mitosciences (Eugene, Oregon,
153 USA). CoxIV antibody (#MA5-31470, 1/1000) was from Thermo Fisher Scientific
154 (Courtaboeuf, France). Cisplatin and cannabidiol and the TBARS and catalase assay kits were
155 purchased from Cayman Chemicals (distributed by INTERCHIM, Montluçon, France).

156 **Cell culture and differentiation.** Mouse C2C12 myoblasts cells were purchased from the
157 ATCC (#CRL-1772, American Type Culture Collection; Manassas, VA). Myoblasts were
158 cultured in a growth medium composed of DMEM containing 4.5 g/L glucose, 2.4 g/L sodium
159 bicarbonate, 10% fetal bovine serum, 100 UI/mL penicillin, and 0.1 mg/mL streptomycin, and
160 incubated at 37°C in humidified air with 5% CO₂. The medium was changed every other day
161 to ensure growth until 90% confluence. Myotube formation was induced by changing the
162 growth medium to a differentiation medium consisting of DMEM supplemented with 2% horse
163 serum, 100 UI/mL penicillin, and 0.1 mg/mL streptomycin for 5 days before cell treatment.
164 Passages between 4 and 10 were used for the experiments.

165 **Cell treatments.** For all experiments, C2C12 cells were incubated in differentiation medium.
166 Cisplatin was prepared extemporaneously in PBS to obtain a 0.5 mg/mL stock solution, then
167 administered to the cells at a final concentration of 50 μ M for 24–48 h. A stock solution of CBD
168 was prepared in ethanol and stored at -80°C. For each experiment, cells were pre-treated for 2
169 h prior to cisplatin treatment with CBD concentrations corresponding to those used in

170 subsequent cisplatin conditions (ranging from 1 to 5 μ M of CBD). After this pre-treatment, cells
171 were incubated with cisplatin with or without a CBD concentration corresponding to the pre-
172 treatment CBD concentration. Control experiments were conducted with the equivalent amount
173 of PBS and ethanol used in the cisplatin and CBD settings. C2C12 viability was estimated using
174 the CellTiter-Glo® luminescent assay (Promega, France).

175 **C2C12 myotube morphology analysis.** Myotubes were photographed directly in the culture
176 plates without fixation, using an AxioCam ERc5s digital camera coupled to an AxioVert.A1
177 microscope and ZEN 2.3 software (Zeiss, Germany). Myotube diameter was measured from
178 three independent experiments on myotubes in each condition. Three random measurements
179 were performed along the length of each myotube (n=3 measurements/myotube) using the ZEN
180 2.3 software, and the average of the three measures was considered as a single value.

181 **Western blotting and measurement of protein synthesis rate.** Protein synthesis was assessed
182 according to the SUNSET method. The SUNSET technique uses puromycin, which incorporates
183 into nascent polypeptide chains and terminates the elongation, resulting in an accumulation of
184 puromycin-conjugated peptides that reflects the rate of protein synthesis. Briefly, C2C12 cells
185 were incubated with 1 μ M puromycin for the last 30 min of experimental treatments, then
186 washed twice with ice-cold PBS and homogenized in ice-cold buffer (50 mM HEPES pH 7.4,
187 150 mM NaCl, 10 mM EDTA, 10 mM NaPPi, 25 mM β -glycerophosphate, 100 mM NaF, 2
188 mM Na orthovanadate, 10% glycerol, 1% Triton X-100) containing 1% protease inhibitor
189 cocktail (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Homogenates were centrifuged at
190 13,000 $\times g$ for 10 min at 4°C. Denatured proteins were separated by SDS-PAGE and transferred
191 to a PVDF membrane (Millipore, Molsheim, France). Immunoblots were blocked with 0.1%
192 TBS-Tween-20 containing 5% dry milk, and then probed overnight at 4°C with primary
193 antibodies. After several washes with 0.1% TBS-Tween-20, the immunoblots were incubated
194 with a horseradish peroxidase-conjugated secondary antibody (DAKO, Trappes, France) for
195 one hour at room temperature. Immune-reactive bands or whole lanes were visualized by
196 chemiluminescence (ECL Western blotting substrate, Thermo Fisher Scientific, Courtaboeuf,
197 France). escent secondary antibodies were visualized using an MF-ChemiBIS 2.0 camera
198 (Fusion Solo, Vilber Lourmat, France). Band densities were quantified using MultiGauge 3.2
199 software (Fujifilm Corporation, FSVT, Courbevoie, France). An internal control was used on
200 each gel to normalize signal intensities between gels.

201 **RNA extraction and quantitative real-time PCR.** Total RNA was extracted using Trizol
202 reagent (Invitrogen) according to the manufacturer's instructions. RNA was quantified by

203 measuring optical density at 260 nm. The concentrations of the mRNAs corresponding to genes
204 of interest were measured by reverse transcription followed by real-time PCR using an AriaMX
205 Real-Time PCR System (Agilent, Les-ULis, France). One microgram of total RNA was reverse-
206 transcribed using SuperScript® III reverse transcriptase and a combination of random hexamer
207 and oligo-dT primers (Invitrogen). PCR amplification was performed in a 20 µL total reaction
208 volume. The real-time-PCR mixture contained 5 µL of diluted cDNA template, 10 µL of 2x
209 ONE Green® Fast qPCR premix (Ozyme, Saint-Cyr-l'École, France), and 0.5 µM of forward
210 and reverse primers. The amplification profile was initiated by 3 min incubation at 95°C to
211 activate the hot-start Taq DNA Polymerase, followed by 40 cycles of two steps: 95°C for 5 sec
212 (denaturation step) and 60°C for 30 sec (annealing/extension step). Relative mRNA
213 concentrations were analyzed using the AriaMX software. Relative mRNA abundance was
214 calculated using the 2- $\Delta\Delta$ CT method with 18S as housekeeping gene. Mitochondrial DNA
215 (mtDNA) was quantified by measuring the ratio between the expression of mitochondrial ND1
216 DNA and nuclear actin DNA as reference. Details of the primers used in the PCR can be found
217 in table 1.

218 **Statistical analysis.** Data are expressed as mean \pm SE. Between group differences were
219 analyzed using one-way ANOVA and Tukey's test for post hoc comparisons. Statistical
220 significance was set at $P < 0.05$ for all analyses.

221

222 RESULTS

223 **CBD prevents cisplatin-induced C2C12 myotube atrophy and death.** In a first set of
224 experiments, we studied whether CBD was able to prevent the atrophy of C2C12 myotubes in
225 response to cisplatin treatment. In absence of CBD, cisplatin induced myotube atrophy, as
226 exemplified by a $\approx 30\%$ reduction ($p < 0.01$) in myotube diameter (Fig. 1A,B). However, co-
227 treatment with CBD prevented myotube atrophy in a dose-dependent manner (Fig. 1A, B). As
228 muscle morphology analysis revealed a high degree of cell death in response to cisplatin (Fig.
229 1A), we measured ATP content as an index of cell viability. We observed that myotube viability
230 was marginally affected by cisplatin and CBD after 24 h of treatment (data not shown) but was
231 reduced by $\approx 30\%$ after 48 h of treatment (Fig. 1C). CBD restored myotube viability in a dose-
232 dependent manner, reaching full protection at a $3 \mu\text{M}$ concentration (Fig. 1C). Western blot
233 analysis revealed that the reduction in cell viability observed in response to cisplatin was
234 associated with the appearance of the cleaved form of caspase 3 indicating induction of
235 apoptosis (Fig. 1D,E). Similar results were observed in myoblast (supplemental figure). As
236 expected, CBD treatment prevented the apoptotic process, as evidenced by the dose-dependent
237 reduction in protein expression of the cleaved-caspase 3 (Fig. 1D,E).

238

239 **CBD restores protein homeostasis in cisplatin-treated C2C12 myotubes.** To investigate
240 whether cisplatin alters protein synthesis and proteolysis and whether CBD is able to counteract
241 this effect, we incubated C2C12 myotubes for 24 h in differentiation media in the presence of
242 cisplatin and increasing concentrations of CBD, and then measured protein synthesis using the
243 SUnSET technique. As shown in Fig. 2A and B, puromycin incorporation was reduced by
244 $\approx 75\%$ ($p < 0.01$) in response to cisplatin treatment. Protein synthesis is mainly controlled by the
245 Akt/mTOR/S6K and eIF2 α signaling pathways, where anabolic conditions lead to increased
246 phosphorylation of Akt on Ser473 and S6K on Thr389 and decreased phosphorylation of
247 eIF2 α on Ser51 residue. As shown in Fig. 2A, C-E, cisplatin treatment was associated with
248 decreased phosphorylation of both Akt and S6K and increased phosphorylation of eIF2 α , in
249 agreement with the observed reduced protein synthesis. Co-treatment with CBD was able to
250 restore protein synthesis (Fig. 2A, B), which was associated with a dose-dependent increase in
251 Akt phosphorylation on Ser473 and S6K phosphorylation on Thr389, and a dose-dependent
252 decrease of Ser51 phosphorylation on eIF2 α (Fig. 2A, C-E). AMPK kinase is activated by an
253 elevated AMP/ATP ratio due to cellular and environmental stress. We observed that cisplatin
254 treatment was associated with an increase in AMPK phosphorylation on Thr172 that was

255 corrected by CBD co-treatment (Fig. 2A). To investigate how cisplatin and cannabidiol impact
256 proteolysis, we evaluated the level of protein poly-ubiquitination and the mRNA expression of
257 MAFBx and MuRF1. As shown in Fig. 3A,B, cisplatin treatment increased protein
258 polyubiquitination by $\approx 70\%$ ($p < 0.01$) and induced a ≈ 2 -fold increase in mRNA expression of
259 Atrogin1/MAFBx (Fig. 3C) and MuRF1 (Fig. 3D). CBD reduced protein polyubiquitination
260 levels in a dose-dependent manner (Fig. 3A,B), and CBD treatment at a 5 μM concentration
261 restored the mRNA expression of both Atrogin1/MAFBx and MuRF1 to control levels (Fig.
262 3C,D).

263

264 **CBD prevents cisplatin-induced oxidative stress in C2C12 myotubes.** The toxicity of
265 cisplatin has been described as a function of DNA binding followed by single-stranded DNA
266 breaks. More recently, cisplatin has been shown to generate oxidative stress, which can also
267 contribute to its anti-tumor effects (32). To determine whether cisplatin-induced C2C12
268 myotube atrophy was associated with oxidative stress, we measured the level of TBARS, a
269 marker of lipid peroxidation, in C2C12 myotubes in response to cisplatin and CBD treatments.
270 We found a two-fold increase in TBARS content in response to 24 h cisplatin treatment that
271 was prevented by co-treatment with 5 μM CBD (Fig. 4A). Catalase is one of the main enzymes
272 responsible for the detoxification of hydrogen peroxide, a reactive oxygen species. We therefore
273 measured catalase activity in response to cisplatin and CBD treatments (Fig. 4B). A 24 h
274 cisplatin treatment induced a $\approx 90\%$ increase in catalase activity, indicating severe oxidative
275 stress (0.236 ± 0.020 vs 0.125 ± 0.013 nmol/min/ μg prot, $p < 0.01$) that was partially restored by
276 co-treatment with CBD (0.172 ± 0.010 vs 0.236 ± 0.020 nmol/min/ μg prot, $p < 0.05$). We then
277 measured the mRNA expression levels of several anti-oxidant systems. As shown in Fig. 4C,
278 mRNA expression of GPX1, SOD1, SOD2 and CAT were all increased in response to 24 h
279 cisplatin treatment. Co-treatment with 5 μM CBD was unable to correct the mRNA expression
280 levels of SOD2 and CAT (Fig. 4C) but decreased GPX1 and SOD1 mRNA expression levels
281 compared to cisplatin treatment (Fig. 4C).

282

283 **Effect of cisplatin and CBD treatment on mitochondria.** In order to analyze the early effects
284 of cisplatin and CBD on mitochondrial biogenesis and quality control, we estimated
285 mitochondrial density by measuring mtDNA (mitochondrial DNA) content and we measured
286 the mRNA expression levels of genes involved in mitochondrial biogenesis (PGC1 α), fission
287 (DRP1, FIS1), fusion (OPA1), and mitophagy (PRKN) in response to 24 h cisplatin and CBD
288 treatments. In these conditions, there were no significant changes in mtDNA content (Fig. 5A)

289 or mRNA expression levels of PGC1 α , FIS1, DRP1 and OPA1 (Fig. 5B-E), but we observed a
290 dramatic \approx 95% reduction in PRKN mRNA expression compared to controls (Fig. 5F). Adding
291 5 μ M CBD to the cisplatin treatment did not result in any changes in mtDNA content or mRNA
292 expression of PGC1 α , OPA1, DRP1, FIS1 and PRKN compared to cisplatin treatment alone
293 (Fig. 5A-F). In a second step, we studied the effect of cisplatin and CBD treatments on the
294 expression of the different mitochondrial respiratory chain complexes. As shown in Figure 6A-
295 B, western blot quantification showed a marked increase in the content of several mitochondrial
296 proteins in C2C12 myotubes treated with 50 μ M cisplatin, with significant increases in
297 NDUFB8 (complex I, p <0.01), UQCRC2 (complex III, p <0.05) and COX4 (complex IV,
298 p <0.05) that were restored to control levels by co-treatment with CBD. Finally, we studied the
299 expression of VDAC1, which is a major mitochondrial transporter that plays a key role in ATP
300 production and is recognized as a key protein in mitochondria-mediated apoptosis (33).
301 Cisplatin treatment of C2C12 myotubes led to a 5-fold increase in VDAC1 protein level, which
302 was partly corrected by CBD treatment (Fig. 6C-D). In a final step, we measured citrate
303 synthase and COX activities. Treatment of C2C12 myotubes with 50 μ M cisplatin resulted in a
304 significant \approx 15% increase in citrate synthase activity (Fig. 6E, p <0.05) and a \approx 50% increase in
305 COX activity (Fig. 6F, p =0.07) that were not corrected by CBD co-treatment.

306

307 **DISCUSSION**

308 The objective of this study was to investigate whether treatment with CBD was able to prevent
309 chemotherapy-induced skeletal muscle atrophy in a cisplatin-treated model of myotubes in
310 culture. In a first set of experiments, we demonstrated that CBD was able to prevent cisplatin-
311 induced apoptosis and myotube atrophy. The increased protein synthesis and decreased
312 proteolysis induced by CBD in cisplatin-treated myotubes explain the anti-atrophic effects of
313 CBD. In a second set of experiments, we showed that cisplatin-induced atrophy was associated
314 with the induction of oxidative stress that was prevented by CBD co-treatment. Finally, we
315 observed early cisplatin-induced alterations in the expression of several mitochondrial proteins
316 involved in mitochondrial respiration and control of apoptosis, and these alterations were also
317 prevented by CBD co-treatment.

318

319 Mitochondria plays an important role in regulating many cellular functions, including ATP
320 production, generation of reactive oxygen species (ROS), and induction of apoptosis (34). Cells
321 maintain optimal mitochondrial health through several pathways, including mitochondrial
322 biogenesis, mitochondrial dynamics (fusion and fission processes shaping mitochondrial

323 morphology), and mitophagy (the process that removes defective mitochondria through
324 autophagy) (35). Alterations in mitochondrial distribution, morphology and function have been
325 reported in many conditions that lead to skeletal muscle wasting, including cachexia and
326 sarcopenia (36, 37). Here, we analyzed the mRNA expression levels of several markers of
327 mitochondrial dynamics in response to cisplatin and CBD treatments in myotubes in culture.
328 We observed no major effect of cisplatin or CBD on the mRNA expression levels of genes
329 involved in mitochondrial biogenesis (PGC1 α), fusion (Opa1), and fission (DRP1, FIS1).
330 However, there was a drastic reduction in the expression of the PRKN gene encoding the Parkin
331 protein. This could reflect inhibition of PARKIN-dependent mitophagy, leading to an
332 accumulation of dysfunctional mitochondria and, ultimately, muscle wasting (38). Previous
333 studies have shown that cisplatin (and chemotherapy in general) causes a decrease in
334 mitochondrial respiration and alterations in mitochondrial biogenesis and fusion/fission
335 processes in models of cancer or chemotherapy, but also in cancer patients (13, 16, 18, 19). Our
336 findings therefore seem to contradict the literature, but there are several possible explanations
337 for this divergence. The majority of studies characterizing the effect of cisplatin on muscle
338 atrophy were carried out *in vivo*, either in rodents or in cancer patients, and treatments were
339 carried out over periods of several days, sometimes with tumor implantation. One limitation of
340 our *in vitro* myotube model is that it does not represent the cellular heterogeneity found in
341 skeletal muscle, which consists of satellite cells, myoblasts, fibro-adipogenic progenitor cells,
342 and immune cells. Further research is needed to investigate the impact of CBD on this
343 heterogeneity and the coordinated functioning of different cell types.

344

345 In the present study, we also documented alterations in the expression of several proteins of the
346 mitochondrial electron transport chain (ETC). Cisplatin treatment was associated with
347 increased protein expression of NDUFB8 (complex I), UQCRC2 (complex III), and COX4
348 (complex IV) that were corrected by CBD, while protein expression of SDHB (complex II) and
349 ATP5A (complex V) was unaffected. In sarcopenia, deficient ETC activity is associated with
350 loss of muscle mass (39). Given that normal ETC function requires proportionally balanced
351 activities of these different complexes (40), the increased expression of NDUFB8, UQCRC2
352 and COX4 could reflect imbalanced ETC activity and reduced energy production. The reduced
353 ATP content observed in cisplatin-treated myotubes and the increased level of AMPK
354 phosphorylation (indicating energy depletion) supports this hypothesis, but further
355 investigation is needed to fully understand the effects of cisplatin and CBD treatments on the
356 activity of the different mitochondrial complexes and on mitochondrial respiration. One of the

357 key proteins that control mitochondrial function is VDAC1, which plays a crucial role in the
358 release of ROS and in regulating apoptosis through the release of mitochondrial pro-apoptotic
359 factors such as cytochrome C and subsequent cleavage of caspase 3 (41). Several studies have
360 shown that the cytotoxic effect of cisplatin on cancer cell lines is associated with upregulation
361 of VDAC1 and excessive production of ROS (16, 42). To our knowledge, our study is the first
362 to demonstrate increased expression of VDAC1 in response to cisplatin in a model of skeletal
363 muscle in vitro, and its correction by CBD. It is still unclear how CBD counters these
364 mitochondrial alterations, but the answer may lie in its antioxidative properties.

365

366 Cisplatin is widely used to combat multiple types of cancers, but it has side effects such as
367 oxidative stress in muscle cells (12, 16). Antioxidative strategies are expected to be useful in
368 limiting oxidative stress-induced skeletal muscle damage (43). As expected, cisplatin treatment
369 resulted in increased TBARS levels (index of lipid peroxidation) together with increased
370 catalase activity and mRNA expression levels of several antioxidant systems (GPX1, SOD1/2,
371 CAT) aimed at detoxifying excessive ROS production. To our knowledge, our study is the first
372 to demonstrate the antioxidative properties of CBD in cisplatin-treated myotubes in vitro.
373 Previous studies have shown that CBD has an antioxidant function (22) that comes from its
374 ability to capture free radicals or transform them into less active forms. For example, CBD was
375 shown to directly prevent the formation of superoxide radicals in a renal nephropathy model
376 using cisplatin-treated mice (22), to reduce nitric oxide (NO) levels in the liver of doxorubicin-
377 treated mice (22), and to suppress ROS production by chelating transition metal ions involved
378 in the Fenton reaction (22). Note too that CBD modifies cellular redox status by modulating
379 both the expression and enzymatic activity of antioxidant systems (22). Our results
380 demonstrated that CBD was able to prevent the cisplatin-induced accumulation of TBARS. The
381 fact that CBD reduced the mRNA expression of antioxidant systems (GPX1, SOD1/2, CAT)
382 and catalase activity in response to cisplatin strongly suggests a direct effect of CBD by
383 capturing ROS and reducing NO production in response to cisplatin, or by reducing their
384 production by increasing the levels of antioxidants such as glutathione (22).

385 On top of these direct antioxidant effects, CBD also indirectly modulates redox state by
386 interacting with several molecular targets, including the EC receptors CB1 and CB2, and
387 PPAR γ which controls the expression of antioxidant systems such as catalase and SOD2 (22).
388 CBD is not only a PPAR γ receptor agonist, but can also increase enzymatic antioxidant
389 activities (22). CBD could also improve oxidative state by modulating the activity of the CB1
390 and CB2 receptors. Indeed, CB1 activation increases ROS production whereas CB2 activation

391 decreases ROS production (44), and it has been shown that CBD is a negative allosteric
392 modulator of the CB1 receptor (45). Further studies are needed to fully understand which of
393 these targets mediate the protective effect of CBD in our *in vitro* model.

394 Oxidative stress is one of the primary causes of skeletal muscle atrophy in several patho-
395 physiological conditions, including muscle inactivity, muscular dystrophy, sarcopenia, and
396 cachexia (43). Regulation of skeletal muscle mass depends on the balance between protein
397 synthesis and degradation. Protein synthesis is mainly controlled by the Akt/mTOR
398 (mammalian target of rapamycin) and GCN2/eIF2 α pathways (46, 47). Excessive production
399 of ROS is known to activate PERK, a key endoplasmic reticulum stress transducer of the
400 unfolded protein response pathway (47) that activates eIF2 α and inhibits protein synthesis. In
401 skeletal muscle, excessive oxidative stress is also known to impair insulin signaling and Akt
402 activation upstream of mTOR, which is important for controlling both protein synthesis and
403 degradation (46). Given the drastic reduction of lipid peroxidation observed here with CBD in
404 cisplatin-treated myotubes, it is highly conceivable that CBD helps maintain protein
405 homeostasis by preventing excessive oxidative stress.

406
407 In summary, using a model of cultured C2C12 myotubes, we demonstrated that CBD prevented
408 cisplatin-induced atrophy by maintaining protein homeostasis (i.e. promoting protein synthesis
409 and limiting proteolysis) by reducing oxidative stress. In cancer patients, muscle mass is
410 predictive of survival but also of response to chemotherapy, which makes it crucial to develop
411 strategies for maintaining muscle mass in these patients. Cisplatin is an antineoplastic agent
412 that is commonly used in the treatment of solid tumors such as ovarian carcinoma and in head
413 and neck squamous cell carcinoma (HNSCC) (48). The toxicity of cisplatin is due to its DNA
414 binding followed by single-stranded DNA breaks, but also its ability to generate oxidative stress
415 in tumor cells. Consequently, some may consider that using CBD for its antioxidant activity
416 could undermine the efficacy of cisplatin treatment. However, rather than reducing cisplatin
417 toxicity, CBD was recently found to potentiate the antineoplastic effect of cisplatin in a model
418 of HNSCC (48). Taken together, the evidence suggests that CBD could be used as an adjuvant
419 in the treatment of cancer cachexia to help maintain muscle mass and improve patient quality
420 of life.

421

422 **GRANTS**

423 This study was supported by the INRAE AlimH (Alimentation Humaine) Department.

424 **CONFLICT OF INTEREST**

425 The authors declare they have no conflict of interest.

426

427 **FIGURE LEGENDS**

428 **Figure 1.** CBD prevents cisplatin-induced atrophy and apoptosis in C2C12 myotubes. **a)**
429 Representative pictures of myotube morphology at 24 h of incubation. **b)** Myotube diameter of
430 cisplatin and CBD treated cells. **c)** 48 h Myotube viability. **d)** Representative western blot
431 showing level of cleaved caspase 3 in response to 24 h treatments. **e)** Quantification of cleaved
432 caspase 3 level from (d). Results are expressed as mean \pm sem. ** $p < 0.01$ vs. CTL, \blacklozenge $p < 0.05$
433 vs. CIS, \blacklozenge $p < 0.01$ vs. CIS, $\#\#$ $p < 0.01$ between CBD conditions.

434 **Figure 2.** CBD prevents cisplatin-induced decrease in protein synthesis in C2C12 myotubes.
435 **a)** Representative western blot of puromycin incorporation and the phosphorylation state of Akt
436 (Ser473-phospho-Akt), S6K (Thr389-phospho-S6K) and eIF2 α (Ser51-phospho- eIF2 α) in
437 response to 24 h treatment with cisplatin and CBD. Quantification of **(b)** puromycin
438 incorporation signal, **(c)** phospho-S6K, **(d)** phospho-Akt, and **(e)** phospho- eIF2 α levels.
439 Results are expressed as mean \pm sem. ** $p < 0.01$ vs. CTL, * $p < 0.05$ vs. CTL, \blacklozenge $p < 0.05$ vs. CIS,
440 \blacklozenge $p < 0.01$ vs. CIS, $\#\#$ $p < 0.01$ between CBD conditions.

441 **Figure 3.** CBD prevents cisplatin-induced proteolysis and atrogene expression. **a)**
442 Representative western blot showing the level of protein poly-ubiquitination in response to 24
443 h treatment with cisplatin and CBD. **b)** Quantification of total protein polyubiquitination from
444 (a). **c)** Real-time PCR quantification of *Atrogin/MAFbx* mRNA expression. **d)** Real-time PCR
445 quantification of *MuRF1* mRNA expression. Results are expressed as mean \pm sem. ** $p < 0.01$
446 vs. CTL, * $p < 0.05$ vs. CTL, \blacklozenge $p < 0.05$ vs. CIS.

447 **Figure 4.** CBD prevents cisplatin-induced oxidative stress. **a)** TBARS content in C2C12
448 myotubes treated for 24 h with cisplatin and CBD. **b)** Catalase activity. **c)** Real-time PCR
449 quantification of *Gpx1*, *Sod1*, *Sod2* and *Cat* mRNA expression. Results are expressed as mean
450 \pm sem. ** $p < 0.01$ vs. CTL, * $p < 0.05$ vs. CTL, \blacklozenge $p < 0.05$ vs. CIS. \blacklozenge $p < 0.01$ vs. CIS.

451 **Figure 5.** Effect of cisplatin and CBD co-treatment on C2C12 myotube mitochondrial
452 dynamics. **a)** Real-time PCR quantification of mtDNA expression in C2C12 myotubes treated
453 for 24 h with cisplatin and CBD. Real-time PCR quantification of *Pgc1alpha* **(b)**, *Fis1* **(c)**,

454 *Drp1* (d), *Opal* (e), and *Prkn* (f) mRNA expression. Results are expressed as mean \pm sem. **
 455 $p < 0.01$ vs. CTL.

456 **Figure 6.** Effect of cisplatin and CBD on C2C12 myotube mitochondrial protein content and
 457 activity. **a)** Representative western blot showing the expression levels of proteins related to
 458 mitochondrial oxidative phosphorylation (OXPHOS) in C2C12 myotubes treated for 24 h with
 459 cisplatin and CBD. **b)** Quantification of several subunits of each mitochondrial complex from
 460 (a). **c)** Representative western blot showing the expression levels of VDAC1. **d)** Quantification
 461 of VDAC1 protein content from (c). **(e)** Citrate synthase activity and **(f)** COX activity. Results
 462 are expressed as mean \pm sem. ** $p < 0.01$ vs. CTL, * $p < 0.05$ vs. CTL, \blacklozenge $p < 0.05$ vs. CIS. \blacklozenge
 463 $p < 0.01$ vs. CIS.

464 **Table 1.** List of primers used for real-time qPCR

Gene name	5'- Sense primer -3'	5'- Antisense primer -3'
Actin (DNA primer)	TACAGCTTCACCACCACAGC	AAGGAAGGCTGGAAAAGAGC
Atrogin/MAFBx	AAGCTTGTGCGATGTTACCCA	CACGGATGGTCAGTGCCCTT
Cat	CCTTCAAGTTGGTTAATGCAGA	CAAGTTTTTGATGCCCTGGT
Drp1	TGCTCAGATCGTCGTAGTG	TGACCACACCAGTTCCTCTG
Fis1	GCCTGGTTCGAAGCAAATAC	CACGGCCAGGTAGAAGACAT
Gpx1	GTGAGCCTGGGCTCCCTGCG	ACTTGAGGGAATTCAGAATC
MuRF1	AGGTGTCAGCGCAAAGCAGT	CCTCCTTTGTCCTCTTGCTG
Nd1 (DNA primer)	GGCCCCCTTCGACCTGACAGA	TAACGCGAATGGGCCGGCTG
Opal	GATGACACGCTCTCCAGTGAAG	CTCGGGGCTAACAGTACAACC
Ppargc1a	GAAGTGGTGTAGCGACCAATC	AATGAGGGCAATCCGTCTTCA
Prkn	ATTCCAAACCGGATGAGTGG	TTGTCTGAGGTTGGGTGTGC
Sod1	CAGGACCTCATTTTAATCCTCAC	TGCCCAGGTCTCCAACAT
Sod2	GACCTGCCTTACGACTAT	TACTTCTCCTCGGTGACG
18S	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT

465

466

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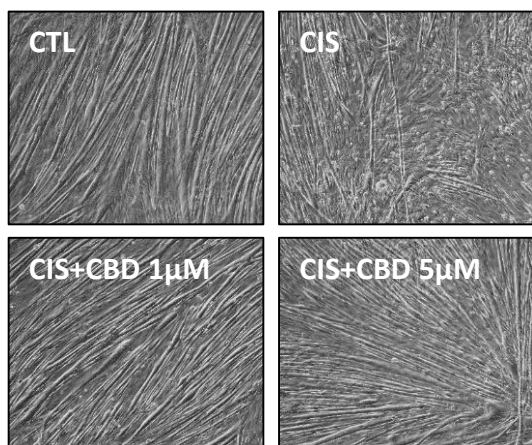
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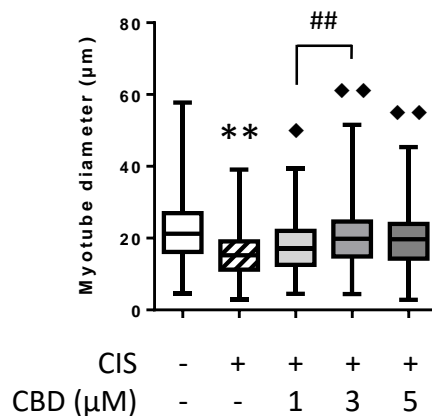
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Figure 1

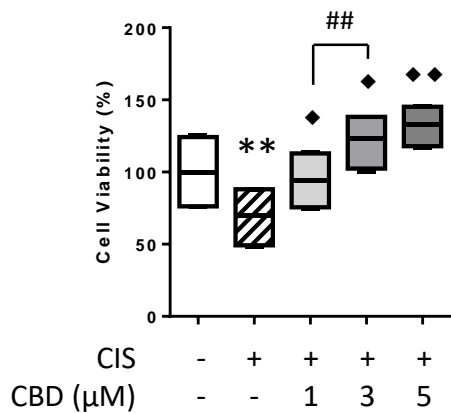
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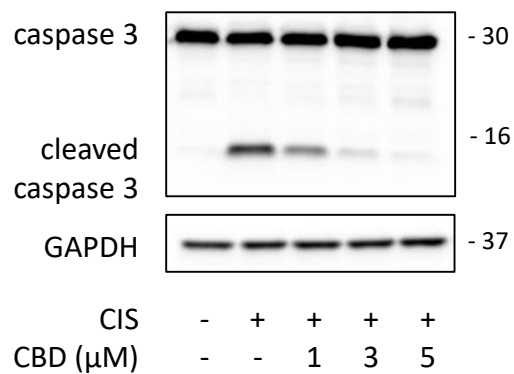
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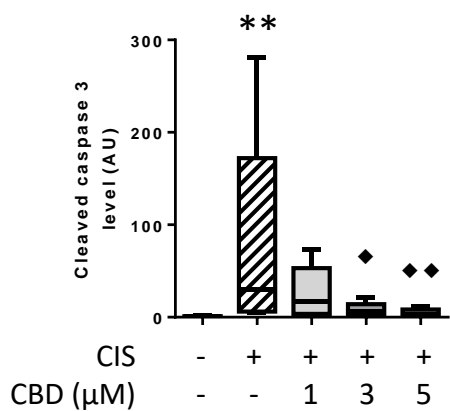
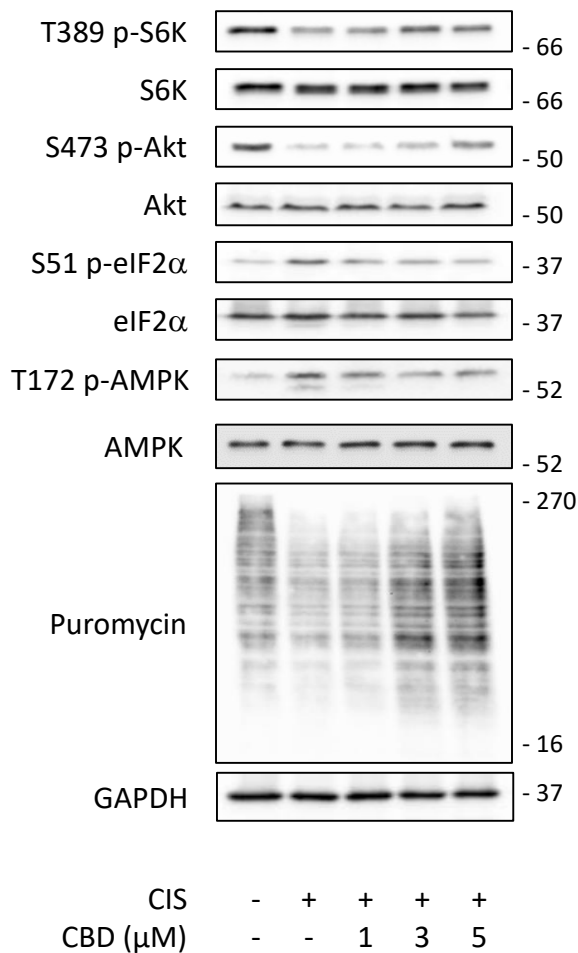
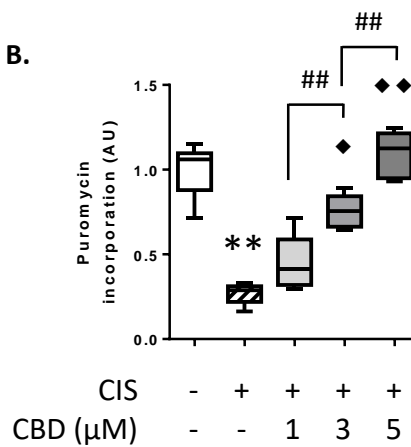


Figure 2

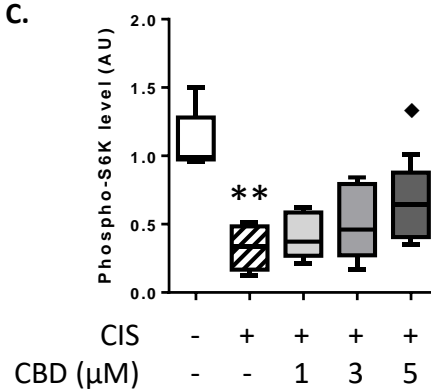
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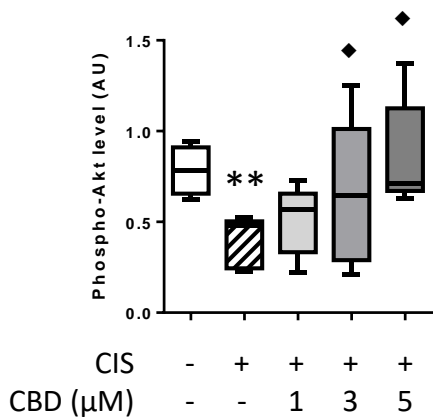
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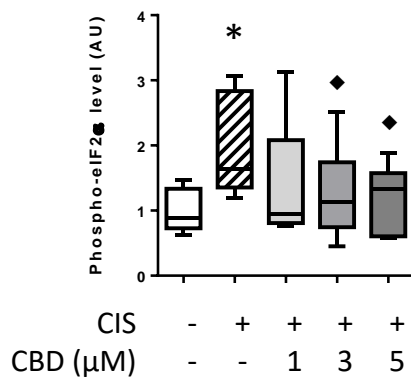
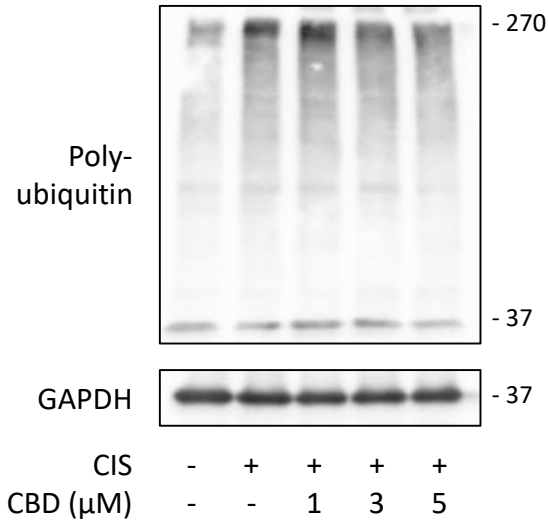
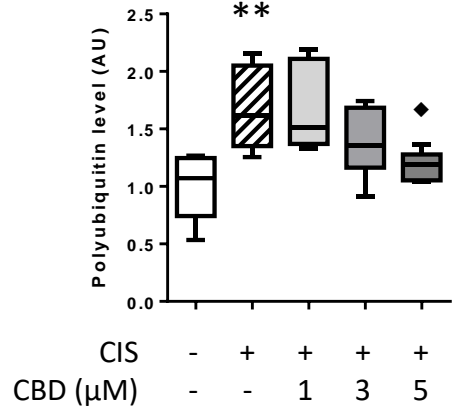


Figure 3

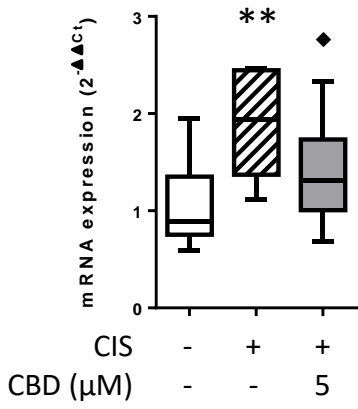
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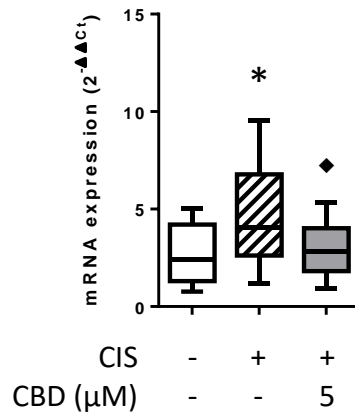


C.



MAFBx

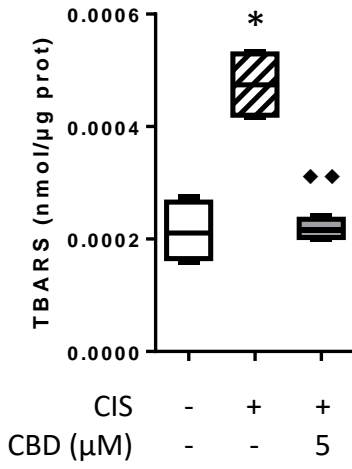
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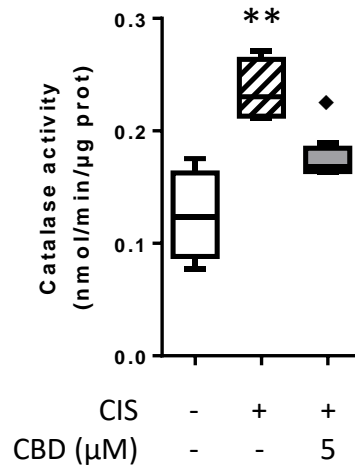
MuRF1

Figure 4

A.



B.



C.

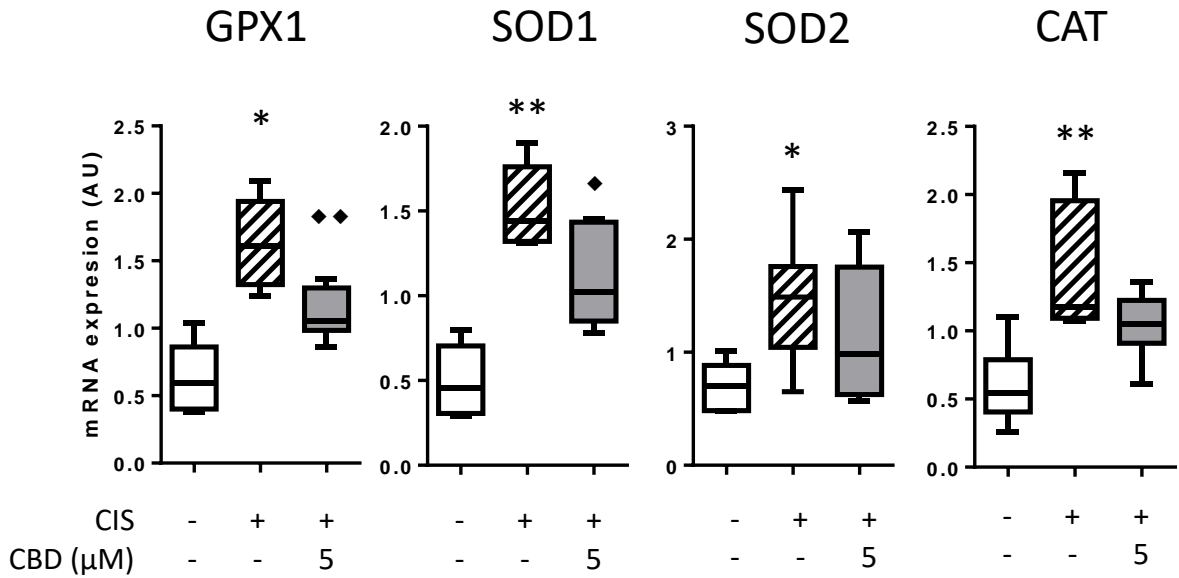


Figure 5

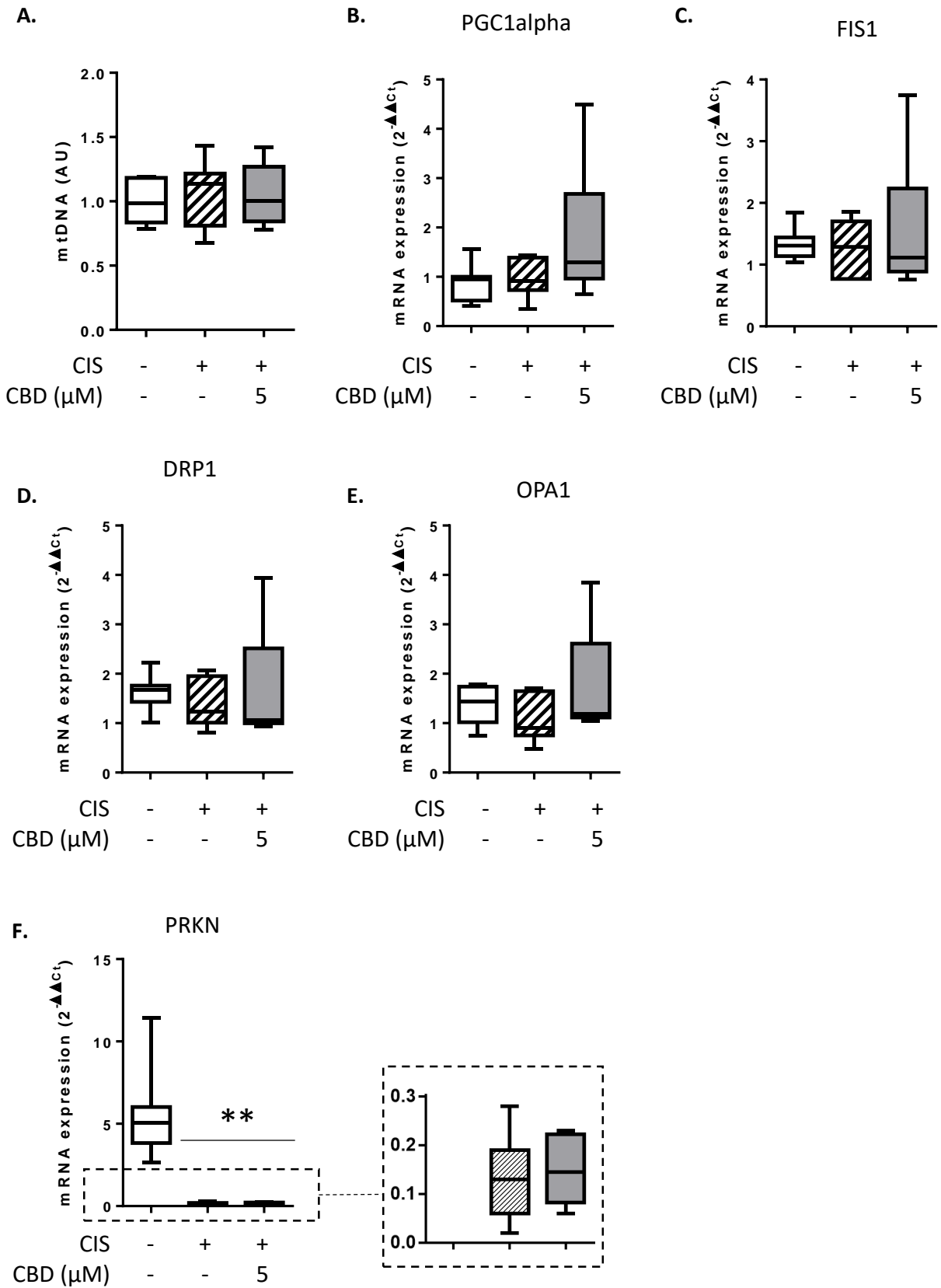


Figure 6

