

## **NDP-MSH reduces oxidative damage induced by palmitic acid in primary astrocytes**

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## ABSTRACT

Recent findings relate obesity to inflammation in key hypothalamic areas for body weight control. Hypothalamic inflammation has also been related to oxidative stress. Palmitic acid (PA) is the most abundant free fatty acid found in food, and *in vitro* studies indicate that it triggers a pro-inflammatory response in the brain. Melanocortins are neuropeptides with proven anti-inflammatory and neuroprotective action mediated by melanocortin receptor 4 (MC4R), but little is known about the effect of melanocortins on oxidative stress. The aim of this study was to investigate whether melanocortins could alleviate oxidative stress induced by a high fat diet (HFD) model. We found that NDP-MSH treatment decreased PA-induced reactive oxygen species (ROS) production in astrocytes, an effect blocked by the MC4R inhibitor JKC363. NDP-MSH abolished nuclear translocation of Nrf2 induced by PA and blocked the inhibitory effect of PA on superoxide dismutase (SOD) activity and glutathione (GSH) levels while it also *per se* increased activity of SOD and  $\gamma$ -glutamate cysteine ligase ( $\gamma$ -GCL) antioxidant enzymes. However, HFD reduced hypothalamic MC4R and BDNF mRNA levels, thereby preventing the neuroprotective mechanism induced by melanocortins.

## 1. INTRODUCTION

Obesity has developed as a major health concern, its rate of incidence tripling since 1975. According to the World Health Organization, in 2016 more than 1.9 billion adults 18 years and older were overweight and over 650 million were obese. If this trend continues, it is estimated that 51% of the world's adult population will be obese by 2030<sup>1</sup>.

Consumption of high fat diets (HFD) is crucial for the development of obesity. Excessive intake of fatty acids induces a chronic low-grade inflammatory response that affects both peripheral tissues and central nervous system (CNS)<sup>2</sup>. *In vivo* studies have shown that high-fat feeding increases cytokine levels and pro-inflammatory signaling in the hypothalamus through TLR4 activation<sup>3</sup>, leading to neuronal death<sup>4</sup> and leptin and insulin signaling impairment<sup>2,5</sup>. Interestingly, hypothalamic inflammation appears long before the onset of peripheral inflammation, metabolic disturbances, and weight gain<sup>6</sup>.

Inflammation and oxidative stress are linked in obesity. Pro-inflammatory signaling pathways activated in obesity increase production of reactive oxygen species (ROS)<sup>7</sup> which, in turn, exacerbate the inflammatory response<sup>8</sup>. Moreover, disturbances in antioxidant enzyme activity and increase in oxidative stress markers have been found in plasma of obese animals<sup>9</sup>. Saturated fatty acids are known to induce neurotoxicity and oxidative stress in the CNS. Palmitic acid (PA) is the most abundant free fatty acid found in food, and *in vitro* studies indicate that PA induces TNF- $\alpha$ , IL-1 $\beta$  and IL-6 release in astrocytes and microglia<sup>10,11</sup>. In addition, PA increases ROS production in astrocytes<sup>12</sup>. In the hypothalamus, astroglial cells are involved in detection and transport of nutrients<sup>13</sup> and also express receptors for hormones and neuropeptides whose activation directly affects neurons that control metabolic homeostasis<sup>14</sup>. HFD also induces reactive gliosis, a phenomenon that persists after the onset of obesity<sup>2</sup>. Activated astrocytes promote blood brain barrier disruption<sup>15</sup>, enhancement of diet reward properties in the mesolimbic system<sup>16</sup>, and synaptic changes in the melanocortin system<sup>17</sup>, thereby perpetuating diet-induced obesity. Melanocortins are a group of neuropeptides that includes adrenocorticotrophic hormone and  $\alpha$ ,  $\beta$  and  $\gamma$ -melanocyte-stimulating hormones (MSH) which have protective actions in the brain<sup>18,19</sup>. Melanocortin actions are mediated by their five receptor subtypes (MC1R to MC5R). In the CNS, MC4R is the most widely distributed subtype.  $\alpha$ -MSH, the endogenous MC4R agonist, has a physiological effect on feeding and energy homeostasis.  $\alpha$ -MSH also exerts neuroprotective effects in different *in vivo* models of ischemia and inflammation by decreasing the expression of pro-inflammatory cytokines<sup>20</sup> and inducing neurogenesis with functional recovery<sup>21</sup>. Our group determined that both astrocytes and microglia express MC4R<sup>22,23</sup> and we also demonstrated that MC4R mediates the anti-inflammatory effect of melanocortins in astrocytes<sup>22</sup>, microglia<sup>23</sup>, and hypothalamus<sup>19</sup>. While anti-inflammatory actions of melanocortins in the CNS have been studied, the melanocortin effect on antioxidant response was explored only in skin cells where  $\alpha$ -MSH reduces ROS production and stimulates antioxidant defenses<sup>24</sup>. Considering that melanocortins have proven anti-inflammatory and neuroprotective actions, we tested the effect of [Nle<sup>4</sup>,D-Phe<sup>7</sup>]- $\alpha$ -

melanocyte-stimulating hormone (NDP-MSH), an  $\alpha$ -MSH analogue, on oxidative damage induced by saturated fatty acids *in vitro* and *in vivo*, in order to evaluate its potential as a modulator of metabolic disorders.

## 2. MATERIALS AND METHODS

### 2.1. Reagents

[Nle<sup>4</sup>,D-Phe<sup>7</sup>]- $\alpha$ -melanocyte-stimulating hormone (NDP-MSH) was purchased from Bachem (CA, USA). Sodium palmitate was purchased from Santa Cruz Biotechnology (TX, USA). Bovine serum albumin, Fraction V, fatty acid free was obtained from Roche (Grenzach, Germany). JKC363 was purchased from Tocris Bioscience (MO, United States). Fetal bovine serum (FBS) was obtained from Natocor (Cordoba, Argentina). Nrf2 antibody (Novus) was kindly provided by Dr. Cymeryng (CEFYO, UBA-CONICET). DMEM/F-12, DMEM, L-Glutamine and antibiotics were purchased from Invitrogen Life Technologies (CA, USA). All other media and supplements were obtained from Sigma-Aldrich Corporation unless specified otherwise.

### 2.2. Cell cultures

**Primary astrocyte culture:** Astrocytes were prepared from rat cerebral cortex tissue of 1- to 2-day old postnatal Wistar rat pups as described previously<sup>22</sup>. Cells were maintained in DMEM/F-12 medium containing 10% FBS, 50  $\mu$ g/ml streptomycin, 50 U penicillin in 75 cm<sup>2</sup> poly-L-lysine coated culture flasks at 37°C in 5% CO<sub>2</sub>. Cell culture medium was changed twice a week. Cells were trypsinized and subcultured and, after 2–3 days of stabilization, incubated with the drugs in MEM containing 6 mM L-glutamine, 50  $\mu$ g/ml streptomycin, and 50 U/ml penicillin. Cultures were routinely more than 95% pure astrocytes as assessed by glial fibrillary acidic protein (GFAP) immunostaining.

**Cell treatments:** Sodium palmitate was conjugated to bovine serum albumin (BSA) after both components were first solubilized separately as described by Pike Winer<sup>25</sup>. Briefly, sodium palmitate was solubilized in 150 mM NaCl by heating it to 70°C in a water bath. Fatty

acid-free BSA was dissolved in 150 mM NaCl and warmed up to 37°C with continuous stirring. Solubilized PA was combined with BSA at a molar ratio of 6:1 (PA/BSA), stirring at 37°C for 1 h, and the final concentration was set at 1 mM. The conjugated PA–BSA was aliquoted and stored at –20°C. Vehicle stock solution was prepared using the same protocol without PA. Astrocytes were incubated with PA and NDP-MSH alone or in combination for 2 h to determine ROS generation, for 6 h to evaluate Nrf2 nuclear translocation and gene expression and for 24 h to determine enzymatic activity. Control astrocytes were incubated with vehicle solution.

### 2.3. *In vivo* experiments

**Animals:** Two month old male Wistar Kyoto rats were obtained from the School of Veterinary Science, National University of La Plata. At the start of the experiment the animals were weighted (mean body weight: 286.22 ±4.64 g) and were randomly assigned to either a standard diet (SD, Asociación Cooperativas Argentinas, having the following composition, w/w: 23% proteins, 5% fat, 6% fibre, 10% minerals and 56% starch and vitamin supplements) or a high-fat diet (HFD; 50% w/w bovine fat added to SD), as described by Lee et al<sup>26</sup>. There were no significant differences in body weight between groups before the experimental diets were administered, as all rats had been kept on a regular chow diet. Rats were housed in standard caging with a 12/12 h light/dark cycle and *ad libitum* access to water and their respective diet unless otherwise noted. HFD was replaced daily to avoid rancidity. Rats were administered the HFD or SD for 8 weeks. Energy concentrations were 2900 kcal/kg for SD and 5950 kcal/kg for HFD.

**Cannulations:** After 7 weeks on the HFD or SD diet, cannulas were placed under stereotaxic guidance into either the right or left lateral cerebral ventricle of the rats as described by Caruso<sup>19</sup>. Briefly, rats were anesthetized with Xylazine (10 mg/kg) and Ketamine (50 mg/kg) and placed into a stereotaxic frame. A 22-gauge guide sleeve was lowered into either the right or the left lateral cerebral ventricle according to the coordinates of Paxinos and Watson<sup>27</sup>(coordinates: A-P -0.6 mm, L -/+2 mm, D-V –3.2 mm)and then

secured to the skull with screws and dental acrylic. Animals were placed in individual cages and allowed to recover for 7 days.

**Procedure:** Animals were fasted overnight and on the day of euthanasia rats received an intracerebroventricular (ICV) injection of either saline (5  $\mu$ l/rat) or NDP-MSH (1  $\mu$ g/5  $\mu$ l/rat). During the next 4 h, animals were fed and then euthanized by decapitation. Trunk blood was collected, allowed to clot at RT and spun at 3000 rpm for 10 min; plasma was separated and stored at  $-20^{\circ}\text{C}$  for further biochemical assay. After euthanasia, a hypothalamic fragment (MBH) that included the arcuate and periventricular nuclei and the median eminence was dissected and immediately processed for either RT-qPCR or enzymatic activity assay.

**Caloric intake:** Caloric intake was calculated by multiplying the amount (g) of food consumed per day by the caloric concentration (kcal/g) of each diet.

**Serum biochemical determinations:** Serum glucose, triglycerides, cholesterol, and insulin were measured in a biochemical analysis lab (Buenos Aires, Argentina) that processes animal samples. Measurements were performed using enzymatic assays: glucose oxidase assay for glucose (sensitivity: 0.54 mg/dl), glycerol-3-phosphate oxidase/peroxidase assay for triglycerides (sensitivity: 0.8 mg/dl), cholesterol esterase/oxidase/peroxidase assay for cholesterol (sensitivity: 0.63 mg/dl) and chemiluminescence assay for insulin (sensitivity: 0.03  $\mu$ UI/ml).

**TNF- $\alpha$  level:** TNF- $\alpha$  concentration was measured using a commercial kit (Invitrogen) following the manufacturer's instructions. Assay sensitivity was 4 pg/ml. All samples were run in duplicate in the same assay for all analyses.

#### **2.4. Superoxide dismutase (SOD) activity**

Total SOD activity was measured by the method described by Misra and Fridovich<sup>28</sup>. This method is based on the inhibition of superoxide-dependent epinephrine auto-oxidation in a spectrophotometer adjusted at 480 nm. A 50 % inhibition of epinephrine auto-oxidation is defined as one unit of SOD, and the specific activity (U SOD/mg protein) was expressed as a percentage of the control group.

## 2.5. $\gamma$ -Glutamate cysteine ligase ( $\gamma$ -GCL) activity

The activity of  $\gamma$ -GCL was measured by a fluorescence-based microtiter plate assay as described by Correa et al.<sup>29</sup> with minor modifications. Briefly, after cell lysis and centrifugation, an aliquot was reserved for protein content analysis. Another aliquot was mixed with the reaction cocktail (400 mM Tris buffer, 20 mM L-glutamic acid, 2 mM EDTA, 20 mM sodium borate, and 40 mM ATP freshly added). The reaction was initiated by adding 2 mM cysteine. After incubation, reaction was stopped with 200 mM 5-sulfosalicylic acid. The amount of  $\gamma$ -glutamylcysteine ( $\gamma$ -GC) formed was measured by 2,3-naphthalenedicarboxaldehyde (NDA, Invitrogen) fluorescence. Fluorescence was read in a Synergy HT microplate reader (Biotek, USA) at 485 nm/528 nm.  $\gamma$ -GCL activity was calculated in moles  $\gamma$ -GC/min/mg protein and presented as a percentage of control conditions.

## 2.6. Glutathione (GSH) measurement

GSH levels were determined by a fluorescence based assay<sup>30</sup> with modifications. Astrocytes were treated for 2 or 24 h, then conditioned medium was saved and cells were collected in Locke Buffer (10 mM HEPES, 5.5 mM KCl, 10 mM glucose, 5 mM NaHCO<sub>3</sub>, and 130 mM NaCl). After sonication, cells were centrifuged at 12,000 rpm for 30 min. Supernatant aliquots were saved for protein determination by the Bradford assay. Conditioned medium and supernatant samples were diluted with an equal volume of 200 mM 5-sulfosalicylic acid and kept on ice for 15 min. After vigorously vortexing, samples were centrifuged at 12,000 rpm for 3 min. 20  $\mu$ l standard curve of GSH, supernatants and conditioned medium samples were loaded in a 96-well plate in duplicate. 180  $\mu$ l/well of NDA solution (50 mM Tris pH 10, 0.5 N NaOH, 10 mM NDA) were added to every well and the plate was incubated for 30 min in the dark at RT. Fluorescence was read in a Synergy HT microplate reader (Biotek, USA) at 485 nm/528 nm.

## 2.7. Reactive oxygen species (ROS) determination

Intracellular ROS levels were detected using 2'-7'-dichlorofluorescein diacetate (DCFH-DA) which when hydrolyzed within the cell is oxidized to fluorescent dichlorofluorescein (DCF) as described by Saba et al.<sup>31</sup>. Cells were seeded onto 96-well plates at a density of  $3 \times 10^4$  cells per well. They were pre-incubated with 10  $\mu$ M DCFH-DA for 30 minutes at 37°C. Then, the cells were exposed to PA with or without 0.1  $\mu$ M NDP-MSH for 2 h. Fluorescence intensity was measured using a Synergy HT microplate reader with filters at 485 nm excitation and 520 nm emission.

## 2.8. Cell viability assay

Cell viability was assessed by the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) reduction assay<sup>32</sup>. Briefly, cells were washed in Krebs buffer and incubated for 4 h with 110  $\mu$ l of a 0.5  $\mu$ g/ml MTT-Krebs buffer solution. Formazan crystals obtained from MTT reduction were dissolved in 100  $\mu$ l of a 0.04 N HCl-isopropanol solution. OD was measured in a microplate spectrophotometer (Bio-Rad) at 595 nm and presented as a percentage of control conditions.

## 2.9. Immunocytochemistry

To examine the nuclear translocation of Nrf2, immunocytochemistry was performed following the Kundu et al. protocol<sup>33</sup> with minor modifications. Cells were washed in PBS and fixed in 4% paraformaldehyde (PFA) for 10 minutes at RT, permeabilized with PBS-triton X 0.2%, and blocked with PBS-Tween 0.1%+10% goat serum. Then, slides were incubated overnight at 4°C with anti-Nrf2 antibody (1:50, Novus). After washing in PBS, cells were further incubated with goat anti-rabbit IgG conjugated with fluorescein (1:200, Vector Laboratories) for 1 hour at RT. Slides were mounted in Vectashield mounting medium (Vector Laboratories). Negative control slides were incubated with blocking solution instead of the



primary antibodies. Staining was visualized in a fluorescence microscope (Axiophot; Carl Zeiss, Jena, Germany). Quantification of fluorescence intensity was done with ImageJ Software (NIH, USA).

### **2.10. Reverse transcriptase-real time polymerase chain reaction (RT-qPCR)**

Total RNA from cultured astrocytes ( $1 \times 10^6$  cells) and from hypothalamus was extracted using Trizol reagent (Invitrogen) following the manufacturer's protocol. Two micrograms of total RNA were treated with 1 U RQ1 RNase free DNase (Promega Corporation, WI, USA) at 37°C for 10 min, and reverse-transcribed as described before<sup>34</sup>. Products of the RT reaction were amplified using specific primers and SYBR Green Select Master Mix (Invitrogen Life Technologies) on a StepOne™ Real-Time PCR System (Applied Biosystems). Primer sequences are detailed in Table 1. PCR product specificity was verified by melting curve analysis. No-RT controls were performed by omitting the reverse transcriptase enzyme in the RT reaction, and no-template controls were performed by addition of nuclease-free water instead of cDNA. Gene expression was normalized to the endogenous reference gene HPRT by the  $\Delta\Delta C_t$  method<sup>35</sup> using Step-One Software (Applied Biosystems), and expressed as fold-changes relative to the control group.

### **2.11. Statistical analysis**

Data were analyzed by one sample t test, Student's t test, one-way analysis of variance (ANOVA) or two-way ANOVA followed by Bonferroni's multiple comparisons test, as required by the experimental design. GraphPad Prism 5 Software was used (GraphPad Software, CA, USA). Differences with a value of  $p < 0.05$  were considered statistically significant.

### **2.6. Ethics Statement**

Experimental procedures were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of the School of Medicine, University of Buenos Aires,

Argentina (resolution n° 2746/2013) and were carried out in compliance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals

### **3. RESULTS**

#### **3.1. NDP-MSH prevented PA-induced ROS production in astrocytes**

In order to determine PA working concentration, we tested cell viability and ROS production in astrocytes exposed to different doses of PA. Astrocyte viability was not significantly different from control after treatment with PA at 50  $\mu$ M and 100  $\mu$ M. However, concentrations of 200  $\mu$ M or higher induced cell death in a dose-dependent manner (Fig. 1a). PA also induced astrocyte ROS production in a concentration-dependent manner (Fig. 1b).

To test the effect of NDP-MSH on ROS production, cells were co-incubated for 2 h with PA (100  $\mu$ M) and NDP-MSH (0.1  $\mu$ M). We observed that ROS production was significantly decreased by NDP-MSH. To determine whether MC4R is involved in NDP-MSH effects on ROS production we treated cells with JKC363 (10 nM), a potent and selective MC4R antagonist. JKC363 completely blocked NDP-MSH effect on ROS levels induced by PA (Fig. 1c). Neither NDP-MSH nor JKC363 alone modified basal ROS levels (Fig. 1c).

#### **3.2. PA activated the Nrf2 pathway in astrocytes**

Nrf2 is the master transcription factor that regulates basal and inducible expression of antioxidant proteins, detoxifying enzymes and xenobiotic transporters<sup>36</sup>. In basal conditions, Nrf2 is located in the cytoplasm; when activated, it migrates to the nucleus<sup>36</sup>. Since PA exhibits pro-oxidant properties, we evaluated the effect of PA on Nrf2 activation by determining its intracellular localization by immunocytochemistry. Astrocytes were exposed to 100  $\mu$ M PA with or without 0.1  $\mu$ M NDP-MSH co-incubation for 6 h. Treatment with PA strongly increased Nrf2 nuclear translocation and NDP-MSH co-treatment reduced nuclear fluorescence intensity compared to PA alone (Fig. 2a). Interestingly, NDP-MSH alone decreased Nrf2 nuclear translocation compared to control conditions (Fig. 2b).

Next, we investigated whether these results correlated with mRNA levels of Nrf2-dependent genes.  $\gamma$ -GCL catalytic subunit (GCLc),  $\gamma$ -GCL modifier subunit (GCLm) and heme-oxygenase 1 (HO-1) gene expression were evaluated by RT-qPCR. PA increased mRNA levels of GCLc (Fig. 2c), GCLm (Fig. 2d), and HO-1 (Fig. 2e), whereas PA+NDP-MSH-treatment decreased them. Surprisingly, NDP-MSH alone did not modify their expression.

Brain derived neurotrophic factor (BDNF) plays an important role in neuronal survival and it has been reported to prevent oxidative damage in primary neuronal cultures and neuronal cell lines by increasing GSH levels and activity of antioxidant enzymes<sup>37</sup>. Our group demonstrated that melanocortins stimulate BDNF expression in astrocytes<sup>34</sup>, and therefore we tested NDP-MSH effect on PA-challenged astroglial cells. PA reduced BDNF gene expression, an effect significantly prevented by NDP-MSH co-incubation. As we reported before, NDP-MSH alone increased BDNF mRNA levels (Fig. 2f)

### **3.3. NDP-MSH enhanced SOD and $\gamma$ -GCL activity in astrocytes**

To determine the mechanism by which NDP-MSH reduced ROS levels we evaluated the activity of SOD and  $\gamma$ -GCL antioxidant enzymes. NDP-MSH increased SOD activity 2-fold compared to control conditions after 24 h of incubation (Fig. 3a). Under PA-oxidative insult, SOD activity was reduced by about 65% compared to basal value and this effect was completely prevented by NDP-MSH (Fig. 3a). NDP-MSH markedly increased the enzymatic activity of  $\gamma$ -GCL in a dose-dependent manner (Fig. 3b). Notably, a remarkable 5-fold increase was observed in the presence of PA+NDP-MSH (Fig. 3c), even though PA alone did not change  $\gamma$ -GCL activity.

### **3.4. NDP-MSH increased GSH levels in astrocytes**

GSH is the main endogenous antioxidant compound, its rate of synthesis depending on  $\gamma$ -GCL activity. Considering that NDP-MSH reduced PA-induced ROS production while increasing  $\gamma$ -GCL activity, as a next step, we evaluated the effect of this melanocortin on intracellular and extracellular levels of GSH. 24 h-treatment with NDP-MSH did not modify

GSH intracellular levels but stimulated GSH extracellularly (Fig. 4a). However, when NDP-MSH was co-incubated with PA for 2 h, it enhanced both intracellular and extracellular GSH concentrations (Fig. 4b and c). In both cases, PA itself did not modify GSH production (Fig. 4b and c).

### 3.5. Effect of HFD and NDP-MSH on calorie intake and biochemical parameters

$\alpha$ -MSH plays a key role in energy homeostasis, inhibiting feeding behavior through MC4R activation. Our group has studied anti-inflammatory actions of melanocortins in the CNS, showing that MC4R is involved in this effect, but little is known about a possible effect of melanocortins on the oxidative component of inflammation. Therefore, we decided to investigate the effect of NDP-MSH treatment on the antioxidant response in animals fed a HFD. To do so, rats were exposed to either SD (5% fat) or HFD (50% fat) for 8 weeks. Daily calorie intake and weight gain were registered during this period. As expected, HFD-fed rats consumed more calories than SD group (Fig. 5a). However, after the 8th week of exposure to each diet, there were no differences in weight gain (Fig. 5b). At the end of the experiment, animals were fasted overnight, and then injected ICV with saline or NDP-MSH, re-fed, and euthanized 4 h later.

Figure 6a depicts 4-h calorie intake in response to NDP-MSH in SD and HFD groups. Consistent with reported anorexigenic actions of melanocortins, NDP-MSH treatment reduced calorie intake in SD-fed rats. However, this effect was not observed in animals exposed to HFD. Biochemical determinations showed that HFD *per se* did not modify any parameter. NDP-MSH reduced serum glucose levels in SD and HFD groups (Fig. 6b). Triglyceride levels markedly decreased upon NDP-MSH stimulation in SD-fed rats. In contrast, this effect was not observed in animals fed a HFD (Fig. 6c). NDP-MSH impact on serum insulin levels differed in function of the diet administered, inducing an increase in SD group (Fig. 6d) and a decrease in HFD group ( $p=0.0603$ ). Treatment did not modify serum cholesterol levels (Fig. 6e) in either SD or HFD-fed animals. As we mentioned before, high fat-feeding triggers inflammation impacting on key hypothalamic areas involved in body

weight regulation<sup>2</sup>. Considering the anti-inflammatory actions of melanocortins, we measured TNF- $\alpha$  production in our model. Surprisingly, neither diet nor NDP-MSH altered serum levels of this cytokine.

### **3.6. Effect of HFD and NDP-MSH on the antioxidant response**

We investigated the effect of NDP-MSH treatment on antioxidant response in the hypothalamus. We observed that after 4 h of treatment NDP-MSH increased SOD activity in SD-fed rats (Fig. 7a), but had no effect on animals exposed to HFD. Moreover, HFD by itself did not modify SOD activity (Fig. 7a). Next, we studied Nrf2 pathway by RT-PCR, determining Nrf2, GCLm, and GCLc mRNA levels. Neither NDP-MSH nor HFD significantly modified expression of the aforementioned genes (Fig. 7b, c and d).

### **3.7. Effect of HFD and NDP-MSH on BDNF and MC4R expression**

BDNF has been reported to exert antioxidant action, and its hypothalamic expression augments in response to melanocortins<sup>38</sup>. For this reason, we evaluated BDNF and MC4R expression in our model. HFD reduced mRNA levels of BDNF (Fig. 8a) and MC4R (Fig. 8b) and, consistent with the lack of effect of NDP-MSH on biochemical and antioxidant parameters upon HFD exposure, NDP-MSH did not revert the decrease in MC4R and BDNF expression induced by the diet. Surprisingly, NDP-MSH did not increase expression of these genes in the SD group, an effect we previously detected when  $\alpha$ -MSH was administered by intraperitoneal injection<sup>39</sup>. On the contrary, we found that in cultured astrocytes PA (100  $\mu$ M) induced a 34% reduction in MC4R mRNA levels compared to control group, while co-incubation with NDP-MSH (0.1  $\mu$ M) induced a significant 46.6% increase in MC4R expression compared to control group, indicating that NDP-MSH might act differentially in whole hypothalamic tissue than in astrocytes.

#### 4. DISCUSSION

Obesity has been associated with chronic low-grade inflammation that affects peripheral tissues and the CNS. Saturated fatty acids are elevated in plasma of obese patients<sup>40</sup>. Since they can cross the blood brain barrier they contribute to activation of glial cells by triggering neuroinflammation<sup>41</sup> which may lead to neurodegeneration. Oxidative damage is involved in the pathogenesis of metabolic diseases and HFD has been reported to trigger oxidative stress<sup>42</sup>. Moreover, PA induces expression of pro-inflammatory cytokines in astrocytes<sup>10</sup> and apoptosis of neurons and astrocytes by increasing oxidative stress<sup>43</sup>. Astrocytes perform several physiological functions to maintain brain homeostasis, and their involvement in obesity-related processes is beginning to be understood. Melanocortins exert anti-inflammatory and neuroprotective effects on the CNS<sup>18,22</sup>. However, antioxidant effects of melanocortins in the brain are unknown. Therefore, we investigated the effect of NDP-MSH, an  $\alpha$ -MSH analogue, on oxidative stress in PA-treated astrocytes and in a model of HFD. In the present work we demonstrated that melanocortins exerted antioxidant actions in astrocyte and in the hypothalamus of male rats.

PA-stimulated ROS production has been studied in different cells including astrocytes<sup>12</sup>. In this study, we report for the first time that NDP-MSH, through MC4R, reduces ROS levels in astrocytes challenged with PA. Astrocytes are key players in defense against oxidative insults<sup>37</sup>. In an oxidative context, astroglial cells respond by changing the expression of several genes regulated by Nrf2, the master redox transcription factor. Previous studies have shown that PA induces Nrf2 expression in hepatocyte primary cultures<sup>44</sup> and in the normal liver cell line QZG<sup>45</sup>. Furthermore, PA activates Nrf2 pathway in mouse hepatoma and in mouse embryonic fibroblast cell lines<sup>46</sup>.  $\alpha$ -MSH also reverses oxidative stress induced by UV radiation through Nrf2 activation in human skin cells<sup>24</sup>. Our experiments showed that Nrf2 and its target genes GCLc, GCLm and HO-1 were positively regulated by PA and that NDP-MSH reversed this effect. Considering that both pro-oxidant compounds and ROS stimulate Nrf2 nuclear translocation, PA-induced Nrf2 activation may be mediated by

increased ROS levels. Given that NDP-MSH reduces ROS production, we suggest that ROS reduction could be responsible for NDP-MSH inhibitory action on Nrf2 nuclear translocation. Interestingly, NDP-MSH alone reduced Nrf2 translocation without modifying ROS levels. JNK and p38 MAPK have been reported to induce Nrf2 phosphorylation, facilitating nuclear translocation of this transcription factor<sup>47</sup>. In other studies, melanocortins were shown to inhibit JNK and p38 activity in HEK293 cells<sup>48</sup> and in a model of Alzheimer's disease<sup>49</sup>, respectively. Considering this background, we could suggest that reduction in Nrf2 translocation induced by NDP-MSH could be a consequence of inhibition of JNK and p38 MAPK.

SOD activity is reduced in embryonic cardiomyocytes exposed to PA<sup>50</sup>. Concordantly, in our model PA lowered SOD activity and NDP-MSH treatment restored enzymatic activity to control levels in cultured astrocytes. Even in basal conditions NDP-MSH increased SOD activity 2 fold, thereby indicating that melanocortins have antioxidant action *per se*. Since NDP-MSH did not activate the Nrf2 pathway in our model, it is likely that the aforementioned transcription factor is not involved in the NDP-MSH effect on SOD activity. Studies indicate that PPAR- $\gamma$  activation increases SOD activity and synthesis in hippocampus<sup>51</sup>. Considering that our group has demonstrated that melanocortins induce expression of PPAR- $\gamma$  in astrocytes<sup>23</sup>, we hypothesize that increased SOD activity could be mediated by this nuclear receptor. Further studies are needed to prove this hypothesis.

GSH is the main antioxidant compound of the cell, its synthesis rate limiting step catalyzed by  $\gamma$ -GCL. Our results show that NDP-MSH protected astrocytes against oxidative damage, stimulating GSH synthesis by increasing  $\gamma$ -GCL activity. Increased GSH release from astrocytes could also support survival of neurons<sup>52</sup>. Contrary to observations in other cell types<sup>53</sup>, in our experiments PA did not modify  $\gamma$ -GCL activity despite increasing mRNA levels of both  $\gamma$ -GCL subunits. Conversely, NDP-MSH stimulated enzymatic activity without modifying mRNA levels. Given that  $\gamma$ -GCL activity is regulated at multiple levels<sup>54</sup>, it is possible that PA affects transcriptional activity whereas NDP-MSH induces post-transcriptional changes. Moreover, we recently reported that BDNF induces GSH synthesis

in astrocytes<sup>31</sup>, and in the present work, we show that NDP-MSH prevented the reduction in BDNF expression induced by PA, suggesting that BDNF could be a mediator of NDP-MSH antioxidant effect in astrocytes.

Hypercaloric diets have extensive harmful effects, impacting multiple organs and systems including the CNS. *In vivo* studies show that high-fat feeding compromises learning and memory<sup>55,56</sup>, induces neuron death in the hippocampus and hypothalamus<sup>4</sup>, and triggers oxidative stress in different brain areas<sup>42</sup>. Therefore, we decided to test the effect of melanocortins on antioxidant defense using an *in vivo* model of high-fat feeding. Interestingly, animals fed a HFD did not gain more weight than SD-fed rats despite consuming more calories per day. Similar to our results several studies have shown no difference in body weight between animals, from different species and strains, fed a SD or a HFD for 8 weeks or longer. For instance, HFD exposure during 8 weeks affected body weight in Long-Evans and Wistar rats but not in Sprague-Dawley rats<sup>57</sup>. Similarly, Marques et al. indicate that Wistar rats increased body weight after consuming HFD for 4 weeks while Sprague-Dawley rats showed significant weight difference after 7 weeks. In addition, neither triglycerides nor insulin levels were affected by HFD in both strains<sup>58</sup>. C57BL/6J mice showed weight gain after 8 week in males and after 15 week in females<sup>59</sup>. On the other, no difference in body weight in C57BL/6J male mice after 16 weeks of HFD exposure has also been reported<sup>60</sup>. The same could apply for changes in cytokines release. We found no changes in TNF- $\alpha$  release in our experimental model. Accordingly, an increased in TNF- $\alpha$  levels was found after prolonged HFD feeding (24 weeks) in C57BL/6J mice<sup>61</sup>. Contradictory results about changes in IL-1 $\beta$  release have also been reported. HFD did not increased plasma levels of IL-1 $\beta$  in Sprague-Dawley rats after 8 weeks feeding a HFD<sup>57</sup> while serum levels of IL-1 $\beta$  were markedly increased after 4 weeks but not at 16 weeks of HFD consumption in C57BL/6J mice<sup>60</sup>, indicating once more differences due to the experimental model. It is noteworthy that different HFD formulations have been used in the reports mentioned above, suggesting that several experimental issues could explain the absence of



weight difference, including the use of different fat sources (lard, vegetable oil, margarine, etc), the different duration of diet exposure, species, and animal strain.

Since HFD did not produce alterations in biochemical or in hypothalamic parameters and did not increase TNF- $\alpha$  levels, we conclude that our *in vivo* model did not develop metabolic syndrome characteristics. However, given that high-fat feeding induces hypothalamic changes before the onset of peripheral inflammation, metabolic disturbances, and weight gain<sup>6</sup>, our model could be useful to evaluate early stages of this process. ICV treatment with NDP-MSH reduced calorie intake in SD-fed animals, as it was described previously<sup>62</sup>. This calorie intake reduction may account for reduced plasma glucose and triglyceride levels in SD-animals. Insulin concentration tended to be higher in HFD-fed rats, suggesting that these animals might develop insulin resistance, given that higher levels of insulin are needed to maintain glucose concentrations similar to control animals. It has been reported that central administration of  $\alpha$ -MSH improves insulin action on glucose uptake and production<sup>63</sup>. In view of the aforementioned, we could suggest that lower insulin levels are able to reduce blood glucose in HFD group treated with NDP-MSH. However, this effect is opposite to NDP-MSH action in SD-fed rats which show elevated insulin concentration.

Reported data on the impact of HFD on MC4R expression are contradictory. Some studies indicate that high-fat feeding does not change MC4R expression<sup>64</sup>, whereas others show that mice exposed to a HFD have higher mRNA levels of the receptor and better responsiveness to short-term treatments with MC4R agonists<sup>65</sup>. It is noteworthy that clinical studies with potent MC4R agonists were ineffective to treat obesity<sup>66</sup>. In line with this finding, several studies reported that HFD-fed rats showed a reduction in MC4R mRNA levels<sup>67</sup>, central resistance to MC4R agonists<sup>64</sup>, and less binding to radio-labeled MC4R agonists in the hypothalamus<sup>68</sup>. Concordant with these studies, our results show that HFD exposure for 8 weeks reduced hypothalamic MC4R expression despite neither increasing body weight nor inducing metabolic syndrome, which reinforces the suggestion that hypothalamic changes occur before the onset of clinical symptoms.

Regarding antioxidant response, we observed that NDP-MSH increased SOD activity in animals exposed to SD. Astrocytes could contribute, at least partially, to this effect, considering results observed *in vitro*. Although PA reduced SOD activity in astrocytes, HFD did not change hypothalamic SOD activity. Interestingly, in HFD group, NDP-MSH did not increase SOD activity. So, it is likely that the lack of melanocortin action on SOD activity in HFD-fed animals could be due to decreased MC4R expression in the hypothalamus. Expression of antioxidant genes was not modified by either diet or treatment, differing from *in vitro* experiments where PA stimulated Nrf2 pathway. This data could be explained by the presence of neurons and other cells that may be affected differently than astrocytes when challenged with components of a HFD, so that the effect of the diet on astrocytes alone is masked when the whole hypothalamus is studied.

Learning deficits detected in obese animals have been linked to decreased BDNF expression<sup>69</sup>. In our experimental model, we observed that HFD reduced BDNF gene expression and NDP-MSH treatment did not reverse this effect. Surprisingly, melanocortin ICV injection did not modify BDNF expression even in animals fed a SD, which differs from the effect we detected previously when  $\alpha$ -MSH was administered by intraperitoneal injection<sup>39</sup> and suggests that peripheral mediators may be needed for  $\alpha$ -MSH action on central BDNF expression. MC4R has been reported to be expressed in the gastrointestinal system, its activation inducing glucagon-like peptide 1 (GLP-1) expression<sup>70</sup>; an analogue of this hormone was shown to stimulate BDNF expression in the brain<sup>71</sup>. Considering these data, GLP-1 could be considered as a possible mediator of the melanocortin effect on hypothalamic BDNF expression.

The hypothalamus is a key player in modulating feeding behavior and energy expenditure. Several studies have linked obesity with mitochondrial alterations which leads to oxidative stress<sup>7</sup>. Then, excessive ROS production induces NF $\kappa$ B activation, the main mediator of the inflammatory response<sup>72</sup>, and modifies neuronal activity in the hypothalamus<sup>73</sup>. High-fat feeding and obesity are associated with hypothalamic inflammation and reactive gliosis in rodents. However, the role of astrocyte activation in response to metabolic and dietary clues

is not fully understood. The present study demonstrated that NDP-MSH increased antioxidant enzyme activity and GSH levels in astrocytes, alleviating PA-induced oxidative stress and contributing to prevention of neuronal damage. *In vivo*, NDP-MSH exerts an antioxidant role by increasing SOD activity in SD-fed animals, suggesting that melanocortin antioxidant action added to their anti-inflammatory activity could protect neurons. However, NDP-MSH fails to show the same effect in the HFD group, in which hypothalamic MC4R expression is reduced. These results underscore the importance of  $\alpha$ -MSH in hypothalamic function and point to early disruptions in the melanocortin circuit in an HFD context as potential contributors to hypothalamic malfunction which may lead to metabolic disorders. Further research is needed in order to elucidate the mechanism of HFD-induced damage in the hypothalamus and how alteration of the melanocortin system contributes to perpetuate food intake dysregulation. Better understanding of these processes will allow us to develop new therapeutic strategies for metabolic diseases.

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## FIGURE LEGENDS

### Figure 1 - NDP-MSH prevented PA-induced ROS production through MC4R. (a)

Astrocytes were treated with increasing PA doses for 24 h. Cell viability was determined by MTT assay. Values are the mean  $\pm$  SEM of 8 determinations per group of 1 representative experiment of 2 independent ones. Data were analyzed by comparing each dose of PA with its respective control by Student's t test \*\*\* $p < 0.001$  vs. control. (b) Astrocytes were treated with increasing concentrations of PA for 2 h and ROS production was determined. Values are the mean  $\pm$  SEM of 8 determinations per group of 1 representative experiment of 2 independent ones. Data were analyzed by comparing each dose of PA with its respective control by Student's t test \*\*\* $p < 0.001$  vs. control. (c) Astrocytes were incubated with PA (100  $\mu$ M) in presence or absence of NDP-MSH (0.1  $\mu$ M) and/or JKC363 (10 nM) for 2 h and ROS production was determined. Values are the mean  $\pm$  SEM of fluorescence intensity (arbitrary units) of 8 determinations per group of 1 representative experiment of 3 independent ones. Data were analyzed by one way ANOVA followed by Bonferroni's multiple comparison test  $F_{(5, 42)} = 99.47$ ; \*\*\* $p < 0.001$  vs. control;  $\wedge p < 0.05$ ;  $\wedge p < 0.01$  vs. PA+NDP-MSH.

### Figure 2 - NDP-MSH prevented Nrf2 nuclear translocation and Nrf2-dependent gene

expression induced by PA and stimulated BDNF expression. Astrocytes were incubated for 6 h with PA (100  $\mu$ M) in presence or absence of NDP-MSH (0.1  $\mu$ M). (a) Representative images for Nrf2 immunostaining of 1 representative experiment of 3 independent ones. (b) Nuclear fluorescence intensity (arbitrary units) was quantified using ImageJ Software. Data represent mean  $\pm$  SEM of 100-175 cells per group of 1 representative experiment of 3 independent ones and were analyzed by one way ANOVA followed by Bonferroni's multiple comparison test  $F_{(3, 577)} = 235.4$ ; \*\*\* $p < 0.001$  vs. control;  $\wedge\wedge p < 0.001$  vs. PA. (c) GCLc, (d) GCLm, and (e) HO-1 expression was determined by RT-qPCR after 6 h-treatment with PA (100  $\mu$ M) in presence or absence of NDP-MSH (0.1  $\mu$ M). Data represent mean  $\pm$  SEM of 4-5 independent experiments and were analyzed by one way ANOVA, GCLc:  $F_{(2, 14)} = 16.42$ ,

GCLm:  $F_{(2, 9)} = 1.92$ , HO-1: GCLm:  $F_{(2, 15)} = 3.818$ , \* $p < 0.05$ , \*\*\* $p < 0.001$  vs. control;  $\wedge p < 0.01$  vs. PA. **(f)** Astrocytes were incubated for 1 h with PA (100  $\mu\text{M}$ ) in presence or absence of NDP-MSH (0.1  $\mu\text{M}$ ) and BDNF expression was determined by RT-qPCR. Data represent mean  $\pm$  SEM of 3 independent experiments and were analyzed by one way ANOVA,  $F_{(3, 12)} = 7.188$ ; \* $p < 0.05$  vs. control;  $\wedge p < 0.05$  vs. PA.

**Figure 3 – NDP-MSH increased SOD and  $\gamma$ -GCL activity.** **(a)** Astrocytes were treated for 24 h with PA (100  $\mu\text{M}$ ) in presence or absence of NDP-MSH (0.1  $\mu\text{M}$ ) and SOD activity was measured using Fridovich's technique as described in Material and Methods. Data represent mean  $\pm$  SEM of 3 independent experiments and were analyzed by one way ANOVA followed by Bonferroni's multiple comparison test,  $F_{(3, 12)} = 20.49$ , \* $p < 0.05$ , \*\* $p < 0.01$  vs. control;  $\wedge p < 0.05$  vs. PA. **(b)** Cells were incubated with increasing doses of NDP-MSH for 24 h and  $\gamma$ -GCL activity was measured by Correa's technique as described in Material and Methods. Data represent mean  $\pm$  SEM of 3 independent experiments and were analyzed by one way ANOVA followed by Bonferroni's multiple comparison test,  $F_{(3, 10)} = 15.66$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. control. **(c)** Astrocytes were incubated with PA (100  $\mu\text{M}$ ) in presence or absence of NDP-MSH (0.1  $\mu\text{M}$ ) for 24 h and  $\gamma$ -GCL activity was measured by Correa's technique as described in Material and Methods. Data represent mean  $\pm$  SEM of 4 independent experiments and were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test \* $p < 0.05$  vs. control;  $\wedge p < 0.05$  vs. PA.

**Figure 4 – NDP-MSH increased GSH levels.** **(a)** Astrocytes were treated with NDP-MSH (0.1  $\mu\text{M}$ ) for 24 h and GSH levels were determined by fluorescence assay. Data represent mean  $\pm$  SEM of 4 independent experiments and were analyzed by Student's t test \*\*\* $p < 0.001$  vs. control. Astrocytes were exposed to PA (100  $\mu\text{M}$ ) in presence or absence of NDP-MSH (0.1  $\mu\text{M}$ ) for 2 h to determine intracellular **(b)** and extracellular **(c)** GSH levels. In both cases, data represent mean  $\pm$  SEM of 3 independent experiments and were analyzed

by one way ANOVA followed by Bonferroni's multiple comparison test, intracellular GSH  $F_{(3, 23)} = 4.187$ , extracellular GSH:  $F_{(3, 24)} = 2.821$ , \* $p < 0.05$  vs. control.

**Figure 5 – Calorie intake and weight gain of animals fed a SD or a HFD. (a)** Calorie intake was calculated by multiplying energy concentration of each diet (kcal/g) by the amount of food (g) consumed per day. **(b)** Weight gain was calculated as the difference between initial and final body weight. Data represent mean  $\pm$  SEM of  $n=11-15$  animals per group and were analyzed by Student's t test. \*\*\* $p < 0.001$  vs. SD

**Figure 6 – Effect of NDP-MSH on calorie intake, biochemical parameters and TNF- $\alpha$  levels in animals fed SD or HFD.** Animals were exposed to either SD (5% fat) or HFD (50% fat) for 8 weeks. Rats were fasted overnight and on the day of euthanasia were injected ICV with either saline or NDP-MSH. After 4 h animals were killed. **(a)** Calorie intake was calculated by multiplying energy concentration of each diet (kcal/g) by the amount of food (g) consumed in the last 4 h of the experiment. **(b)** Glucose, **(c)** tryglicerides, **(d)** insulin, and **(e)** cholesterol were measured in trunk blood after 4 h treatment with either saline or NDP-MSH. **(f)** Hypothalamic TNF- $\alpha$  levels were determined by ELISA. Data represent the mean  $\pm$  SEM of  $n=5-8$  animals per group and were analyzed by two way ANOVA \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  vs. SD saline; ^^ $p < 0.001$  vs. HFD saline.

**Figure 7 – Effect of NDP-MSH on hypothalamic antioxidant response in animals fed with SD or HFD. (a)** SOD activity was measured using Fridovich's technique as described in Materials and Methods. **(b)** Nrf2, **(c)** GCLc, and **(d)** GCLm gene expression was determined by RT-qPCR. Data represent the mean  $\pm$  SEM of  $n=5-8$  animals per group and were analyzed by two way ANOVA \* $p < 0.05$  vs. SD saline.

**Figure 8 – Effect of NDP-MSH on hypothalamic BDNF and MC4R gene expression in animals fed SD or HFD. (a) BDNF and (b) MC4R expression was determined by RT-qPCR.**

Data represent the mean  $\pm$  SEM of n=5-8 animals per group and were analyzed by two way ANOVA \*p<0.05 vs. SD saline; ^p<0.05 vs. SD NDP-MSH.

**Figure 9 - Graph depicting the antioxidant action of NDP-MSH in astrocytes. *In vitro* stimulation of astroglial MC4R increased SOD and  $\gamma$ -GCL activity, BDNF expression and GSH levels, alleviating PA-induced ROS production in astrocytes and possibly contributing to neuron protection.**

**Table 1.**Primer sequences used for RT-qPCR

Gene	Forward primer sequence	Reverse primer sequence
HO-1	5'-TCTATCGTGCTCGCATGAACA-3'	5'-TCTTCTGTCACCCTGTGCTTGA-3'
GCLc	5'-TCTGCCCAATTGTTATGGCTTT-3'	5'-GTCTGACACGTAGCCTCGGTAA-3'
GCLm	5'-CGTCACTGAGGCGTGAATGT-3'	5'-CGCCAGGGAGGTACTCAAAC-3'
Nrf2	5'-CAAATCCCACCTTGAACACAGAT-3'	5'-TGGGCTCTGCTAGGAAAGCA-3'
MC4R	5'-CCAACTGCAGCCTTCCAACT-3'	5'-GCCCCGAGTGGTTTTTCTC-3'
BDNF	5'-GATGAGGACCAGAAGGTTTCG-3'	5'-TCCAGCAGAAAGAGCAGAGG-3'
HPRT	5'-CTCATGGACTGATTATGGACAGGAC-3'	5'-GCAGGTCAGCAAAGAACTTATAGCC-3'



















