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Reductive stress impairs myoblasts mitochondrial function and triggers mitochondrial hormesis

François Singh [a,b,c], Anne-Laure Charles [a], Anna-Isabel Schlagowski [a,b], Jamal Bouitbir [c], Annalisa Bonifacio [c], François Piquard [a], Stephan Krähenbühl [c], Bernard Geny [a,b], Joffrey Zoll [a,b,*]

[a] University of Strasbourg, Faculty of Medicine, Fédération de Médecine Translationelle, EA 3072, 11 rue Humann, Strasbourg, France
[b] CHRU of Strasbourg, Physiology and Functional Explorations Department, New Civil Hospital, B.P. 426, 67091 Strasbourg, France.
[c] Department of Clinical Pharmacology & Toxicology, Department of Biomedicine, University Hospital, Hebelstrasse 20, 4031 Basel, Switzerland

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ABSTRACT

Even though oxidative stress damage from excessive production of ROS is a well known phenomenon, the impact of reductive stress remains poorly understood. This study tested the hypothesis that cellular reductive stress could lead to mitochondrial malfunction, triggering a mitochondrial hormesis (mitohormesis) phenomenon able to protect mitochondria from the deleterious effects of statins. We performed several in vitro experiments on L_6 myoblasts and studied the effects of N-acetylcysteine (NAC) at different exposure times. Direct NAC exposure (1 mM) led to reductive stress, impairing mitochondrial function by decreasing maximal mitochondrial respiration and increasing H_2O_2 production. After 24 h of incubation, the reactive oxygen species (ROS) production was increased. The resulting mitochondrial oxidation activated mitochondrial biogenesis pathways at the mRNA level. After one week of exposure, mitochondria were well-adapted as shown by the decrease of cellular ROS, the increase of mitochondrial content, as well as of the antioxidant capacities. Atorvastatin (ATO) exposure (100 μM) for 24 h increased ROS levels, reduced the percentage of live cells, and increased the total percentage of apoptotic cells. NAC exposure during 3 days failed to protect cells from the deleterious effects of statins. On the other hand, NAC pretreatment during one week triggered mitochondrial hormesis and reduced the deleterious effect of statins. These results contribute to a better understanding of the redox-dependant pathways linked to mitochondria, showing that reductive stress could trigger mitochondrial hormesis phenomenon.

1. Introduction

The mitochondrion is the most important organelle in determining continued cell survival and cell death. In most cell types, mitochondria are the major source of reactive oxygen species (ROS), which affect apoptosis both directly and indirectly via the activation of transcription factors [1]. For a long time, ROS were considered exclusively unwanted by-products of oxidative phosphorylation, as high concentrations of ROS cause lipid peroxidation and damage to cell membranes, proteins, carbohydrates, and DNA [2]. In the last decade, it has been shown that low, non-cytotoxic concentrations of ROS can serve as signals, triggering the activation of specific pathways [3–10]. The preservation of this non-cytotoxic level of ROS is ensured by antioxidant systems, which play a major role in cellular redox homeostasis. A deficiency or defect in these systems leads to an increase in tissue damage. In such cases, ROS can act either as second messengers or as a source of cellular damage, depending on the level of ROS production [4,6,7,11]. The concept of mitochondrial hormesis, or "mitohormesis", proposes that low doses of mitochondrial ROS can activate mitochondrial biogenesis and antioxidant capacities in order to counteract oxidative stress and to re-establish homeostasis [4,5,7,8,12].

The development of chronic oxidative stress has been implicated in the metabolic myopathy that is a secondary symptom of numerous pathologies such as diabetes mellitus, heart failure, or chronic obstructive pulmonary disease (COPD) [13,14]. This myopathy is also the most common adverse event encountered in patients treated with statins (HMG-CoA inhibitors, used to lower the plasmatic cholesterol levels), the most frequently prescribed treatment in developed countries. Recently, we found that statins protect mitochondria in the highly oxidative cardiac muscle by triggering a mitohormesis mechanism but impair mitochondrial function in glycolytic skeletal muscle [4].

Therefore, strategies involving moderate mitochondrial stress, inducing a mitohormesis mechanism may be used for the development of new ROS-targeting drugs. This would result in reducing the symptoms of metabolic myopathies by strengthening muscular mitochondrial function [11,15].

Even though oxidative stress damage from excessive production of ROS is a well known phenomenon, the impact of reductive stress

* Corresponding author at: Université de Strasbourg, Faculté de Médecine, EA 3072, 11 rue Humann, 67000 Strasbourg, France. Tel.: +33 3 68 85 34 36; fax: +33 3 68 85 34 44.
E-mail address: zolljoffrey@yahoo.com (J. Zoll).

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remains poorly understood. Reductive stress can be defined as an excess of reducing equivalents (NAD(P)H and/or GSH) in the presence of intact oxido-reductive systems [2]. N-acetylcysteine (NAC) is the acetylated form (efficiently metabolized) of cysteine and is the most immediate precursor of glutathione (GSH). The GSH system is the main mechanism of detoxification of free radicals and ionized metabolites [16,17], and it is partly localized in the mitochondria. NAC is mainly used for its mucolytic action on the disulfide bonds of mucoproteins [18] and its modulating action on oxidative stress [19]. Interestingly, it has been shown that NAC treatment in H_9C_2 cells led to reductive stress [20]. This condition paradoxically increased the level of mitochondrial oxidation. However, the consequences as well as the potential beneficial effects following this reductive stress-inducing mitochondrial oxidation remained undiscovered [2].

We hypothesized that NAC treatment could change the redox environment of the cell, inducing mitochondrial oxidation (as described by [2,20]) and triggering a mitohormesis phenomenon, protecting L_6 myoblasts from the deleterious effects of statins.

Therefore, we investigated the effects of N-acetylcysteine on L_6 myoblasts. We found that NAC induced cellular reductive stress, which had for consequence an inhibitory effect on the mitochondrial respiratory chain, leading to an increase in mitochondrial ROS production and mitochondrial oxidation. This triggered mitochondrial biogenesis pathways and led to an increase in the number of mitochondria and in antioxidant capacities after 7 days of incubation. Finally, we showed that mitochondrial hormesis, triggered by NAC, protected cells against statin-induced apoptosis.

2. Materials and methods

2.1. Cell culture

L_6 rat myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM Low glucose, Milerium, VWR International) supplemented with 20% fetal calf serum (FCS) and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Gibco) at 37 °C under a humidified 5% CO_2 atmosphere. These cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

The acute effects of N-acetylcysteine (Sigma) were studied at several doses (50 μM, 500 μM, 1 mM, and 5 mM) for mitochondrial respiration, H_2O_2 production, and for the detection of superoxide production by electron spin resonance (ESR).

The chronic effects of N-acetylcysteine on L_6 myoblasts were studied by incubation with 1 mM of NAC in the culture medium for 24 h, three days, and one week. For the three days and one-week experiments, cells were also incubated with 100 μM atorvastatin for the last 24 h, in DMEM containing 10% FCS and 1% antibiotics.

2.2. Cell viability assays

Cell viability assays were performed after 24 h or one week of incubation with or without 1 mM NAC in DMEM containing 20% FCS, using a Muse™ Cell Analyzer (Merck Millipore), with the Muse Count and Viability Assay kit (Merck Millipore, Cat. Number: MCH100102), following the manufacturer's indications.

2.3. Study of mitochondrial respiration by oximetry

Mitochondrial respiration was studied in saponin-skinned cells to keep mitochondria in their architectural environment [4]. The analysis took place in a thermostated oxygraphic chamber at 37 °C with continuous stirring (Oxygraph-2 k, Oroboros instruments, Innsbruck, Austria). Cells were collected with trypsin and placed in R^+ medium (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 6.56 mM $MgCl_2$, 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 50 mM K-methane sulfonate, 5 mM glutamate, 2 mM malate, 3 mM phosphate, and 2 mg/ml of BSA; pH = 7) in the oxygraphic chamber with saponin (0.125 mg/ml per chamber). Maximal respiration rate (V_{max}) was measured in the presence of saturating amounts of succinate (25 mM) and ADP (2 mM) as substrates. V_{max} characterizes the electron flow through complexes I, II, III, and IV. NAC was directly injected into the oxygraphic chamber after V_{max}. After the experiments, cells were collected for total protein content determination. Results are expressed as a percentage of the V_{max} or of the control group.

2.4. H_2O_2 production in permeabilized cells

H_2O_2 production was measured with the Amplex Red reagent (Invitrogen), which reacted with H_2O_2 in a 1:1 stoichiometry catalyzed by HRP (Horse Radish Peroxidase; Fluka Biochemika) to yield the fluorescent compound resorufin and molar equivalent O_2 [21]. Resorufin has excitation/emission characteristics of 563/587 nm and is extremely stable once formed. Fluorescence was measured continuously [change in fluorescence (F)/s] with a Fluoromax-4 (Jobin Yvon) spectrofluorometer with temperature control (set at 37 °C) and under continuous magnetic stirring.

Approximately 300,000 cells were added to 600 μl of buffer Z (110 mM K-MES, 35 mM KCl, 1 mM EGTA, 5 mM K_2HPO_4, 3 mM $MgCl_2$, and 0.5 mg/ml BSA) with HRP (0.5 U/ml) and Amplex Red (5 μM). Saponin was directly added to allow cell permeabilization (0.125 mg/ml saponin). H_2O_2 production was then measured with glutamate (5 mmol/l), malate (2 mmol/l), succinate (25 mM), and ADP (2 mM) to stimulate electron flow through complexes I, II, III, and IV. To study the acute effects of NAC, 1 mM of NAC was then added in the spectrofluorometer's quartz cell. H_2O_2 production rate was calculated from the slope of F/s, after subtracting background, from a standard curve established with the appropriate reaction conditions. At the conclusion of each experiment, cells were collected for total protein content determination. Values are expressed as percentage of the control group.

2.5. Mitochondrial free radical leak (FRL)

H_2O_2 production and O_2 consumption were measured in parallel in the same sample under similar experimental conditions. This allowed the calculation of the fraction of electrons out of sequence which reduce O_2 to ROS in the respiratory chain (the percentage of free radical leak) instead of reaching cytochrome oxidase to reduce O_2 to water [21]. Because two electrons are needed to reduce 1 mol of O_2 to H_2O_2, whereas four electrons are transferred in the reduction of 1 mol of O_2 to water, the percent of FRL was calculated as the rate of H_2O_2 production divided by twice the rate of O_2 consumption, and the result was multiplied by 100.

2.6. NAD^+/NADH determination

NAD^+/NADH ratio was determined after 1H incubation with NAC 1 mM, using the EnzyChrom™ NAD^+/NADH assay kit (BioAssay Systems, Cat. Number: E2ND-100), following the manufacturer's instructions.

2.7. Electron spin resonance measurement of superoxide production

Superoxide ($O_2^{\bullet -}$) production was determined after incubation with 1 mM NAC for either 24 h, three days, or one week. For the three days and one-week experiments, cells were also incubated with 100 μM atorvastatin for the last 24 h, in DMEM containing 10% FCS and 1% antibiotics. Cells were placed into a 24-well plate with Krebs–Hepes Buffer containing 25 μmol/l deferoxamine and 5 μmol/l DETC. Cells were then incubated at 37 °C with the spin probe CMH (1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine HCl, 200 μM) for 30 min under 2.7% oxygen, and 20 mm Hg partial pressure using a Gas-Controller (Noxygen Sciences Transfer, Elzach, Germany). The reaction was then stopped on ice. All ESR experiments measuring the

concentration of oxidized CM were conducted at 15 °C in a Bruker e-scan M (Bruker biospin) in disposable capillary tubes, as previously described [22]. Detection of superoxide was conducted under the following ESR settings: center field g = 3482.579; sweep width 60 G; microwave power 21.85 mW; modulation amplitude 2.40 G; sweep time 5.24 s; number of lag curve points 1.

2.8. Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was obtained from cells using TRIzol™ (Invitrogen Life Technologies, Rockville, MD, USA), as previously described [23] and following the manufacturer's instructions. RNA was stored at −80 °C until the reverse transcription reaction was performed. cDNA was synthetized from total RNA with the SuperScript First-Strand Synthesis System (Invitrogen) and random hexamer primers. To perform the real-time PCR reaction, 1 μg of cDNA was mixed with 10 μM of each primer (sense and antisense), SYBR Green (Invitrogen Life Technologies, Rockville, MD, USA) as a fluorescent dye and H_2O. The real-time PCR measurement of individual cDNAs was performed in triplicate using SYBR Green dye to measure duplex DNA formation with the LightCycler System (Roche Diagnostics, Meylan, France). The primer sequences were designed using information contained in the public database GenBank of the National Center for Biotechnology Information (NCBI). The sequences of the primer sets used are listed in Table 1. Quantification of gene expression was performed by the method described in [24], using the β-actin gene as the internal control because it is a stable gene for real-time RT-PCR measurements in muscles. The amplification efficiency of each sample was calculated as described by Ramakers et al. [25].

2.9. ATP monitoring assay

The intracellular concentration of ATP in myoblasts was measured with the ATPLite 1-step kit from PerkinElmer Life and Analytical Sciences (Shelton, CT, USA). Luminescence was detected with a Victor3 Wallac 1420 multilabel counter (PerkinElmer). ATP concentrations were quantified according to a standard curve and expressed as percentage of the control group.

Table 1
Primer sequences used for quantitative real-time PCR amplification.

Target gene	Organism	Forward primer 5′ → 3′ Reverse primer 5′ → 3′	Accession number
PGC-1α	Rat	CACCAAACCCACAGAGAACAG GCAGTTCCAGAGAGTTCCACA	NM_031347
PGC-1β	Rat	CCCCAGTGTCTGAAGTGGAT TCTGGAACTGAGGCTGGTCT	NM_176075
NRF1	Rat	GGCCCTTAACAGTGAAGCTG CATCTGGGCCATTAGCATCT	NM_001100708
NRF2a	Rat	TACAATTGACCAGCCTGTGC ATCCTTGGGGACCTTTGAAC	NP_001102311
NRF2b	Rat	CCCGATGGACAGCAAGTATT CCGGTTCTCAATTATTTCCA	XM_006254075
TFAm	Rat	GAAAGCACAAATCAAGAGGA CTGCTTTTCATCATGAGACAG	NM_031326
Catalase	Rat	AGATGGCACACTTTGACAGAGAG GAGAATCGGACGGCAATAGGAG	NM_012520
SOD1	Rat	AGATGACTTGGGCAAAGGTG CAATCCCAATCACACCACAA	NM_017050
SOD2	Rat	CTGGACAAACCTGAGCCCTA GAACCTTGGACTCCCACAGA	NM_017051
Citrate synthase	Rat	TATGGCATGACGGAGATGAA CATGGACTTGGGCCTTTCTA	DQ403126
Cox1	Rat	CCAGAGTCATGAGTCGAAGGA AGGCGCATGAGTACTTCTCGG	YP_665631
Cox4i1	Rat	GTTGGCTACCAGGGCACTTA CACATCAGGCAAGGGGTAGT	NP_058898
GPx	Rat	GCCGAGTGTGGTTTACGAAT GGCTGCAAACTCCTTGATTT	NM_030826
Cytb	Rat	GCAGCTTAACATTCCGCCCAATCA TACTGGTTGGCCTCCGATTCATGT	J01436
Pyruvate kinase	Rat	TGTGGGTGATCTGGTGATTGTGGT AGGCATTTCAGGATACGCTCAGCA	NM_012624

2.10. mtDNA content

DNA was isolated from L_6 myoblasts that had been treated with 1 mM NAC for one week. Briefly, cells were placed in KTT lysis buffer and vortexed. After centrifugation, phenol/chloroform premixed with isoamyl alcohol (25:24:1; pH = 8; Interchim Uptima) was added to the supernatant, followed by a centrifugation. Then chloroform was added to the supernatant before another centrifugation. The aqueous supernatant was collected; ammonium acetate (4 M, pH = 7) and absolute ethanol were added. Samples were precipitated for 1 h at −20 °C. After centrifugation, pellets of DNA were washed two times with 70% ethanol. Each pellet was dried and resuspended in 10 μl Tris–EDTA buffer (TE). DNA in samples was quantified spectrophotometrically at 260 nm. The DNA was subjected to real-time PCR in duplicate (100 ng/μl). Relative amounts of nuclear and mtDNA were determined by comparison of the amplification kinetics of pyruvate kinase and cytochrome b (Primer sequences in Table 1). Results were expressed as percentage of the control group.

2.11. Measurement of total glutathione

Total glutathione was measured in cells after one week of incubation with NAC 1 mM according to the Akerboom and Sies method [26] by monitoring the reduction of 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) to 5-thio-2-nitrobenzoate (TNB) by GSH at 412 nm. Results were expressed as μM/mg protein.

2.12. Expression of PGC-1α, and SOD2

After one week incubation with NAC 1 mM, cells were lysed on ice for 15 min with 200 μl of NET lysis buffer (0.05 M Tris–HCl pH 8.0, 50 mM NaCl, 5 mM EDTA, 1% NP-40 and a protease inhibitor tablet from Roche, Basel, Switzerland). After lysis, the mixture was vortexed and centrifuged for 10 min at 4 °C at 10,000 rpm. The supernatant was collected and the protein concentration determined using the Pierce BCA protein assay kit (Darmstadt, Germany). For each sample, 20 μg of protein was separated on a denaturing SDS polyacrylamide gel (4%). The antibodies against PGC-1α, and SOD2 were used at dilutions of 1:1000, and 1:500, respectively (Goat polyclonal anti-PGC-1α antibody (ab106814) from Abcam PLC, Cambridge, UK; and Rabbit polyclonal anti-SOD2 antibody (ab13533) from Abcam PLC, Cambridge, UK). Peroxidase-labeled anti-rabbit IgG or anti-goat IgG (1:2000 dilution) in combination with a chemiluminescent substrate (ECL Western Blot detection kit, GE Healthcare, Amersham, UK) were used for the analysis. Quantification was performed using the ImageJ software (National Institutes of Health). Results were expressed as percentage of the control group.

2.13. Apoptosis measurement assays

Apoptosis measurement assays were performed after three days and one week of incubating L_6 myoblasts with or without 1 mM NAC and/or 100 μM atorvastatin for the final 24 h, in DMEM containing 10% FCS and 1% antibiotics. Atorvastatin (Tahor) was generously provided by Pfizer. Staurosporine (200 nM) was used as a positive control (data not shown). The experiments were performed using a Muse™ Cell Analyzer (Merck Millipore), with the Muse Annexin V and dead cell kit (Merck Millipore, Cat. Number: MCH100105), following the manufacturer's instructions.

2.14. Statistics

Data are represented as means ± SEM. Statistical analyses were performed using Student's t test or 1-way ANOVA followed by a Tukey post test (GraphPad Prism 5, Graph Pad Software, Inc., San Diego, CA, USA). Statistical significance was displayed as *, $p < 0.05 or **,$$p < 0.01 or ***, $$$p < 0.001.

3. Results

3.1. Acute N-acetylcysteine exposure impaired mitochondrial function

The effects of NAC were first observed at the cellular level: after 1 h exposure of L_6 cells with NAC 1 mM, NAD^+/NADH ratio was decreased compared to control (−72%; $p < 0.05$; Fig. 1A).

At the mitochondrial level, maximal mitochondrial respiration rates (V_{max}) and H_2O_2 production in saponin skinned cells were studied after direct exposure. NAC decreased V_{max} in a concentration-dependent manner (Fig. 1B). The diminution of V_{max} became significant for a concentration of 0.5 mM (−19%; $p < 0.05$) and reached −28% at a concentration of 1 mM ($p < 0.01$).

Fig. 1C shows mitochondrial H_2O_2 production from permeabilized L_6 cells. When cells were exposed to NAC 1 mM, there was an increase of H_2O_2 production (+40%, $p < 0.05$) compared to control. The concentration of 1 mM was used for further experiments because this concentration corresponded to a significant decrease in V_{max} combined with a significant increase of H_2O_2 production.

When L_6 cells were directly exposed to NAC, the free radical leak (FRL) was significantly increased (+174%; $p < 0.05$; Fig. 1D) compared to control.

3.2. N-acetylcysteine increased mitochondrial ROS production and triggered activation of mitochondrial biogenesis pathways after 24 h

L_6 cells were treated with 1 mM NAC for 24 h. ESR measurements showed an increase of superoxide production (+42%; $p < 0.001$) (Fig. 2A). The cell viability assay did not show any difference between CTL and NAC groups (Fig. 2B). The maximal mitochondrial oxidative capacity of L_6 cells was not altered (Fig. 2C) following 24 h of exposure. It is important to underscore the fact that contrary to the acute time point, we did not add NAC in the oxygraph chamber. The acceptor control ratio (ACR) was not changed between the two groups (Fig. 2D).

The relative mRNA expression levels of the main actors in mitochondrial biogenesis, mitochondrial proteins, and of the main enzymatic antioxidant systems were measured by quantitative RT-PCR (Fig. 2E) after this 24 h incubation with NAC. The relative expression level of PGC-1α was decreased by 50% ($p < 0.01$), whereas the other actors of mitochondrial biogenesis were highly increased: PGC-1β (+240%; $p < 0.001$), NRF1 (+174%; $p < 0.001$), and TFAm (+179%; $p < 0.001$) compared to CTL. We observed significant increases in the relative mRNA expression levels of COX1 (+372%; $p < 0.001$), COX4i1 (+281%; $p < 0.01$), citrate synthase (+484%; $p < 0.01$), and SOD1 (+260%; $p < 0.01$) after NAC exposure. In contrast, concerning the genes implicated in antioxidant systems, there was no modification of the relative mRNA expression levels of SOD2 or GPx.

3.3. One week of NAC incubation induced mitochondrial biogenesis pathways, increasing mitochondrial content as well as antioxidant enzymatic capacities, and reducing basal ROS production

After one week of incubation with 1 mM NAC, the mRNA expression levels of the main actors of mitochondrial biogenesis were increased

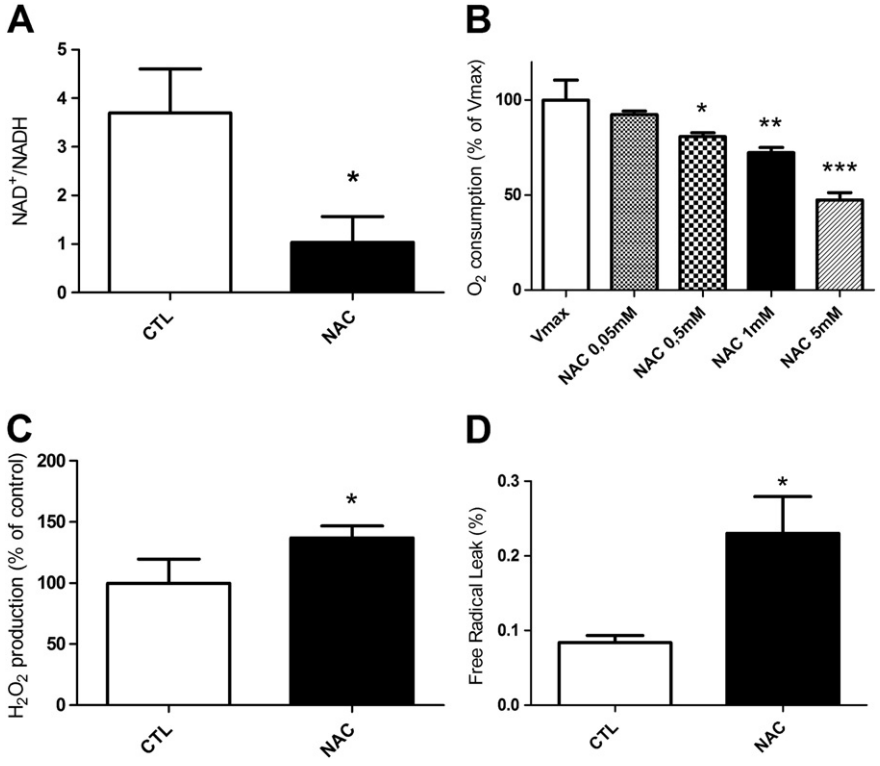

Fig. 1. Acute effects of NAC on mitochondrial function. (A) NAD^+/NADH ratio measurement after 1 H incubation with NAC 1 mM (n = 4). (B) Dose–response of NAC effects on mitochondrial maximal respiratory rates (V_{max}) (n = 12). (C) Measurement of mitochondrial H_2O_2 production in the presence of NAC 1 mM (n = 12). (D) Free radical leak (FRL) measurement in the presence of NAC 1 mM, (n = 6). Values are expressed as means ± SEM, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; One way ANOVA with repeated measures for (B) and unpaired t-test for (A, C and D).

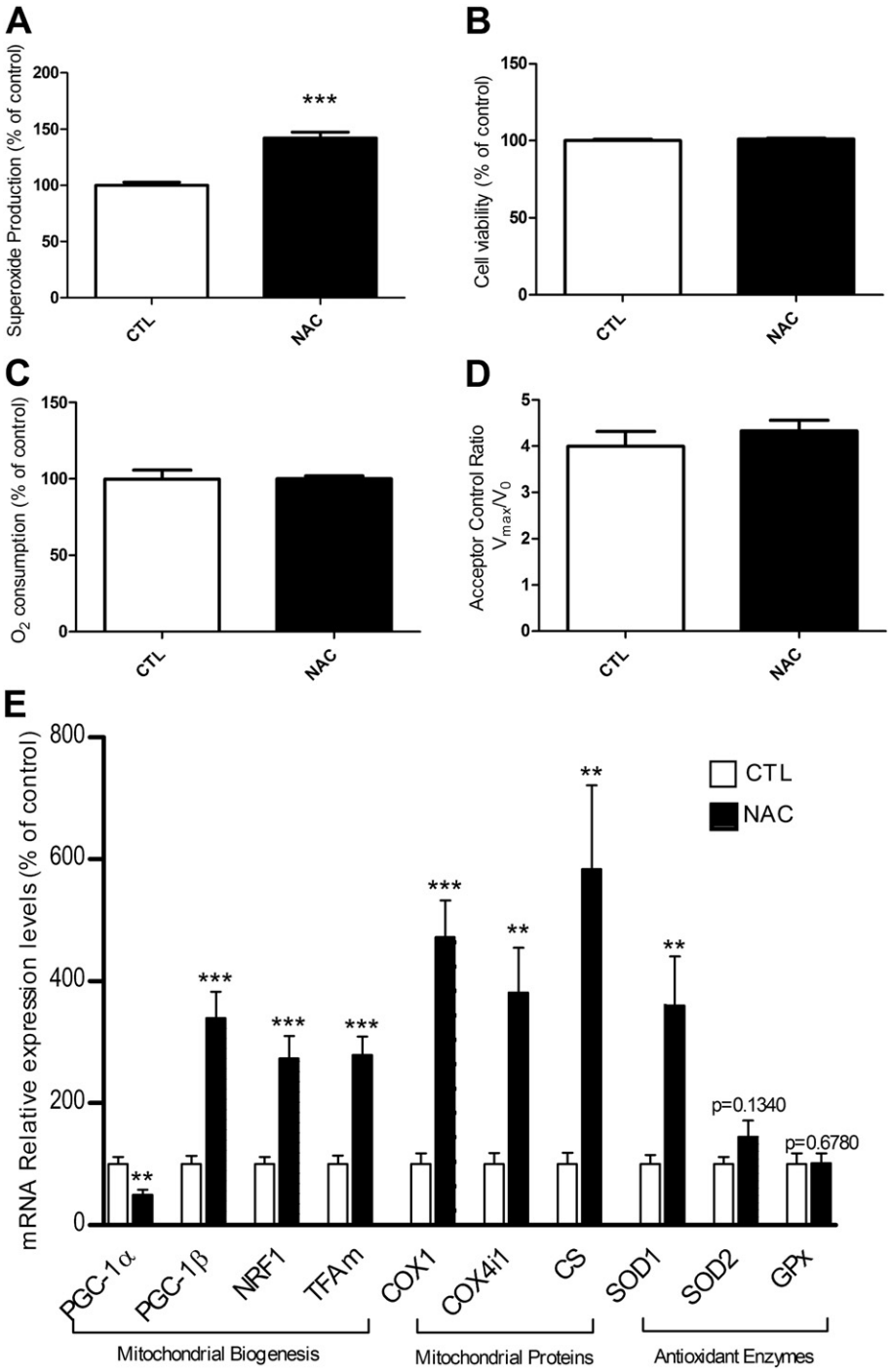

Fig. 2. Chronic effects of NAC 1 mM (24 h). (A) Superoxide production was measured by ESR (CTL: n = 16; NAC: n = 23). (B) Cell viability assay realized with the MUSE Cell Analyzer (n = 6). (C) Maximal mitochondrial oxidative capacities (V_{max}) (n = 4). (D) Acceptor control ratio. (n = 4). (E) mRNA relative expression levels of factors involved in mitochondrial biogenesis (PGC-1α, PGC-1β, NRF1, and TFAm), mitochondrial proteins (COX1, COX4i1, and citrate synthase), and antioxidant proteins (SOD1, SOD2, and GPx) were explored with or without NAC (n = 11). Values are expressed as means ± SEM, **p < 0.01; ***p < 0.001; unpaired t-test.

(Fig. 3A): PGC-1α (+64%; p = 0.06), PGC-1β (+123%; p < 0.05) as well as NRF1 (+121%; p < 0.05), NRF2a (+62%; p < 0.05), NRF2b (+106%; p < 0.01), and TFAm (+179%; p < 0.01). The relative mRNA expression levels of several mitochondrial proteins were also upregulated, including COX1 (+124%; p < 0.05), COX4i1 (+165%; p < 0.001), and citrate synthase (+87%; p < 0.05). Concerning the antioxidant systems, NAC exposure non-significantly increased the level of catalase (+41%; p = 0.08) and significantly increased the relative mRNA expression levels of SOD1 (+95%; p < 0.05), SOD2 (+107%; p < 0.01) and GPx (+145%; p < 0.001).

NAC treatment enhanced the protein expression of PGC-1α (+67%; p < 0.05) confirming the activation of the mitochondrial biogenesis pathways (Fig. 3B). The increase in SOD2 protein expression with NAC (+44%; p = 0.0683) confirmed the augmentation of antioxidant capacities obtained at the mRNA level (Fig. 3B).

The increase in mitochondrial content was shown with several techniques (Western blotting, oxygraphy, quantification of mitochondrial DNA (mtDNA), and an ATP production assay).

Interestingly, the maximal mitochondrial oxidative capacity (V_{max}, +17%; p < 0.05) (Fig. 4A) as well as the mtDNA content were increased

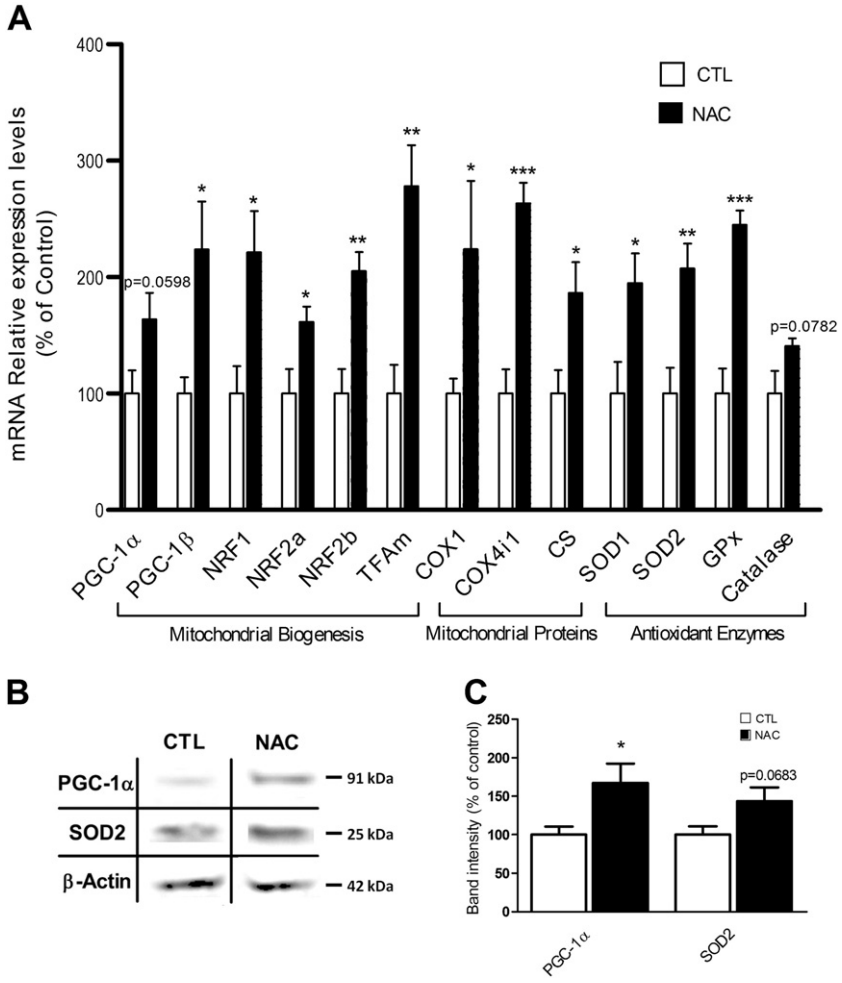

Fig. 3. Chronic effects of NAC 1 mM (1 week) on mRNA expression as well as protein expression levels. (A) mRNA relative expression levels of factors involved in mitochondrial biogenesis (PGC-1α, PGC-1β, NRF1, NRF2a, NRF2b, and TFAm), mitochondrial proteins (COX1, COX4i1, and citrate synthase), and antioxidant proteins (SOD1, SOD2, GPx, and catalase) (n = 5–6). (B–C) Band intensity of Western blots of PGC-1α and of SOD2 (n = 6–7). Values are expressed as means ± SEM, *p < 0.05; **p < 0.01; ***p < 0.001; unpaired t-test.

(+144%; p < 0.05) after one week NAC treatment (Fig. 4C). The acceptor control ratio was not changed between the two groups (Fig. 4B). The ATP monitoring assay showed an important increase in the ATP content after treatment with 1 mM NAC (+303%; p < 0.001) (Fig. 4D). These results strongly suggest an activation of the cellular metabolism after NAC exposure, leading to the increase in mitochondrial content as well as enzymatic antioxidant capacities.

We investigated the redox state of cells by studying the superoxide production by ESR and the total cellular glutathione content. In cells treated with NAC, it appeared that superoxide production was significantly diminished (−40%; p < 0.001; Fig. 4E) compared to CTL. Total cellular glutathione content was increased after treatment (+38%; p < 0.05; Fig. 4F) compared to CTL. No deleterious effects on cell viability were observed after chronic NAC exposure (Fig. 4G).

3.4. Short NAC treatment (3 days) failed to protect cells from deleterious effects of statins

Incubation of myoblasts with 100 μM atorvastatin for 24 h (ATO) impaired mitochondrial function (Fig. 5). Atorvastatin decreased the maximal mitochondrial oxidative capacity of cells (−37%; p < 0.001; Fig. 5A) and the acceptor control ratio (−31%; p < 0.05; Fig. 5B). NAC pretreatment for three days did not change either V_{max} or ACR compared to CTL. Short pretreatment with NAC did not protect maximal mitochondrial oxidative capacity of cells from the deleterious effect of ATO (NAC + ATO, −37%; p < 0.001; Fig. 5A), and did not improve the ACR (−40%; p < 0.01; Fig. 5B) compared to CTL. Cellular ATP content (Fig. 5C) decreased after 24 h of statin treatment (−29%; p < 0.05). NAC pretreatment for three days did not change ATP content in cells. ATP content in NAC + ATO group was reduced compared to CTL (−61%; p < 0.001), as well as to ATO (p < 0.05). Incubation of myoblasts with ATO increased superoxide production (+37%; p < 0.05; Fig. 5D). Superoxide production was not altered in NAC group compared to CTL, whereas three days of pretreatment with NAC 1 mM did not protect L_6 cells from this high ROS production (NAC + ATO; +65%; p < 0.001).

Apoptosis-measurement assays showed that atorvastatin incubation for 24 h decreased cell viability (−21%; p < 0.001; Fig. 5E). Three days of NAC pretreatment (NAC + ATO) did not prevent these deleterious effects of statins (−20%; p < 0.001 compared to CTL). NAC + ATO group had a higher percentage of early apoptotic cells compared to the ATO group (13.4% vs. 7%; p < 0.001; Fig. 5F). The percentage of late apoptotic cells (Annexin V^+/7-AAD^+; Fig. 5G) was increased in both ATO and NAC + ATO compared to CTL (20.41% and 14.78%, respectively; p < 0.001). Moreover, the percentage of total apoptotic cells (early apoptotic and late apoptotic; Fig. 5H) was increased in the ATO condition compared to CTL (27.5% vs. 7%, respectively; p < 0.001) as well as in the NAC + ATO condition (28.2%; p < 0.001 compared to CTL). NAC pretreatment alone did not change the percentage of total apoptotic cells compared to CTL.

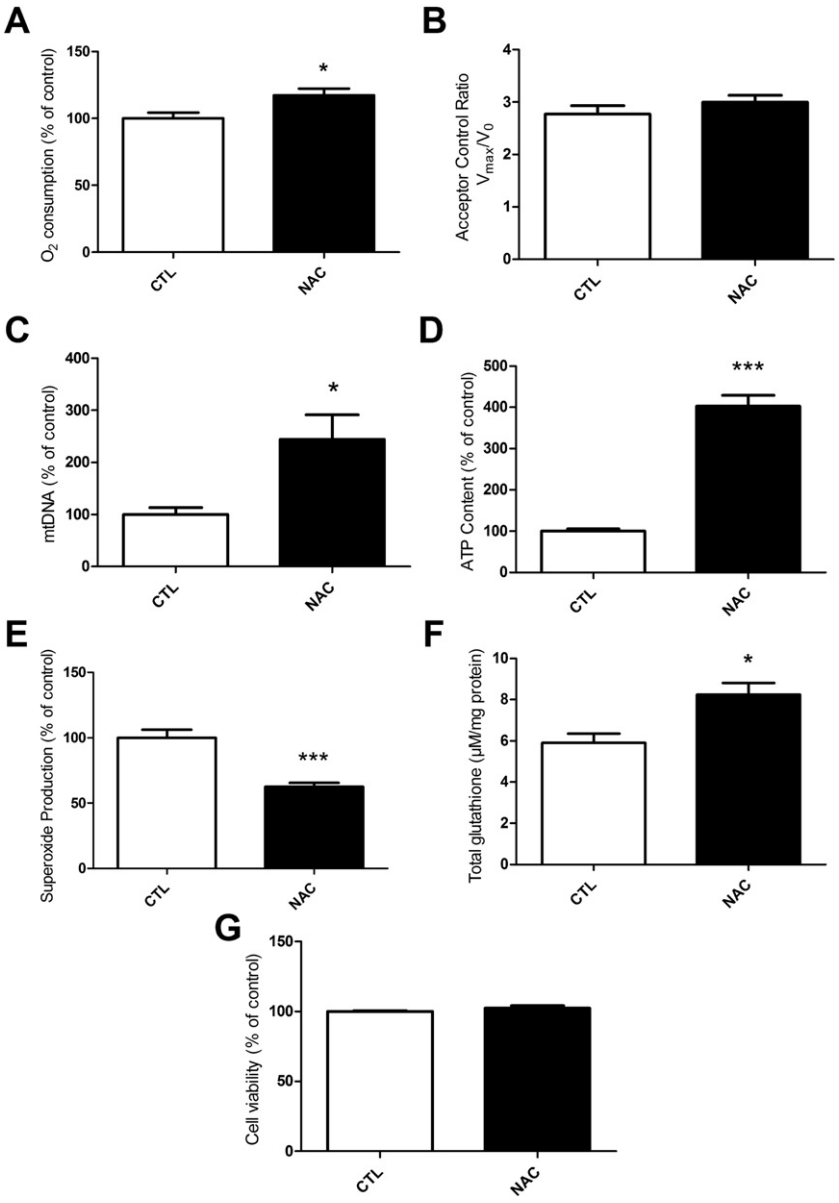

Fig. 4. Chronic effects of NAC 1 mM (1 week) on mitochondrial respiration, mitochondrial content and efficiency, oxidative stress and cell viability. (A) Maximal mitochondrial oxidative capacities (V_{max}) (n = 14–15). (B) Acceptor control ratio (n = 14–15). (C) mtDNA quantification (n = 10–11). (D) ATP content determination assay (n = 12). (E) Superoxide production measured by ESR (CTL: n = 14; NAC: n = 9). (F) Total glutathione content (n = 4). (G) Cell viability assay realized with the MUSE Cell Analyzer (n = 6). Values are expressed as means ± SEM, *p < 0.05; ***p < 0.001; unpaired t-test.

3.5. One week of NAC treatment protected cells from the deleterious effects of statins

NAC pretreatment for one week, then 24 h with growth medium containing 10% of FCS, increased maximal mitochondrial respiratory rates (+22%; p < 0.05; Fig. 6A), without affecting the ACR (Fig. 6B), and increased cellular ATP content (+59%; p < 0.001; Fig. 6C). Seven days of pretreatment with NAC (NAC + ATO) prevented the alteration of these parameters from the deleterious effects of ATO without improving the ACR (+35% maximal respiratory rates compared to ATO, p < 0.05; +29% ATP content compared to ATO, p < 0.001). Interestingly, this NAC pretreatment protected L_6 cells from this Atorvastatin-induced ROS production (NAC + ATO, −26% compared to CTL, p < 0.05; −41% compared to ATO, p < 0.001; Fig. 6D).

Apoptosis-measurement assays showed that atorvastatin incubation for 24 h decreased cell viability (−10%; p < 0.001; Fig. 6E). The NAC + ATO group had a higher percentage of viable cells compared to the ATO group (83.2% vs. 78.4%, respectively; p < 0.001), although this percentage remained lower than the control condition without atorvastatin (−5%; p < 0.001). Atorvastatin incubation tend to increase the percentage of cells in early apoptosis (Annexin V^+/7-AAD^-; Fig. 6F) compared to the control group (13.8% vs. 10.4%, respectively; p < 0.05). The NAC + ATO group had a lower percentage of early apoptotic cells compared to the ATO group (10.4% vs. 13.8%; p < 0.05). The percentage of late apoptotic cells (Annexin V^+/7-AAD^+; Fig. 6G) was increased in both ATO and NAC + ATO compared to the control (+323% and +276%, respectively; p < 0.001). The co-treatment did not change the percentage of late apoptotic cells compared to the ATO condition (6.4% vs. 7.2%, respectively). However, the percentage of total apoptotic cells (early apoptotic and late apoptotic; Fig. 6H) was increased in the ATO condition compared to CTL (21.5% vs. 13.5%, respectively; p < 0.001) and NAC + ATO had a lower percentage of total apoptotic cells compared to the ATO group (16.7% vs. 21.5%, respectively; p < 0.05).

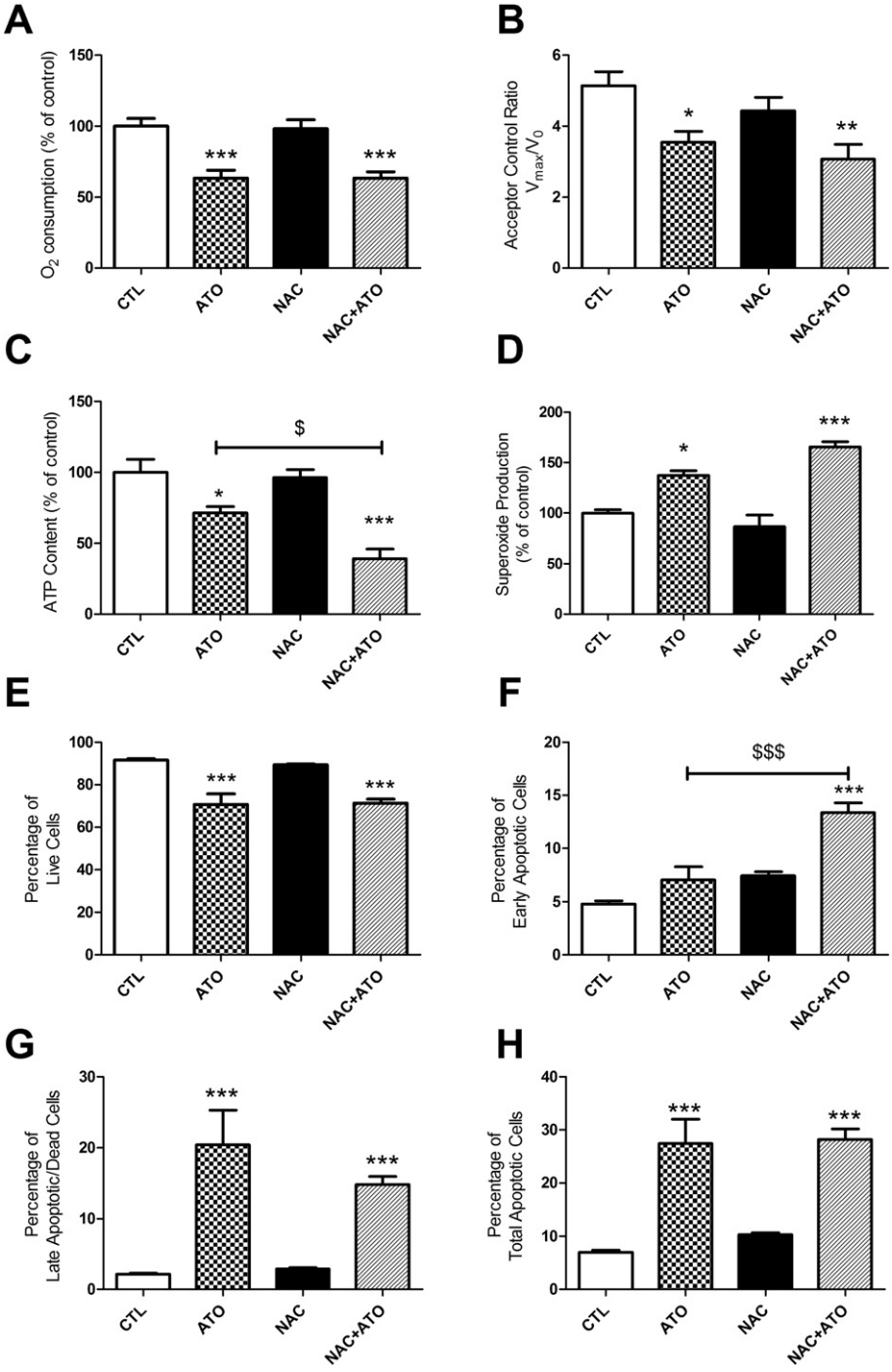

Fig. 5. NAC pretreatment for 3 days failed at protecting cells from deleterious effects of statins. (A) Maximal mitochondrial oxidative capacities (V_{max}) (n = 7). (B) Acceptor control ratio (n = 7). (C) ATP content determination assay (n = 6). (D) Superoxide production measured by ESR (n = 6). (E–H) Annexin V assay (n = 6): (E) Percentage of live cells (Annexin V-PE^-/7-AAD^-). (F) Early apoptotic cells (Annexin V-PE^+/7-AAD^-). (G) Late apoptotic cells (Annexin V-PE^+/7-AAD^+). (H) Total apoptotic cells. Values are expressed as means ± SEM, *p < 0.05; **p < 0.01; ***p < 0.001 compared to CTL; $p < 0.05; $$$p < 0.001 compared to ATO. One way ANOVA followed by a Tukey post test.

4. Discussion

This study showed that: (I) Acute treatment with N-acetylcysteine induced a cellular reductive stress that impaired mitochondrial function of myoblasts; (II) following this reductive stress, there was a rapid activation of mitochondrial biogenesis pathways (after 24 h of incubation); (III) three days of NAC treatment was not sufficient to induce a phenotypic adaptations allowing to protect cells from statins toxicity; (IV) after one week of NAC exposure, the mitochondrial biogenesis pathways remained activated, allowing an increase of mitochondrial content as well as antioxidant enzymatic capacities, corresponding to a mitohormesis mechanism; (V) the activation of this mitohormesis phenomenon clearly protected cells from apoptosis triggered by short statins treatment.

4.1. The antioxidant molecule N-acetylcysteine induced cellular reductive stress, and mitochondrial oxidation by inhibiting the mitochondrial respiratory chain

Our results showed that NAC treatment induced a cellular reductive stress, as suggested by the reduction of the NAD^+/NADH ratio. Our

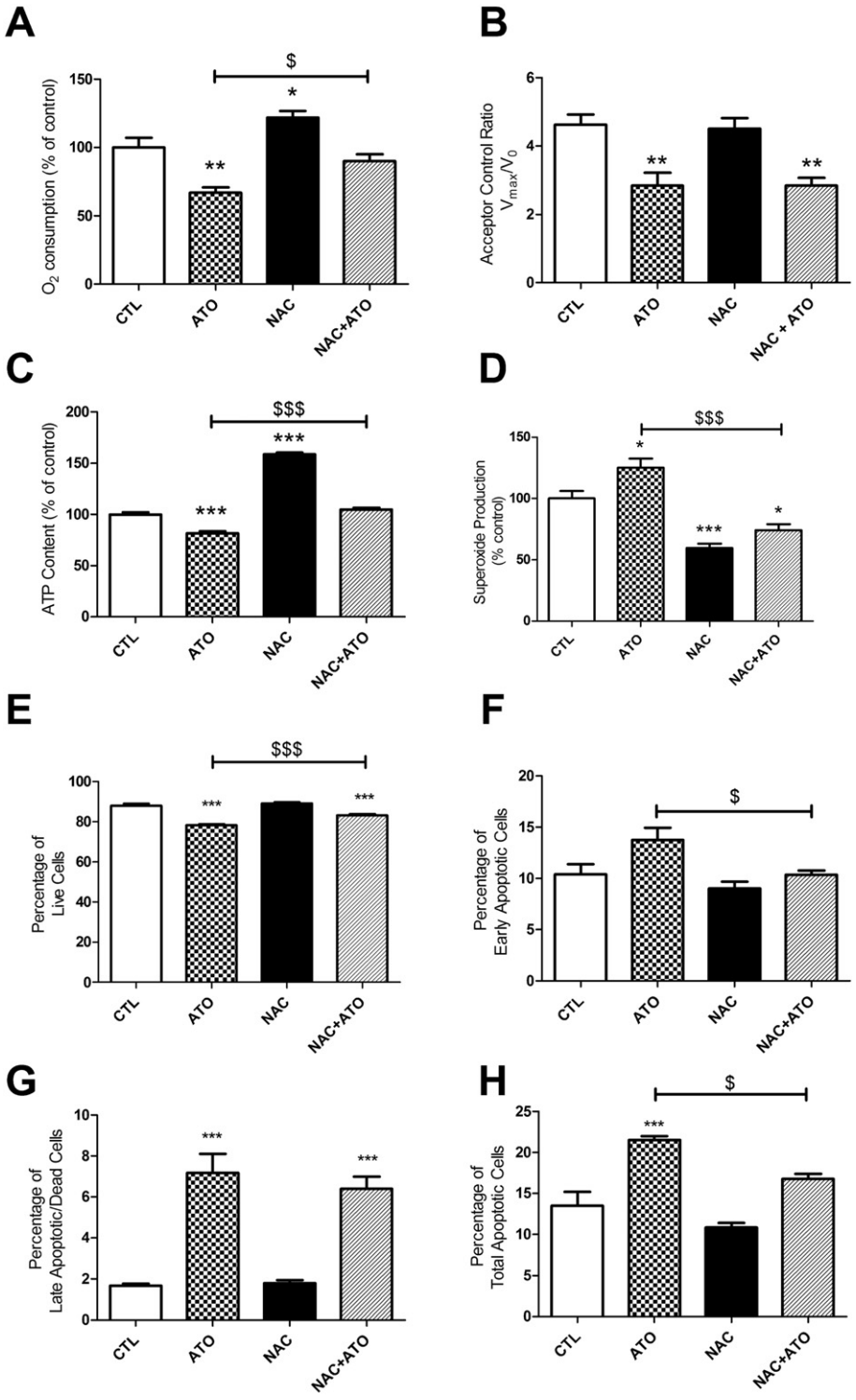

Fig. 6. Protective effects of NAC-induced mitohormesis phenomenon against deleterious effects of statins. (A) Maximal mitochondrial oxidative capacities (V_{max}) (n = 6–8). (B) Acceptor control ratio (n–6-8). (C) ATP content determination assay (n = 6). (D) Superoxide production measured by ESR (n = 6). (E–H) Annexin V assay (n = 6): (E) Percentage of live cells (Annexin V-PE^-/7-AAD^-). (F) Early apoptotic cells (Annexin V-PE^+/7-AAD^-). (G) Late apoptotic cells (Annexin V-PE^+/7-AAD^+). (H) Total apoptotic cells. Values are expressed as means ± SEM, *p < 0.05; **p < 0.01; ***p < 0.001 compared to CTL; $p < 0.05; $$$p < 0.001 compared to ATO. One way ANOVA followed by a Tukey post test.

results are in accordance with some other studies showing that NAC could provoke glutathionylation of mitochondrial proteins due to a NAC-induced reductive stress effect on H9C2 cardiomyocytes [2,20,27]. Zhang et al. observed, that causing reductive stress with NAC (4 mM) treatment for 60 min, induced an oxidation of the redox state of mito-roGFP and that Trx 2 correspondingly shifted from 56% reduced form to 36% reduced form after NAC exposure compared with controls [20].

Then, subunits of the respiratory chain complexes could be altered by this mechanism, leading to oxidative phosphorylation (OXPHOS) dysfunction and then to an increase in ROS production as shown by the decrease of V_{max} and increase of H_2O_2 production, leading to an

increase of the free radical leak (FRL) [21,28,29]. The FRL represents the fraction of electrons out of sequence that reduce O_2 to ROS in the respiratory chain (the percent of free radical leak) instead of reaching cytochrome oxidase to reduce O_2 to water [30]. The FRL is a good index of mitochondrial oxidative stress [21] because it is independent of the mitochondrial content, which is known to influence the production of ROS [30]. Inhibition of mitochondrial respiration-induced mitochondrial ROS production has already been shown in several other in vitro models [4,31–33], and both complex I and complex III should be potential sites of $O_2^{\bullet-}$ production [29,34,35]. Then, in contrast to the general model in which NAC functions solely as an antioxidant, we and others observed that NAC could increase mitochondrial ROS production [2,20,36]. Our findings underscore the importance of considering the dynamic events related to oxido-reductive signals and redox-dependent pathways.

4.2. Reductive stress triggered a mitochondrial hormetic mechanism

Mitohormesis is based on the assumption that a moderate increase in ROS production during mitochondrial activity leads to the activation of cellular defense systems [4–9]. Our results suggest that NAC induced perturbations of the mitochondrial redox potential, triggering this mitohormesis phenomenon.

The transcriptional control of mitochondrial biogenesis depends on many factors, and the members of the PGC-1 family (PGC-1α; PGC-1β, and PRC) are considered to be key regulators of this process [37–39]. The conventional wisdom attributes oxidative stress response to PGC-1α, however the regulation and function of PGC-1β is still unclear in the literature. Both are enriched in mitochondria-rich tissues, but PGC-1β is thought to preferentially induce genes involved in the removal of reactive oxygen species and is supposed to play a role in constitutive mitochondrial biogenesis [40]. However, only PGC-1α is thought to respond to metabolic challenges. Indeed, we found a rapid transcriptional activation leading to the increased expression of several genes implicated in mitochondrial biogenesis pathways, including PGC-1β, the NRF family, and TFAm [12,37–39,41–43]. We observed however a significant increase in the expression of the PGC-1α protein at 7 days despite what we observed at the mRNA level. That is why we can suggest that the adaptive response observed in our study could be driven through PGC-1α, but we cannot exclude that this adaptive response does only affect PGC-1α protein levels and not PGC-1β. Then, the mRNA results suggest that the establishment of the mitohormesis phenotype at 7 days needs both PGC-1α and β induction, with PGC-1β expression being required earlier in the mitohormesis time course than PGC-1α. Mitochondrial adaptations could be considered as a sequential program, requiring 6 to 10 days to obtain an effective mitochondrial biogenesis [44]. We studied the effects of one week of NAC treatment to assess the phenotypic consequences of this activation of biogenesis. In our study, we clearly showed that the activation of mitochondrial biogenesis led to an increase in cellular levels of mitochondrial content as well as cellular antioxidant capacities. These phenotypic changes were demonstrated with several techniques. First, we showed an augmentation of the relative mRNA expression of mitochondrial proteins, such as citrate synthase, as well as the simultaneous expression of nuclear and mitochondrial genomes, with an increase in the relative mRNA expression of subunits of cytochrome c oxidase (complex IV), COX1 (encoded by the mitochondrial genome) and COX4 (encoded by the nuclear genome). This simultaneous expression of both genomes could be explained by TFAm overexpression. These observations were corroborated by Western blot results showing an effective upregulation at the protein level of mitochondrial biogenesis pathways, as well as by an increase in the mtDNA copy number. At the functional level, we observed an augmentation of the mitochondrial maximal oxidative capacities (V_{max}). Interestingly, the antioxidant enzymatic systems were clearly enhanced, allowing a decrease in the cellular ROS content measured by electron spin resonance. These results are consistent with numerous findings indicating that ROS may evoke cellular signaling that promotes metabolic health and longevity. Indeed, ROS serve as essential signaling molecules delivering messages from the mitochondria to other cellular compartments in response to physiological or pathophysiological changes [6,35,45–49]. Classically, N-acetylcysteine (NAC) is considered to be an antioxidant molecule because it is the most immediate precursor of glutathione (GSH), which is the main mechanism of detoxification of free radicals and ionized metabolites [16,17], especially in mitochondria. Our findings are quite novel in demonstrating that NAC has powerful mitochondrial and antioxidant effects, not only via its properties as a glutathione precursor (increase in GSH content) but also by enhancing the levels of mitochondrial content and antioxidant enzymes by triggering mitohormesis. According to Calabrese et al., this phenomenon could be defined as *chemical conditioning hormesis* [50]. Moreover, our results could partially explain the divergent and sometimes disappointing effects encountered with chronic NAC treatment and antioxidant supplementation [51–57]. Indeed, reductive stress could lead to an increase in mitochondrial oxidation due to these so-called antioxidants and have dramatic effects on redox pathways and on cell survival.

4.3. Mitochondrial hormesis protected cells from pro-apoptotic effects of statins

Statins are the most prescribed and most efficient medication used to lower plasma cholesterol levels [58] and are considered relatively clinically safe. However, these molecules show numerous adverse effects ranging from myalgia to rhabdomyolysis. These deleterious effects have been shown to be linked to oxidative stress in glycolytic skeletal muscles, whereas cardiac oxidative muscle, which has a higher mitochondrial content as well as higher antioxidant properties, was preserved [4]. An increase of mitochondrial content in L_6 myoblasts has been shown to protect against both caspase-dependent and caspase-independent apoptosis [59]. Therefore, statin-induced mitochondrial ROS overproduction may lead to the triggering of apoptotic pathways and lead to the appearance of statin-induced myopathy. We observed that three days of pretreatment with NAC 1 mM was not sufficient to induce mitochondrial hormesis. At this time point, NAC pretreated cells seems to be weakened by the induced mitochondrial oxidation, and so more affected by a subsequent mitochondrial stress with statins. Thus, in our conditions we observed that the switch to an effective hormetic protective phenotype occurs between an incubation time superior to 3 days, and an incubation time inferior or equal to one week. Indeed, seven days of NAC exposure seem to be necessary in order to increase mitochondrial content via mitohormesis. This phenomenon was able to protect cells against statin-induced toxic effects. Moreover, the protection provided by NAC does not occur through its known antioxidant action, but seems more likely due to the triggering of adaptive pathways. Even though NAC pretreatment does not remove totally the deleterious of statins, it enabled to maintain a mitochondrial functionality comparable to the CTL group. Statins are known to alter mitochondrial function via a direct inhibitory mechanism of the mitochondrial respiratory chain, thus decreasing ATP content and increasing ROS production [4]. The improvement of the mitochondrial function observed after NAC pretreatment protected cells against a subsequent mitochondrial stress with statins by diminishing cell death and the triggering of apoptotic pathways. Mitochondrial phenotype appears to be a key factor in the triggering of statins' myopathy, and switching muscle fiber type from glycolytic to oxidative by increasing mitochondrial content could represent a potential therapy to protect muscles from statins' myopathy.

5. Conclusions

We showed that the antioxidant N-acetylcysteine provoked a cellular reductive stress, which led to mitochondrial malfunction. The resulting mitochondrial oxidation leads to the triggering of mitochondrial biogenesis pathways via a mitohormesis mechanism, leading to

an increase of mitochondrial content combined with an improvement of antioxidant capacities in L_6 myoblasts. This process allowed a decrease in oxidative stress and provided protection against apoptosis, as previously described in another model [59]. This improved cell survival after atorvastatin treatment. To our knowledge, this is the first study showing that reductive stress could trigger the mitochondrial hormesis phenomenon.

Moreover, our findings underscore the importance of considering the dynamic events related to both oxidative and reductive stress within cells. Thus, molecules chemically considered as antioxidants might have dramatic effects on oxido-reductive signals and redox-dependant pathways. Mitochondrial content and cellular antioxidant power seem to be determining factors in the appearance of statin-induced myopathy; thus, the activation of this mitohormesis phenomenon represents a potential new therapeutic solution to reduce the toxicity of compounds affecting mitochondrial function. For example, finding a molecule able to increase mitochondrial content in skeletal muscle in vivo could provide a protection against statin-induced myopathy.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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