The Effects of a Theacrine-based Supplement on mRNAs Related to Various Metabolic Processes and Sirtuin Activity in vitro

Petey Mumford
Lindenwood University
Shelby Osburn
Auburn University
Michael D. Roberts (mdr0024@auburn.edu)
Auburn University https://orcid.org/0000-0002-7359-5362

Short report

Keywords: theacrine, mRNAs, sirtuins, mitochondria

Posted Date: August 4th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-51095/v1

Abstract

There is evidence in rodents to suggest theacrine-based supplements modulate tissue sirtuin activity as well as other biological processes associated with aging. Herein, we examined if a theacrine-based supplement (NAD3) altered sirtuin activity *in vitro* while also affecting markers of mitochondrial biogenesis and the mRNA expression of genes related to various cellular processes in muscle. The murine C2C12 myoblast cell line was used for experimentation. Following 7 days of differentiation, myotubes were treated with 0.45 mg/mL of NAD3 (containing ~ 2 mM theacrine) for 3 and 24 hours (n=6 treatment wells per time point). Control treatments consisted of cellulose-only treatments at the same time points. Relative to CTL-treated cells, NAD3 treatments increased ($p<0.05$) *Sirt1* mRNA levels at 3 hours, as well as global sirtuin activity at 3 and 24 hours. While NAD3 treatments decreased mRNA levels of *Nfe2l2* at 3 hours and increased levels at 24 hours relative to CTL-treated cells (a gene involved in mitochondrial biogenesis, $p<0.05$), citrate synthase activity levels (a surrogate of mitochondrial density) remained unaltered between treatments. NAD3 treatments for 3 and 24 hours decreased *Nlrp3* mRNA levels relative to CTL-treated cells (an inflammatory marker, $p<0.05$). Additionally, NAD3 treatments decreased *Map1lc3b* mRNA levels (an autophagy marker) after 24-hour treatments ($p<0.05$). Although these data are limited to select biomarkers *in vitro*, these preliminary findings suggest a theacrine-based supplement can modulate various skeletal muscle biomarkers related to sirtuin activity, inflammation, and autophagy. Muscle biopsy studies in humans are needed to confirm these current findings.

Introduction

Skeletal muscle aging is associated with numerous factors including (but not limited to) age-associated alterations in circulating hormones, low-grade inflammation, mitochondrial perturbations via free radicals, and transcriptomic alterations due to epigenetic factors (reviewed in (1-3)). There are various nutritional supplements geared towards affecting biomarkers associated with tissue aging in general, and several of these supplements act through reducing inflammation (e.g., curcumin, (4)), diminishing oxidative stress (e.g., vitamins C and E, (5)), and enhancing mitochondrial function (e.g., coQ10 and quercetin, (6, 7)).

Sirtuins are a class of proteins (SIRT1-7) that exist in various subcellular compartments and possess either mono-ADP-ribosyltransferase or deacetylase activity (8). Research dating back to the 1970s across several organismal models have indicated sirtuins are involved in the tissue aging process; specifically, stressors that activate sirtuin activity are largely thought to promulgate anti-aging effects (9). Thus, there has also been a widespread interest in identifying nutritional supplements that increase sirtuin activity.

Theacrine is a purine alkaloid that structurally similar to caffeine, and various studies have examined its efficacy as a neuroactive ingredient (10-12). Aside from potential neurotrophic effects, there are limited data in rodents suggesting theacrine-based supplements can reduce inflammation (13, 14), modulate mitochondrial function (15), and activate sirtuins (15). However, it is currently unclear how theacrine-based supplements affect biomarkers related to these processes in skeletal muscle. Therefore, the purpose of this rapid report was to assess how a theacrine-based supplement (NAD3) affected markers

of sirtuin activity, mitochondrial biogenesis, and mRNAs related to other metabolic processes in skeletal muscle cells *in vitro*.

Methods

Cell culture

Cell culture methods were performed similar to other studies published by our laboratory (16, 17). Briefly, C2C12 myoblasts (passage 6; American Type Culture Collection, Manassas, VA, USA), were seeded in growth medium (DMEM, 10% FBS, 1% penicillin/streptomycin, and 0.1% gentamycin) on six-well plates at a seeding density of 3 x10^5 under standard culture conditions (37°C in a 5% CO_2 atmosphere). Once myoblast growth reached 80-90% confluence ~48 h after seeding, differentiation was induced by removing growth medium and replacing it with differentiation medium [DM; DMEM, 2% (vol/vol) horse serum, 1% penicillin/ streptomycin, and 0.1% gentamycin]. DM was then replaced every 24 h for 7 days to allow for adequate myotube growth.

NAD3 versus control treatments

Treatments occurred on day 7 post-differentiation for either 3 hours or 24 hours with either 0.45 mg/mL of NAD3 (containing ~ 2 mM theacrine; Compound Solutions, Carlsbad, CA, USA) (n=6 treatment wells per time point) or a weight-equivalent of cellulose-only (n=6 treatment wells per time point) suspended in DM. Notably, we opted not to resuspend the treatments in a vehicle prior to adding these agents to DM (e.g., ethanol or DMSO) given the potential cytotoxic effects of vehicle compounds. Rather, NAD3 or cellulose were added to DM in 50 mL tubes, tubes were centrifuged for 5 minutes at 100 *g* to remove any insoluble material, and the supernatant was used as treatment media. Notably, the investigators were blinded to treatments until the conclusion of the study and after statistical analyses were performed.

Post-treatment processing of cells and real-time PCR for mRNA expression analyses

After all treatments, differentiation/treatment media was removed, and cells were washed once with phosphate-buffered saline (PBS). Thereafter, PBS was siphoned off and then a subset of cells were scraped from plate and transferred into 250 µL of Trizol for RNA isolation. Following Trizol-based RNA isolation methods, total RNA concentrations were analyzed using a Nanodrop Lite spectrophotometer (Thermo Fisher Scientific, Waltham, MS, USA), and 1 µg of cDNA were synthesized using a commercial qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) per the manufacturer's recommendations. Real-time PCR was performed using gene-specific primers and SYBR green chemistry, and all PCR reactions were confirmed to produce only one melt product. Relative expression values were performed using the $2^{-\Delta\Delta CT}$ method where $2^{-\Delta CT}$ [housekeeping gene CT – gene of interest CT] and $2^{-\Delta\Delta CT}$ (or fold change) = [$2^{-\Delta CT}$ value/$2^{-\Delta CT}$ average of control treatment]. Cyclophilin (*Ppia*) was used as a housekeeping gene. Primer sequences from all genes of interest are available upon request.

After the RNA scrape described above, 250 µl of ice-cold cell lysis buffer was applied to each well [20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM Na-EDTA, 1 mM EGTA, 1% Triton, 20 mM sodium pyrophosphate, 25 mM sodium fluoride, 1 mM β-glycerophosphate, 1 mM Na3VO4, and 1 µg/ml leupeptin; Cell Signaling; Danvers, MA, USA]. Plates were then scraped, and the cell slurry was removed and placed in 1.7 mL tubes. Cells were homogenized via micropestles, and homogenates were subsequently centrifuged at 500 g for 5 min. After centrifugation, insoluble proteins were pelleted, and supernatants containing solubilized cell material were placed in new 1.7 mL tubes and stored at -80°C until global SIRT activity and citrate synthase assays were performed.

Global SIRT activity and citrate synthase activity assays

Global SIRT activity levels were performed on cell lysis buffer lysates using a commercial assay (Abcam, Danvers, MA, USA; catalog#: Ab156915) similar to previous methods published by our laboratory (18). Prior to performing the assay, protein concentrations of supernatants were determined using a BCA assay (Thermo Fisher Scientific). Lysates (8 µL) were loaded in duplicate onto 96-well plates provided by the kit for enzymatic reactions as well as a no SIRT co-factor control reaction (NNC reaction, or an internal negative control reaction). Following execution of the assay per manufacturer's recommendations, absorbances were read at OD450 using a plate reader (BioTek Synergy H1). NNC OD values were subtracted from enzymatic reaction OD values, and these values are presented in the results section as OD450 per µg protein. The average coefficient of variation (CV) values for all duplicates was 10.3%.

Citrate synthase activity levels were determined in duplicate on lysates from 24-hour treatments only; notably, these methods are similar to previous methods used by our laboratory (18). This metric was used as a surrogate for mitochondrial density per the findings of Larsen et al. (19) suggesting citrate synthase activity strongly correlates with transmission electron micrograph images of intracellular space occupied by mitochondria ($r=0.84$, $p<0.001$). The assay principle is based on the reduction of 5,50-dithiobis (2-nitrobenzoic acid) (DTNB) at 412 nm (extinction coefficient 13.6 mmol/L/cm) coupled to the reduction of acetyl-CoA by the citrate synthase reaction in the presence of oxaloacetate. Briefly, protein obtained from lysates was added to a mixture composed of 0.125 mol/L Tris–HCl (pH 8.0), 0.03 mmol/L acetyl-CoA, and 0.1 mmol/L DTNB. All reactions occurred in 96-well plates, reactions were initiated by the addition of 5 µL of 50 mmol/L oxaloacetate per well, and the absorbance change was recorded for 60 seconds in a spectrophotometer (BioTek Synergy H1). The average CV values for all duplicates was less than 10%.

Statistics

All statistical analyses were performed using SPSS v25.0 (IBM Corp, Armonk, NY, USA). The 3-hour and 24-hour data were analyzed independently between treatments using independent samples t-tests. All data are presented in figures and tables as means ± standard deviation (SD) values, and statistical significance was established as $p<0.050$.

Results

Effect of NAD3 treatments on SIRT markers

Data in Figure 1 illustrate that 3-hour NAD3 treatments increased the mRNA expression of *Sirt1* relative to CTL-treated cells ($p<0.05$), while not affecting *Sirt4* or *Sirt6* mRNA expression levels (Fig. 1a/b). Additionally, 3- and 24-hour NAD3 treatments increased global SIRT activity (Fig. 1c/d).

Effect of NAD3 treatments on mitochondrial biogenesis markers

Data in Figure 2 illustrate that, while 3- and 24-hour NAD3 treatments did not affect *Ppargc1a* mRNA levels, 3-hour NAD3 treatments decreased and 24-hour NAD3 treatments increased *Nfe2l2* mRNA levels relative to CTL-treated cells ($p<0.05$) (Fig. 2b). However, 24-hour NAD3 treatments did not affect citrate synthase activity levels (Fig. 2c).

Effect of NAD3 treatments on mRNAs related to other metabolic processes

Data in Figure 3 illustrate that 3-hour NAD3 treatments increased *Map1lc3b* mRNA levels while decreasing *Nlrp3* mRNA levels relative to CTL-treated cells (Fig. 3a).These same trend for *Nlrp3* levels was observed in 24-hour treatments, and NAD3 additionally decreased *Map1lc3b* mRNA levels at this time point (Fig. 3b).

Discussion

This is the first study to examine how NAD3, a theacrine-based supplement, affects molecular markers in skeletal muscle cells. Notably, NAD3 treatments increased *Sirt1* mRNA levels as well as global sirtuin activity relative to CTL-treated cells. Moreover, NAD3 treatments increased mRNA levels of genes related to mitochondrial biogenesis (*Nfe2l2*), while decreasing *Nlrp3* mRNA levels (an inflammatory marker) and *Map1lc3b* (an autophagy marker).

As mentioned prior, there is widespread interest in ways to augment cellular sirtuin activity given that sirtuins have been implicated in tissue aging. The NAD3-induced increase in global sirtuin activity and Sirt1 mRNA levels agrees, in part, with prior literature. For instance, Wang et al. (15) determined that theacrine activates SIRT3 activity in liver cells *in vitro*. Song et al. (20) similarly reported that theacrine increases SIRT3 protein levels in myocardial tissue. Due to cell lysate constraints, we opted to measure global sirtuin activity rather than the individual activity of various sirtuins. However, it is possible that NAD3 preferentially activated SIRT3 activity leading to the observed increases in global sirtuin activity herein. This finding is promising given the role that sirtuins have in skeletal muscle; specifically, their role in enhancing mitochondrial function and the transcriptional control of various metabolic genes (18).

In spite of the NAD3-induced increase in global sirtuin activity as well as the NAD3-induced increase in *Nfe2l2* mRNA levels, mitochondrial biogenesis (as assessed through citrate synthase activity) remained unaltered. Although this finding is difficult to reconcile, it may be possible that NAD3, rather than appreciably affecting mitochondrial biogenesis, enhanced mitochondrial function. While we did not assess metrics of mitochondrial function (e.g., oxidation rates), it is notable Wang et al. (15) reported that

theacrine enhanced mitochondrial fat oxidation in liver cells. Moreover, the authors noted that these effects were likely mediated through theacrine-induced increases in SIRT3 activity given that this enzyme acts to deacetylate various mitochondrial protein in order to increase their activity. Thus, in light of these findings, future research is needed to determine if NAD3 enhances mitochondrial function in skeletal muscle.

What should finally be noted is the differential effects NAD3 had on mRNAs related to various metabolic processes. Our mRNA targets were not extensive due to limited RNA yield from culture; thus, we chose to examine select mRNAs related to inflammation (*Nlrp3*) and autophagy (*Atg12* and *Map1lc3b*). The most profound effect observed was the decrease in *Nlrp3* mRNA at both 3 and 24 hours relative to CTL-treated cells. The NLRP3 protein acts as an intracellular sensor for endogenous (e.g., oxidized mitochondrial DNA and ceramides) and exogenous pro-inflammatory molecules (e.g., lipopolysaccharide and other bacterial as well as viral particles) (21). Once activated, the NLRP3 protein coalesces with other proteins to form the NLRP3 inflammasome, and this protein complex then acts to generate proinflammatory cytokines. While not widely studied in skeletal muscle, Boursereau et al. (22) have shown that NLRP3 protein exists in skeletal muscle, and levels are induced through lipopolysaccharide stimulation. A more recent investigation suggests that that angiotensin II increases skeletal muscle NLRP3 inflammasome formation, and these events lead to mitochondrial dysfunction and muscle atrophy (23). It is difficult to interpret the significance of our findings given that the cells were not challenged with pro-inflammatory compounds. Notwithstanding, our data warrant further examination as to whether theacrine-based supplements can be used to inhibit inflammasome formation in situations where low-grade inflammation may increase the activity of this pathway.

Experimental considerations

There are limitations to the current data. First, these data are *in vitro*, and muscle biopsy studies in humans are needed to confirm and refine the current findings. Another limitation to these data are that only select biomarkers were interrogated in one cell type. Thus, more studies are needed to extensively assess the molecular alterations induced by NAD3 treatments in multiple cell types.

Conclusions

Although these data are *in vitro*, this preliminary evidence suggests a theacrine-based supplement can modulate SIRT activity while also affecting various mRNAs related to metabolic processes. However, and as mentioned prior, human studies are needed to validate the current findings.

Abbreviations

mRNA, messenger ribonucleic acid

Nlrp3, NLR Family Pyrin Domain Containing 3

OD, optical density

SIRT, sirtuin

Declarations

Ethics approval and consent to participate

All procedures described herein were approved by the Auburn University Institutional Biosafety Committee.

Consent for publication

Not applicable

Availability of data and material

Not applicable

Competing interests

None of the authors has competing interests to declare.

Funding

Costs for cells and reagents were funded by a grant provided to M.D.R by Compound Solutions (Carlsbad, CA, USA) and JUVN3 Holdings. M.D.R. has been a paid consultant in the past by the funding entity for scientific writing endeavors. Additionally, the funding entities have committed a multiple-year laboratory donation to the laboratory of MDR for graduate student development, assay development, and equipment purchases. However, M.D.R. affirms that the current data do not present a conflict of interest with these additional activities.

Authors' contributions

This experiment was performed at Auburn University's School of Kinesiology in the Molecular and Applied Sciences Laboratory. M.D.R. was responsible for the conception and design of the experiment, and primarily drafted the manuscript. P.W.M., S.C.O., and M.D.R. performed all experiments. All authors read and approved the final manuscript.

Acknowledgements

We thank Dr. Andreas Kavazis and Hailey Parry (School of Kinesiology, Auburn University) for running citrate synthase activity assays.

References

1. Johnson ML, Robinson MM, Nair KS. Skeletal muscle aging and the mitochondrion. Trends Endocrinol Metab. 2013;24(5):247-56.
2. Gensous N, Bacalini MG, Franceschi C, Meskers CGM, Maier AB, Garagnani P. Age-Related DNA Methylation Changes: Potential Impact on Skeletal Muscle Aging in Humans. Front Physiol. 2019;10:996.
3. Demontis F, Piccirillo R, Goldberg AL, Perrimon N. Mechanisms of skeletal muscle aging: insights from Drosophila and mammalian models. Dis Model Mech. 2013;6(6):1339-52.
4. Bielak-Zmijewska A, Grabowska W, Ciolko A, Bojko A, Mosieniak G, Bijoch L, et al. The Role of Curcumin in the Modulation of Ageing. Int J Mol Sci. 2019;20(5).
5. Harrison FE. A critical review of vitamin C for the prevention of age-related cognitive decline and Alzheimer's disease. J Alzheimers Dis. 2012;29(4):711-26.
6. Quinzii CM, Hirano M. Coenzyme Q and mitochondrial disease. Dev Disabil Res Rev. 2010;16(2):183-8.
7. Anand David AV, Arulmoli R, Parasuraman S. Overviews of Biological Importance of Quercetin: A Bioactive Flavonoid. Pharmacogn Rev. 2016;10(20):84-9.
8. Dang W. The controversial world of sirtuins. Drug Discov Today Technol. 2014;12:e9-e17.
9. Grabowska W, Sikora E, Bielak-Zmijewska A. Sirtuins, a promising target in slowing down the ageing process. Biogerontology. 2017;18(4):447-76.
10. Cesareo KR, Mason JR, Saracino PG, Morrissey MC, Ormsbee MJ. The effects of a caffeine-like supplement, TeaCrine(R), on muscular strength, endurance and power performance in resistance-trained men. J Int Soc Sports Nutr. 2019;16(1):47.
11. Kuhman DJ, Joyner KJ, Bloomer RJ. Cognitive Performance and Mood Following Ingestion of a Theacrine-Containing Dietary Supplement, Caffeine, or Placebo by Young Men and Women. Nutrients. 2015;7(11):9618-32.
12. Bello ML, Walker AJ, McFadden BA, Sanders DJ, Arent SM. The effects of TeaCrine(R) and caffeine on endurance and cognitive performance during a simulated match in high-level soccer players. J Int Soc Sports Nutr. 2019;16(1):20.
13. Gao M, Zheng J, Zheng C, Huang Z, Huang Q. Theacrine alleviates chronic inflammation by enhancing TGF-beta-mediated shifts via TGF-beta/SMAD pathway in Freund's incomplete adjuvant-induced rats. Biochem Biophys Res Commun. 2020;522(3):743-8.
14. Li WX, Li YF, Zhai YJ, Chen WM, Kurihara H, He RR. Theacrine, a purine alkaloid obtained from Camellia assamica var. kucha, attenuates restraint stress-provoked liver damage in mice. J Agric Food Chem. 2013;61(26):6328-35.
15. Wang GE, Li YF, Zhai YJ, Gong L, Tian JY, Hong M, et al. Theacrine protects against nonalcoholic fatty liver disease by regulating acylcarnitine metabolism. Metabolism. 2018;85:227-39.
16. Mumford PW, Romero MA, Mao X, Mobley CB, Kephart WC, Haun CT, et al. Cross talk between androgen and Wnt signaling potentially contributes to age-related skeletal muscle atrophy in rats. J

Appl Physiol (1985). 2018;125(2):486-94.

17. Romero MA, Mumford PW, Roberson PA, Osburn SC, Parry HA, Kavazis AN, et al. Five months of voluntary wheel running downregulates skeletal muscle LINE-1 gene expression in rats. Am J Physiol Cell Physiol. 2019;317(6):C1313-C23.
18. Lamb DA, Moore JH, Mesquita PHC, Smith MA, Vann CG, Osburn SC, et al. Resistance training increases muscle NAD(+) and NADH concentrations as well as NAMPT protein levels and global sirtuin activity in middle-aged, overweight, untrained individuals. Aging (Albany NY). 2020;12(10):9447-60.
19. Larsen S, Nielsen J, Hansen CN, Nielsen LB, Wibrand F, Stride N, et al. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. J Physiol. 2012;590(14):3349-60.
20. Song LL, Zhang Y, Zhang XR, Song YN, Dai HZ. Theacrine attenuates myocardial fibrosis after myocardial infarction via the SIRT3/beta-catenin/PPARgamma pathway in estrogen-deficient mice. Eur Rev Med Pharmacol Sci. 2019;23(12):5477-86.
21. Swanson KV, Deng M, Ting JP. The NLRP3 inflammasome: molecular activation and regulation to therapeutics. Nat Rev Immunol. 2019;19(8):477-89.
22. Boursereau R, Abou-Samra M, Lecompte S, Noel L, Brichard SM. Downregulation of the NLRP3 inflammasome by adiponectin rescues Duchenne muscular dystrophy. BMC Biol. 2018;16(1):33.

23. Liu Y, Bi X, Zhang Y, Wang Y, Ding W. Mitochondrial dysfunction/NLRP3 inflammasome axis contributes to angiotensin II-induced skeletal muscle wasting via PPAR-gamma. Lab Invest. 2020;100(5):712-26.

Figures

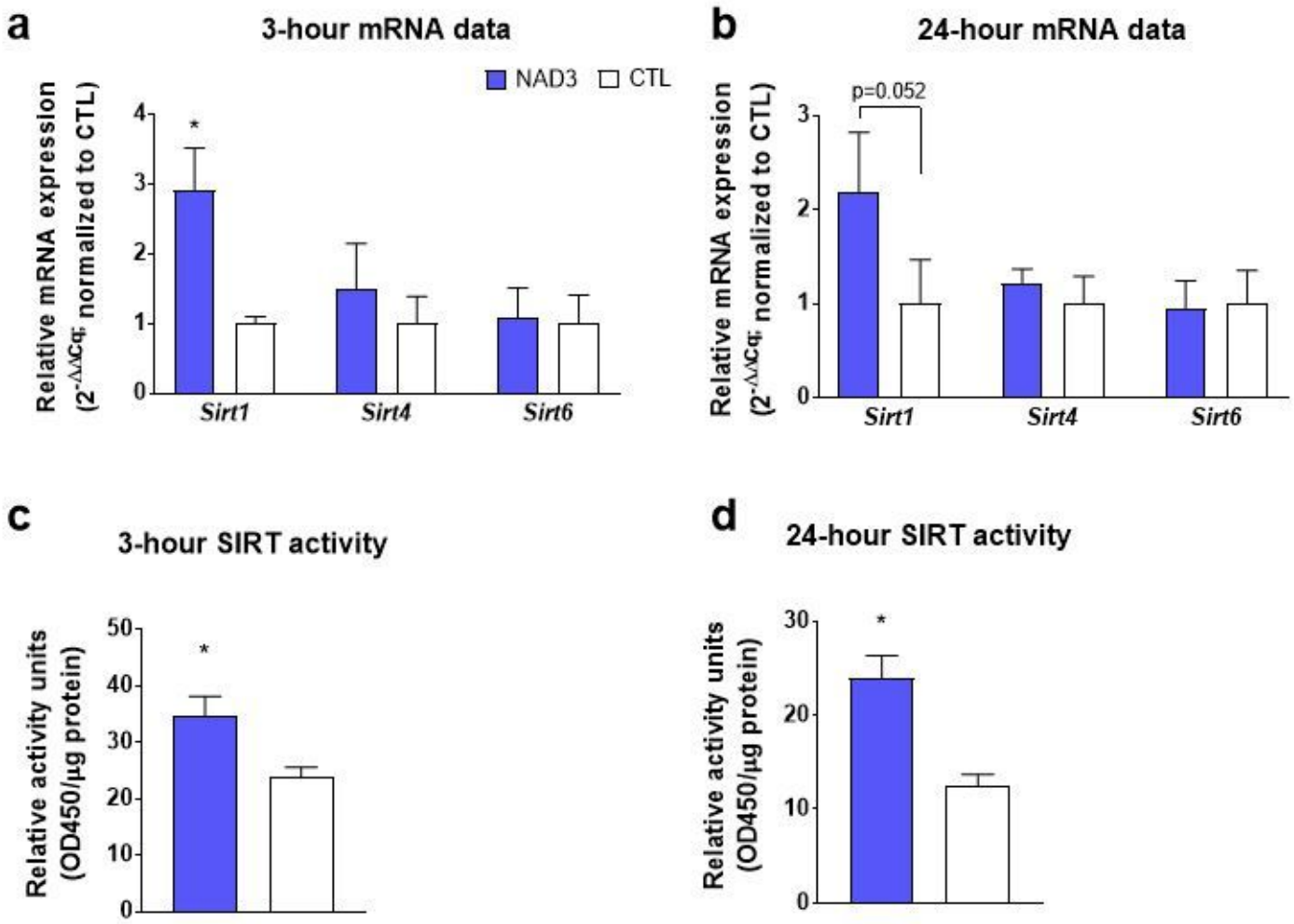

Figure 1

Effects of NAD3 treatments on SIRT markers Legend: These data illustrate 3-hour treatments on SIRT-related mRNAs (panel a), 24-hour treatments on SIRT-related mRNAs (panel b), 3-hour treatments on global SIRT activity (panel c), and 24-hour treatments on global SIRT activity (panel d). All data are presented as means ± standard deviation values, and significance was set at $p<0.05$. Symbol: *, indicates more highly expressed in NAD3-treated versus control (CTL)-treated cells.

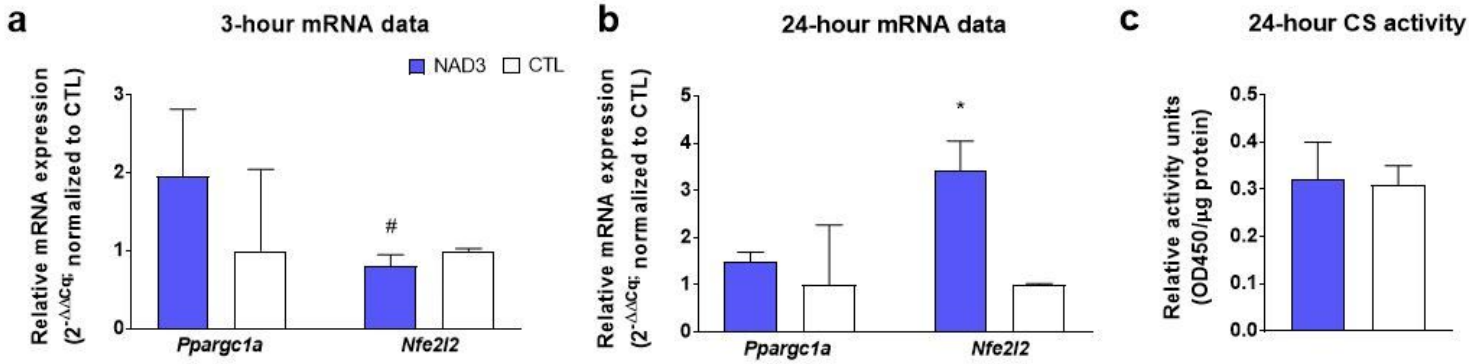

Figure 2

Effects of NAD3 treatments on mitochondrial biogenesis markers Legend: These data illustrate 3-hour treatments on mRNAs related to mitochondrial biogenesis (panel a), 24-hour treatments on mRNAs related to mitochondrial biogenesis (panel b), and 24-hour treatments on a surrogate marker of mitochondrial volume (panel c). All data are presented as means ± standard deviation values, and significance was set at $p<0.05$. Symbols: *, indicates more highly expressed in NAD3-treated versus control (CTL)-treated cells; #, indicates more highly expressed in control (CTL)-treated versus NAD3-treated cells.

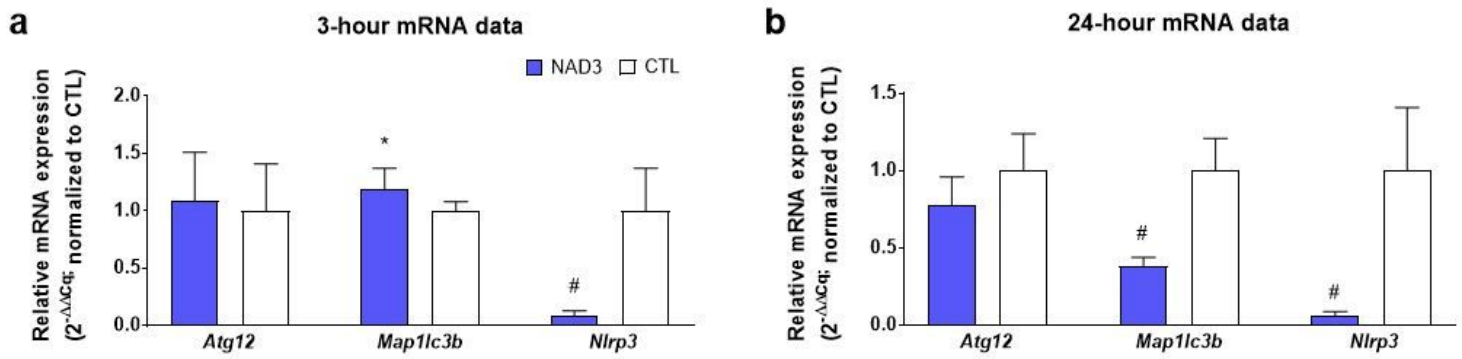

Figure 3

Effects of NAD3 treatments on mRNAs related to autophagy and inflammation Legend: These data illustrate 3-hour (panel a) and 24-hour (panel b) treatments on mRNAs related to various metabolic processes (panel a) All data are presented as means ± standard deviation values, and significance was set at $p<0.05$. Symbols: *, indicates more highly expressed in NAD3-treated versus control (CTL)-treated cells; #, indicates more highly expressed in control (CTL)-treated versus NAD3-treated cells.