Pharmacological Research 157 (2020) 104851

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Antioxidant modulation of sirtuin 3 during acute inflammatory pain: The ROS control

Sara Ilari[a], Luigino Antonio Giancotti[b], Filomena Lauro[a,b], Concetta Dagostino[a], Micaela Gliozzi[a], Valentina Malafoglia[a,c], Luigi Sansone[d], Ernesto Palma[a], Marco Tafani[e], Matteo Antonio Russo[f], Carlo Tomino[g], Massimo Fini[g], Daniela Salvemini[b], Vincenzo Mollace[a], Carolina Muscoli[a,*]

[a] Institute of Research for Food Safety & Health (IRC-FSH), Department of Health Sciences, University "Magna Graecia" of Catanzaro, 88201 Catanzaro, Italy
[b] Department of Pharmacology and Physiology and the Henry and Amelia Nasrallah Center for Neuroscience, Saint Louis University School of Medicine, 1402 South Grand Blvd, St. Louis, MO 63104, USA
[c] Institute for Research on Pain, ISAL Foundation, Torre Pedrera, RN, Italy
[d] Department of Cellular and Molecular Pathology, IRCCS San Raffaele Pisana, 00163 Rome, Italy
[e] Department of Experimental Medicine, University of Rome "Sapienza", 00161 Rome, Italy
[f] MEBIC Consortium, San Raffaele Rome Open University, 00163 Rome, Italy
[g] Scientific Direction, IRCSS San Raffaele Pisana, 00163 Rome, Italy

ARTICLE INFO

Chemical compounds studied in this article:
Carrageenan (Pubchem CID: 91972149)
Resveratrol (Pubchem CID: 445154)
Mn (III) tetrakis (4-benzoic) porfirin (MnTBAP) (Pubchem CID: 16760566)
4-hydroxynonenal (4-HNE) (Pubchem CID: 5283344)
2,4 dinitrophenylhydrazine (DNPH) (Pubchem CID: 3772977)

Keywords:
SIRT3
Inflammatory hyperalgesia
Oxidative stress
Natural antioxidants
Mitochondrial dysfunction

ABSTRACT

Oxidative stress induced post-translational protein modifications are associated with the development of inflammatory hypersensitivities. At least 90% of cellular reactive oxygen species (ROS) are produced in the mitochondria, where the mitochondrial antioxidant, manganese superoxide dismutase (MnSOD), is located. MnSOD's ability to reduce ROS is enhanced by the mitochondrial NAD^+-dependent deacetylase sirtuin (SIRT3). SIRT3 can reduce ROS levels by deacetylating MnSOD and enhancing its ability to neutralize ROS or by enhancing the transcription of MnSOD and other oxidative stress-responsive genes. SIRT3 can be post-translationally modified through carbonylation which results in loss of activity. The contribution of post-translational SIRT3 modifications in central sensitization is largely unexplored. Our results reveal that SIRT3 carbonylation contributes to spinal MnSOD inactivation during carrageenan-induced thermal hyperalgesia in rats. Moreover, inhibiting ROS with natural and synthetic antioxidants, prevented SIRT3 carbonylation, restored the enzymatic activity of MnSOD, and blocked the development of thermal hyperalgesia. These results suggest that therapeutic strategies aimed at inhibiting post-translational modifications of SIRT3 may provide beneficial outcomes in pain states where ROS have been documented to play an important role in the development of central sensitization.

1. Introduction

Peripheral and central inflammation play key roles in the development and maintenance of pathological pain states [1]. Over the last decade substantial evidence has been gathered to support the roles of reactive oxygen (superoxide; SO) and nitrogen (peroxynitrite; PN) species (collectively referred to as ROS and RNS) in the development of peripheral and central sensitization associated with acute and chronic pain states including in the development of carrageenan-induced thermal hyperalgesia [2–11]. Increased formation of ROS/RNS in the spinal cord contributes to the development of central sensitization that governs inflammatory hyperalgesia through several mechanisms [2]. In particular PN post-translationally modifies several proteins that are directly linked to the development of persistent pain transmission

Abbreviations: ROS, reactive oxygen species; MnSOD, manganese superoxide dismutase; SIRT3, sirtuin 3; SO, superoxide; PN, peroxynitrite; RNS, reactive nitrogen species; etc, electron transport chain; 4-HNE, 4-hydroxynoneal; FoxO3, forkhead box protein O3; GPX, glutathione peroxidase; PGC1alpha, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; Tfam, mitochondrial transcription factor A; RSV, resveratrol; MnTBAP, Mn (III) tetrakis (4-benzoic acid) porphyrin; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; NTB, nitrobluetetrazolium; BCA, bicinchoninic acid; DNPH, 24-dinitrophenylhydrazine; EDTA, ethylenediaminetetraacetic acid; PARP, poly (ADP-ribose) polymerase; NAD^+, nicotinamide adenine dinucleotide; WT, wild type; shRNA, short hairpin RNA

* Corresponding author.
E-mail address: muscoli@unicz.it (C. Muscoli).

https://doi.org/10.1016/j.phrs.2020.104851
Received 4 November 2019; Received in revised form 18 April 2020; Accepted 19 April 2020
Available online 11 May 2020

[12–16]. Thus, PN seems to be a key mediator for therapeutic strategies. Several lines of evidence demonstrated that pharmacologic removal of PN can reverse and prevent the characteristic findings associated with pain of several etiologies including inflammatory pain, neuropathic pain or morphine-induced hyperalgesia and tolerance [2,4,17].

Our previous studies demonstrated that post-translational nitration and modification of manganese superoxide dismutase (MnSOD) are implicated in the development of inflammatory pain [18,19]. In this context, more than 90% of cellular ROS are produced in the mitochondria, where electrons escape the electron transport chain (ETC) and combine with molecular O_2 to form superoxide. MnSOD is the mitochondrial antioxidant within a network of detoxification enzymes which neutralize highly reactive SO [20]. Excess superoxide can lead to DNA, lipid, and protein damage [21]. In particular, SO combines with nitric oxide to form PN which in turn nitrates and inactivates MnSOD; this sustains the formation of SO and PN resulting in the development and maintenance of central sensitization in nociceptive signaling [13,18].

Superoxide also plays a central role in lipid peroxidation, from which 4-hydroxynoneal (4-HNE) represents the most abundant and effective end product causing cellular damage [22]. This 4-HNE readily reacts with target molecules, enhances cell damage [23], and represents the main precursor for protein carbonylation [24], an irreversible post-translational modification formed by covalent adduction of lipid aldehydes to nucleophilic protein groups [25]. Recent studies have proven that 4-HNE accumulation quickly modifies the activity of a new class of proteins called sirtuins [26]. Sirtuins are a family of epigenetic proteins able to deacetylate histone and non-histone proteins including transcription factors, DNA repair proteins, and signaling factors [27]. Thus, sirtuins control different vital functions and influence several pathologies such as metabolic diseases, neurodegenerative disorders, inflammation and cancer [28,29]. It is well known that the nuclear protein sirtuin 1 (SIRT1) modulates the activity of nuclear factor-kB (NF-kB) via post-translational modification of the NF-kB p56 subunit [30]. Accordingly, SIRT1 activators have anti-inflammatory effects in both *in vitro* and *in vivo* models of inflammation [30]. Furthermore, sirtuins are also target proteins of post-translational modifications.

In particular, carbonylation of mitochondrial sirtuin 3 (SIRT3) by 4-HNE at Cys (280) was reported in liver mitochondrial extracts of ethanol-consuming mice, resulting in allosteric inhibition of SIRT3 activity [24]. SIRT3 is emerging as a pivotal regulator of oxidative stress, deacetylating the substrates involved in both ROS production and detoxification. Notably, SIRT3 deacetylates and activates many mitochondrial enzymes involved in fatty acid β-oxidation, amino acid metabolism, and the electron transport chain (ETC) [31,32]. Interestingly, SIRT3 activity can reduce ROS levels by modulating key antioxidants [31]. In particular, SIRT3 directly deacetylates the MnSOD 122-lysine residue in the mitochondria [33] significantly enhancing its ability to neutralize ROS [33,34]. SIRT3 enhances the transcription of MnSOD and other oxidative stress-responsive genes, as well as forkhead box protein O3 (FoxO3), catalase, glutathione peroxidase (GPX), and isocitrate dehydrogenase 2 [35,36]. In fact, enzyme dysfunction in antioxidant defense systems, through the downregulation of SIRT3 signaling, can trigger oxidative stress [33]. In particular, SIRT3 binds FoxO3a which in turn regulates antioxidants include Mn-SOD [37,38]. Moreover, SIRT3-mediated deacetylation of FOXO3 triggers both mitochondrial biogenesis and mitophagy processes. On the one hand, SIRT3-FOXO3 axis upregulates Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 alpha (PGC1alpha; the mastergene of mitochondrial biogenesis) and mitochondrial transcription factor A (Tfam), thus controlling mitochondrial mass and ATP production; on the other hand the main mitochondrial autophagic mediators (Bnip3, Nix and LC3) are positively controlled by FOXO3 deacetylation [39]. We propose that alterations in mitochondrial SIRT3 disrupt mitochondrial homeostasis contributing to the development of hyperalgesia.

Synthetic or natural antioxidant agents are known to show anti-hyperalgesic effects [40,41]. Recent study have shown that dietary polyphenols, including resveratrol (RSV), have antioxidant and long-lasting anti-nociceptive effects [40,42,43]. It has been demonstrated that resveratrol attenuates the development of neuropathic pain by alleviating the reduction of SIRT1 deacetylase activity [44]. Moreover, resveratrol exhibited an anti-hyperalgesic effects through the down-regulation of ROS level and pERK/ERK ratio, indicating that anti-hyperalgesic effect of resveratrol might be correlated with antioxidant activity [40].

Additionally, our previous studies [11,16,18] reported the use of synthetic antioxidants for the removal of free radicals. Among these, Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) is a potent inhibitors of lipid peroxidation [45] and exerts a protective effect against some of the detrimental effects associated with endotoxic and hemorrhagic shock [46]. Its efficacy is probably due to its peroxynitrite-scavenging activity in addition to its superoxide-scavenging activity [18].

The contribution of post-translational SIRT3 modifications in central sensitization is largely unexplored and the modulation of key endogenous antioxidant enzymes is not well known in inflammatory pain. Accordingly, in the present study, we investigated the impact of oxidative stress and the role of antioxidants (resversatrol and MnTBAP) on SIRT3 signaling in the spinal cord in a well-characterized model of inflammatory hyperalgesia in rats [6,13,47–49].

2. Material and methods

2.1. Animals

Male Sprague-Dawley rats (225 − 250 g, 8 weeks old, Envigo) were used for these studies. All animals were housed and cared in accordance with the NIH Guidelines on Laboratory Animal Welfare following the Italian regulations for the protection of animals used for experimental and other scientific purposes (D.L. 26/2014), and with European Economic Community regulations (2010/63/UE) with authorization number 577-2016-PR. The numbers of animals used were the minimum necessary to achieve statistical significance at $p < 0.05$. Rats were housed two per cage and maintained under identical conditions of temperature (21 ± 1 °C) and humidity (60% ± 5%), with a 12 h light/12 h dark cycle and allowed food *ad libitum* and all experiments took place during the light period in a quiet room. All the drugs were purchased from Sigma Aldrich and dissolved in saline (sodium chloride 0.9%).

2.2. Experimental groups

Rats were allocated into one of the following experimental groups:

2.2.1. Vehicle group

Rats (n = 15) received an intraperitoneal injection of saline, 15 min before intraplantar injection of saline into the right hindpaw.

2.2.2. Carrageenan group

Rats (n = 15) received an intraperitoneal injection of saline 15 min before intraplantar injection of carrageenan (1% suspension in 0.85% NaCl; Calbiochem, cat. 22049) into the right hindpaw.

2.2.3. Drug groups

Rats received an intraperitoneal injection of different doses of Resveratrol (RSV; 10 mg/kg; 30 mg/kg; or 50 mg/kg; for each dose n = 15; Sigma, cat. R5010), Mn (III) Tetrakis (4-Benzoic acid) porphyrin (MnTBAP; 5 mg/kg; 10 mg/kg; or 30 mg/kg; for each dose; n = 15; Cayman, cat. 75850) 15 min before intraplantar (i.pl.) injection of carrageenan (1% suspension in 0.85% NaCl; Calbiochem, cat. 22049) into the right hindpaw.

The dose of the compounds has been chosen according with the bibliography [16,40,50,51].

For all groups, the lumbar enlargement segment of the spinal cord (L4–L6) was removed six hours after the second injection, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent analysis.

2.3. Measurements of thermal hyperalgesia and edema after carrageenan administration

Hyperalgesic responses to heat were determined as described by Hargreaves [47] and a cut off latency of 20 s was chosen to prevent tissue damage in non-responsive animals. Rats were individually allowed to acclimate for 30 min within plexiglass chambers on a clear glass plate in a quiet testing room (Ugo-Basile, Varese, Italy). A mobile foot stimulus unit, consisting of a high-intensity projector bulb, was positioned to deliver a thermal stimulus directly to a single hindpaw from beneath the chamber. The withdrawal latency period of injected and contralateral paws was determined to the nearest 0.1 s with an electronic clock circuit and thermocouple. Each successful test point represented the difference (sec) in withdrawal latency between paws [withdrawal latency of contralateral (left paw) minus withdrawal latency of injected paw (right paw)] at each time point. Results were expressed as Paw-withdrawal latency changes (sec). Changes in paw volume were measured as previously described [52]. Paw volume was measured with a plethysmometer (Ugo-Basile, Varese, Italy) immediately before the injection of carrageenan and at 6 h. Edema was expressed as the increase in paw volume (mL) after carrageenan injection, relative to the paw's pre-injection volume. Results are expressed as paw volume change (mL). All experimenters were blinded to treatment conditions. Data were unblinded during data analysis.

2.4. Cell cultures

Mechanistic studies were performed *in vitro* using the breast cancer cell line MDA-MB-231. In fact, such a cell line has been extensively used in our laboratory as an *in vitro* system to identify or validate SIRT3 metabolic functions as well as to confirm SIRT3 activity on new and old targets [29]. In particular, our previous work on MDA-MB-231 allowed us to obtain and validate, from a protein and enzymatic point of view, clones overexpressing and silenced for SIRT3 and to discover the role of SIRT3 in regulating ROS production, the mitochondrial permeability transition (MPT) by interacting with hexokinase II (HKII) as well as the intracellular acidification by deacetylating mitochondrial carbonic anhydrase Vb (CAVB) [29]. In this work, the MDA-MB-231 human breast carcinoma cell line was purchased from LGC Standards (Milan, Italy). MDA-MB-231 was maintained in 75-cm^2 polystyrene flasks (Corning Costar Corp., Oneonta, NY, USA) with RPMI 1640 medium (Mediatech, Inc., Herndon, VA, USA), containing 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 10% by volume of heat-inactivated fetal bovine serum. The medium was changed every other day. The cells were passaged up to six times. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.5. Cell treatment

To evaluate the involvement of SIRT3, MDA-MB-231 human cells were incubated with 50 μM 4-HNE (Abcam Biochemicals, cat. ab141502) in DMSO for 3 h. In the experiments with antioxidants, cells were incubated with 50 μM MnTBAP (Cayman, cat. 75850) and 50 μM Resveratrol (Sigma, R5010) for 15 min before exposure to 4-HNE. The dose of the compounds has been chosen according with the bibliography [53–55].

2.6. Generation of SIRT3 transfectants

MDA-MB-231 cells were stably transfected with the pcDNA 3.1 expression vector encoding for human SIRT3-Flag (Addgene Inc., Cambridge, MA, USA) as previously reported [29]. Briefly, stable clones were generated by delivering plasmid DNA constructs into cells using TurboFectin 8.0 (Origene Technologies, Rockville, MD, USA), according to manufacturer recommendations. Cells (5×10^4) were seeded on a 24-well plate and were transfected the following day. TurboFectin reagents (0.5 μL) were first mixed with serum-free RPMI at room temperature for five minutes. Subsequently, plasmid DNA (0.3 μg) was added to the TurboFectin-containing media and incubated at room temperature for 30 min. After that, the transfection mixtures were added to the cells. The selection of stable clones started 24 h later with the addition of 500 μg/mL of Geneticin (Sigma-Aldrich).

2.7. Lentiviral transduction

Mission™ TRC shRNA lentiviral transduction particles expressing short hairpin RNA (shRNA) targeting SIRT3 (NM 012239 TRC N0000038889) and lentiviral negative control particles (SHC016 V) were purchased from Sigma-Aldrich. Stably transduced clones were generated according to manufacturer instructions. Cells were seeded on a 24-well plate and infected the following day. After 24 h, the medium was replaced with fresh RPMI. The selection of stable clones started 24 h later with the addition of 3 μg/mL of puromycin. The expression of SIRT3 was confirmed by western blotting.

2.8. Tissue or cells preparation for mitochondrial extraction

For mitochondrial extraction, tissues or cells were homogenized with lysis buffer at 1:3 w/v ratio. The lysis buffer (250 mM Sucrose; 10 mM Tris; 1 mM EDTA; pH 7.8) contained 1% protease inhibitor cocktail (v/v) (Sigma, cat. P8340). Solubilized extracts were centrifuged at 1600*g* for 10 min at 4 °C to obtain supernatants that were re-centrifuged at 12,000*g* for 15 min at 4 °C. The resulting pellets were collected and suspended for 10 min with mitochondrial lysis buffer (1% Triton; 1:100 Protease inhibitor cocktail) and centrifuged at 10,000*g* for 10 min at 4 °C. The resulting supernatants were immediately stored at −80 °C for subsequent analysis. Protein concentration was determined using the Bicinchoninic Acid (BCA) protein assay (Thermo Scientific, cat. 23225).

2.9. Immunoprecipitation and Western blot analyses

Mitochondrial fractions obtained as previously described were used for immunoprecipitation and western blot analyses. For immunoprecipitation, Protein A-sepharose 4B resin (Sigma, cat. P3391) was prepared by washing 50 μL of resin four times in PBS. Each wash consisted of mixing resin with fresh PBS and collecting the cleaned resin after centrifugation (14,000 rpm at 4 °C for 1 min). The cleaned resin was re-suspended in Lysis Buffer (1% Triton; 1:100, IBI Scientific cat. IB07100; Protease inhibitor cocktail, Sigma, cat. P8340) and coated with specific antibodies against the protein of interest (anti-SIRT3, Cell Signaling, cat. 26275 or anti-MnSOD, Millipore, cat. 06984) and gently mixed overnight at 4 °C. Beads were washed four times with PBS and were added to every 300 μg of tissue or cell homogenate supernatant and incubated overnight at 4 °C. Samples were centrifuged (14,000 rpm at 4 °C for 20 min) and each pellet was collected for SDS-PAGE analysis.

Western blot analyses of the immunoprecipitated protein complex and total lysates were made using specific antibodies. The immunoprecipitated proteins were resolved in 12% SDS-PAGE minigels and transferred to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature in 1% BSA/0.1% thimerosal in 50 mM Tris−HCl (pH 7.4)/150 mM NaCl/0.01% Tween-20 (TBS/T), followed by overnight incubation at 4 °C with: anti-Lys-Acetylated antibodies (1:1000; Cell Signaling, cat. 9441); anti-SOD2/MnSOD (acetyl K122) (1:1000; abcam, cat. ab214675); anti-4-HNE (1:1000; Millipore, cat. MAB3249); anti-SIRT3 (1:1000; Cell Signaling, cat. 26275); or anti-MnSOD (1:1000; Millipore, cat. 06984). Membranes were then washed

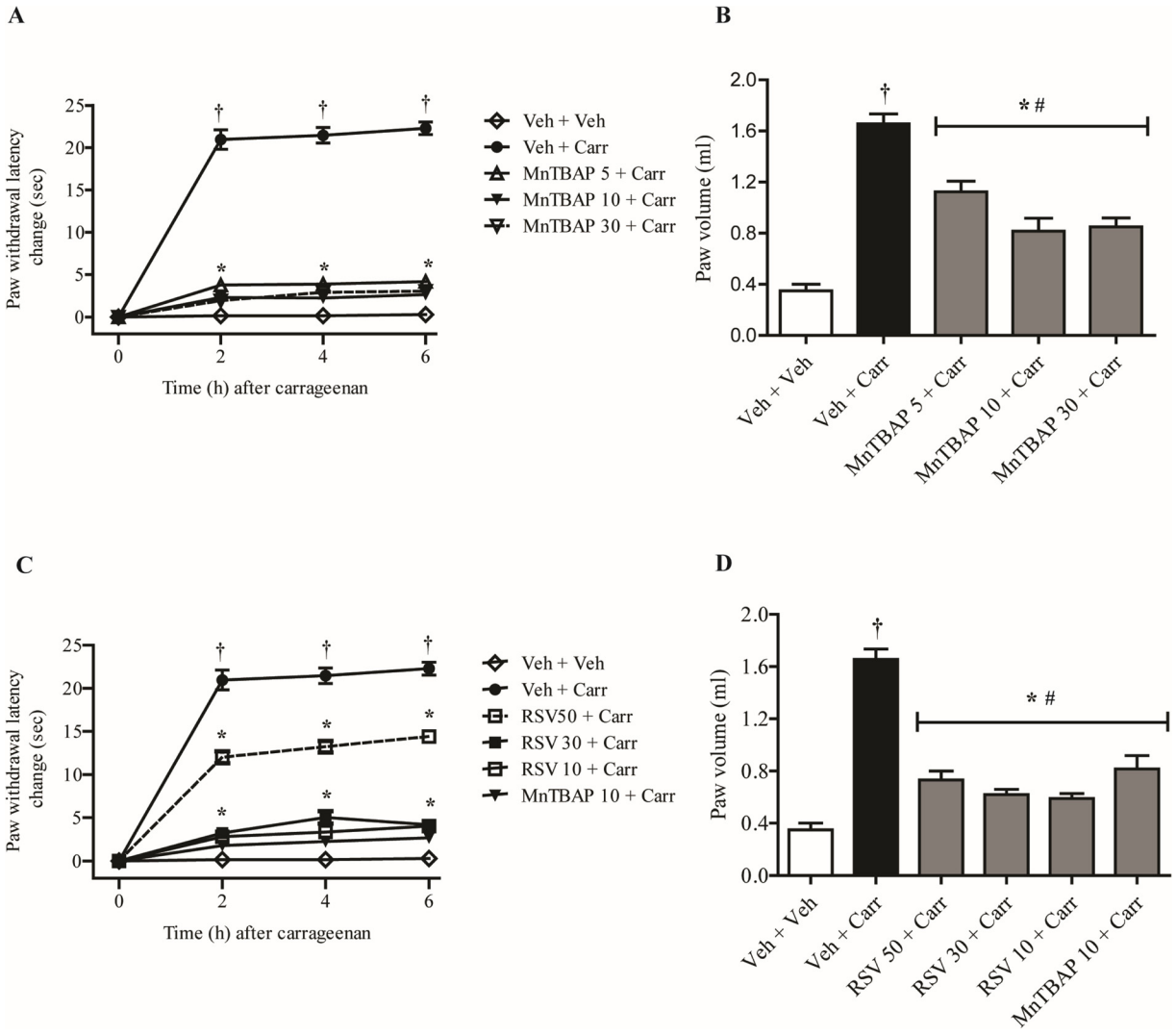


Fig. 1. Oxidative stress causes thermal hyperalgesia and edema that are induced by intraplantar carrageenan injection. (A–D) Intraperitoneal injections of MnTBAP (5–30 mg/kg) or RSV (10–50 mg/kg) 15 min prior to each carrageenan injection inhibit thermal hyperalgesia (A and C respectively) and inflammatory response (B and D respectively) induced by intraplantar carrageenan injection. Results are expressed as means ± SEM for 15 rats in each group. *P < 0.05 compared to Veh + carr; †P < 0.05, ♯P < 0.05 compared to Veh + Veh.

with TBST and incubated with monoclonal (anti-mouse 1:10000; GE Healthcare, cat. NA931) or polyclonal (anti-rabbit 1:30000; GE Healthcare, cat. NA934) secondary antibodies conjugated to horseradish peroxidase, for 1 h at room temperature. After washes, proteins were visualized by enhanced chemiluminescence (ECL; GE Healthcare, cat. RPN2232). No difference for Prohibitin (1:1000, Thermo Scientific, cat. MS261) was detected among the lanes. All densitometry data were normalized against prohibitin for each lane and expressed as the ratio of nitrated to unnitrated proteins. Quantitation of protein bands of interest was determined by densitometry using ImageQuant 5.2 software (Molecular Dynamics).

2.10. Analytical analysis of 4-HNE

4-HNE was monitored by liquid chromatography-mass spectrometry (LC/MS/ESI) equipped with a double analyzer, which allows the fragmentation of molecular ions by collision with a gas. In the present work, we focused on ion 137 *m/z* to 4-HNE (devoid of a water molecule). LC–MS measured the ratio of mass with respect to charge in conditions of very low pressure (10^{-6} Torr). A spray (electrospray, liquid pumped to a determined pressure) was generated with charged molecules. Chromatogram elaboration was made through Windows® version of the software Borwin, provided by Jasco.

2.11. Determination of MnSOD activity

MnSOD activity was measured as previously described [56,57].

Briefly, the tissues were homogenized with 10 mM phosphate-buffered saline (pH7.4) in a Polytron homogenizer, sonicated on ice for one minute, and subsequently centrifuged for 10 min at 1100*g*. To determinate SOD activity, a competitive inhibition assay using xanthine oxidase-generated O_2 was performed by reducing nitrobluetetrazolium (NTB) to blue tetrazolium salt, as previously described [6]. The reaction was performed in sodium carbonate buffer (50 mM, pH 10.1) containing EDTA (0.1 mM), nitrobluetetrazolium (25 μM), xanthine (0.1 mM), and xanthine oxidase (2 nM). The rate of NBT reduction was monitored at 560 nm. MnSOD activity was calculated by preincubating the sample for 30 min with 2 mM NaCN. The amount of protein required to inhibit the rate of NTB reduction by 50% was defined as 1 unit of enzymatic activity. Enzymatic activity was expressed in units per milligram of protein. All determinations were performed in triplicate.

2.12. Determination of protein carbonylation

The presence of protein carbonyl groups was determined using the Oxyblot protein oxidation kit (Millipore, cat. 57150). Protein carbonyl derivatives were detected by reaction with 2,4-dinitrophenylhydrazine (DNPH), according to manufacturer instructions. The level of carbonyl protein groups was analyzed by Western Blot. SIRT3 and prohibitin levels were detected after stripping the membrane, using stripping buffer solution (Thermo scientific, cat. 21059) following the manufacturer protocol.

2.13. SIRT3 deacetylase activity assay

SIRT3 deacetylase activity was determined using the SIRT3 Fluorimetric Activity Assay/Drug Discovery Kit (EnzoLife, cat. BMLAK5570001) following the manufacturer protocol. The mitochondrial extract (5 μg) was incubated with the Fluor de Lys substrate buffer at 37 °C for 1 h, followed by Fluor de Lys Developer at 37 °C for 40 min. After excitation at 360 nm, the emitted light was detected at 460 nm, using an Infinite 200 microplate fluorometer (Tecan).

2.14. Statistical analysis

Data are expressed as means ± SEM for n animals. Data from the time course studies were analyzed *via* two-way repeated measures analysis of variance (ANOVA) with Bonferroni comparisons.

One-way ANOVA followed by the Newman-Keuls test was used to analyze other data. Statistically significant differences were defined as a $P < 0.05$. All statistical analyses were performed using GraphPad Prism (v8.00; GraphPad Software, Inc).

3. Results

3.1. Carrageenan-induced thermal hyperalgesia and edema are associated with 4-hydroxinonenal (4-HNE) production and post-translational modifications of mitochondrial proteins

Intraplantar injection of carrageenan in rats caused a time-dependent development of thermal hyperalgesia and edema which peaked within 2–3 h and plateau up to 6 h (Fig. 1A–D). This was associated with lipid mitochondrial damage quantified by measuring 4-HNE accumulation in the spinal cord (Fig. 2A–B). Polyunsaturated fatty acids within the cellular membrane are primary targets of free radicals, and 4-HNE is a major lipid peroxidation product of omega-6 fatty acids [22]. Our analysis revealed significant 4-HNE production in mitochondria of the lumbar spinal cord of carrageenan treated rats (2A-B).

Intraperitoneal (i.p.) injection of different doses of RSV (10 − 50 mg/kg, 15 min before carrageenan) alleviated the development of thermal hyperalgesia (Fig. 1C) supporting previous studies [40,58–60]. These results were comparable to the action of MnTBAP, the SOD mimetic, a potent inhibitors of lipid peroxidation (5 − 30 mg/kg, administered i.p. 15 min before carrageenan) (Fig. 1A, C).

In addition, RSV (10 − 50 mg/kg) and MnTBAP (5 − 30 mg/kg) have significant beneficial effects against tissue damage and inflammation as shown by the reduction of edema (Fig. 1B, D) and may provide a different therapeutic approach avoiding the typically associated NSAIDs side effects. Furthermore, the SOD mimetic and resveratrol inhibited enhanced 4-HNE production in the spinal cord of carrageenan-treated rats (Fig. 2A–C).

4-HNE interferes with mitochondrial function by inflicting protein damage by carbonylation *via* addition reactions with lysine amino groups, cysteine sulfhydryl groups, and histidine imidazole groups [61]. Modifications of proteins by reactive aldehydes contribute to cellular damage and neurodegenerative disorders [62]. To evaluate mitochondrial protein damage induced by oxidative stress *via* 4-HNE production *in vivo*, we evaluated carbonylation levels of mitochondrial proteins. Spinal cord segments from carrageenan-treated rats revealed significant increases in carbonylated proteins that were decreased in rats treated with MnTBAP and resveratrol (Fig. 2C).

3.2. Thermal hyperalgesia and edema are associated with SIRT3 carbonylation and inactivation

SIRT3 is considered the major mitochondrial deacetylase [63]. Thus, alteration of SIRT3 activity alters mitochondrial pathways. We examined SIRT3 post-translational modification by reactive aldehydes. Our data demonstrated that although SIRT3 expression levels remained unaltered in rat spinal cords during carrageenan-induced inflammation and hyperalgesia, SIRT3 carbonylation levels were significantly increased (Fig. 3A). Carbonylated SIRT3 lost its enzymatic activity. Resveratrol and MnTBAP restored the SIRT3 enzymatic activity (Fig. 3B).

3.3. Inhibition of SIRT3 activity caused hyperacetylation of mitochondrial proteins and MnSOD inactivation

Next, we analyzed mitochondrial acetylated proteins as an additional marker of SIRT3 inactivation. Our data demonstrated a significant increase in mitochondrial protein acetylation in the lumbar spinal cord at the time of peak edema and hyperalgesia (Fig. 4A). MnTBAP and resveratrol reduced acetylation of mitochondrial proteins (Fig. 4A). Targets of SIRT3 deacetylation are wide spread and are implicated in a host of cellular pathways, including oxidative phosphorylation, fatty acid metabolism, alcohol metabolism, and the oxidative stress response proteins, such as MnSOD that limit accumulation of mitochondrial ROS [64,65]. SIRT3 carbonylation and subsequent inactivation is responsible for spinal MnSOD hyperacetylation (Fig. 4B) and its subsequent inactivation (Fig. 4C). Pretreatments with MnTBAP and resveratrol attenuated MnSOD acetylation and restored its enzymatic activity (Fig. 4B, C).

3.4. SIRT3 regulates mitochondrial MnSOD activity

To further dissect the endogenous role of SIRT3 on MnSOD activity, we used SIRT3-over-expressing (Sirt3 OE), SIRT3 knockdown (SIRT3 KD), and SIRT3 Wild Type (WT) cells (Fig. 5A). First, we compared MnSOD acetylation levels in SIRT3 KD and SIRT3 WT cells and showed that acetylated MnSOD levels were significantly lower in SIRT3 WT than in SIRT3 KD cells (Fig. 5B). These acetylated MnSOD levels were linked to endogenous SIRT3 activity; indeed, our data demonstrated a significant reduction of SIRT3 activity (Fig. 5C) and a concomitant increase in MnSOD acetylation and inactivation in SIRT3 KD cells (Fig. 5B, D). No significant difference in MnSOD acetylation was observed following the administration of MnTBAP and resveratrol in both cellular models (Fig. 5B).

Gels are representative of results from 6 different experiments and the histograms represent densitometric analysis of the MnSOD acetylation/MnSOD ratio and SIRT3/Prohibitin ratio of the gels shown. Results are expressed as mean ± SEM. $^{*}P < 0.05$ *vs.* WT cells. $^{\dagger}P < 0.05$ *vs.* SIRT3 OE. SIRT3 KD, SIRT3-knockdown cells; WT, SIRT3-Wild Type cells; SIRT3 OE, SIRT3-over-expressed cells; cell line used: human breast cancer cells MDA-MB-231; AFU, arbitrary fluorescence unit.

3.5. Oxidative stress inhibits SIRT3 activity

Finally, we analyzed MnSOD acetylation levels in SIRT3 OE cells treated with 4-HNE to confirm the role of oxidative stress on SIRT3 activity. Our study demonstrated that 4-HNE treatment increased MnSOD acetylation levels and inactivation of MnSOD and SIRT3 (Fig. 6A–C).

Addition of MnTBAP maintained SIRT3 activity and inhibited MnSOD hyperacetylation, restoring its activity (Fig. 6A–C). This effect

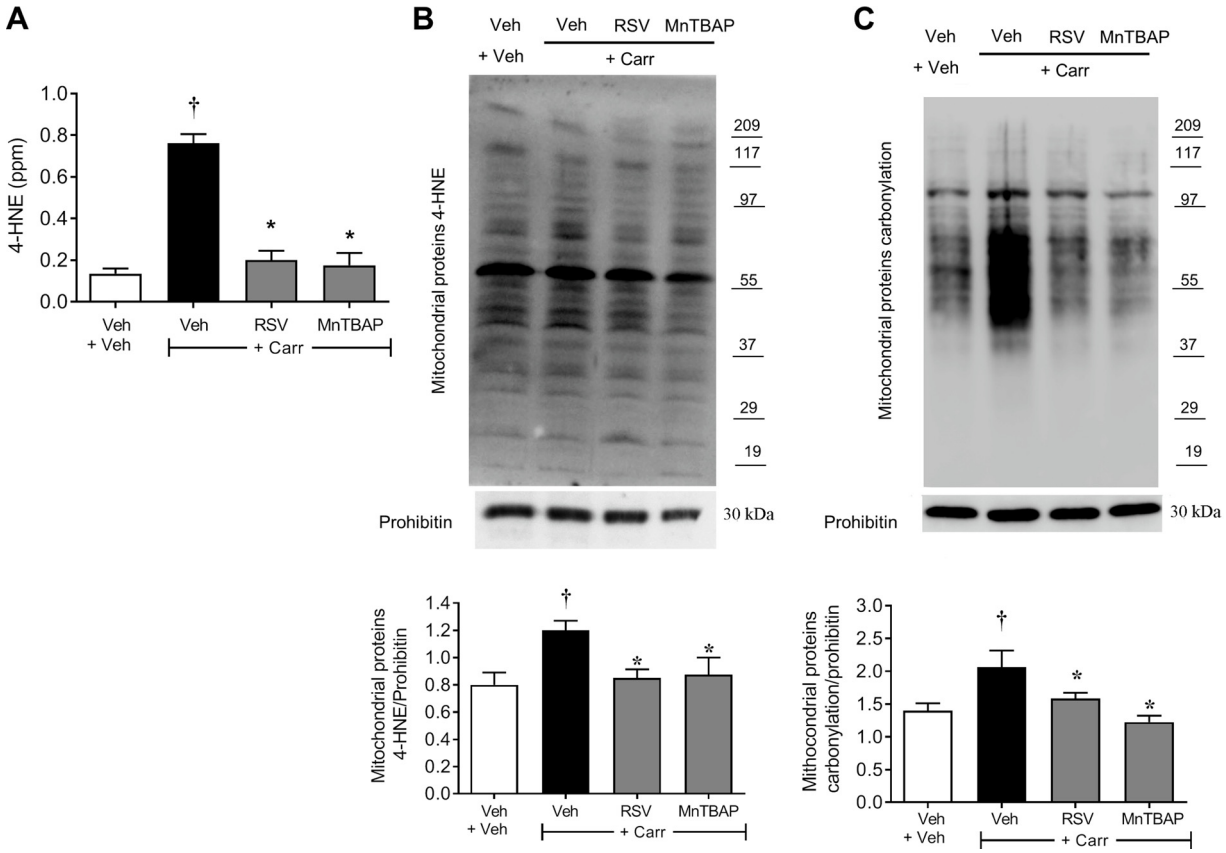
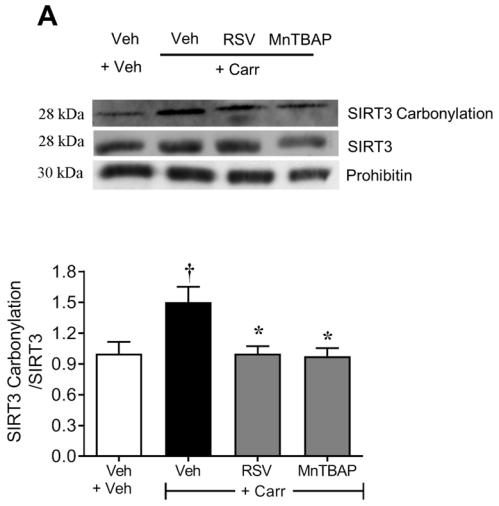


Fig. 2. Inflammatory pain is associated with oxidative stress damage. Intraplantar (i.pl.) injection of carrageenan increased 4-HNE levels (A, B) and 4-HNE-carbonylated mitochondrial proteins (C) in the spinal cord (L4-L5) of rats as measured by LC/MS/ESI (A) and Western Blot (B, C). Intraperitoneal (i.p.) administration of MnTBAP (10 mg/kg; 15 min prior to each carrageenan injection) or RSV (10 mg/kg; 15 min prior to each carrageenan injection) both attenuated 4-HNE expression (A, B) and protected mitochondrial proteins against such 4-HNE attack (C). No differences for prohibitin expression were detected among the lanes in these conditions. Gels are representative of results from 5 animals and the histogram represents densitometric analysis of total mitochondrial proteins 4-HNE/prohibitin ratio of the gel shown (B), or total mitochondrial proteins carbonylation/prohibitin ratio of the gel shown (C). Results are expressed as means ± SEM for 5 rats in each group. †P < 0.05 compared to Veh + Veh; *P < 0.05 compared to Veh + carrageenan. Ppm, parts per million.



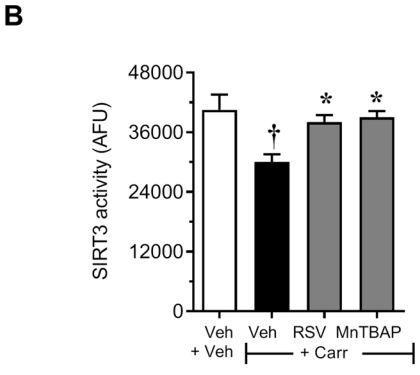


Fig. 3. Thermal hyperalgesia is associated with SIRT3 carbonylation. (A) Carrageenan injection significantly increases carbonylation of mitochondrial SIRT3 in the lumbar spinal cord segment. Pre-treatment with MnTBAP (10 mg/kg) or resveratrol (10 mg/kg), 15 min prior to each carrageenan injection, prevents such SIRT3 carbonylation. No differences for prohibitin or SIRT3 expression were detected among the lanes in these conditions. Gels are representative of results from 5 animals and the histogram represents densitometric analysis of SIRT3 carbonylation/SIRT3 ratio of the gel shown. Results are expressed as means ± SEM for 5 rats per group. †P < 0.001 *vs.* Veh + Veh; *P < 0.001 *vs.* Veh + carrageenan. (B) Intraplantar (i.pl.) injection of carrageenan is linked to the inactivation of SIRT3 enzymatic function. Compared with the vehicle group, animals receiving carrageenan injection showed lower levels of SIRT3 activity. Pre-treatment of MnTBAP (10 mg/kg; 15 min prior to each carrageenan injection) or resveratrol (10 mg/kg; 15 min prior to each carrageenan injection) maintained SIRT3 activity. Results are expressed as means ± SEM for 5 rats. †P < 0.05 *vs.* Veh + Veh; *P < 0.05 *vs.* Veh + carrageenan. AFU, arbitrary fluorescence unit.

has been compared to that one of RSV, showing high similarities between the natural and the synthetic antioxidant action, even if RSV was not able to totally restore the activity of SIRT3 to the level control (Fig. 6B).

4. Discussion

Reactive species, including superoxide and peroxynitrite, are implicated in both the development and maintenance of inflammatory pain, through multiple mechanisms [2,5,19]. More recently,

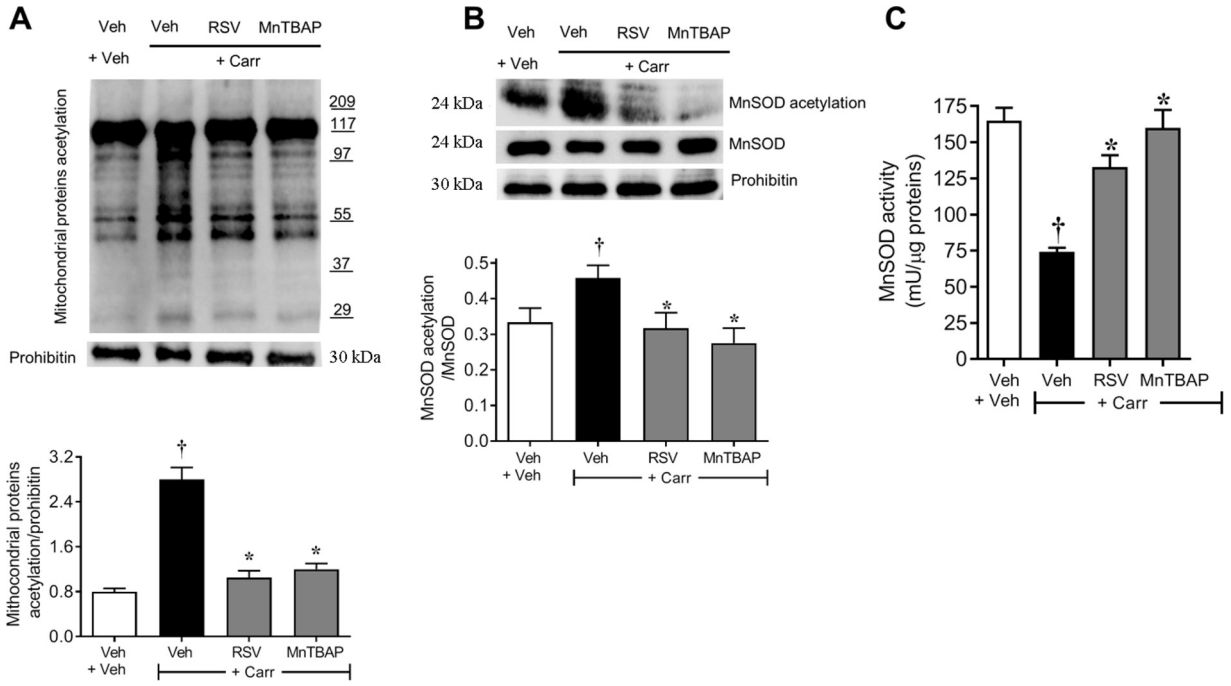


Fig. 4. Sirt3 inhibition caused acetylation of mitochondrial proteins and inactivation of MnSOD. (A) Western blot analyses demonstrated significant acetylation of mitochondrial proteins in the spinal cord of animals that received intraplantar carrageenan injections. Pre-treatment of such animals with MnTBAP (10 mg/kg) or resveratrol (10 mg/kg) before carrageenan injections inhibited such post-translational modification of proteins. No differences for the marker protein prohibitin's expression were detected among the lanes in these conditions. (B) Inactivation of SIRT3 by intraplantar carrageenan administration induced mitochondrial MnSOD acetylation in the animals' lumbar spinal cords. Rats pretreated with either MnTBAP (10 mg/kg) or resveratrol (10 mg/kg) prevented carrageenan-induced MnSOD acetylation. No difference for prohibitin or MnSOD expression was detected among the lanes in these conditions. Gels are representative of results from 5 animals and the histogram represents densitometric analysis of total mitochondrial protein acetylation/prohibitin (A) or MnSOD acetylation/MnSOD (B) ratio of the gel shown. Results are expressed as means ± SEM for 5 rats. *$P < 0.05$ *vs.* Veh + Veh; †$P < 0.05$ *vs.* Veh + carrageenan. (C) Acetylation of MnSOD is linked to the inactivation of MnSOD's enzymatic function. Compared with the saline-injected vehicle group, animals that received a carrageenan injection showed lower levels of MnSOD activity in their lumbar spinal cords. MnTBAP (10 mg/kg) or resveratrol (10 mg/kg) pretreatment maintained MnSOD activity in carrageenan-inflamed rats. Results are expressed as means ± SEM for 5 rats. †$P < 0.05$ *vs.* Veh + Veh; *$P < 0.05$ *vs.* Veh + carrageenan.

mitochondrial dysfunction has been considered to be an important component in inflammatory pain [66,67]. In particular, proper mitochondrial function requires careful regulation of multiple enzymes [68]. SIRT3 is localized to the inner mitochondrial membrane and it is implicated in metabolic regulation of physiopathological processes, such as inflammation, metabolism and cell cycle regulation and it also seems to be involved in the epigenetic regulation [69]; its activity appears to be regulated by oxidative stress agents, and it is responsive to aberrant or increased mitochondrial levels of SO [70]. Within the antioxidant defense system [36], SIRT3 is a main regulator of cell defense and survival in response to oxidative stresses induced by various injuries [39]. As an experimental corollary, SIRT3 signaling down-regulation leads to mitochondrial antioxidant enzyme dysfunction and thus increased oxidative stress [36]. In this regard, SIRT3 can be considered as a crucial mitochondrial fidelity protein able to resist oxidative stresses, counteract oxidative stresses [29,70,71], and protect cells against ROS. However, the role of SIRT3 in modulating inflammatory pain has not yet been investigated.

As shown in numerous studies, inflammatory hyperalgesia evoked by carrageenan induces, also at the spinal cord level, various ROS species formation, presumably in response to glutamate, which play a major role in the development of pain through central sensitization [3,6–8].

Our results strongly suggest that 4-HNE formation is a key element in the generation of inflammatory hyperalgesia. In an animal model of inflammatory pain we have demonstrated an increase of 4-HNE along with the oxidative stress marker MDA and nitrotyrosine level in the spinal cord [11,13,19]. The enhanced presence of 4- HNE could be responsible for the carbonylation and inactivation of SIRT3 [24,72,73] that in turn lead to mitochondrial hyperacetylation and MnSOD inactivation.

These effects were significantly attenuated by a single prior antioxidant treatment with MnTBAP and resveratrol, showing the pivotal role of free radicals as mediators of hyperalgesia and inflammation.

To confirm the direct link between SIRT3 and oxidative stress, we used a validate cell line in which SIRT3 was either overexpressed or knockdown. The SIRT3-knockdown cells showed reduced basal or carrageenan-induced SIRT3 activity compared with wild type cells and this reduction is followed by a SIRT3-dependent MnSOD inactivation. To confirm the relationship between oxidative stress and SIRT3, we analyzed the levels of MnSOD acetylation in SIRT3-overexpressing cells that were treated with 4-HNE. We observed a significant increase of MnSOD acetylation followed by a MnSOD inactivation after treatment with 4-HNE, and this protein alteration was prevented or greatly reduced by pre-treatment with MnTBAP or resveratrol.

Our findings demonstrate, for the first time, deactivation of sirtuins is involved in the development of hyperalgesia, and SIRT3 protection by antioxidants is beneficial during oxidative stress-induced hyperalgesia and inflammation. Notably, SIRT3 appears to be the major deacetylase in the mitochondria [74,75], but until now little was known about the mechanism of its regulation during nociception. Collectively, our results indicate that the effects of MnSOD deacetylation on the suppression of inflammation and inflammatory pain are canonically mediated by the SIRT3/MnSOD pathway.

Previous studies demonstrated that SIRT3 down-regulation resulted in reduced poly (ADP-ribose) polymerase (PARP) activity. SIRT3 and PARP use the same cofactor Nicotinamide Adenine Dinucleotide (NAD^+) and PARP hyperactivity also causes chronic inflammation by

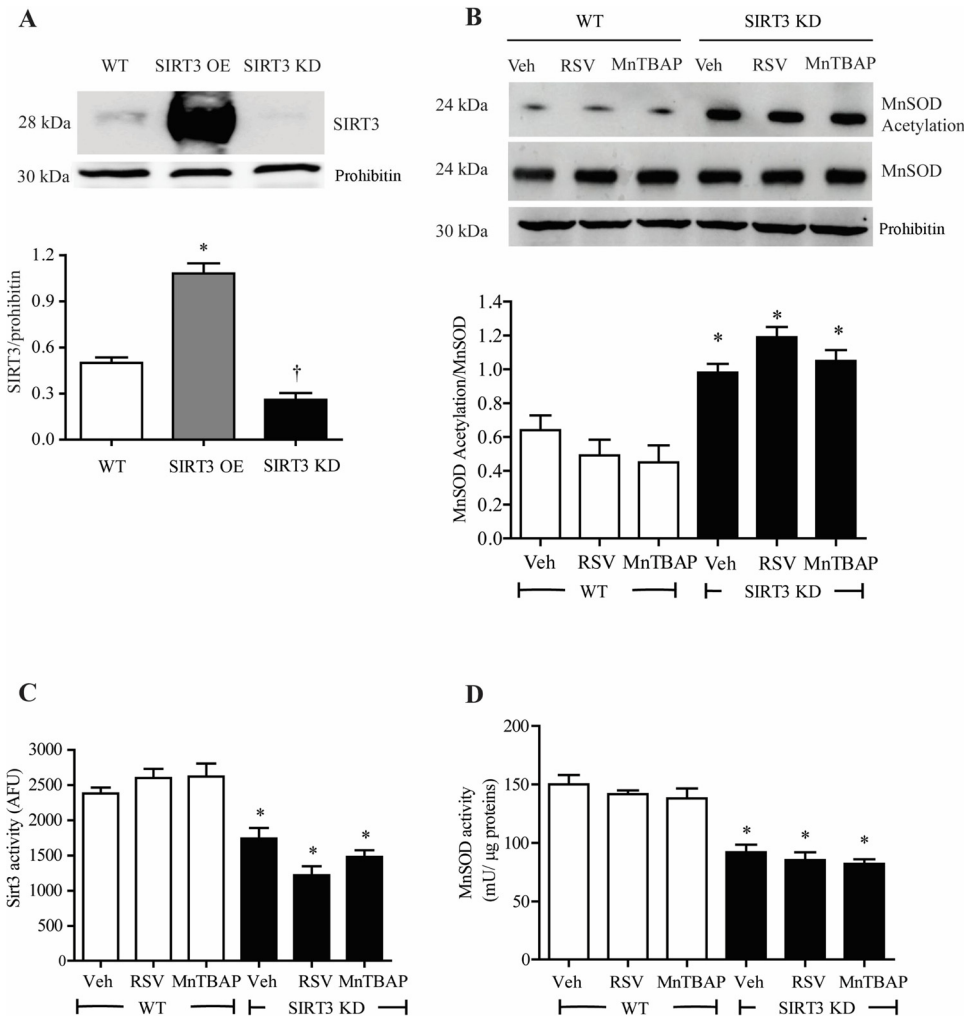


Fig. 5. MnSOD acetylation depends upon SIRT3 activity. (A) To confirm SIRT3 values in over-expressed and knockdown cells, MDA-MB-231 cell extracts were blotted with anti-SIRT3 antibodies. (B) SIRT3-knockdown cells induced mitochondrial MnSOD acetylation when compared with WT cells. No differences for either MnTBAP (50 µM) or RSV (50 µM) expression were detected among the lanes in these conditions. (C) The increase of MnSOD acetylation, and then its inactivation (D), in SIRT3-knockdown cells was closely linked to decreased SIRT3 activity.

depleting NAD^+, which subsequently decreases endogenous SIRT activity [76]. In addition, mitochondrial Ca^{2+} influx has a crucial role in the down regulation of the NAD^+/NADH ratio and in the NAD^+-dependent deacetylase activity of SIRT3 to inhibit MnSOD activity. Thus, increasing the production of mitochondrial ROS [77,78].

These interactions could create both an amplified auto-feedback loop that regulates SIRT3 activity and a positive feedback signal that further enhances oxidative damage and inflammatory processes. Therefore, it can be deduced that both natural or synthetic antioxidants could inhibit inflammatory pain by maintaining SIRT3 activity and thereby limit endogenous ROS production.

5. Conclusion

In conclusion, our data identify mitochondrial dysfunction as crucial player in the pathogenesis of inflammatory pain.

We demonstrate, for the first time, that SIRT3 carbonylation contributes to spinal MnSOD inactivation during carrageenan-induced thermal hyperalgesia in rats. Moreover, inhibiting ROS with antioxidants prevented SIRT3 carbonylation, restored the enzymatic activity of MnSOD and blocked the development of pain. These results suggest that therapeutic strategies aimed at inhibiting post-translational modifications of SIRT3 may provide beneficial outcomes in pain states where ROS have been documented to play an important role in the development of central sensitization. Restoring mitochondrial sirtuins pathway may also represent an innovative approach for therapeutic intervention in specific patient populations in rehabilitation treatment who present with inflammatory pain.

Funding

This study was funded by grants from PON03PE_00078_1, PON03PE_00078_2 and sponsored by IBSA Foundation and ISAL Foundation. This study is also supported by the Italian Ministry of Health [ricerca corrente].

Declaration of Competing Interest

All authors declare no conflict of interest.

Acknowledgments

We would like to thank Dr. A. J. Lechner (Department of Pharmacology and Physiology, Saint Louis University, Saint Louis, MO) and Alexandra Patterson (University of South Carolina College of Engineering and Computing, Columbia, SC) for their valuable input and careful editorial review of our work.

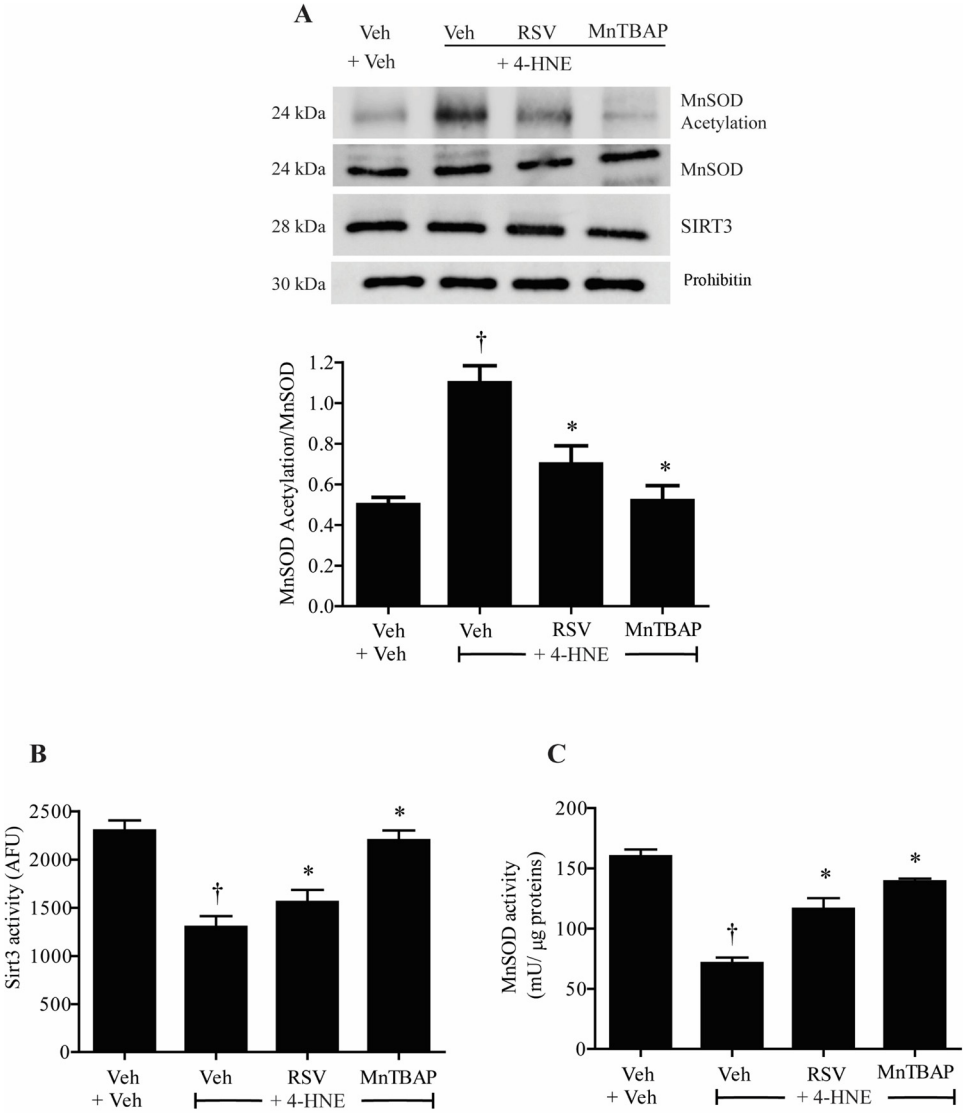


Fig. 6. Oxidative stress affects SIRT3 activity. SIRT3 OE cells treated with 4-HNE showed an increase in MnSOD acetylation and inactivation levels (A, C) and a decrease in SIRT3 activity (B). Pre-treatment with the antioxidants MnTBAP (50 µM) or resveratrol (50 µM), maintaining the activity of SIRT3, prevented MnSOD acetylation (A) and its inactivation (C). No differences for prohibitin, MnSOD, and SIRT3 expression were detected among the lanes in these conditions. Gels are representative of results from 6 different experiments and the histogram (A) represents densitometric analysis of MnSOD acetylation/MnSOD ratio for the gels shown. Results are expressed as means ± SEM. $^{\dagger}P < 0.05$ *vs.* Veh + Veh; *P < 0.05 *vs.* Veh + 4-HNE. SIRT3 OE, SIRT3-overexpressed cells; WT, SIRT3-Wild Type cells; AFU, arbitrary fluorescence unit.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2020.104851.

References

[1] J.M. Zhang, J. An, Cytokines, inflammation, and pain, Int. Anesthesiol. Clin. 45 (2) (2007) 27–37.

[2] D. Salvemini, J.W. Little, T. Doyle, W.L. Neumann, Roles of reactive oxygen and nitrogen species in pain, Free Radic. Biol. Med. 51 (5) (2011) 951–966.

[3] D. Salvemini, W. Neumann, Targeting peroxynitrite driven nitroxidative stress with synzymes: a novel therapeutic approach in chronic pain management, Life Sci. 86 (15–16) (2010) 604–614.

[4] P.M. Grace, A.D. Gaudet, V. Staikopoulos, S.F. Maier, M.R. Hutchinson, D. Salvemini, L.R. Watkins, Nitroxidative signaling mechanisms in pathological pain, Trends Neurosci. 39 (12) (2016) 862–879.

[5] J.W. Little, T. Doyle, D. Salvemini, Reactive nitroxidative species and nociceptive processing: determining the roles for nitric oxide, superoxide, and peroxynitrite in pain, Amino Acids 42 (1) (2012) 75–94.

[6] Z.Q. Wang, F. Porreca, S. Cuzzocrea, K. Galen, R. Lightfoot, E. Masini, C. Muscoli, V. Mollace, M. Ndengele, H. Ischiropoulos, D. Salvemini, A newly identified role for superoxide in inflammatory pain, J. Pharmacol. Exp. Ther. 309 (3) (2004) 869–878.

[7] T. Doyle, Z. Chen, C. Muscoli, L. Bryant, E. Esposito, S. Cuzzocrea, C. Dagostino, J. Ryerse, S. Rausaria, A. Kamadulski, W.L. Neumann, D. Salvemini, Targeting the overproduction of peroxynitrite for the prevention and reversal of paclitaxel-induced neuropathic pain, J. Neurosci. 32 (18) (2012) 6149–6160.

[8] J. Yowtak, K.Y. Lee, H.Y. Kim, J. Wang, H.K. Kim, K. Chung, J.M. Chung, Reactive oxygen species contribute to neuropathic pain by reducing spinal GABA release, Pain 152 (4) (2011) 844–852.

[9] M.M. Khattab, TEMPOL, a membrane-permeable radical scavenger, attenuates peroxynitrite- and superoxide anion-enhanced carrageenan-induced paw edema and hyperalgesia: a key role for superoxide anion, Eur. J. Pharmacol. 548 (1–3) (2006) 167–173.

[10] Z. Chen, C. Muscoli, T. Doyle, L. Bryant, S. Cuzzocrea, V. Mollace, R. Mastroianni, E. Masini, D. Salvemini, NMDA-receptor activation and nitroxidative regulation of the glutamatergic pathway during nociceptive processing, Pain 149 (1) (2010) 100–106.

[11] C. Muscoli, C. Dagostino, S. Ilari, F. Lauro, M. Gliozzi, E. Bardhi, E. Palma, V. Mollace, D. Salvemini, Posttranslational nitration of tyrosine residues modulates glutamate transmission and contributes to N-methyl-D-aspartate-mediated thermal hyperalgesia, Mediators Inflamm. 2013 (2013) 950947.

[12] N.A. Duggett, L.A. Griffiths, O.E. McKenna, V. de Santis, N. Yongsanguanchai, E.B. Mokori, S.J. Flatters, Oxidative stress in the development, maintenance and resolution of paclitaxel-induced painful neuropathy, Neuroscience 333 (2016) 13–26.

[13] F. Lauro, L.A. Giancotti, S. Ilari, C. Dagostino, M. Gliozzi, C. Morabito, V. Malafoglia, W. Raffaeli, M. Muraca, B.M. Goffredo, V. Mollace, C. Muscoli, Inhibition of spinal oxidative stress by bergamot polyphenolic fraction attenuates the development of morphine induced tolerance and hyperalgesia in mice, PLoS One 11 (5) (2016) e0156039.

[14] B. Alvarez, G. Ferrer-Sueta, B.A. Freeman, R. Radi, Kinetics of peroxynitrite reaction with amino acids and human serum albumin, J. Biol. Chem. 274 (2) (1999) 842–848.

[15] B. Alvarez, H. Rubbo, M. Kirk, S. Barnes, B.A. Freeman, R. Radi, Peroxynitrite-dependent tryptophan nitration, Chem. Res. Toxicol. 9 (2) (1996) 390–396.

[16] V. Mollace, C. Muscoli, C. Dagostino, L.A. Giancotti, M. Gliozzi, I. Sacco, V. Visalli, S. Gratteri, E. Palma, N. Malara, V. Musolino, C. Carresi, S. Muscoli, C. Vitale, D. Salvemini, F. Romeo, The effect of peroxynitrite decomposition catalyst MnTBAP on aldehyde dehydrogenase-2 nitration by organic nitrates: role in nitrate tolerance,

Pharmacol. Res. 89 (2014) 29–35.
[17] D. Salvemini, T.M. Doyle, S. Cuzzocrea, Superoxide, peroxynitrite and oxidative/nitrative stress in inflammation, Biochem. Soc. Trans. 34 (Pt 5) (2006) 965–970.
[18] C. Muscoli, V. Mollace, J. Wheatley, E. Masini, M. Ndengele, Z.Q. Wang, D. Salvemini, Superoxide-mediated nitration of spinal manganese superoxide dismutase: a novel pathway in N-methyl-D-aspartate-mediated hyperalgesia, Pain 111 (1-2) (2004) 96–103.
[19] F. Lauro, S. Ilari, L.A. Giancotti, C.A. Ventura, C. Morabito, M. Gliozzi, V. Malafoglia, E. Palma, D. Paolino, V. Mollace, C. Muscoli, Pharmacological effect of a new idebenone formulation in a model of carrageenan-induced inflammatory pain, Pharmacol. Res. 111 (2016) 767–773.
[20] O. Ozden, S.H. Park, H.S. Kim, H. Jiang, M.C. Coleman, D.R. Spitz, D. Gius, Acetylation of MnSOD directs enzymatic activity responding to cellular nutrient status or oxidative stress, Aging (Albany NY) 3 (2) (2011) 102–107.
[21] D. Salvemini, D.P. Riley, S. Cuzzocrea, SOD mimetics are coming of age, Nat. Rev. Drug Discov. 1 (5) (2002) 367–374.
[22] A. Ayala, M.F. Munoz, S. Arguelles, Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal, Oxid. Med. Cell. Longev. 2014 (2014) 360438.
[23] G. Barrera, S. Pizzimenti, E.S. Ciamporcero, M. Daga, C. Ullio, A. Arcaro, G.P. Cetrangolo, C. Ferretti, C. Dianzani, A. Lepore, F. Gentile, Role of 4-hydroxynonenal-protein adducts in human diseases, Antioxid. Redox Signal. 22 (18) (2015) 1681–1702.
[24] K.S. Fritz, J.J. Galligan, R.L. Smathers, J.R. Roede, C.T. Shearn, P. Reigan, D.R. Petersen, 4-Hydroxynonenal inhibits SIRT3 via thiol-specific modification, Chem. Res. Toxicol. 24 (5) (2011) 651–662.
[25] M. Fedorova, R.C. Bollineni, R. Hoffmann, Protein carbonylation as a major hallmark of oxidative damage: update of analytical strategies, Mass Spectrom. Rev. 33 (2) (2014) 79–97.
[26] V. Calabrese, C. Cornelius, A.T. Dinkova-Kostova, I. Iavicoli, R. Di Paola, A. Koverech, S. Cuzzocrea, E. Rizzarelli, E.J. Calabrese, Cellular stress responses, hormetic phytochemicals and vitagenes in aging and longevity, Biochim. Biophys. Acta 1822 (5) (2012) 753–783.
[27] G. Marfe, M. Tafani, B. Pucci, C. Di Stefano, M. Indelicato, A. Andreoli, M.A. Russo, P. Sinibaldi-Salimei, V. Manzi, The effect of marathon on mRNA expression of anti-apoptotic and pro-apoptotic proteins and sirtuins family in male recreational long-distance runners, BMC Physiol. 10 (2010) 7.
[28] V. Carafa, A. Nebbioso, L. Altucci, Sirtuins and disease: the road ahead, Front. Pharmacol. 3 (2012) 4.
[29] L. Pellegrini, B. Pucci, L. Villanova, M.L. Marino, G. Marfe, L. Sansone, E. Vernucci, D. Bellizzi, V. Reali, M. Fini, M.A. Russo, M. Tafani, SIRT3 protects from hypoxia and staurosporine-mediated cell death by maintaining mitochondrial membrane potential and intracellular pH, Cell Death Differ. 19 (11) (2012) 1815–1825.
[30] T. Yoshizaki, S. Schenk, T. Imamura, J.L. Babendure, N. Sonoda, E.J. Bae, D.Y. Oh, M. Lu, J.C. Milne, C. Westphal, G. Bandyopadhyay, J.M. Olefsky, SIRT1 inhibits inflammatory pathways in macrophages and modulates insulin sensitivity, Am. J. Physiol. Endocrinol. Metab. 298 (3) (2010) E419–28.
[31] B. Kincaid, E. Bossy-Wetzel, Forever young: SIRT3 a shield against mitochondrial meltdown, aging, and neurodegeneration, Front. Aging Neurosci. 5 (2013) 48.
[32] A. Cheng, Y. Yang, Y. Zhou, C. Maharana, D. Lu, W. Peng, Y. Liu, R. Wan, K. Marosi, M. Misiak, V.A. Bohr, M.P. Mattson, Mitochondrial SIRT3 mediates adaptive responses of neurons to exercise and metabolic and excitatory challenges, Cell Metab. 23 (1) (2016) 128–142.
[33] X. Qiu, K. Brown, M.D. Hirschey, E. Verdin, D. Chen, Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation, Cell Metab. 12 (6) (2010) 662–667.
[34] R. Tao, M.C. Coleman, J.D. Pennington, O. Ozden, S.H. Park, H. Jiang, H.S. Kim, C.R. Flynn, S. Hill, W. Hayes McDonald, A.K. Olivier, D.R. Spitz, D. Gius, Sirt3-mediated deacetylation of evolutionarily conserved lysine 122 regulates MnSOD activity in response to stress, Mol. Cell 40 (6) (2010) 893–904.
[35] K.M. Jacobs, J.D. Pennington, K.S. Bisht, N. Aykin-Burns, H.S. Kim, M. Mishra, L. Sun, P. Nguyen, B.H. Ahn, J. Leclerc, C.X. Deng, D.R. Spitz, D. Gius, SIRT3 interacts with the daf-16 homolog FOXO3a in the mitochondria, as well as increases FOXO3a dependent gene expression, Int. J. Biol. Sci. 4 (5) (2008) 291–299.
[36] S.P. Yoon, J. Kim, Poly(ADP-ribose) polymerase 1 contributes to oxidative stress through downregulation of sirtuin 3 during cisplatin nephrotoxicity, Anat. Cell Biol. 49 (3) (2016) 165–176.
[37] L.O. Klotz, C. Sanchez-Ramos, I. Prieto-Arroyo, P. Urbanek, H. Steinbrenner, M. Monsalve, Redox regulation of FoxO transcription factors, Redox Biol. 6 (2015) 51–72.
[38] G.J. Kops, T.B. Dansen, P.E. Polderman, I. Saarloos, K.W. Wirtz, P.J. Coffer, T.T. Huang, J.L. Bos, R.H. Medema, B.M. Burgering, Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress, Nature 419 (6904) (2002) 316–321.
[39] A.H. Tseng, S.S. Shieh, D.L. Wang, SIRT3 deacetylates FOXO3 to protect mitochondria against oxidative damage, Free Radic. Biol. Med. 63 (2013) 222–234.
[40] A.K. Singh, M. Vinayak, Resveratrol alleviates inflammatory hyperalgesia by modulation of reactive oxygen species (ROS), antioxidant enzymes and ERK activation, Inflamm. Res. 66 (10) (2017) 911–921.
[41] N. Singh, M. Agrawal, S. Dore, Neuroprotective properties and mechanisms of resveratrol in in vitro and in vivo experimental cerebral stroke models, ACS Chem. Neurosci. 4 (8) (2013) 1151–1162.
[42] I.C. Arts, P.C. Hollman, Polyphenols and disease risk in epidemiologic studies, Am. J. Clin. Nutr. 81 (1 Suppl) (2005) 317S–325S.
[43] E. Middleton Jr., C. Kandaswami, T.C. Theoharides, The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer, Pharmacol. Rev. 52 (4) (2000) 673–751.
[44] H. Shao, Q. Xue, F. Zhang, Y. Luo, H. Zhu, X. Zhang, H. Zhang, W. Ding, B. Yu, Spinal SIRT1 activation attenuates neuropathic pain in mice, PLoS One 9 (6) (2014) e100938.
[45] B.J. Day, I. Batinic-Haberle, J.D. Crapo, Metalloporphyrins are potent inhibitors of lipid peroxidation, Free Radic. Biol. Med. 26 (5–6) (1999) 730–736.
[46] B. Zingarelli, B.J. Day, J.D. Crapo, A.L. Salzman, C. Szabo, The potential role of peroxynitrite in the vascular contractile and cellular energetic failure in endotoxic shock, Br. J. Pharmacol. 120 (2) (1997) 259–267.
[47] K. Hargreaves, R. Dubner, F. Brown, C. Flores, J. Joris, A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia, Pain 32 (1) (1988) 77–88.
[48] E.M. Rock, C.L. Limebeer, L.A. Parker, Effect of cannabidiolic acid and (9)-tetrahydrocannabinol on carrageenan-induced hyperalgesia and edema in a rodent model of inflammatory pain, Psychopharmacology (Berl) 235 (11) (2018) 3259–3271.
[49] T. Mert, E. Sahin, S. Yaman, M. Sahin, Pain-relieving effectiveness of Co-treatment with local tramadol and systemic minocycline in carrageenan-induced inflammatory pain model, Inflammation 41 (4) (2018) 1238–1249.
[50] S. Nistico, D. Ventrice, C. Dagostino, F. Lauro, S. Ilari, M. Gliozzi, C. Colica, V. Musolino, C. Carresi, M.C. Strongoli, I. Vecchio, M. Rizzo, V. Mollace, C. Muscoli, Effect of MN (III) tetrakis (4-benzoic acid) porphyrin by photodynamically generated free radicals on SODs keratinocytes, J. Biol. Regul. Homeost. Agents 27 (3) (2013) 781–790.
[51] A.Y. Berman, R.A. Motechin, M.Y. Wiesenfeld, M.K. Holz, The therapeutic potential of resveratrol: a review of clinical trials, NPJ Precis. Oncol. 1 (2017).
[52] D. Salvemini, Z.Q. Wang, P.S. Wyatt, D.M. Bourdon, M.H. Marino, P.T. Manning, M.G. Currie, Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation, Br. J. Pharmacol. 118 (4) (1996) 829–838.
[53] S.O. Abarikwu, A.B. Pant, E.O. Farombi, 4-Hydroxynonenal induces mitochondrial-mediated apoptosis and oxidative stress in SH-SY5Y human neuronal cells, Basic Clin. Pharmacol. Toxicol. 110 (5) (2012) 441–448.
[54] Q. Zhou, M. Einert, H. Schmitt, Z. Wang, F. Pankratz, C.B. Olivier, C. Bode, J.K. Liao, M. Moser, MnTBAP increases BMPR-II expression in endothelial cells and attenuates vascular inflammation, Vascul. Pharmacol. 84 (2016) 67–73.
[55] N.N. Xiao, Effects of resveratrol supplementation on oxidative damage and lipid peroxidation induced by strenuous exercise in rats, Biomol. Ther. (Seoul) 23 (4) (2015) 374–378.
[56] C. Beauchamp, I. Fridovich, Superoxide dismutase: improved assays and an assay applicable to acrylamide gels, Anal. Biochem. 44 (1) (1971) 276–287.
[57] S. Nishida, K. Teramoto, S. Kimoto-Kinoshita, Y. Tohda, S. Nakajima, T.T. Tomura, K. Irimajiri, Change of Cu,Zn-superoxide dismutase activity of guinea pig lung in experimental asthma, Free Radic. Res. 36 (6) (2002) 601–606.
[58] E.S. Schwartz, H.Y. Kim, J. Wang, I. Lee, E. Klann, J.M. Chung, K. Chung, Persistent pain is dependent on spinal mitochondrial antioxidant levels, J. Neurosci. 29 (1) (2009) 159–168.
[59] O.Y. Kim, J.Y. Chung, J. Song, Effect of resveratrol on adipokines and myokines involved in fat browning: perspectives in healthy weight against obesity, Pharmacol. Res. 148 (2019) 104411.
[60] W.S. Cheang, W.T. Wong, L. Wang, C.K. Cheng, C.W. Lau, R.C.W. Ma, A. Xu, N. Wang, Y. Huang, X.Y. Tian, Resveratrol ameliorates endothelial dysfunction in diabetic and obese mice through sirtuin 1 and peroxisome proliferator-activated receptor delta, Pharmacol. Res. 139 (2019) 384–394.
[61] H. Esterbauer, R.J. Schaur, H. Zollner, Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes, Free Radic. Biol. Med. 11 (1) (1991) 81–128.
[62] K. Uchida, 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress, Prog. Lipid Res. 42 (4) (2003) 318–343.
[63] W. He, J.C. Newman, M.Z. Wang, L. Ho, E. Verdin, Mitochondrial sirtuins: regulators of protein acylation and metabolism, Trends Endocrinol. Metab. 23 (9) (2012) 467–476.
[64] Y. Zhu, S.H. Park, O. Ozden, H.S. Kim, H. Jiang, A. Vassilopoulos, D.R. Spitz, D. Gius, Exploring the electrostatic repulsion model in the role of Sirt3 in directing MnSOD acetylation status and enzymatic activity, Free Radic. Biol. Med. 53 (4) (2012) 828–833.
[65] C. Quijano, M. Trujillo, L. Castro, A. Trostchansky, Interplay between oxidant species and energy metabolism, Redox Biol. 8 (2016) 28–42.
[66] B.D. Sui, T.Q. Xu, J.W. Liu, W. Wei, C.X. Zheng, B.L. Guo, Y.Y. Wang, Y.L. Yang, Understanding the role of mitochondria in the pathogenesis of chronic pain, Postgrad. Med. J. 89 (1058) (2013) 709–714.
[67] Z. Yan, S. Zhu, H. Wang, L. Wang, T. Du, Z. Ye, D. Zhai, Z. Zhu, X. Tian, Z. Lu, X. Cao, MOTS-c inhibits Osteolysis in the Mouse Calvaria by affecting osteocyte-osteoclast crosstalk and inhibiting inflammation, Pharmacol. Res. 147 (2019) 104381.
[68] J. Skoda, K. Borankova, P.J. Jansson, M.L. Huang, R. Veselska, D.R. Richardson, Pharmacological targeting of mitochondria in cancer stem cells: an ancient organelle at the crossroad of novel anti-cancer therapies, Pharmacol. Res. 139 (2019) 298–313.
[69] H. Jing, H. Lin, Sirtuins in epigenetic regulation, Chem. Rev. 115 (6) (2015) 2350–2375.
[70] S.H. Park, O. Ozden, H. Jiang, Y.I. Cha, J.D. Pennington, N. Aykin-Burns, D.R. Spitz, D. Gius, H.S. Kim, Sirt3, mitochondrial ROS, ageing, and carcinogenesis, Int. J. Mol. Sci. 12 (9) (2011) 6226–6239.
[71] D. Candas, J.J. Li, MnSOD in oxidative stress response-potential regulation via mitochondrial protein influx, Antioxid. Redox Signal. 20 (10) (2014) 1599–1617.
[72] H. Zhong, H. Yin, Role of lipid peroxidation derived 4-hydroxynonenal (4-HNE) in

cancer: focusing on mitochondria, Redox Biol. 4 (2015) 193–199.
[73] N.P. Visavadiya, S.P. Patel, J.L. VanRooyen, P.G. Sullivan, A.G. Rabchevsky, Cellular and subcellular oxidative stress parameters following severe spinal cord injury, Redox Biol. 8 (2016) 59–67.
[74] H.J. Weir, J.D. Lane, N. Balthasar, SIRT3: a central regulator of mitochondrial adaptation in health and disease, Genes Cancer 4 (3–4) (2013) 118–124.
[75] Y. Chen, L.L. Fu, X. Wen, X.Y. Wang, J. Liu, Y. Cheng, J. Huang, Sirtuin-3 (SIRT3), a therapeutic target with oncogenic and tumor-suppressive function in cancer, Cell Death Dis. 5 (2014) e1047.
[76] J. Gao, Z. Zheng, Q. Gu, X. Chen, X. Liu, X. Xu, Deacetylation of MnSOD by PARP-regulated SIRT3 protects retinal capillary endothelial cells from hyperglycemia-induced damage, Biochem. Biophys. Res. Commun. 472 (3) (2016) 425–431.
[77] T. Ren, H. Zhang, J. Wang, J. Zhu, M. Jin, Y. Wu, X. Guo, L. Ji, Q. Huang, H. Zhang, H. Yang, J. Xing, MCU-dependent mitochondrial Ca(2+) inhibits NAD(+)/SIRT3/SOD2 pathway to promote ROS production and metastasis of HCC cells, Oncogene 36 (42) (2017) 5897–5909.
[78] C. Echem, T.J.D. Costa, V. Oliveira, L. Giglio Colli, M.A. Landgraf, S.F. Rodrigues, M. Franco, R.G. Landgraf, R.A. Santos-Eichler, G.F. Bomfim, E.H. Akamine, M.H.C. de Carvalho, Mitochondrial DNA: a new driver for sex differences in spontaneous hypertension, Pharmacol. Res. 144 (2019) 142–150.