1 **Full Title**

2 Microtubule Defect Involved in 'Mitophagy Resistance' Under Subacute
3 Oxidative Stress - Potential Mechanism for Cellular Inflammation

4

5 **Short Title**

6 Microtubule Defect Involved in 'Mitophagy Resistance' Under Subacute
7 Oxidative Stress

8

9 **Author names and affiliations**

10 Takahiko Tamura^{1,2}, M.D., Ph.D. Nobuo Yasuda¹, Ph.D. Tomoharu Shakuo¹,
11 M.D., Ph.D. Aki Kashiwagi¹, M.D., Ph.D. Jeevendra A, J, Martyn¹, M.D., Ph.D.
12 Masataka Yokoyama², M.D., Ph.D. Shingo Yasuhara¹ M.D., Ph.D.

13

14 1. Department of Anesthesia, Critical Care and Pain Medicine
15 Shriners Hospitals for Children Boston, Massachusetts General Hospital and
16 Harvard Medical School. 51 Blossom Street, Boston, MA 02114, USA
17 2. Department of Anesthesiology and Intensive Care Medicine
18 Kochi Medical School. Oko-cho Kohasu, Nankoku, Kochi, 7838508, Japan

19

20 **Corresponding Author**

21 Shingo Yasuhara

22 51 Blossom Street, Boston, MA 02114

23 Phone number: (617)314-5109

24 Email address: syasuhara@mgh.harvard.edu

25

26 **Author contributions**

27 Takahiko Tamura: Data Curation, Formal Analysis, Investigation, Methodology,

28 Visualization, Writing-Original Draft Preparation

29 Nobuo Yasuda: Data Curation, Formal Analysis, Investigation, Methodology,

30 Visualization

31 Tomoharu Shakuo: Data Curation, Investigation, Methodology

32 Aki Kashiwagi: Data Curation, Investigation, Methodology

33 Jeevendra A, J, Martyn: Finding Acquisition, Project Administration, Resources,

34 Supervision, Writing-Review & Editing

35 Masataka Yokoyama: Finding Acquisition, Project Administration, Supervision

- 36 Shingo Yasuhara: Conceptualization, Finding Acquisition, Methodology, Project
- 37 Administration, Resources, Software, Supervision, Writing-Review & Editing

38 [Abstract]

39 **Introduction:** Oxidative stress is considered an essential mechanism in
40 ICU-acquired weakness. The roles of oxidative stress in autophagy/mitophagy
41 dysfunction remains elusive. Microtubule serves as an essential guide rail for
42 auto/mitophagosome trafficking required for proper maturation of
43 auto/mitophagosomes in normal circumstances, and microtubules network
44 formation is regulated by signal transduction mechanisms involving Akt, GSK3 β ,
45 and the microtubule plus-end tracking molecule, EB1. We have investigated (1)
46 whether oxidative stress affects this pathway, leading to the defective mitophagy
47 response, and (2) whether trehalose, an auto/mitophagy modulator, can
48 ameliorate these pathological conditions.

49 **Methods:** By stably transfecting markers for auto/mitophagy or MT synthesis,
50 we have established a few new C2C12 myocyte cell lines, expressing, GFP-LC3,
51 EB1-GFP, and/or tandem-fluorescence LC3 (tfLC3). To monitor microtubule
52 network, the cells were stained by SiR-tubulin. The cells were cultured in the
53 presence or absence of oxidative stress by hydrogen peroxide (H₂O₂) and
54 treated with or without trehalose. The response of mitophagy parameters
55 including vesicle motion and the maturation status was monitored by stimulating

the cells with carbonyl cyanide m-chlorophenyl hydrazone (CCCP), an established mitophagy inducer, under a time-lapse confocal microscopy. Signal transduction mechanisms linking mitophagy to microtubule formation was analyzed by Western Blotting against Akt and GSK3 β .

Results: Cells under the oxidative stress, showed abolished MT network formation, decreased microtubule synthesis by EB1, and a decrease in CCCP-invoked response of mitophagosome motion, perturbed mitophagosome maturation, and increased superoxide production. Signal resistance of Akt/GSK3 β pathway to mitophagic stimulation, was documented. Trehalose treatment reversed signal resistance, diminished MT synthesis, ameliorated the disturbed MT network, and improved maturation defects, suppressing the production of superoxide.

Conclusions: Oxidative stress decreases the response of mitophagy and abolishes microtubule network. Trehalose improves the synthetic ability of microtubule and normalized the disturbed microtubule network, resulting in the improvement of the perturbed mitophagosomes maturation under the oxidative stress.

74 **[Keywords]**

75 Muscle weakness, ICU-acquired weakness, Autophagy, Mitophagy, Oxidative
76 stress, Microtubule, Akt, GSK3 β , Trehalose, Mitochondria

77 [Introduction]

78 Muscle wasting and muscle weakness is one of the major complications among
79 many types of critical illnesses including sepsis, burn, and major trauma [1]. In
80 critical illnesses, muscle wasting occurs mostly in chronic phase. More recently,
81 however, ICU- acquired weakness (ICU-AW), which occurs rapidly in the
82 critically ill patients, has acquired both scientific and clinical attention in the field
83 of critical illness studies [2] and the onset of muscle wasting/dysfunction is
84 regarded to develop at relatively earlier stage of the disease than previously
85 considered. When the disease persists, the muscle wasting and weakness
86 arising from these illnesses lead to prolonged mechanical ventilation [3], with
87 increased morbidity and mortality [4]. Oxidative stress and mitochondria
88 dysfunctions are considered one of the key mechanisms in sepsis or burn
89 injury-induced organ dysfunctions, and in ICU-AW. [5,6]

90 Mitochondrial dysfunction has been reported to occur concomitantly in
91 many forms of muscle dysfunctions in critical illnesses. Perturbation of
92 mitochondrial network integrity and their functions leads to systemic catabolism,
93 affecting adenosine triphosphate (ATP) production, decreased organelle
94 biogenesis, elevated proteolysis and often resulting in the loss of muscle size [2].

In addition to their roles in bioenergetic homeostasis, mitochondria are involved in various signal transduction. Dysregulation of these signals lead to cellular malfunction directly or indirectly. Damaged mitochondria release catastrophic mediators including reactive oxygen species (ROS), cytochrome-c, which can initiate the process of cell death and induce proteolysis [7]. Mitochondrial quality control is therefore essential not only for energy homeostasis but also in the determination of cell fate.

Autophagy is a cellular housekeeping system that mediates either bulk removal of cellular components or selective degradation of damaged organelles and protein aggregates [8]. Its defect or derangement in signals is associated with pathogenesis of various diseases such as cancer, neurodegeneration, metabolic diseases and multiple organ failure of critically illness [9]. Autophagy plays an essential role in the skeletal muscle system, maintaining muscle fiber integrity [10]. Dysregulation of autophagy [5,11] has been associated with the development of ICU-AW, or muscle wasting syndrome in critical illnesses, but the precise pathophysiological mechanism has remained elusive. Particularly, damaged mitochondria are removed by a form of selective autophagic degradation, or mitophagy. Mitophagy thus plays essential roles of cellular and

113 mitochondrial homeostasis, but the causative relationship with muscle wasting
114 and mitophagy has not been fully clarified in critical illness-induced muscle
115 dysfunctions or ICU-AW despite the reports of mitochondrial dysfunctions. [12]

116 The relationship between oxidative stress and auto/mitophagy has been
117 studied intensively mostly with the conclusion that oxidative stress, mediated by
118 ROS, activates auto/mitophagy [13,14]. Given that auto/mitophagy are cell
119 protective systems acquired by eukaryotes during evolution [8,15], findings
120 about ROS activating auto/mitophagy is rational. The cells need to adapt to
121 acute stress by reinforcing auto/mitophagy. In this context, however, the effect of
122 oxidative stress on the basal level of, (but not the responsiveness or
123 competency of), auto/mitophagy, has been the focus of study [16]. The effect of
124 prolonged oxidative stress on the 'response', or competency, of auto/mitophagy
125 has not been investigated in detail. Clinically, it has been recognized that
126 competency of various organs for stress adaptation is diminished in many
127 chronic inflammatory diseases or in critical illnesses. [17,18] In this setting, it can
128 be advocated that examining the competency of, but not just the basal level of,
129 auto/mitophagy, is critical to fully understand the pathological symptoms of many
130 diseases. This study, therefore, focused on the analyses of the capability of cells

131 for turning mitophagy flux, in response to mitophagy-inducing stimulation and
 132 investigated how oxidative stress affects this response. Herein, by focusing on
 133 the response of mitophagy, a new disease entity of compromised response to
 134 mitophagy-inducing stimuli ('resistance to mitophagic stimuli', or operationally
 135 simplified as 'mitophagy resistance') will be discussed. Similar to insulin
 136 resistance in type 2 diabetes mellitus (T2DM) or in chronic illnesses, we have
 137 noted there is a pathological condition where signal response for mitophagy flux
 138 turnover is defective, thus yielding poor response to mitophagy-invoking stimuli
 139 and thus resulting in decompensated quality control of mitochondria and in
 140 diminished safety margin in the homeostasis of mitochondria. [19,20]

141 Furthermore, despite the intensive studies about the relationship
 142 between ROS and auto/mitophagy, the molecular target of ROS in
 143 auto/mitophagy pathway remained somewhat elusive [16]. Previous studies tried
 144 to link ROS signal to pathways for activating the basal level of auto/mitophagy
 145 signals [13,21], but studies for explaining the link between the ROS and the
 146 responsiveness of auto/mitophagy have been limited. Microtubule (MT)s are
 147 hollow cytoskeletal rods with a diameter of approximately 25nm, and their
 148 network is maintained by a dynamic equilibrium, via constantly undergoing

assembly and disassembly to fulfill cellular needs. They function both to determine cell morphology and participate in a variety of cell locomotion, the intracellular transport of organelles, and the separation of chromosomes during mitosis. Accumulating numbers of recent studies demonstrated that MT serves as an essential guide rail for auto/mitophagosome vesicle trafficking and thus are the key component of auto/mitophagy pathway [22]. The roles of MTs in auto/mitophagy dysregulation, however, have less been investigated especially in the context of diseases. In this study, we analyzed whether MTs and its regulation are affected by oxidative stress and how it leads to the defective mitophagy response.

There have been attempts of therapeutic approaches by stimulating the autophagy in disease models where defects of autophagy and/or mitophagy is reported. Many of these approaches are aimed at activating the upstream autophagy initiating mechanisms. However, there has been no discussion regarding the validity of stimulating the upstream event where the downstream can be defective and thus the flux potentially be blocked due to the disease. The difficulty in such discussion derives partly due to the elusiveness of pathophysiology of auto/mitophagy defect in many diseases. In this study, after

evaluating the flux blockade of mitophagy response, the efficacy of a non-conventional auto/mitophagy modulator is examined. Trehalose, a non-reducing disaccharide composed of two D-glucose units linked α -1,1, is present in many organisms, including bacteria, fungi, plants, yeast, and invertebrates including tardigrades and brine shrimps. These organisms use trehalose to augment their adaptation competency against extremely severe environmental conditions including frigidity, dehydration, starvation, and UV-radiation. [23,24] Supplementation with trehalose also improves the survival of mammalian cells [25,26]. Recent studies demonstrated that trehalose induces autophagy via mammalian (mechanistic) target of rapamycin (mTOR)-independent pathway [27,28]. Some studies have suggested that trehalose helps maintain MT-forming function of tubulins [29]. By employing the cell-protective and autophagy-augmenting proficiency of trehalose, therapeutic studies have been proposed to treat several diseases in which autophagy plays an important role [9]. This study therefore examined whether normalizing the disturbed MTs with trehalose can prevent the mitophagy dysfunction under the subacute oxidative stress.

[Materials and Methods]

Ethics Statement and Animal Research

There is no animal research involved in the current study.

Reagents, Transgene Plasmid Constructs, Antibodies

The reagents used in this study are; MitoSOX (Thermo Fisher Scientific), LysoTracker (Thermo Fisher Scientific), SiR-tubulin (Cytoskeleton), CCCP (Millipore Sigma), E64d (Millipore Sigma), pepstatin A (Millipore Sigma), Lipofectamine 3000 (Thermo Fisher Scientific), G418 (Millipore Sigma), Mammalian expression plasmids for GFP-LC3 (kindly provided by Dr. N. Mizushima), tandem-fluorescent LC3 (Addgene), GFP-EB1 (Addgene), antibodies against LC3 (Sigma), phospho-Akt (Ser473, Cell Signaling Technology, #9271), phospho-GSK3 β (Ser9, Cell Signaling Technology, #9322), Anti-GAPDH (#9484 abcam, #2275-PC-100 Trevigen)

Cell Culture and Staining

C2C12 murine myocytes were obtained from ATCC and maintained in DMEM (Gibco/Thermo Fisher Scientific) with 10% fetal bovine serum (ATCC) and

203 50U/mL penicillin-streptomycin (Gibco/Thermo Fisher Scientific). By stably
204 transfecting auto/mitophagy markers in C2C12 myocyte, new muscle cell lines
205 were established, expressing GFP-LC3 or EB1-GFP or tfLC3.

206

207 ***Stable Transfection***

208 Mammalian expression transgene constructs were transfected into C2C12 cells
209 by lipofection using Lipofectamine 3000 according to the manufacturer's
210 instructions. After 24 hours of transfection, stable transfectants were selected
211 using G418 selection for one week and fluorescently labeled cells were cloned
212 according to the method described previously [30].

213

214 ***Drug Treatment, Mitophagy Induction, and Oxidative stress***

215 To impose subacute oxidative stress, myocytes were exposed to 500μM of
216 hydrogen peroxide (H₂O₂) for 12-18 hours. To test the therapeutic efficacy of
217 trehalose, the cells were treated with or without 100μM trehalose for 2 hours
218 before the observation. To monitor the response of mitophagy, the cells were
219 stimulated with 5μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), an

220 established mitophagy inducer. To disrupt microtubule network, the cells were
221 treated with 5 μ M colchicine for 1 hour.

222

223 ***Cell Staining***

224 To monitor lysosomes, the cells were stained by LysoTracker Blue. To observe
225 microtubule, the C2C12 myocyte were stained by SiR-tubulin. To investigate the
226 superoxide production from mitochondria, the C2C12 cells were stained by
227 MitoSOX at 5 μ M.

228

229 ***Image Data Analysis***

230 For detailed time-lapse image analyses, C2C12 cells were observed using a
231 confocal microscopy (Nikon Eclipse, A1R HD) with a 2 second interval, and the
232 captured images were analyzed by Image J. To investigate the mechanism of
233 the beneficial function of trehalose on MT, the synthesis speed of MT was
234 monitored using EB1-GFP, the plus-end tracking reporter. To investigate the
235 mitophagy response induced by CCCP, the motion and maturation of
236 mitophagosome were monitored. The vesicle motion was measured by using the
237 tracking plugins of ImageJ. The maturation of mitophagosome was defied by the

238 ratio of RFP/GFP. To measure fluorescent light intensity, a wide-field fluorescent
239 microscope (Nikon Eclipse 800) calibrated with a standard fluorescent beads
240 (LinearFlow, Thermo Fisher)

241

242 ***Cell Experiment Procedure for Western Blot***

243 The C2C12 cells were cultured on 6cm dishes to 80-90% confluence. Cells were
244 serum starved, treated with or without oxidative stress by using 500μM H₂O₂ for
245 12-18 hours. After that, the cells were treated with or without 100μM trehalose
246 for 2 hours. The response of mitophagy was monitored by stimulating the cells
247 with 5μM CCCP.

248

249 ***Cell Homogenization***

250 Cell samples were frozen under liquid nitrogen and homogenized in ice-cold
251 homogenization buffer (50 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 2 mM
252 EDTA, 1% Nonidet P-40, 10% glycerol, 10 mM sodium fluoride, 2 mM sodium
253 vanadate, 1 mM phenylmethyl- sulfonyl fluoride, 10 mM sodium pyrophosphate,
254 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The homogenized samples were

centrifuged at 13000 rcf for 30 min. Aliquots of supernatant containing same

amounts of protein, determined by BCA protein assay method.

Western Blot

Cell homogenates containing equal amounts of protein were subjected to 10%

SDS-PAGE after the addition of sample buffer and boiling for 3 min. After being

transferred electrophoretically to nitrocellulose membrane (Li-COR, Lincoln, NE),

the membranes were blocked in 5% skim milk for 1 h at room temperature,

followed by incubation with primary antibodies for overnight at 4°C.

Anti-phospho-Akt (p-Akt), anti-phospho-GSK-3 β (p- GSK-3 β), anti-GAPDH (Cell

Signaling, Beverly, MA), anti-LC3 (Sigma Aldrich, St Louis, MO) antibodies were

used as primary antibodies. The anti-rabbit or -mouse IgG antibody was used as

a secondary antibody at a dilution of 1:10,000. Bands of interest were scanned

and quantified with the use of Odyssey CLx (Li-COR, Lincoln, NE).

Statistical Analysis

Data are expressed as mean $\pm$ standard deviation (SD) and analyzed with

Student's t-test for two-group comparison, one-way ANOVA and Tukey's

method for more than three-group comparison. A value of $p < 0.05$ was

considered significant.

[Results]

Subacute Oxidative Stress-Induced Perturbation in Mitophagy Response

To investigate the non-acute effect of oxidative stress on the responsiveness of

mitophagy, C2C12 cells were treated with H_2O_2 for 16 hours. Cells were then

stimulated by CCCP to induce mitophagy in the presence or absence of

auto/mitophagy flux blockade with E64d and pepstatin A. Purified mitochondria

fraction was analyzed for mitophagy induction by Western Blotting against LC3.

As shown in Fig.1, H_2O_2 treatment elevated the basal level of mitophagy, but

when CCCP-stimulated response of mitophagy was compared between controls

(H_2O_2 -) and the oxidative stress group (H_2O_2 +), mitophagy flux was significantly

blocked, indicating that prolonged exposure to oxidative stress confers signal

resistance to mitophagic stimulation in C2C12 cells.

Figure 1: Mitophagy flux response to CCCP stimulation is diminished under

oxidative stress.

(a-d) Western blotting pictures either of the mitochondrial fraction (a, b) or the input of total homogenate (c) are shown. C2C12 myocytes were cultured in the absence ("H₂O₂ -") or presence ("H₂O₂+") of hydrogen peroxide for 18 hours. Cells were stimulated by CCCP and the response of mitophagy was monitored with or without mitophagy flux blockade by E64d plus pepstatin A ("E&A-" vs. "E&A+"). Shown in the WB pictures are; (a) blotting against LC3, the auto/mitophagy marker in the mitochondrial fraction, (b) mitochondrial residual molecule, Tom20, as an internal control for equal mitochondrial protein load, and (c) GAPDH in the total homogenate as the input loading control for fractionation. (d) Quantification of the band intensity is shown as the ratio of LC3II/Tom20 in the mitochondrial fraction. Despite the tendency of increased basal level of mitophagy under oxidative stress without mitophagy stimulation, (H₂O₂+, CCCP-, E&A-), mitophagy flux is diminished (increment from E&A - to E&A+) under the oxidative stress (H₂O₂+), as compared to that in the control group (H₂O₂-). (e) The ratio of increase in the LC3II/Tom20 before and after the flux blockade is shown. *: p<0.05, N=4. Data are shown as average +/- standard deviation. Note that in the mitochondrial fraction for LC3 (a), LC3II form is predominantly detected, because this form mainly consists of the vesicle attached form.

309

310 ***The Effect of Oxidative Stress on Mitophagy Motion and Its Maturation***

311 To further investigate the mechanisms involved in the disturbed mitophagy
312 response by CCCP under oxidative stress conditions, the movement of
313 mitophagosome was monitored by time-lapse confocal microscopy, using
314 C2C12 cells expressing GFP-LC3. In cells under the subacute oxidative stress,
315 the movement of mitophagosomes invoked by CCCP decreased significantly
316 than that in the control cells (Fig.2, Supplementary Video 1), consistent with the
317 data for the decreased mitophagy flux under CCCP stimulation in Fig.1.
318 Trehalose, however, ameliorated the once diminished movement of
319 mitophagosomes under the subacute oxidative stress (Fig.2, Supplementary
320 Video 1). The increment of mitophagosome motion in response to CCCP was
321 significantly improved by trehalose treatment under the subacute oxidative
322 stress (132 vs. 100%: with vs. without trehalose; $p<0.05$).

323 To investigate the impact of decreased motion of mitophagosomes due
324 to oxidative stress on the mitophagy flux, C2C12 cells expressing
325 tandem-fluorescent LC3 (tfLC3) were monitored for analyzing the maturation
326 status and thus flux of mitophagy after CCCP stimulation. As is shown in Fig.3,

327 tfLC3-expressing cells under the oxidative stress harbored more yellow dots
328 (GFP and RFP both positive) as compared to red-only predominant pattern in
329 controls, demonstrating the inhibition of mitophagosomes maturation under
330 oxidative stress conditions. Note that tfLC3 reporter provides fluorescent signal
331 of both GFP and RFP and thus yield yellow signal when autophagosomes and
332 mitophagosomes are premature. During the process of maturation,
333 autophagosomes and mitophagosomes fuse with lysosomes, and due to the
334 drop of intravesicular pH, GFP signal fades, resulting in red-dominant signal.
335 This finding of defective maturation supports the decreased mitophagy flux
336 under the oxidative stress (Fig.1). Trehalose treatment, however, improved ratio
337 of yellow to red mitophagosomes, and ameliorated the defective maturation
338 under the subacute oxidative stress. Next, to further decipher the mechanism of
339 the beneficial effect of trehalose, colchicine , a drug that destabilizes microtubule,
340 was added to the treatment. Colchicine abolished the effect of trehalose on
341 mitophagy maturation (Fig.3), suggesting that beneficial effect of trehalose is
342 likely mediated by its effect on normalization of microtubules.
343
344 Figure2. The mitophagy movement is diminished under oxidative stress.

345 (Top) (a-c) Confocal microscopic images of C2C12 cells expressing GFP-LC3,
346 co-stained with LysoTracker Blue. Pseudo colors are added with green for
347 GFP-LC3, and red for LysoTracker Blue. (a) In controls, GFP-LC3 dots are fine
348 granular and show vigorous movement. (b) In H₂O₂ treated cells, GFP-LC3
349 vesicles are swollen and the motions diminished. (c) Trehalose treatment
350 partially reverts the size of GFP-LC3 dots and reactivates the vesicle motion.
351 White scale bar at the right bottom corner of each image represents 10µm. (d)
352 The movement of mitophagosomes was analyzed by ImageJ and shown as the
353 average value of area with standard deviation (N=3-8). *:p<0.05 by ANOVA with
354 Tukey's comparison. For video information, refer to Supplementary Video 1.
355
356 Supplementary Video 1: Time-lapse confocal video image of C2C12 myocytes
357 expressing GFP-LC3, stained by LysoTracker Blue.
358 Live video information for Fig.2 is shown here.
359 Cells are incubated in the presence or absence of H₂O₂ with or without trehalose
360 treatment. In controls (H₂O₂ -, trehalose -, far left), GFP-LC3 dots are fine
361 granular and show vigorous movement. In H₂O₂ treated cells without treatment
362 (H₂O₂ +, trehalose -, middle), GFP-LC3 vesicles are swollen and the motions

markedly diminished. Trehalose treatment (H_2O_2 +, trehalose +, far right) partially reverts the size of GFP-LC3 dots and reactivates the vesicle motion. Yellowish dots represent the mature vesicles after LC3-GFP dot (green) fuses with lysosomes (red), and also shows similar pattern of motion changes. In this video, pseudo colors are added with green for GFP-LC3, and red for LysoTracker Blue.

Figure3. The mitophagosome maturation is disturbed under oxidative stress. (Top) (a-h) Confocal microscopic images of C2C12 cells expressing tf-LC3 are shown to document the effect of H_2O_2 and Trehalose on the maturation of mitophagosomes after CCCP stimulation (GFP Green, RFP; Red). (a,e) Control group, (b,f) H_2O_2 group, (c,g) H_2O_2 +trehalose group, (d,h) H_2O_2 +trehalose +colchicine group. Scale bar represents 10 μ m.

(i) The maturation status of mitophagosomes was analyzed by ImageJ and shown as the average value of the ratio of areas of GFP/RFP with the standard deviation (N=12). *:p<0.05, ‡:p<0.05 vs colchicine group by ANOVA with Tukey's comparison.

381 ***The Effect of Oxidative Stress on Microtubule Network Formation***

382 Next, to investigate the roles of MT network in the regulation of mitophagy in
 383 C2C12 myocytes under the oxidative stress, the MT network of C2C12 cells
 384 were stained by SiR-tubulin (Fig.4). C2C12 cells under the oxidative stress
 385 showed disturbed MT network formation as compared to normal cells,
 386 suggesting that the molecular target of oxidative stress likely involves pathways
 387 related to MT formation. In line with this finding, it has been reported that
 388 trehalose augments autophagy/mitophagy by mTOR-independent pathway [27],
 389 but its precise target has not been fully investigated. As shown above, trehalose
 390 improves the disturbed maturation process apparently through working on the
 391 MT network. The effect Trehalose treatment on MT was thus monitored using
 392 SiR-tubulin. Treatment by trehalose of cells exposed to oxidative stress
 393 ameliorated the disturbed MT network, suggesting that trehalose can work on
 394 maintaining the healthy MT network. To examine whether the signal amelioration
 395 of MTs by SiR under trehalose treatment was not due to artifact of measurement,
 396 C2C12 cells with trehalose were treated by colchicine. Treatment by colchicine
 397 abolished, further supporting that trehalose indeed helps maintain the MT
 398 network under oxidative stress condition.

399

400 Figure4. Microtubule network formation is disturbed under oxidative stress.

401 (Top) (a-d) Confocal microscopic images of C2C12 cell stained by SiR-tubulin

402 are shown for the potential effect of H₂O₂, trehalose and colchicine on the

403 microtubule network. (a) Control group, (b) H₂O₂ group, (c) H₂O₂ +trehalose

404 group, and (d) H₂O₂ +trehalose+colchicine group. Scale bar =10μm.

405 (e) The area of SiR-tubulin was analyzed by ImageJ and shown as the average

406 value of area with standard deviation (N=7). *:p<0.05, **:p<0.05 vs colchicine

407 group by ANOVA with Tukey's comparison.

408

409 ***The Effect of Oxidative Stress on the Synthesis of Microtubule***

410 To investigate the mechanism of the beneficial function of trehalose on MTs, the

411 synthesis extent and speed of microtubule was monitored by time-lapse confocal

412 microscopy, using EB1-GFP, the plus-end tracking reporter (Fig.5,

413 Supplementary Video 2). In cells under the subacute oxidative stress, the

414 movement and the number of EB1 significantly decreased as compared to

415 control cells. When cells are treated with trehalose, the diminished movement

416 and number of EB1 was significantly increased in cells under the subacute

oxidative stress. These results suggest that trehalose normalizes MT network in cells under oxidative stress conditions either by augmenting the MT synthesis or by inhibiting the MT degradation and thus secondarily affecting the synthesis.

Figure 5. The synthesis ability of microtubule is diminished by oxidative stress (TOP) (a-c) Confocal microscopic images of C2C12 cell expressing EB1-GFP are shown to analyze the effect of H₂O₂ and trehalose on extent and speed of MT synthesis. (a) Control group, (b) H₂O₂ group, (c) H₂O₂ +trehalose group. Scale bar =10μm.

(d-f) Magnified images of the boxed areas in the top (a-c) shown. Synthesized overlay images of ten stacked images of time-lapse observation are shown to document the trajectory of EB1 motion. (d) Control group, (e) H₂O₂ group, (f) H₂O₂ +trehalose group. Scale bar =10μm.

(g, h) The movement speed (g) and number (h) of EB-1 analyzed by ImageJ and shown as the average value of area with standard deviation (N=5). *:p<0.05, **:p<0.05 vs colchicine group by ANOVA with Tukey's comparison. For video information, refer to Supplementary Video 2.

Supplementary Video 2: Time-lapse confocal video image of C2C12 myocytes expressing GFP-EB1 for measuring MT synthesis. Cells are incubated in the presence or absence of H₂O₂ with or without trehalose treatment. In controls (H₂O₂ -, trehalose -, far left), GFP-EB1 dots, representing the plus-end of elongating MTs, show vigorous movement with greater majority of dots trafficking in the efferent direction. In H₂O₂ treated cells without treatment (H₂O₂ +, trehalose -, middle), both the numbers and the speed of GFP-EB1 motion are markedly decreased. Trehalose treatment (H₂O₂ +, trehalose +, far right) partially rescues the EB1 trafficking.

Signaling Mechanisms Involved in Disturbed Mitophagy Response

Previous lines of evidence suggested the involvement of Akt/GSK3 β signaling in CCCP-induced mitophagy [31] and in the elongation signals of MTs via EB1 activation [32]. Activated Akt functions through the phosphorylation and inhibition of Glycogen Synthase Kinase-3 β (GSK3 β) [33] leading to the activation of EB1-mediated MT synthesis through CLAPS2 recruitment. We first analyzed the time course of Akt/GSK3 β phosphorylation in normal C2C12 cells in response to mitophagy-inducing stimulation (CCCP 5 μ M and 12.5 μ M) using

Western blot with antibodies against phosphorylated form of Akt (Ser473 p-Akt) and GSK3 β (Ser9 p-GSK3 β). (Fig.6-a). Both 5 μ M and 12.5 μ M stimulation caused rapid phosphorylation of Akt and GSK3 β peaking at 15 minutes of stimulation. Since there was no essential difference between 5 μ M and 12.5 μ M stimulation, 5 μ M stimulation was used thereafter unless otherwise stated. Next, to investigate the effect of oxidative stress on the activation of Akt/GSK3 β pathway, phosphorylation status of Akt after CCCP stimulation was monitored. Under the oxidative stress, the level of p-Akt and p-GSK3 β was significantly decreased as compared to control cells (Fig.6-b, 0.82 ± 0.39 vs. 1.6 ± 0.26 ; $p < 0.05$; $n = 5$, and 0.70 ± 0.03 vs. 1.17 ± 0.06 ; $p < 0.05$; $n = 5$, for p-Akt and p-GSK3 β , respectively). Trehalose treatment to cells exposed to oxidative stress rescued the deficient signal response of Akt/GSK3 β in response to CCCP, as compared to that in cells without treatment (Fig.6-c).

Figure6. (a-c) Western blot analysis for phospho-Akt (p-Akt) and phospho-GSK3 β (p-GSK3 β) in the C2C12 myocyte after carbonyl cyanide m-chlorophenyl hydrazone (CCCP) stimulation. In both p-Akt and p-GSK3 β

expression, CCCP treatment induced rapid phosphorylation of Akt and GSK3 β ,
peaking at 15min.

(d) Western blot analysis for p-Akt and p-GSK3 β in the C2C12 myocyte with or
without H₂O₂, trehalose. The p-Akt and p-GSK3 β expression significantly
decreased in C2C12 cells under the oxidative stress as compared to the control
group (n=5, * p<0.05). Trehalose treatment partially reversed the decreased
p-Akt and p-GSK3 β expression. The results of the GAPDH analysis are shown
as an internal control.

(e) Western blot analysis for mitophagy response (the increase rate before and
after CCCP) in the C2C12 myocyte under the oxidative stress with or without
trehalose treatment. Trehalose treatment significantly ameliorated the disturbed
p-Akt and p-GSK3 β expression (n=5, * p<0.05) under the oxidative stress. The
results of the GAPDH analysis are shown as an internal control.

Superoxide Production From Mitochondria

Mitophagy is one of the major quality control systems of mitochondria that
removes defective mitochondria and thus prevents ROS release from the
damaged mitochondria. In line with this notion, the superoxide production from

488 mitochondria in C2C12 myocyte was analyzed as an outcome of poor mitophagy
489 response under subacute oxidative stress using a staining dye for
490 mitochondria-derived superoxide, MitoSOX (Fig.7). The ratio of C2C12 with
491 elevated production of superoxide was significantly increased. Trehalose
492 treatment significantly ameliorated the increase of superoxide production,
493 supporting the hypothesis that amelioration of defective mitophagy by trehalose
494 rescues the mitochondria and helps the quality control of mitochondria.

495

496 Figure7. Superoxide production from mitochondria

497 (a-c) Fluorescence microscopic images of C2C12 cells stained MitoSOX are
498 shown to document the superoxide production from mitochondria after CCCP
499 stimulation. (a) Control group, (b) H₂O₂ group, and (c) H₂O₂ +trehalose group.

500 Scale bar=1000μm.

501 (d) The ratio of C2C12 cells positively stained by MitoSOX was analyzed by
502 ImageJ and shown as the average value of area with standard deviation (N=6).
503 H₂O₂ subacute stimulation increased superoxide production from mitochondria,
504 and H₂O₂ +trehalose ameliorated after CCCP administration. *:p<0.05, **:p<0.05
505 vs control by ANOVA with Tukey's comparison.

506

507

508 **[Discussion]**

509 ***ROS and Mitophagy Response Defect***

510 In this study, we demonstrated that prolonged oxidative stress decreases the

511 response of mitophagy, especially at the maturation stage, possibly due to the

512 perturbed MT network formation. This is among the first detailed systematic

513 study that analyzed the mechanisms of disturbed mitophagy response, not the

514 basal level of mitophagy, in the cells exposed to an oxidative stress.

515 Furthermore, it was demonstrated that trehalose, a mTOR-independent

516 auto/mitophagy modulator, improves defective mitophagy response in myocytes

517 under the oxidative stress, ameliorating the inhibition of mitophagosomes

518 maturation, presumably by normalizing MT network formation and increasing the

519 synthetic ability of MT. The current study opens a possibility for therapeutic

520 approaches for targeting maturation defect in mitophagy response and for

521 modulating defective MT network formation in treating diseases arising from

522 prolonged oxidative stresses.

523

524 ***Oxidative Stress and Autophagy/mitophagy - Upregulation or***

525 ***Downregulation?***

526 Although the relationship between ROS and auto/mitophagy has been studied
 527 previously [16], most of the previous investigations focused on the mechanisms
 528 of ROS-induced upregulation of auto/mitophagy. In these previous studies, the
 529 basal levels of auto/mitophagy were analyzed and few studies focused on their
 530 response. Furthermore, in many of previous studies, cells or tissues were
 531 acutely stimulated for the analyses. Upregulation of auto/mitophagy in such
 532 acutely stressed cells is reasonable given that auto/mitophagy evolved as
 533 adaptive responses to the external and internal stresses. Importantly, the current
 534 finding about the defective mitophagy response is not contradictory to the
 535 previous findings for upregulated auto/mitophagy under oxidative stress. One
 536 has to take into account whether the analyses are performed to quantify the
 537 basal level of auto/mitophagy, or the changes in their responses of
 538 auto/mitophagy to stimulations. In biological systems, these two often show
 539 opposite trends. For example, in type 2 diabetes mellitus (T2DM), despite the
 540 fact that pathophysiology of many disease symptoms can be explained by
 541 insulin resistance, or the defective response of intracellular signals to insulin

stimulation in many organs, even though the basal level of plasma insulin is often elevated in many patients. Despite the serum insulin elevation, suppression or blockade of insulin signal would not be considered a reasonable therapeutic approach. Likewise, although many previous studies reported upregulation of the basal level of auto/mitophagy, blocking autophagy or mitophagy in oxidative stress related diseases may not provide a useful therapeutic approach, especially given that auto/mitophagy are mostly cell protective response in many situations.

Mitophagy Defect and Human Diseases Related to Oxidative Stress

Mitophagy, a form of macroautophagy that selectively degrades damaged mitochondria, is considered as one of the major quality control system of mitochondria, and helps maintaining the integrity and the functions of healthy mitochondria [34]. Mitophagy defect is currently assumed to be an essential pathogenic factor in cancer, neurodegeneration, metabolic disorders, muscle atrophy, inflammation and organ failure caused by sepsis [35,36].

Among many other diseases, prolonged increase of oxidative stress is considered one of the key mechanisms in sepsis or burn injury induced organ

dysfunctions. In our experiments, subacute H₂O₂ stimulation significantly inhibited mitophagy response, after CCCP stimulation (Fig.1,2) blocked mitophagosomes maturation (Fig.3), resulting in elevated superoxide generation from the defective mitochondria (Fig.7). These data suggest that subacute oxidative stress leads to mitophagy defect in myocyte after CCCP stimulation. Trehalose, however, improved the defects in maturation under the subacute oxidative stress (Fig.3) and thus will be a promising drug candidate for treating the mitophagy defect.

Microtubule and Mitophagy Maturation

MT forms a dynamic cytoskeletal structure. Both integrity of healthy MT and the normal functions of MT-based motors have been shown essential in autophagy [22]. The centripetal movement of mature autophagosomes prior to fusion with lysosomes requires stable MT [37]. A key mechanism of auto/mitophagosome flux is the transport of auto/mitophagosomes along MTs toward lysosomes to form mature autophagolysosomes (autolysosomes) [38]. In this study, observation C2C12 cells stained by SiR-tubulin showed that the subacute oxidative stress abolished MT network formation as compared to normal cells

(Fig4). Observation of tfLC3-expressing cells demonstrated that the subacute oxidative stress inhibited mitophagosomes maturation (Fig3), implying that the unstable MT could be one of the important mechanisms of mitophagy defect under the subacute oxidative stress. This notion is supported by the finding that trehalose treatment ameliorated MT defect and mitigated mitophagy maturation defect but that colchicine abolished the beneficial effect of trehalose. These data suggest that MT serves as an essential guide rail for mitophagosome vesicle trafficking and thus are a key component of mitophagy pathway involved in disease conditions

Phosphoinositide-3-Kinase (PI3-K) and the downstream kinases Akt and GSK3 β have recently been implicated in regulating both MT dynamics and organization [39]. Akt and GSK3 β coordinately regulate the phosphorylation status of MT end capping molecules including CLASP2 and EB1, thus regulating their recruitment to the synthesis plus end of MTs[40]. Importantly, auto/mitophagy stimulating signal activate this pathway [31,41]. The results of Western Blot analysis showed that the subacute oxidative stress significantly decreased the pAkt and GSK3 β expression as compared to control cells (Fig.6-b,

c), suggesting that subacute oxidative stress can affect MT synthesis by invoking signal resistance in PI3K/Akt/GSK3 β pathway.

Trehalose Function as a Modulator of Mitophagy

It has been established that trehalose is a modulator of auto/mitophagy pathway, augmenting these stress adaptation functions by mTOR-independent mechanisms in COS-7 cells and mouse embryonic fibroblasts (MEFs) [42,43]. Trehalose also upregulates p62 expression and activates the autophagy flux in Hepa1-6 cells and MEFs [9]. In our study, trehalose can improve the synthesis ability of MT and thus ameliorates the defective MT network under the subacute oxidative stress (Fig.4). Thus repaired MT network improved the oxidative stress-induced inhibition of mitophagosomes maturation in trehalose treated cells. Notably, colchicine, a drug that destabilizes MT, abolished the beneficial effect of trehalose on mitophagy (Fig.3&4). In the detailed analyses of the signal transduction mechanisms, trehalose treatment significantly increased the disturbed Akt/GSK3 β signaling in cells under the oxidative stress (Fig.6-b, c). Many therapeutic approaches have been previously suggested in human diseases or their murine equivalent models where auto/mitophagy are defective.

613 In many of previous studies, one of the proposed strategies was to boost
 614 auto/mitophagy. The current study suggests, however, that auto/mitophagy
 615 defect in diseases can involve maturation defect, or the blocked flux. To
 616 forcefully stimulate the upstream activation of auto/mitophagy in such condition
 617 where the downstream is blocked, (e.g. by disturbed MT network as shown in
 618 the current study), may not serve an effective therapeutic approach. Thus, the
 619 importance of finding the precise target of auto/mitophagy defect and
 620 ameliorating the perturbation, cannot be overemphasized.

621 Oxidative stress-induced MT disturbance can be a pivotal event in the
 622 critical illness-related mitochondrial dysfunction in skeletal muscles, and needs
 623 further investigation. Trehalose can ameliorate the perturbed MTs by increasing
 624 the synthetic ability of MT, and normalizing the disturbed MTs can serve a novel
 625 therapeutic agent in critical illnesses.

626

627 **[Conclusions]**

628 Oxidative stress decreases the response of mitophagy and abolishes MT
 629 network formation. Trehalose improves the synthetic ability of MT by increasing
 630 the number and movement of the MT plus end-capping molecule, EB1,

suggesting that trehalose augments MT network synthesis. Observation of
 tfLC3-expressing cells demonstrates that trehalose also improve the inhibition of
 mitophagosomes maturation under the oxidative stress. Finally, trehalose
 normalizes MT network formation by increasing the synthetic ability of MT. All
 the obtained data suggest that normalizing the disturbed MTs under oxidative
 stress can prevent the mitophagy dysfunction under the oxidative stress and
 serve a novel therapeutic target.

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781

Fig1

control

H_2O_2

H_2O_2
Trehalose

H_2O_2
Trehalose
colchicine

Fig3

Fig4

Fig6

control

H₂O₂

H₂O₂ + Trehalose

Fig7

control

H₂O₂

H₂O₂ + Trehalose

Fig2

Fig5