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ORIGINAL ARTICLE

The ubiquitin–proteasome system and autophagy are defective in the taurine-deficient heart

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Abstract Taurine depletion leads to impaired mitochondrial function, as characterized by reduced ATP production and elevated superoxide generation. These defects can fundamentally alter cardiomyocyte function and if left unchanged can result in cell death. To protect against these stresses, cardiomyocytes possess quality control processes, such as the ubiquitin–proteasome system (UPS) and autophagy, which can rejuvenate cells through the degradation of damaged proteins and organelles. Hence, the present study tested the hypothesis that reactive oxygen species generated by damaged mitochondria initiates UPS and autophagy in the taurine-deficient heart. Using transgenic mice lacking the taurine transporter (TauTKO) as a model of taurine deficiency, it was shown that the levels of ubiquitinated protein were elevated, an effect associated with a decrease in ATP-dependent 26S β5 proteasome activity. Treating the TauTKO mouse with the mitochondria-specific antioxidant, mitoTEMPO, largely abolished the increase in ubiquitinated protein content. The TauTKO heart was also associated with impaired autophagy, characterized by an increase in the initiator, Beclin-1, and autophagosome content, but a defect in the generation of active autophagolysosomes. Although mitoTEMPO treatment only restores the oxidative balance within the mitochondria, it appeared to completely disrupt the crosstalk between the damaged mitochondria and the quality control processes. Thus, mitochondrial oxidative stress is the main trigger initiating the quality control systems in the taurine-deficient heart. We conclude that the activation of the UPS and autophagy is another fundamental function of mitochondria.

Keywords Proteasome activity · Taurine deficiency · Autophagosome formation · Mitochondrial morphology · Consequences of mitochondrial oxidative stress · Protein degradation · Defective autophagolysosome

Introduction

Cells are highly dependent on quality control mechanisms to maintain a proper balance between protein and organelle degradation and biosynthesis. In postmitotic tissues, such as the heart, cellular homeostasis is critical in ensuring cellular viability and functionality. A disturbance in the cell's degradation pathways prevents the removal of defective molecules, causing the accumulation of damaged proteins and organelles that increase a cell's vulnerability to oxidative stress and energy depletion, factors that may trigger apoptosis (Yuan et al. 2009).

The ubiquitin–proteasome system (UPS) and autophagy are among two of the major quality control mechanisms that regulate the degradation of cytoplasmic molecules. The UPS selectively degrades damaged and abnormal proteins through a process in which proteins are first ubiquitinated and then delivered to the proteasome for proteolytic degradation. Among the stimuli that enhance protein ubiquitination are oxidative stress (Shang and Taylor 2011) and AMPK activation (Baskin and Taegtmeyer 2011). Impairment of the UPS, either related to decreased protein ubiquitination (Carvalho et al. 2013) or diminished proteasome activity (Keller et al. 2000), prevents protein clearance and ultimately leads to aberrant protein aggregation, a

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determinant of pathology in degenerative diseases (Keller et al. 2000b), congestive heart failure (Day et al. 2013), cardiomyopathy (Liu et al. 2006) and myopathy (Askanas et al. 2009).

Autophagy, on the other hand, degrades damaged organelles and protein aggregates. When activated, double-membraned vesicles known as autophagosomes are formed to engulf cytoplasmic molecules (Levine and Kroemer 2008). Subsequently, the autophagosome merges with lysosomes to form an autophagolysosome, which degrades engulfed cytoplasmic molecules (Levine and Kroemer 2008).

While the regulation of autophagy remains elusive, several factors are known to induce autophagy, including nutrient and amino acid deprivation (Young et al. 2009), AMPK activation (Roach 2011), endoplasmic reticular (ER) stress (Ogata et al. 2006), hypoxia (Zhang et al. 2008), oxidative stress (Scherz-Shouval et al. 2007) and pathogen infection (Gong et al. 2012). The process of autophagy is flexible, as it is capable of removing specific organelles, such as mitochondria (mitophagy), peroxisomes (pexophagy), ribosomes (ribophagy) and endoplasmic reticulum (reticulophagy), or of degrading cellular molecules, such as lipid droplets (lipophagy), secretory granules (zymophagy) and protein aggregates (aggrephagy) (Klionsky et al. 2007). Aberrant rates of autophagy have been linked to various pathologies, with accelerated degradation of cytoplasmic molecules implicated in atrophy (Penna et al. 2013), and accumulation of damaged cytoplasmic molecules implicated in degenerative diseases (Hara et al. 2006), cardiomyopathy (Taneike et al. 2010) and myopathy (Masiero and Sandri 2010).

Taurine is an ubiquitous sulfur-containing, β-amino acid found in very high concentration in excitable tissues, including the heart (Huxtable 1992). Several physiological actions have been attributed to taurine, with metabolic, antioxidant, anti-inflammatory and cytoprotective activities being the most important (Mozaffari et al. 1986; Jong et al. 2012; Marcinkiewicz and Kontny 2014; Ramila et al. 2015). Maintenance of high tissue taurine levels is required for normal cellular function, as taurine deficiency is associated with the development of cardiomyopathy, neuropathy, retinopathy, developmental defects and pregnancy complications (Knopf et al. 1978; Sturman et al. 1985; Sturman and Messing 1992; Schuller-Levis et al. 1990; Ito et al. 2008). Interestingly, the taurine-deficient phenotype resembles that of the mitochondrial disease, MELAS (Schaffer et al. 2013), tying both phenotypes to the function of $tRNA^{Leu(UUR)}$. In MELAS, mutations in the gene for $tRNA^{Leu(UUR)}$ interferes with a taurine-dependent post-translational modification of the $tRNA^{Leu(UUR)}$ gene, resulting in a weakening of the interaction between the UUG codon for leucine and the AAU anticodon of $tRNA^{Leu(UUR)}$ (Kirino et al. 2004). Decreases in the formation of the taurine conjugation product, 5-taurinomethyluridine-$tRNA^{Leu(UUR)}$, diminish UUG decoding causing impaired expression of UUG-dependent mitochondria-encoded proteins, which serve as key subunits of the respiratory chain. Reductions in their expression lead to diminished respiration and ATP production, as well as an elevation in mitochondrial superoxide generation (Jong et al. 2012).

Mitochondria-linked respiratory dysfunction and oxidative stress also appear to be central mechanisms involved in the development of taurine-deficient cardiomyopathy (Jong et al. 2012); however, the taurine-deficient heart also contains disrupted and unorganized myofilaments, defective endoplasmic reticulum and cellular vacuolization (Ito et al. 2008), anomalies that are indicative of altered quality control. Although oxidative stress appears to play a prominent role in the induction of cellular quality control processes (Scherz-Shouval et al. 2007), there have been no studies examining cellular quality control in either taurine deficiency or MELAS. Because it is widely thought that aberrant function of the quality control processes is associated with cellular deterioration (Hara et al. 2006; Liu et al. 2006; Askanas et al. 2009; Masiero and Sandri 2010; Taneike et al. 2010), we examined the hypothesis that taurine depletion-mediated mitochondrial dysfunction leads to impaired autophagy and dysfunction of the UPS. The present data show that treatment with the mitochondria-specific antioxidant, mitoTEMPO, reverses defects in autophagy and the UPS of transgenic mice lacking the taurine transporter (TauTKO), the transporter responsible for the maintenance of normal taurine levels in the heart.

Methods

Mice

Wild-type (WT) and homozygous taurine transporter knockout (TauTKO) mice were produced by breeding heterogenous taurine transporter knockout ($TauTKO^{+/-}$) C57BL/6 mice. Animal handling and experimental procedures followed the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the University of South Alabama.

Preparation of isolated neonatal rat cardiomyocytes

Isolated neonatal rat cardiomyocytes were prepared according to the methods described by Jong et al. (2012). In brief, hearts were removed from 2- to 3-day-old Wistar rats, subjected to enzymatic homogenization and pre-plated for non-myocyte attachment. After 1.5 h, the unattached

cardiomyocytes were plated in 6-well plate in minimum essential medium (MEM), supplemented with 10 % newborn calf serum and 0.1 mM 5-bromo-2-deoxyuridine and incubated overnight at 37 °C in 5 % CO_2, 95 % O_2 incubator. On the next day, the serum-containing medium was removed and cardiomyocytes were incubated with serum-substituted medium for 2 days. Then cardiomyocytes were treated with either 0 mM (Control) or 5 mM β-alanine for 48 h before subjected to mitochondrial morphology assessment.

Examination of mitochondrial morphology

Cardiomyocytes grown on 35 mm glass bottom culture dishes were stained with 150 nM MitoTracker Deep Red in Krebs buffer (0.12 M NacL, 0.025 M $NaHCO_3$, 4 mM KCl, 1.2 mM $MgSO_4{\cdot}7H_2O$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2{\cdot}2H_2O$) containing 10 mM glucose and 10 g/L dextran (pH adjusted to 7.4) for 30 min at 37 °C. Mitochondrial morphology was examined under fluorescence microscope at excitation wavelength of 633 nm and emission wavelength of 650 nm.

Isolation of mitochondrial proteins

Isolation of mitochondrial proteins was conducted following the methods described by Cheng et al. (2011) with a brief modification. In brief, hearts were homogenized in mitochondrial buffer containing 70 mM sucrose, 190 mM mannitol, 20 mM HEPES, 1 mM EDTA, 1 mM PMSF, 1 mM NaV_2O_5, 1 mM NaF and 1X protease inhibitor cocktail. Homogenates were centrifuged at 600*g* for 10 min. The supernatant was then centrifuged for 12,000*g* for 30 min, producing a pellet that was defined as the mitochondrial fraction. The pellet was washed and finally resuspended in radio-immunoprecipitation assay (RIPA) lysis buffer (50 mM Tris Base, pH 8.0, 150 mM NaCl, 0.5 % deoxycholic acid, 1 % NP-40, 0.1 % sodium dodecyl sulfate) supplemented with 1 mM PMSF, 1 mM NaV_2O_5, 1 mM NaF and 1X protease inhibitor cocktail.

Western blot analysis

Total lysates were prepared from hearts homogenized in RIPA lysis buffer. Homogenates were centrifuged at 10,000*g* for 10 min and supernatants were collected as total lysates. Protein concentration was measured by the bicinchoninic assay (BCA). Protein (20–30 μg) was mixed with an equal volume of 5× sample buffer (1.25 mM Tris HCl, pH 6.8, 1 % sodium dodecyl sulfate, 10 % glycerol, 5 % β-mercaptoethanol) and then boiled for 5 min. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membranes then were blocked in blocking buffer (5 % milk in Tris buffered saline with Tween 20) and incubated with an appropriate primary antibody overnight at 4 °C. Membranes were washed before incubating with an appropriate secondary antibody. After washing, Western blots were analyzed by enhanced chemiluminescent reagents.

β5 26S proteasome activity

Proteasome activity was assayed according to the methods described by Iorga et al. (2012). In brief, hearts were homogenized in a homogenization buffer containing 50 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT, pH 7.5. Homogenates were centrifuged at 12,000*g* for 10 min and the supernatant was collected. 20 μg proteins were used to measure ATP-dependent 26S β5 proteasome activity using β5 fluorescence substrate (Suc-Leu-Leu-Val-Tyr-AMC) in the assay buffer (50 mM Tris, 1 mM EDTA, 150 mM NaCl, 10 mM $MgCl_2$, 0.1 mM ATP, pH 7.5) with or without 20 uM epoxomicin, a β5 proteasome inhibitor. The fluorescence of released AMC was measured using a plate reader at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Analysis of autophagy flux

Wild-type and TauTKO mice were administered either PBS (vehicle) or chloroquine (60 mg/kg/day) by intraperitoneal injection for 5 consecutive days. On the 5th day, mice were killed and hearts were rapidly removed and snap frozen in liquid nitrogen and stored at −80 °C. Autophagy flux was assessed by measuring LC3-II levels by Western blotting.

Analysis of the role of mitochondrial oxidative stress

Wild-type and TauTKO mice were administered via intraperitoneal injection for 7 consecutive days either PBS (vehicle) or mitoTEMPO (1.4 mg/kg/day). On the 7th day, the mice were killed and hearts were rapidly removed and snap frozen in liquid nitrogen and stored at −80 °C.

Statistical analysis

All results were reported as mean ± SEM. The statistical significance of the data was determined using the Student's *t* test for comparison within two groups. Analysis of variance combined with the Newman–Keuls test was used to establish significance when comparing multiple groups. Values of $p < 0.05$ were considered statistically significant.

Results

Taurine depletion decreases ubiquitin–proteasome system

As part of the quality control process, damaged proteins are commonly degraded by the UPS. The first step in the UPS process is the ubiquitination of damaged proteins (Shang and Taylor 2011). As shown in Fig. 1a, TauTKO hearts demonstrate a 1.75-fold increase in ubiquitinated protein content, a finding consistent with proteins either being targeted for degradation or a defective UPS process that allows the accumulation of ubiquitinated proteins. Because the UPS also regulates mitochondrial health by degrading mitochondrial outer membrane proteins (Hammerling and Gustafsson 2014), the ubiquitin content of mitochondrial proteins was assessed. Analogous to the changes in total tissue lysate, the ubiquitin content of isolated mitochondria prepared from TauTKO hearts was significantly elevated (1.5-fold) (Fig. 1b). A common cause of excessive accumulation of ubiquitinated proteins in the diseased heart is impaired proteasome function. Because the β5 subunit of the 26S proteasome functions as the primary protease involved in the degradation of oxidized proteins (Ferrington et al. 2005; Hammerling and Gustafsson 2014), the effect of taurine deficiency on β5 subunit activity was evaluated. As seen in Fig. 1c, the protease activity of the β5 subunit is significantly diminished in the TauTKO heart, suggesting that the accumulation of ubiquitinated proteins

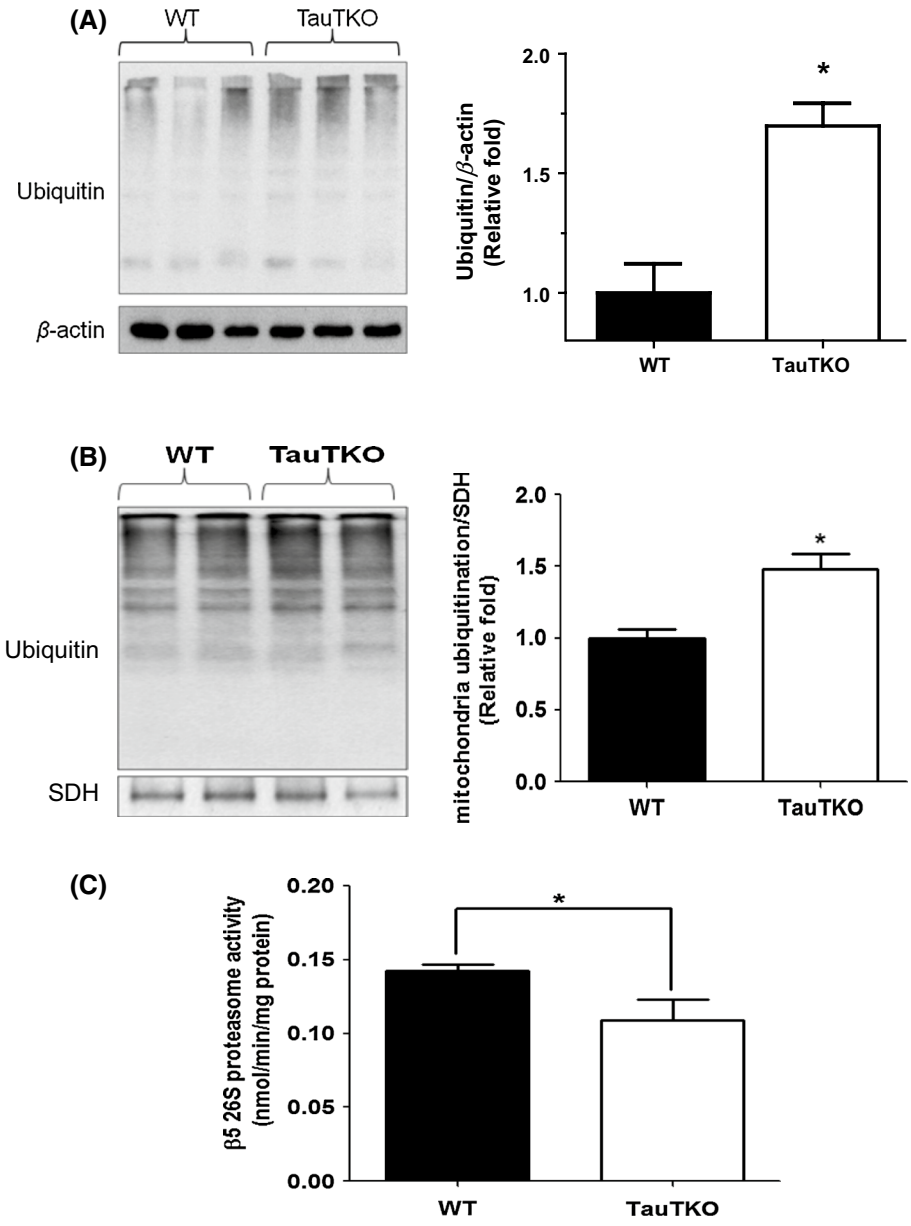

Fig. 1 Effect of taurine deficiency on the UPS. Hearts from taurine transporter knockout (TauTKO) and wild-type (WT) mice were homogenized in RIPA lysis buffer. **a** Total lysates, which were prepared following centrifugation of the homogenate, were subjected to SDS-PAGE and then transferred to nitrocellulose membrane for Western blot analyses. Shown in the *left hand panel* is a representative Western blot of ubiquitinated proteins and the β-actin loading control. Values shown in the *right hand panel* represent means ± SEM of 6–9 different preparations. **b** Isolated mitochondria were prepared from the homogenate. In the *left hand panel* are representative Western blots for ubiquitinated proteins of wild-type and TauTKO heart mitochondria. Values shown in the *right hand panel* represent means ± SEM of 6–9 different preparations. **c** ATP-dependent 26S β5 proteasome activity of wild-type and TauTKO heart lysates using a β5 fluorescent substrate. Values shown represent means ± SEM of 4–5 different preparations. *Asterisks* denote significant differences between the wild-type and TauTKO heart samples ($p < 0.05$). Taurine deficiency was associated with an elevation in ubiquitinated protein in total heart lysates and mitochondria, an effect related to a significant decrease in 26S β5 proteasome activity

is mediated in part by a decrease in proteolytic activity of the proteasome.

Taurine depletion initiates autophagy but decreases autophagolysosome formation and degradation

Autophagy plays an important role in the degradation of organelles and protein aggregates. Therefore, in the absence of robust removal of damaged proteins by the UPS, autophagy is activated. To determine whether the beginning steps of autophagy are initiated in the TauTKO heart, the levels of Beclin-1, which is an initiator of autophagosomal membrane formation, and LC3-II, a protein that binds to the autophagosomal membrane and is commonly used as a marker of autophagy, were examined in TauTKO and wild-type mice. In total lysates of the TauTKO heart, the levels of both Beclin1 and LC3-II were significantly elevated relative to those of the wild-type heart lysates (Fig. 2a, b). While these data support the idea that taurine deficiency might induce the formation of the autophagosome, the

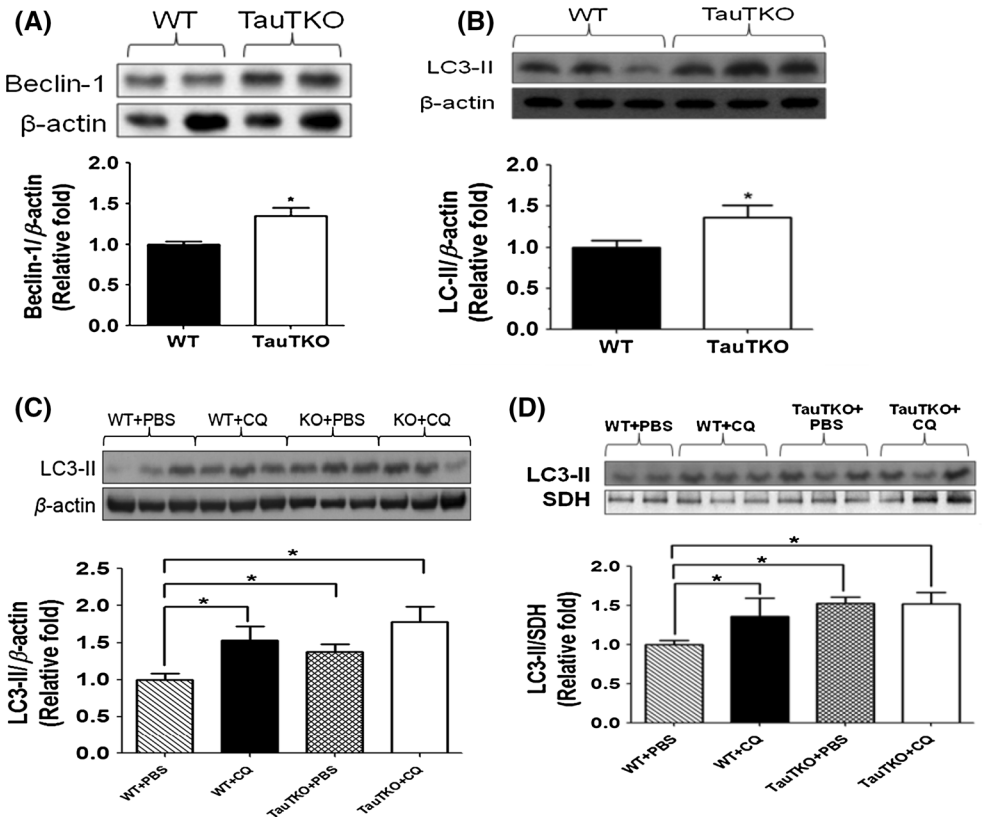

Fig. 2 Taurine deficiency induces autophagy but downstream steps in the degradation process are defective. In **a** and **b**, whole heart lysates and isolated mitochondria were prepared from wild-type and TauTKO hearts. In **c** and **d**, whole heart lysates and isolated mitochondria were prepared from wild-type and TauTKO hearts of mice treated with either a PBS vehicle or chloroquine. **a** Shown in the *upper panel* are representative Western blots of Beclin-1 and the β-actin loading control of hearts obtained from wild-type or TauTKO mice. Values shown in the *lower panel* represent means ± SEM of 6–9 different preparations. The *asterisk* denotes significant difference between wild-type and TauTKO hearts ($p < 0.05$). Taurine deficiency leads to a significant increase in Beclin-1 content. **b** Shown in the *upper panel* are representative Western blots of LC3-II and the β-actin loading control of hearts obtained from wild-type or TauTKO mice. Values shown in the *lower panel* represent means ± SEM of 6–9 different preparations. *Asterisks* denote significant difference between hearts of wild-type and TauTKO mice ($p < 0.05$). **c** Shown in the *upper panel* are representative Western blots of LC3-II and the β-actin loading control of hearts obtained from wild-type or TauTKO mice that were treated with either a PBS vehicle or chloroquine. Values shown in the *lower panel* represent means ± SEM of 4–6 different preparations. *Asterisks* denote significant difference between the wild-type control and the other three groups. Taurine deficiency was associated with an increase in LC3-II (autophagosome) levels of heart lysates but treatment with the lysosome inhibitor, chloroquine, did not further elevate LC3-II levels. Chloroquine treatment increased LC3-II levels of heart lysates obtained from wild-type mice. **d** Shown in the *upper panel* are representative Western blots of LC3-II and the succinate dehydrogenase (SDH) loading control of heart mitochondria obtained from wild-type or TauTKO mice that were treated with either a PBS vehicle or chloroquine. Values shown in the *lower panel* represent means ± SEM of 6–9 different preparations. *Asterisks* denote significant difference between the wild-type control and the other three groups ($p < 0.05$). Taurine deficiency was associated with an increase in LC3-II (autophagosome) levels of heart mitochondria but treatment with the lysosome inhibitor, chloroquine, did not further elevate LC3-II levels. Chloroquine treatment increased LC3-II levels of heart mitochondria obtained from wild-type mice

actual degradation of macromolecules and organelles by autophagy is mediated by autophagolysosomes, which are formed from the merger of an autophagosome with a lysosome (Mizushima et al. 2010). Because the formation of active autophagolysosomes appears to be a limiting step in the process of autophagy, inhibition of autophagolysosome formation elevates the levels of autophagosomes, revealing that the increase in autophagosome levels in the TauTKO heart might arise from either enhanced autophagosome formation or inhibition of autophagolysosome formation and activation. To establish the cause of autophagosome accumulation in the TauTKO heart, the effect of chloroquine, a lysosomal inhibitor, on autophagosome (LC3-II) content was examined (Iwai-Kanai et al. 2008). As seen in Fig. 2c, chloroquine treatment significantly increased LC3-II levels of the wild-type heart but not that of the TauTKO heart. A similar pattern was noted for the effect of taurine deficiency on mitochondria-associated LC3-II, as autophagosome levels rose in wild-type mitochondria but not in TauTKO mitochondria (Fig. 2d). Moreover, chloroquine treatment increased LC3-II content of the heart of the wild-type mouse but not that of the TauTKO heart. The failure of LC3-II levels to rise in hearts of TauTKO mice following chloroquine treatment is attributed to the defect in autophagolysosome formation and activation in the TauTKO heart. Together, these data suggest that the accumulation of autophagosomes in the TauTKO heart and mitochondria is caused by the existence of a bottleneck in the autophagic pathway that restricts active autophagolysosome formation and leads to reduced autophagy flux. This bottleneck acts on the same limiting step as chloroquine treatment; therefore, the lysosome inhibitor is incapable of affecting autophagosome content of the TauTKO heart.

Mitophagy is a unique process in which mitochondria are specifically targeted to the autophagosome for degradation. There are several factors that assist the cell in targeting damaged mitochondria for mitophagy, including oxidative stress, enhanced mitochondrial fission and upregulation of the adapter protein, Parkin (Scherz-Shouval et al. 2007; Narendra et al. 2008; Twig et al. 2008). Although we have previously reported that mitochondria of taurine-depleted cells exist in an oxidatively stressed state (Jong et al. 2012), it remains to be determined if damaged mitochondria of the taurine-deficient heart are capable of undergoing fission, a change in morphology that could promote the formation of mitochondria-associated autophagosomes. Because fission and fusion are difficult to detect in the intact heart, we examined mitochondrial fission in taurine-deficient neonatal cardiomyocytes; taurine deficiency was produced by exposing the cells to medium containing the taurine transport inhibitor, β-alanine (Jong et al. 2012). Control and β-alanine-treated cardiomyocytes were loaded with the mitochondrial marker, Mitotracker Deep Red, which was capable of providing images for both the shape and size of the mitochondria. As shown in Fig. 3a, a 45 % decrease in cardiomyocyte taurine content led to mitochondrial fragmentation, as evidenced by the presence of small, circular-shaped mitochondria, which contrasts with mitochondria of controls cells which are elongated and tubular. These findings raise the possibility that mitochondrial fission in the taurine-deficient cardiomyocyte might assist in the formation of mitochondria-associated autophagosomes.

According to Twig and Shirihai (2011), mitochondrial fission, coupled with cleavage of the mitochondrial fusion protein, Opa1, signals a deterioration of the mitochondria. As seen in Fig. 3b, the levels of Opa1 are reduced in the taurine-deficient heart, a finding consistent with the observation of Chen et al. (2012), who reported that a reduction in Opa1 favors mitochondrial fission. It also is consistent with the view that the taurine-deficient heart exists in a deteriorated state that favors the initiation of autophagy.

The adapter protein, Parkin, also plays a central role in the recruitment of mitochondrial mitophagy. Among its actions are the regulation of mitochondrial fission–fusion and the ubiquitination of mitochondrial proteins, which together not only are indispensable for PINK1-mediated neuronal mitophagy but also determine the magnitude of mitophagy in other cell types (Bingol et al. 2014; Ordureau et al. 2014; Scarffe et al. 2014). However, in contrast to the other regulators of mitochondria targeting, the levels of Parkin are decreased in the TauTKO heart (Fig. 3c), suggesting that fewer mitochondria are likely being recruited for degradation in the taurine-deficient heart. Collectively, these data suggest that while some mitochondria are targeted for mitophagy in the TauTKO heart, a defect in Parkin expression and autophagolysosome turnover limit the removal of damaged mitochondria via mitophagy.

Mitochondrial oxidative stress induces protein ubiquitination and autophagy

Taurine deficiency causes mitochondrial dysfunction, resulting in enhanced generation of superoxide by the respiratory chain (Jong et al. 2012). While ROS can contribute to cellular damage, they can also serve as important signaling agents. Generally, cell damage is associated with high rates of ROS generation while lower rates of ROS generation are commonly associated with cytoprotective events, such as cell signaling and activation of favorable enzymes. However, oxidative cell damage can also initiate quality control mechanisms to remove damaged macromolecules and restore normal cell function. Among the quality control mechanisms activated by oxidative stress is autophagy, which is known to revitalize cells by reducing the number of damaged macromolecules and organelles (Scherz-Shouval et al. 2007; Shang and Taylor 2011). Thus, ROS

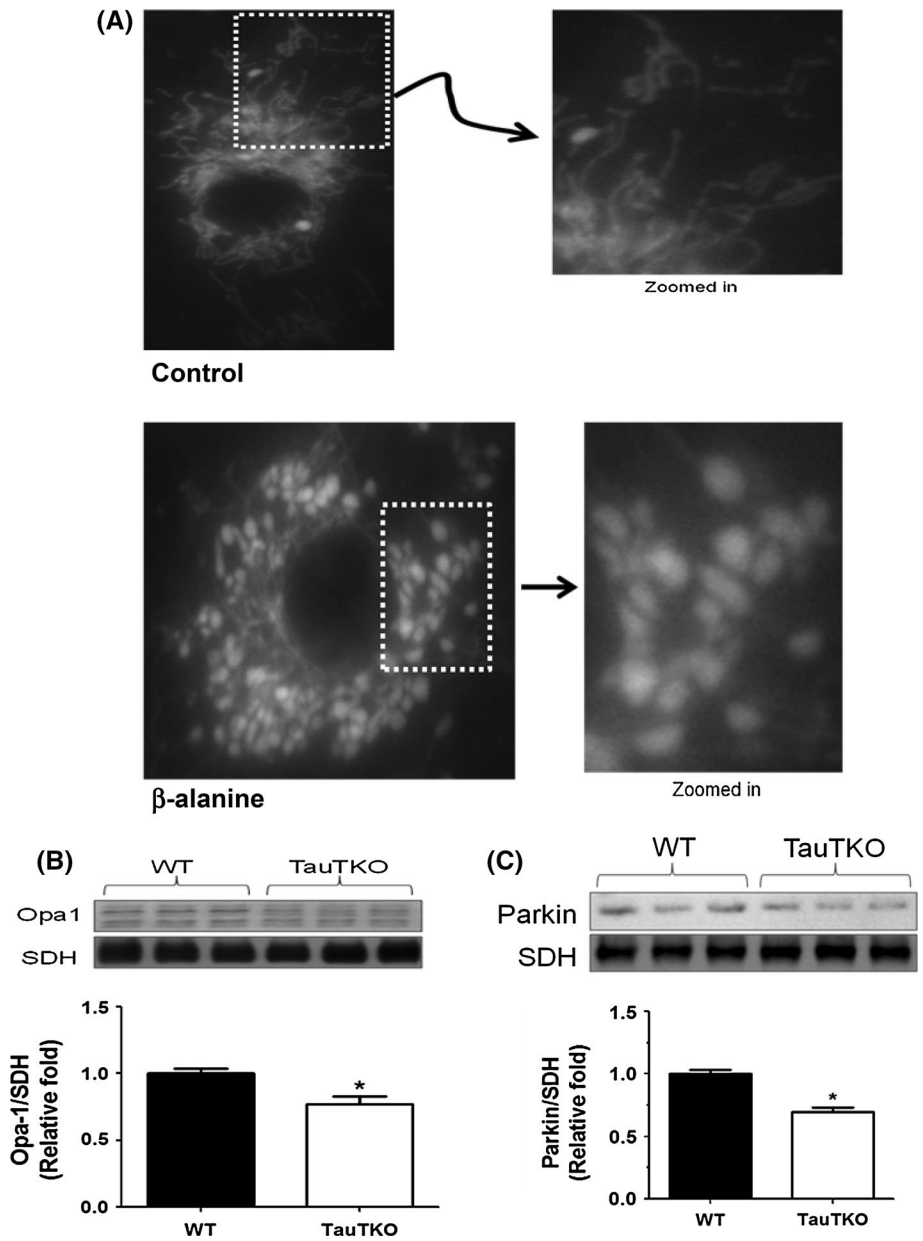

Fig. 3 Taurine deficiency induces mitochondrial fission. **a** Rat neonatal cardiomyocytes were incubated for 48 h with either normal serum substitute medium (SSM) or SSM supplemented with either 0 mM (Control) or 5 mM β-alanine, a taurine transporter inhibitor. Cardiomyocytes in culture were stained with MitoTracker Deep Red and morphology examined by fluorescence microscopy. While the mitochondria of the control cells were elongated and tubular, the taurine-deficient cells (β-alanine group) were small and circular. The data shown are representative images from 4 different preparations. The zoomed in images are the magnified images of each *boxed-off area*. **b** Isolated mitochondria of wild-type and TauTKO hearts were prepared by centrifugation as described in the "Methods" section. The *upper panel* contains representative Western blots for Opa1 and the succinate dehydrogenase (SDH) loading control of mitochondria prepared from wild-type and TauTKO hearts. The *lower panel* represents mean ± SEM for 6–9 different preparations. The *asterisk* denotes a significant difference between Opa1 content of Wild-type and TauTKO mitochondria ($p < 0.05$). Taurine deficiency is associated with a reduction in mitochondrial Opa1 levels. **c** The *upper panel* contains representative Western blots for Parkin and the SDH loading control of mitochondria obtained from wild-type and TauTKO hearts. The *lower panel* represents mean ± SEM for 6–9 different preparations. The *asterisk* denotes a significant difference between the normalized Parkin content of wild-type and TauTKO mitochondria ($p < 0.05$). Taurine deficiency is associated with a decrease in normalized Parkin content

can both damage the cell and correct the source of damage. To provide insight into the crosstalk between mitochondrial oxidative stress and the need to activate autophagy as part of quality control, TauTKO hearts were treated with the mitochondria-specific antioxidant, mitoTEMPO. Figure 4a shows that mitoTEMPO suppresses the increase in

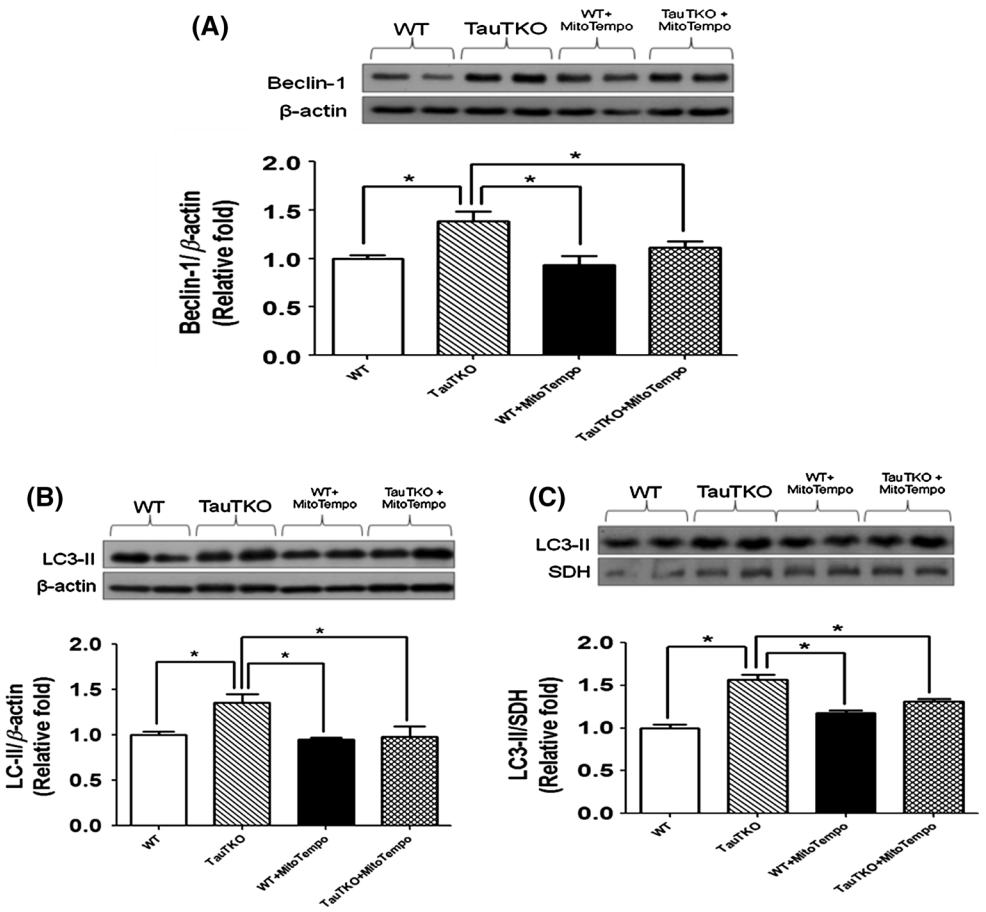

Fig. 4 MitoTEMPO treatment suppresses autophagy in taurine-deficient hearts. Wild-type and TauTKO mice were administered vehicle (PBS) and mitoTEMPO for 7 consecutive days. Hearts were then removed and whole lysates or isolated mitochondria were prepared. **a** Shown in the *upper panel* are representative Western blots of Beclin-1 and the β-actin loading control of whole heart lysates of treated and untreated wild-type and TauTKO mice. Values shown in the *lower panel* represent means ± SEM of 4–6 different preparations. The *asterisks* denote significant differences between the untreated wild-type and untreated TauTKO hearts, as well as between the untreated TauTKO heart and the two mitoTEMPO-treated groups ($p < 0.05$). Taurine deficiency increased normalized Beclin-1 content, an effect blocked by treatment of the TauTKO mice with mitoTEMPO. MitoTEMPO treatment had no effect on normalized Beclin-1 content of the wild-type heart. **b** Shown in the *upper panel* are representative Western blots of LC3-II and the β-actin loading control of whole heart lysates of treated and untreated wild-type and TauTKO mice. Values shown in the *lower panel* represent means ± SEM of 4–6 different preparations. The *asterisks* denote significant differences between the untreated wild-type and untreated TauTKO hearts, as well as between the untreated TauTKO heart and the two mitoTEMPO-treated groups ($p < 0.05$). Taurine deficiency increased normalized LC3-II content, an effect blocked by treatment of the TauTKO mice with mitoTEMPO. MitoTEMPO treatment had no effect on normalized LC3-II content of the wild-type heart. **c** Shown in the *upper panel* are representative Western blots of LC3-II and the succinate dehydrogenase (SDH) loading control of isolated heart mitochondria obtained from treated and untreated wild-type and TauTKO mice. Values shown in the *lower panel* represent means ± SEM of 6–9 different preparations. The *asterisks* denote significant differences between the untreated wild-type and untreated TauTKO hearts, as well as between the untreated TauTKO heart and the two mitoTEMPO-treated groups ($p < 0.05$). Taurine deficiency increased normalized LC3-II content, an effect blocked by treatment of the TauTKO mice with mitoTEMPO. MitoTEMPO treatment had no effect on normalized LC3-II content of the wild-type heart

cellular Beclin-1 levels of the TauTKO heart, supporting the view that oxidative stress plays a role in the initiation of autophagy in the TauTKO heart. Also supporting this view is the observation that mitoTEMPO treatment of TauTKO mice attenuates the increase in the autophagosome marker, LC3-II, in both the cell lysate and isolated mitochondria (Fig. 4b, c).

Oxidative stress is a major cause of protein damage through the oxidation of specific amino acids, carbonylation, fragmentation, glycoxidation or disulfide bridge formation (Hohn et al. 2014). To evaluate the effect of mitochondrial oxidative stress on the accumulation of ubiquitinated, damaged proteins, TauTKO and wild-type mice were treated with the mitochondria-specific antioxidant, mitoTEMPO. Figure 5a, b shows that taurine deficiency

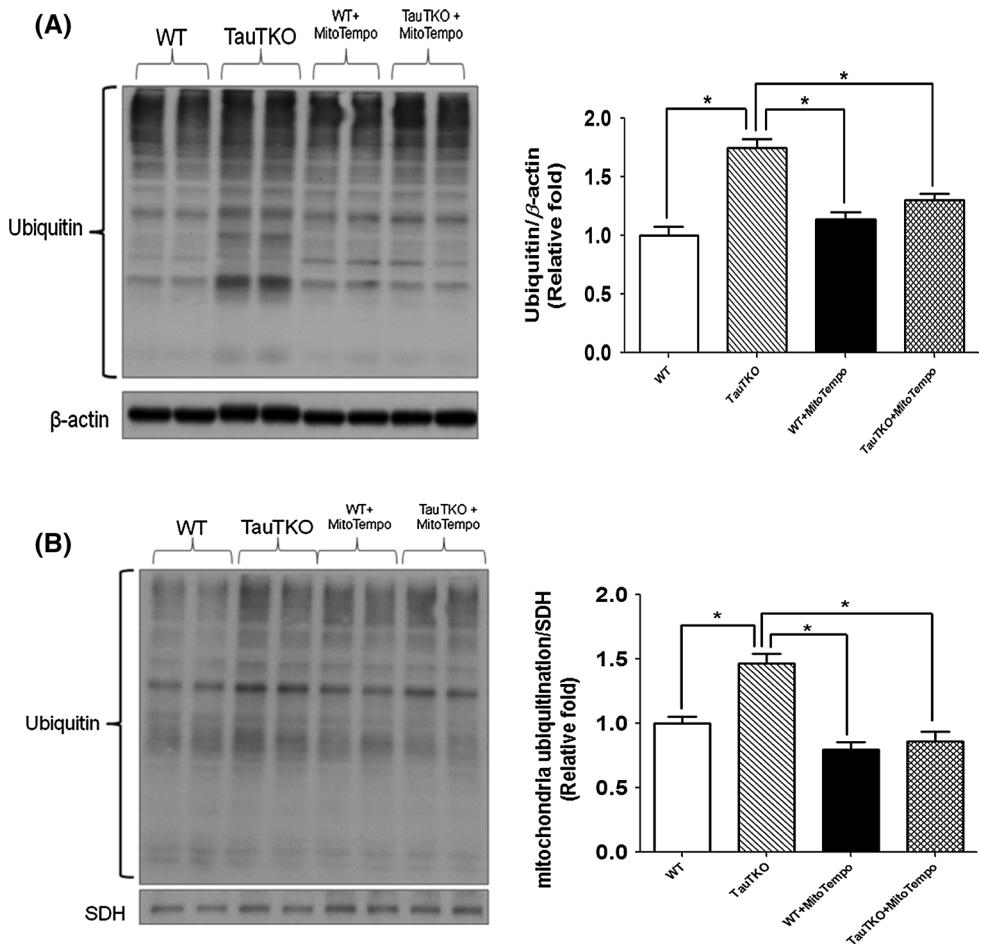

Fig. 5 Treatment of taurine-deficient hearts with the mitochondria-specific antioxidant, mitoTEMPO, reduces the degree of protein ubiquitination. Wild-type and TauTKO mice were administered vehicle (PBS) and mitoTEMPO for 7 consecutive days. Hearts were then removed and whole lysates or isolated mitochondria were prepared. **a** Total lysates of the four groups of hearts (untreated wild-type, untreated TauTKO, treated wild-type and treated TauTKO) were subjected to SDS-PAGE and then transferred to nitrocellulose membrane for Western blot analyses. Shown in the *left hand panel* are representative Western blots of ubiquitinated proteins and the β-actin loading control for the four groups of hearts. Values shown in the *right hand panel* represent means ± SEM of 6–9 different preparations. *Asterisks* denote a significant difference between the untreated wild-type and untreated TauTKO lysates, as well as between the untreated TauTKO and the two treated groups ($p < 0.05$). MitoTEMPO treatment significantly reduced the increase in ubiquitin protein content. **b** Isolated mitochondria were prepared from the homogenate. In the *left hand panel* are representative Western blots of ubiquitinated proteins of wild-type and TauTKO heart mitochondria and their treatment cohorts. Values shown in the *right hand panel* represent means ± SEM of 6–9 different preparations. *Asterisks* denote significant differences between the untreated wild-type and untreated TauTKO groups, as well as between the untreated TauTKO group and the two mitoTEMPO-treated groups ($p < 0.05$). Taurine deficiency increases the levels of ubiquitinated proteins in the mitochondria, an effect prevented by treatment of the TauTKO mouse with the mitochondria-specific antioxidant, mitoTEMPO

increases the content of ubiquitinated proteins in both the cell lysate and mitochondria, but the treatment with mitoTEMPO normalized the levels in the TauTKO heart as well as in the mitochondrial fraction. Interestingly, more ubiquitinated protein bands were present in the gels loaded with the TauTKO heart samples than in the wild-type heart samples, suggesting that taurine deficiency might not only increase the amount of a specific ubiquitinated protein but might actually uncover new protein targets for ubiquitination. Treatment of TauTKO mice with mitoTEMPO decreased the levels of all ubiquitinated proteins in the TauTKO heart samples, eliminating differences between the taurine-deficient and wild-type heart samples (Fig. 5a).

Discussion

Taurine plays a fundamental role in the mitochondria, where it is required for normal respiration, ATP generation and superoxide output (Jong et al. 2012; Mozaffari et al.

1986). The tissues most sensitive to taurine deficiency are those with high energy demand. Based on this criterion, it is not surprising that taurine deficiency is associated with the development of cardiomyopathy, neuropathy, retinopathy and developmental defects (Knopf et al. 1978; Sturman et al. 1985; Schuller-Levis et al. 1990; Sturman and Messing 1992; Ito et al. 2008). Recently, it has been proposed that the taurine-deficient phenotype resembles that of certain mitochondrial diseases, not only because mitochondrial dysfunction is the major defect in both phenotypes but also because they share common symptoms (Schaffer et al. 2013, 2014).

Mitochondria play a critical role in cell survival and normal cellular function; therefore, cells have evolved vibrant quality control mechanisms to degrade abnormal mitochondria and restore mitochondrial function. In the heart, autophagy and UPS are the major mediators of quality control, where they revitalize the cardiomyocytes and prolong their lifespan. The present study reveals that while taurine depletion-mediated mitochondrial oxidative stress induces the initial stages of UPS and autophagy, the downstream degradation processes are dysfunctional in the taurine-deficient heart, severely restricting the effectiveness of the two quality control processes. As a result, the taurine-deficient cardiomyocyte accumulates both ubiquitinated proteins and damaged mitochondria, a condition associated with development of cardiomyopathy. These defects appear to be related in part to excessive generation of ROS by the mitochondria, as mitochondrial levels of ubiquitinated proteins, Beclin-1 and autophagosomes of the TauTKO mouse are restored to normal by treatment with the mitochondrial antioxidant, mitoTEMPO.

Although taurine is approved for the treatment of congestive heart failure in Japan, the mechanism underlying the development of taurine-deficient cardiomyopathy differs from that of classical congestive heart failure, in which the initial insult is a reduction in cardiac output that triggers a series of events that lead to compensatory hypertrophy. An important early event in this cascade is activation of the baroreceptors, which raise plasma catecholamine levels. Another neurohumoral agent that is elevated early in the hypertrophic cascade is angiotensin II, which like the catecholamines, promotes the generation of ROS. Although ROS are capable of damaging macromolecules, they also serve as signaling agents to stimulate protein synthesis. Also contributing to the development of myocardial hypertrophy is an increase in blood volume, which elevates venous return, causing increased cardiomyocyte stretching and stimulation of stretch-related hypertrophic cascades. Further remodeling and cardiomyocyte death lead to additional muscle weakness and eventually the onset of overt congestive heart failure. In contrast to this classic scenario, the taurine-deficient cardiomyocyte does not undergo hypertrophy (Schaffer et al. 1998; Ito et al. 2008). Despite the dilation of the chamber of the taurine-deficient heart, the ventricles themselves undergo atrophy (Ito et al. 2008). Although the focus of the present study was the evaluation of the quality control mechanisms in the taurine-deficient heart, it is interesting that elevations in cytosolic ROS from angiotensin II and catecholamine signaling contribute to the development of cardiac hypertrophy in classical congestive heart failure while the atrophic, taurine-deficient heart is closely associated with enhanced mitochondrial ROS generation.

It is widely recognized that atrophy arises when an imbalance develops between the biosynthesis of proteins and organelles and their degradation. In the normal heart, the UPS accounts for 80–90 % of intracellular protein degradation, including that associated with normal protein turnover as well as the removal of damaged proteins from the cardiomyocyte. Another important function of the UPS is the degradation of regulatory proteins that modulate cellular function. In the taurine-deficient heart, ubiquitinated proteins accumulate in both the mitochondria and the intact cardiomyocyte. The ubiquitination of targeted proteins involves a three-step process (Shang and Taylor 2011), with the first step leading to the formation of a covalent linkage between ubiquitin and an E1 protein (E1 activity). Ubiquitin is subsequently transferred from E1 to an ubiquitin carrier protein, E2 (E2 activity), which then transfers ubiquitin to a lysine residue of the targeted protein substrate (E3 ligase activity). Following polyubiquitination of the targeted protein, the damaged protein is degraded by the 26S proteasome complex (Shang and Taylor 2011). Although taurine deficiency has no effect on atrogin-1 or MuRF1, which are two of the important E3 ligases (data not shown), the levels of ubiquitinated proteins are significantly elevated in the TauTKO heart.

Based on the data obtained in the present study, a mechanism that stands out as a likely cause for the elevation of ubiquitinated proteins in the TauTKO heart is ROS-mediated depression of UPS function. Jong et al. (2012) have previously shown that ROS generation by the mitochondria is enhanced in the taurine-deficient heart, a change shown in the present study to be associated with the accumulation of ubiquitinated proteins. Treatment of TauTKO mice with the mitochondria-specific antioxidant, mitoTEMPO, reduces the level of ubiquitinated proteins to that seen in the wild-type mouse. Two factors could contribute to these changes. It is widely recognized that ROS can interfere with proper protein folding, which often produces improperly cross-linked proteins and protein aggregates that are difficult to digest. Second, there is evidence that ROS can alter the function of the 26S proteasome or one of its basic units, which include a 20S catalytic core and two 19S regulatory units (Powell 2006). The 20S catalytic core contains

α- and β-subunits, with the proteolytic activity mainly residing with the β-subunits. In the heart, the β1, β2 and β5 subunits catalyze the cleavage of proteins at acidic, basic and hydrophobic amino acids, respectively (Powell 2006). Among the three β-subunits, the β5 subunit functions as the primary protease for the degradation of oxidized proteins (Ferrington et al. 2005). Several studies have shown that the proteolytic activity of the 26S proteasome can be diminished by changes in either the composition of the 20S catalytic core (Ferrington et al. 2005; Hwang et al. 2007) or by post-translational oxidative modification of the α- or β-subunits (Ishii et al. 2005; Demasi et al. 2003). In the taurine-deficient heart, the activity of the β-subunit of the 26S proteasome is diminished, likely by the generation of ROS by the mitochondria. In addition to the UPS, the heart also contains a pathway of protein degradation, known as the mitochondria-associated degradation (MAD) pathway. Although MAD is stimulated by oxidative stress, damaged proteins accumulate in the TauTKO heart, suggesting that the MAD pathway must play a minor role in the removal of damaged proteins in the TauTKO heart. Also, the mitochondrial matrix, which is subject to extensive quality control, contains AAA proteases that remove proteins damaged by ROS (Hammerling and Gustafsson 2014). Particularly noteworthy in the present study is the observation that mitoTEMPO treatment normalizes ubiquitinated protein content, suggesting that ROS inhibits protein degradation, a property of the UPS, but not that of the MAD pathway (Taylor and Rutter 2011; Voigt et al. 2013; Reyskens and Essop 2014; Segref et al. 2014). Together, the data suggest that taurine deficiency and mitochondrial oxidative stress are associated with a reduction in protein degradation accompanied by an elevation in the levels of ubiquitinated protein.

Taurine deficiency is also associated with a defect in the biosynthesis of certain proteins, in particular that of ND6, whose mRNA contains 8 UUG codons (Kirino et al. 2004; Jong et al. 2012; Schaffer et al. 2014). According to Jong et al. (2012), a significant reduction in mitochondrial taurine levels diminishes UUG decoding, reducing the expression of ND6 and other UUG-dependent proteins. Because ND6 is a key subunit of complex I, taurine deficiency leads to reduced cellular respiration secondary to impaired complex I activity. The resulting defect in complex I also results in the diversion of electrons to oxygen, increasing superoxide production. Thus, taurine deficiency fits the stereotype of a common mitochondrial stressor that causes morphological and functional defects. Although the effect of taurine deficiency on cytosolic translation has not been examined, it is significant that taurine deficiency reportedly affects ER stress, which among other actions can suppress protein biosynthesis (Naidoo 2009). This suppression of protein biosynthesis is presumably of sufficient magnitude to allow a shift in favor of proteolysis, a key property of the atrophic heart (Razeghi et al. 2003). This pattern is consistent with that found in MELAS, in which both UPS and protein biosynthesis appear to be defective (Segref et al. 2014).

Another important finding of the present study is that autophagy, which degrades organelles and protein aggregates, is initiated in the taurine-deficient heart but downstream degradation activity subsequently slows flux through the autophagy pathway. The initial phase of autophagy focuses on the formation of the autophagosome from rough endoplasmic reticulum, a process that involves 3 stages, initiation, nucleation and expansion (Dunn 1990). The maturation of the autophagosome is mediated by microtubule-associated light chain 3 (LC3), which moves from the cytosol to the autophagosome, where it conjugates with a lipid in the autophagosomal membrane producing LC3-II; LC3-II often serves as a marker of the autophagosome (Mizushima et al. 2010). The next phase in autophagy is the merger of the autophagosome with a lysosome to form an autophagolysosome, which is responsible for degrading targeted proteins and organelles (Mizushima et al. 2010).

A common target for autophagy in the heart is the mitochondria, which are required for cardiomyocyte viability and function. While the proteasome, MAD pathway and the AAA proteases assume important roles in removing mitochondrial proteins when damage is modest, autophagy assumes a key role in removing entire mitochondria during periods of extensive or prolonged damage. Two pathways are known to regulate autophagy, the PINK1/Parkin and the Nix/BNIP3 pathways (Hammerling and Gustafsson 2014). In the PINK1/Parkin pathway, PINK1 (PTEN-induced putative kinase 1) serves as a sensor of mitochondrial damage. In response to damage, it accumulates on the outer mitochondrial membrane, where it recruits Parkin from the cytosol. Parkin, which is activated by PINK1, is an E3 ligase that ubiquitinates several outer mitochondrial membrane proteins of dysfunctional mitochondria, a process that initiates mitophagy or mitochondrial degradation via autophagy (Ordureau et al. 2014). Kubli et al. (2013) found that mitochondria of Parkin null mice are smaller and more disorganized than those in the wild-type heart. Although mitochondrial and contractile function of the Parkin null heart is fairly normal, Parkin deficiency severely compromises the stressed myocardium, decreasing contractile function and reducing survival. Based on the observation that Parkin-deficient hearts accumulate more damaged, highly ubiquitinated mitochondria when undergoing stress, Kubli et al. (2013) concluded that the PINK1/Parkin pathway assumes a central role in the removal of damaged mitochondria from stressed, but not unstressed, hearts. Because the TauTKO heart is both oxidatively and metabolically stressed, one would anticipate that the PINK1/Parkin pathway should function to actively remove damaged mitochondria. Indeed, Wang et al.

(2012) found that ROS-mediated mitochondrial depolarization promotes PINK/Parkin autophagy. Moreover, oxidative stress promotes the translocation of Beclin-1 to the rough endoplasmic reticulum, where it facilitates autophagosome formation (Perrotta et al. 2011). However, the present study is seemingly inconsistent with a central role for the PINK1/Parkin pathway in the taurine-deficient heart. First, Parkin levels are reduced in the TauTKO heart, a change generally associated with impaired mitophagy. Second, Parkin contains several cysteine residues, which could be susceptible to oxidative modification in the taurine-deficient heart (Wani et al. 2015). Third, the formation and activity of the autophagolysosome appear to be impaired in the TauTKO heart. Therefore, despite the presence of damaged mitochondria and the initiation of autophagy by Beclin-1, the TauTKO heart is unsuccessful in overcoming the block in autophagy at the autophagolysosome step. Finally, the mitochondria of the TauTKO heart are irreversibly damaged, as taurine therapy cannot overcome the bottleneck formed by the downregulation of the taurine transporter. Without restoring normal mitochondrial taurine content, newly generated mitochondria would remain severely impaired.

Nix and BNIP3L are pro-apoptotic proteins that are located on the outer mitochondrial membrane and are capable of interacting with LC3 to modulate mitophagy. The binding of BNIP3 to LC3 decreases the mitophagy process while Nix induces autophagy in response to oxidative stress. It has been proposed that the Nix/BNIP3 pathway assumes a central role under baseline conditions, while the PINK1/Parkin pathway is a key initiator of autophagy in the stressed heart (Hammerling and Gustafsson 2014). Nix/BNIP3 also plays a key role in apoptosis when quality control is defective, as it is in the TauTKO heart. The initiation of apoptosis by BNIP3 is potentiated in the oxidatively stressed heart, which may account for the increase in apoptosis in the TauTKO heart (Lee et al. 2015).

The disruption in cellular clearance is often caused by impaired formation of the autophagolysosome or by aberrant lysosomal activity (Grumati et al. 2010; Masiero and Sandri 2010). The resulting increase in damaged mitochondria and protein worsen the severity of several diseases, including cardiomyopathy and myopathy. Not only do damaged mitochondria elevate the risk of cardiomyocyte death, but they also diminish ATP generation and increase ROS production. Recently, Ramila et al. (2015) reported that the taurine-deficient cardiomyopathy is also associated with sarcoplasmic reticular Ca^{2+} mishandling. The present study raises the possibility that an imbalance in the turnover of proteins, coupled with the accumulation of damaged proteins, may further weaken the TauTKO heart. Thus, defects in both UPS and autophagy increase the risk of severe cardiomyopathy.

In conclusion, we demonstrated that taurine deficiency triggers the induction of the UPS and autophagy but the degradation activity that mediates cellular clearance is defective. We have previously shown that taurine deficiency decreases mitochondrial function and causes oxidative stress (Jong et al. 2012). In the present study, we demonstrated a role for mitochondrial oxidative stress in the induction of the UPS and autophagy. These findings reveal that the activation of cellular quality control processes is an adaptive stress response to mitochondrial oxidative stress rather than the other cellular disturbances associated with taurine deficiency. However, defective downstream degradation activity prevents proper clearance of damaged proteins and organelles, a consequence that causes cell death and likely contributes to the development of cardiomyopathy in the taurine-deficient heart. Therefore, this study has major implications for the treatment of certain mitochondrial diseases.

Compliance with ethical standards

Conflict of interest We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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