Thioredoxin-ASK1 complex levels regulate ROS-mediated p38 MAPK pathway activity in livers of aged and long-lived Snell dwarf mice

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ABSTRACT We have proposed that the age-associated increase of reactive oxygen species (ROS) by electron transport chain (ETC) dysfunction may cause the elevated basal level of p38 MAPK stress response pathway activity. However, the mechanism by which ROS activates this pathway is not clear. Here we propose that activation of the p38 MAPK pathway by complex I (CI) generated ROS, in response to rotenone (ROT) treatment, is based on the ability of reduced Trx to bind to and inhibit ASK1 and its release from the complex upon oxidation. This balance of free vs. bound ASK1 regulates the level of p38 MAPK pathway activity. To support this mechanism we demonstrate that the production of ROS by ROT treated AML12 hepatocyte cells dissociates the Trx-ASK1 complex, thereby increasing p38 MAPK pathway activity. This mechanism is supported by the ability of N-acetyl cysteine (NAC) to prevent dissociation of Trx-ASK1 and activation of the p38 MAPK pathway. We also demonstrated that the ratio of ASK1/Trx-ASK1 increases in aged mouse livers and that this correlates with the increased basal activity of the p38 MAPK pathway. The longevity of Snell dwarf mice has been attributed to their resistance to oxidative stress. A comparison of the levels of Trx-ASK1 in young and aged dwarfs showed a higher abundance of the complex than in their age-matched controls. These results, which are indicative of a decreased level of oxidative stress, suggest that increased ROS production in aged liver may alter the ratio of ASK1 and Trx-ASK1, thereby increasing the age-associated basal level of p38 MAPK pathway activity.—Hsieh, C.-C., Papaconstantinou, J. Thioredoxin-ASK1 complex levels regulate ROS-mediated p38 MAPK pathway activity in livers of aged and long-lived Snell dwarf mice. FASEB J. 20, 259–268 (2006)

Key Words: rotenone · MKK3 kinase · MKP-1 levels · MAPK signaling

The Free Radical Theory of Aging proposes that endogenously produced oxygen radicals (ROS) are a basic cause of the progressive age-associated declines in tissue function, and that oxidative stress generated by extrinsic environmental factors accelerate this decline (1–6). Some of the biochemical characteristics of aged tissues are the consequences of an increase in their pro-oxidant state, and it has been hypothesized that this affects the activity and function of key proteins of signal transduction pathways that regulate stress response (7, 8). Specifically, our past studies have shown that the regulation of translation of C/EBPa and C/EBPβ transcription factors and the increased basal levels of activities of p38 MAPK and SAPK/JNK stress response signaling pathways suggest the development of a state of chronic stress in aged tissues (4, 7–13). However, although these studies suggest an interaction between the increased levels of intrinsic oxidative stress and of stress response signal transduction pathway activities, little is known about the mechanism that links these physiological characteristics of aged tissues.

It has been shown that the reduced form of thioredoxin (Trx) interacts with the N-terminal portion of apoptosis stimulating kinase 1 (ASK1) in vitro and in vivo thereby inhibiting the activity of this serine-threonine kinase of the MKKK family (14). Furthermore, formation of the Trx-ASK1 complex occurs only with reduced Trx. Thus, oxidation of the ASK1 bound Trx by certain oxidants, including ROS, disrupts the complex thus enabling ASK1 activation. It has been proposed, therefore, that reduced Trx is a physiological inhibitor of ASK1, and that its oxidation and release from ASK1 link such cytotoxic stresses as TNF, Fas, and H2O2 to activation of the p38 MAPK and SAPK/JNK stress response pathways (14–16). These studies have focused on the role of cytoplasmic and mitochondrial localized ASK1 on activation of distinct apoptotic pathways induced by TNF, and suggested that Trx2, the mitochondrial isoform of Trx, regulates mitochondrial ASK1 while Trx1 regulates cytoplasmic ASK1-mediated apoptosis via distinct pathways (16). The role of the Trx-ASK1 complex levels in the activation of stress pathways by ROS generated by specific mitochondrial electron transport chain (ETC) dysfunction has not been...
been addressed. Thus, we propose that the regulation of the ratio of ASK1/Trx-ASK1 may be altered by ROS generated by ETC dysfunction and that the association and dissociation of the Trx-ASK1 complex may play a key role in the increased endogenous level of activity of the p38 MAPK pathway in aged mice (9, 10). Based on these studies we propose that mitochondrial ROS may cause a decreased level of Trx-ASK1 complex formation (Fig. 1). This would increase the level and activity of ASK1 and the basal level of activity of the components of the p38 MAPK stress response pathway. To test our hypothesis, we first compared the levels of Trx-ASK1 complex formation and corresponding activity of components of the downstream p38 MAPK pathway in hepatic AML12 cells treated with rotenone (ROT), which is a specific inhibitor of ETC complex I (CI) that generates ROS at this site (17, 18). Second, we determined whether the Trx-ASK1 levels in young vs. aged mouse livers correlate with the increased basal levels of the p38 MAPK pathway in aged mice.

Resistance to oxidative stress is a major factor in longevity determination of nematodes (19–22), Drosophila (23–26), and rodents (27). One of the factors in the extension of life span of Snell dwarf mice has been attributed to their resistance to oxidative stress (28–30). This is supported by the observation that Snell dwarf fibroblasts are significantly more resistant to various oxidative stress-producing factors such as UV light, heavy metal (Cd), H2O2, paraquat, and heat shock (30). In view of this, we compared the levels of Trx-ASK1 in young vs. aged controls to those in age-matched long-lived Snell dwarf mice to determine whether the regulation of ASK1 and downstream p38 MAPK activities are indicative of a decreased level of oxidative stress. In these studies we propose a basic mechanism that attributes the increased basal level of p38 MAPK pathway activity in aged livers to the Trx-mediated regulation of the redox state of the cell. We focus on whether the activity of the p38 MAPK pathway in aged tissue may be attributed to regulation by its upstream activators, i.e., ASK1 and MKK3, whose activities depend on the Trx-ASK1 levels, and whether the Trx-ASK1 levels correlate with the decreased activity in the long-lived Snell dwarf mutant. We propose, therefore, that the mechanism by which the dwarf exhibits characteristics of decreased age-associated oxidative stress may involve the intracellular balance between free ASK1 vs. Trx-ASK1 complex that mediates the level of activity of the stress response p38 MAPK pathway. We demonstrate that ROS produced by the specific dysfunction of ETC CI activates the p38 MAPK pathway via the balance of ASK vs. Trx-ASK levels determined by the redox state of the cells.

**MATERIALS AND METHODS**

**Animals and tissues**

All mice were born and reared in a specific pathogen-free colony at The Jackson Laboratory as described (31). Control males were housed in groups of 1–3 littersmates per cage; dwarf males were also housed 1–3 per cage, with at least one control female to provide warmth. Snell dwarf mice were produced by mating DW/J Pit+/+/ parents with C3H/HeJ Pit+/+ parents to produce the compound mutant DWC3F1 Pit1bw/Pit1bw dwJ dwarf and both wild-type (Pit1+/+) and heterozygote (Pit1bw/+) controls. Since the wild-type and heterozygote controls are phenotypically similar with respect to body weight and longevity, they are collectively designated “+/-” controls.

The animals were killed by decapitation. Liver samples of 3- to 6- and 20- to 23-months-old male control and Snell dwarf mice were harvested, sliced, immediately frozen in liquid nitrogen, and stored at –80°C until use. The number of mice used in this study: 3- to 6-months-old control (n = 6), 5- to 6-months-old Snell dwarf (n = 4), 20- to 23-months-old control (n = 4), 20- to 23-months-old Snell dwarf (n = 6).

**Cell cultures and reagents**

AML12 cells, a nontransformed mouse hepatocyte cell line, were obtained from ATCC (#CRL-2254; Gibco, Grand Island, NY, USA). The cells are maintained in DMEM:F12 (1:1) medium (Gibco) supplemented with 10% fetal bovine serum (HyClone; Logan, UT, USA) and ITS (insulin-transferrin-selenium-X; Gibco). Cells were cultured in 100 mm culture
dishes and treated with 5 μM ROT (Sigma; St. Louis, MO, USA) or pretreated with 20 mM N-acetyl cysteine (NAC; Sigma) when the cell density reached ~80% confluency. The cells were harvested at various time points and the proteins were prepared following the protocols described with our modifications (31). Protease inhibitors and phosphatase inhibitors were added as described (32).

Preparation of liver tissue extracts

Liver cytosolic and nuclear extracts were prepared as described previously (32). Protein concentrations were determined using the Bio-Rad protein assay reagent and bovine serum albumin as a standard (Bio-Rad, Hercules, CA, USA).

Western blot analyses and immunoprecipitation assays

Liver or AML12 cytosolic protein (30 μg) was resolved on a precast 4–20% gradient SDS-PAGE (Cambrex; Rockland, ME, USA) and electrotransferred onto an Immobilon PVDF membrane (Millipore; Bedford, MA, USA). After primary and secondary antibody incubations, the proteins were detected according to the procedures recommended by the manufacturers using the Supersignal kits from Pierce (Rockford, IL, USA). In the cases where the level of phosphorylation was measured, the specific band on the blot was detected using the appropriate antiphospho-antibody first. The same blot was then washed extensively, stripped, and probed with the corresponding antibodies to detect the level of total proteins. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) except for P-p38 MAPK (#9211), MKK3 (#9292), p38 MAPK kinase assay kit (#9820; Cell Signaling Technology, Beverly, MA, USA) and phospho-MAPKAPK2 (MK2; #07-155; Upstate, Charlottesville, VA, USA). The bands detected by antibody or antiphospho-antibody were imaged in a MultiImage Light Cabinet (Alpha Innotech) and the density of the image was measured. The densities of the phosphorylated bands were normalized with the densities of the total protein and the ratios were plotted. To ensure equal loading, the gels were stained after transfer with Gel Code (Pierce, Rockford, IL, USA) and the membranes were stained with Coomassie blue after Supersignal detection.

Immunoprecipitation assays were performed following the procedure provided by the manufacturer (Sigma). Briefly, 200 μg of protein from either individual samples or pooled samples was incubated overnight with 2 μL of antibody at 4°C, and 20 μL of protein A-conjugated agarose beads was added and incubated an additional 2–4 h. The beads were washed four times with PBS, resuspended in sample loading buffer, and boiled for 3–5 min. The supernatant was collected and loaded on a precast 4–20% SDS-PAGE. The gel was blotted onto a PVDF membrane and processed as described for Western blots using Supersignal West Pico detection kit. The level of ATF-2 phosphorylated by p38 MAPK was determined using an antiphospho ATF-2 antibody. The intensity of the phosphorylated ATF-2 band represents the relative p38 MAPK kinase activity precipitated by antiphospho-p38 MAPK antibody. The in vitro MKK3 kinase assay was performed using MKK3 antibody to pull down the protein. The immunoprecipitated MKK3 kinase activity was then determined by its ability to phosphorylate recombinant human p38 MAPK. The level of p38 phosphorylation by MKK3 was determined using antiphospho-p38 antibody.

Statistical analysis

Statistical analyses were performed for age-matched comparisons, the single dependent variable being the Snell dwarf mutant. The normalized values of protein and phosphoprotein bands were analyzed using the 2-tailed test to test the difference in means between two age-matched groups at a significance level of 0.05. The symbols (*) between two bars indicate statistical significance for the values represented by the bars.

RESULTS

Roteneone-mediated ROS production and Trx-ASK1 complex formation in AML12 hepatocytes

The mechanism of activation of the p38 MAPK stress response pathway by TNF-generated ROS involves the oxidation and release of reduced Trx bound to the ASK1 N-terminal domain (33). The dissociation of this complex enables the activation of ASK1 in response to TNF-mediated ROS production (34). There are no studies, however, that link the ROS produced by specific mitochondrial ETC dysfunction to activation of either the p38 MAPK or SAPK/JNK pathways. Rotenone, an inhibitor of ETC complex I (CI) has been shown to activate p38 MAPK activity, but the mechanism of this activation is not fully understood (36). To address this, we examined whether ROT treatment of AML12 hepatocytes in culture would affect the Trx-ASK1 complex level. The data in Fig. 2A show that the amount of Trx coimmunoprecipitated with anti-ASK1 antibody is severely decreased by 30 min after ROT treatment and that the complex appears to reform at 120 min after treatment. To correlate the level of the Trx-ASK1 complex to ROS levels, we treated the cells with N-acetyl cysteine (NAC), an anti-oxidant that decreases ROS. The data in Fig. 2A show that the Trx-ASK1 complex does not dissociate in cells treated with ROT and NAC. Furthermore, the data show an increase in the phosphorylation of Ser189/207 of MKK3/MKK6, which is the downstream substrate for ASK1, in response to ROT treatment (Fig. 2B). This phosphorylation suggests activation of MKK3/MKK6, which is the upstream activator of p38 MAPK. In addition, treatment of the cells with NAC prevented the phosphorylation of Ser189/207-MKK3/MKK6 (Fig. 2B). To demonstrate that the p38 MAPK pathway is activated in

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response to ROT treatment we measured the phosphorylation of Thr<sup>180</sup> and Tyr<sup>182</sup> amino acid residues of the catalytic site of this kinase. The data in Fig. 2C show that the ROT treatment stimulates phosphorylation of those amino acids. Furthermore, treatment of the cells with NAC significantly inhibits catalytic site phosphorylations, which suggests that the ROS activation of p38 MAPK may be mediated via the sequential activation of ASK1 and MKK3/6.

Levels of Trx-ASK1 complex formation in young vs. aged control and Snell dwarf mouse livers

Our previous studies have shown that the basal level of activity of the p38 MAPK pathway is up-regulated in aged mouse livers (9). Furthermore, we proposed that this increased activity may be due to the increased level of ROS production due to mitochondrial dysfunction. In this study we asked whether the constitutive levels of the ASK1-Trx-ASK1 complex correlate with the characteristic basal levels of p38 MAPK activity in aged control and Snell dwarf mouse liver. To address this question, we measured the pool levels of the Trx-ASK1 complex in young vs. aged control and long-lived Snell dwarf mouse livers. Using anti-ASK1 antibody, we measured the level of Trx coimmunoprecipitated with ASK1 and found that the dwarf mouse livers exhibit a much greater pool level of the Trx-ASK1 complex at both ages (Fig. 3A; 15, 37). In view of this result (Fig. 3A), we measured the total ASK1 pool levels in these livers (Fig. 3B). The results show that the level of ASK1 is at least 50% lower in both young and aged dwarf livers compared with their age-matched controls and suggest that the lower abundance of ASK1 in the dwarf livers combined with increased level of Trx-ASK1 complex may establish a lower level of activity of the p38 MAPK pathway. The data in Fig. 3C show that the total Trx pool levels are the same in all controls and dwarfs and that the differences in Trx-ASK1 complex levels are not due to the abundance of Trx (Fig. 3C). The significant differences in the levels of this complex in control and dwarf livers indicate that the ASK1-bound and free Trx may play a key role in the regulation of the p38 MAPK response to ROS generated by mitochondrial dysfunction.

MKK3 kinase activities in Snell dwarf mouse livers

MKK3 is an up stream kinase that activate p38 MAPK by phosphorylating the amino acid residues of the catalytic site of this kinase. The data in Fig. 2C show that the ROT treatment stimulates phosphorylation of those amino acids. Furthermore, treatment of the cells with NAC significantly inhibits catalytic site phosphorylations, which suggests that the ROS activation of p38 MAPK may be mediated via the sequential activation of ASK1 and MKK3/6.
lytic site, i.e., Thr^{180}-Tyr^{182} (Fig. 1; 38,39). Our previous studies have shown that the levels of phosphorylated MKK3 (P-Thr) are elevated in the aged mouse livers (9, 10). To further support our hypothesis that the elevated basal activity of components of the p38 MAPK pathway is a characteristic of aging, we measured MKK3 (Fig. 4A) pool levels and kinase activities (Fig. 4B) in the livers of dwarf mice and their age-matched controls. Our results show that the protein pool levels of MKK3 are relatively similar in young and aged controls and dwarfs. To demonstrate whether MKK3 kinase activities are altered in dwarf mouse livers, we measured the in vitro phosphorylation of recombinant p38 MAPK in young vs. aged control and dwarf liver extracts. The in vitro kinase assays showed that MKK3 activities are significantly lower in dwarf liver extracts compared with their age-matched controls and that the activity increases with age in the controls (Fig. 4B).

**Nuclear p38 MAPK levels and activity in young and aged control and Snell dwarf mouse livers**

Phosphorylation of the p38 MAPK catalytic site amino acid residues is indicative of the activation of this stress response signaling pathway. In previous studies we showed that the p38 MAPK pool levels are not altered in young vs. aged controls and dwarfs (11). Furthermore, these studies showed that basal levels of p38 MAPK phosphorylation and kinase activity are significantly higher in young and aged control livers compared with the age-matched controls (9, 11). We proposed that this may be indicative of a state of chronic oxidative stress in the aged mouse livers due to increased basal level of ROS production by mitochondrial dysfunction (9). These studies also indicated that the basal level of oxidative stress in the dwarf livers is significantly lower than the age-matched controls. Similarly, it has been shown that in nematodes (19–22), *Drosophila* (23–26), and mice (27), decreased levels of oxidative stress are associated with increased life span and suggested that the longevity of the Snell dwarf mouse may be associated with a decreased basal level of oxidative stress (28, 30). Thus, we propose that the activity of the p38 MAPK pathway may be a consequence of the redox state of the cells in young vs. aged control and long-lived Snell dwarf mouse livers. To address this we measured the p38 MAPK protein pool and phosphorylation levels of the P-Thr^{180}-P-Tyr^{182} in the nuclear fractions of the Snell dwarf mouse liver extracts and corresponding age-matched controls.

Upon activation of p38 MAPK, the phosphorylated protein is translocated to the nucleus where it interacts with its targeted transcription factors (38, 39). In these experiments we measured the P-p38 MAPK levels in the nuclear extracts of both control and long-lived Snell dwarf mouse livers. The data show that the nuclear p38 MAPK is ~80% lower in young dwarf livers vs. their age-matched controls, and is ~70% lower in aged dwarf livers as compared with their age matched controls (Fig. 5A). Similarly, there is a significant decrease in the level of P-p38 MAPK in both young and aged dwarfs (Fig. 5B). The pool levels (Fig. 5A) and phosphorylation levels (Fig. 5B) of p38 MAPK are significantly higher in aged controls than in young controls. Thus, since the level of nuclear P-p38 MAPK is indicative of stress activation, these data suggest that the basal level of oxidative stress is significantly lower in young and aged dwarfs compared with their age-matched controls.

**p38 MAPK kinase activity in young and aged Snell dwarf mouse livers**

We previously demonstrated an increase in the level of p38 MAPK-mediated phosphorylation of Thr^{71} of ATF-2 in aged mouse liver nuclei (9). Since ATF-2 is a transcription factor activated by P-p38 MAPK (Fig. 1), we used this reaction as an indicator of basal level stress-associated activity of nuclear P-p38 MAPK in young and aged mice. To demonstrate the p38 MAPK kinase activity, we measured the in vitro phosphorylation of Thr^{71} in recombinant ATF-2 using young vs. aged control and dwarf liver extracts. We immunoprecipitated p38 MAPK from the liver extracts and deter-
mined the level of ATF-2 phosphorylated by p38 MAPK. These analyses showed that the aged control livers have the highest p38 MAPK kinase activity (Fig. 6). Furthermore, the kinase activity in young and aged dwarf livers is lower than in young and aged controls, respectively. These results suggest that the age-associated increase in basal level activity of this stress response kinase is attenuated in both young and aged dwarf livers and that this correlates with the expected decreased activity that might be attributed to the higher levels of Trx-ASK1 complexes in the dwarf mice.

The data in Fig. 7A show that the levels of endogenous ATF-2 are higher in young controls vs. dwarfs and in aged controls vs. dwarfs (Fig. 7A). Furthermore, there is a dramatic ~5-fold increase in abundance of ATF-2 in aged controls and dwarfs vs. their age-matched controls. This increase in abundance between the young vs. aged controls and young vs. aged dwarfs indicates that this is an age-associated characteristic (Fig. 7A). However, the data also suggest a lower level of P-Thr71-ATF-2 in the young and aged dwarfs (Fig. 7B), thus suggesting a decreased activity of the p38 MAPK pathway throughout the life span of the dwarf.

Mitogen-activated protein kinase phosphatase-1 (MKP-1) levels in the nuclei of Snell dwarf mouse livers

MKP-1 is a member of the dual specificity phosphatases that play a crucial role in controlling MAPK enzyme activity by dephosphorylating the Tyr-Thr residues of their catalytic sites (Fig. 1; 40). Because of its role in the
down-regulation of p38 MAPK activity, we measured the nuclear pool levels of MKP-1 in control and Snell dwarf mouse livers (Fig. 8). The results show that the pool levels of MKP-1 are dramatically elevated in the nuclei of both young and aged dwarf livers compared with their age matched controls. These data suggest that the higher level of nuclear MKP-1 may play a role in the decreased level of nuclear p38 MAPK activity and may contribute to the decreased level of stress response in young and aged dwarfs.

**MK2•P-p38•ATF-2•MKP complex formation in the nuclei of Snell dwarf mouse livers**

The mitogen-activated protein kinase activated kinases 2 (MK2) and -3 (Apk3) are downstream enzymes that complex with P-p38 MAPK, and transcription factors in the nucleus (Fig. 1). Under normal conditions, unphosphorylated MK2 and Apk3 are rapidly translocated to the nucleus (41). It has been suggested that MK2 may serve as nuclear import-export carriers of P-p38 MAPK as a part of the nuclear activity of the p38 MAPK signaling process (41–44). Furthermore, it has been postulated that this complex consists of a phosphatase, such as MKP, that upon activation of the transcription factor dephosphorylates the complex, thus initiating its export to the cytoplasm (41–47). Thus, the activity of this nuclear complex may play a role in increased basal level of activation of stress response genes in the aged liver. To address this, we examined whether the pool levels and intracellular localizations of MK2 are altered in the young and aged controls and dwarfs. The data in Fig. 9 show that the intracellular distribution of P-MK2 is ~2-fold higher in young dwarf mouse liver nuclei. On the other hand, the levels of P-MK2 in the cytoplasm are ~2-fold higher in the young controls vs. the age-matched dwarfs whereas the levels in young dwarfs vs.

Figure 8. Nuclear pool levels of MKP-1 in young vs. aged control and Snell dwarf mouse livers. The Western blot below the bar graphs consists of individual samples of control and dwarf liver extracts. Lanes 1–4 represent four groups of mouse livers as described in Fig. 3. *P < 0.05 between controls and dwarfs.

Figure 9. The nucleocytoplasmic distribution of phosphorylated MK2 in nuclear (A), cytoplasmic (B) extracts from young and aged control and Snell dwarf mouse livers. The Western blots beneath each bar graph represent individual samples. Lanes 1–4 represent 4 groups of mouse livers as described in Fig. 3. *P < 0.05 between controls and dwarfs. †P < 0.05 between young controls and aged controls. aged dwarfs are similar (Fig. 9B). A dramatic (~3-fold) decrease in cytoplasmic P-MK2 also occurs in aged controls that is not seen in the nuclear protein pool level, suggesting that the turnover of this protein may be altered. Thus, the increased nuclear level of MK2 suggests that the level of stress is lower in the young dwarf. On the other hand, the decrease in cytoplasmic MK2 is not accounted for in the nuclear levels. This suggests that the nucleocytoplasmic trafficking of MK2 may be altered in the normal aged liver (Fig. 9B).

Our model (Fig. 1) shows that a MK2•P-p38•ATF-2•MKP1 complex is formed in the nucleus in response to a challenge or stress factor. Thus, we would expect an increase in the level of this complex in the nucleus due to the increased basal level of stress in control aged mice. The data in Fig. 10A show the levels of coimmunoprecipitation of P-MK2 (Fig. 10A), P-ATF-2 (Fig. 10B) and MKP-1 (Fig. 10C) by anti-p38 MAPK. These data show an increase, with age, of P-MK2 and MKP-1 in the complex in controls and dwarfs. The higher level of p38•P-MK2 in the aged dwarfs suggests a lower level of stress. Furthermore, the level of complexed P-ATF-2 is decreased in the aged dwarf, suggesting that the activation of this stress response gene is decreased in the dwarf. This further supports our hypothesis that the basal level of stress is lower in the aged dwarf (7, 8). It
is also interesting that an MK2 p38 MAPK MKP-1 complex that is deficient in ATF-2 is highest in the aged dwarf, suggesting that the activation of this stress response complex may be reduced.

**DISCUSSION**

We have proposed a mechanism for the activation of p38 MAPK stress response pathway by ROS generated by ETC dysfunction. ROS generated by mitochondrial ETC dysfunction is a major factor in the development of aging characteristics (48). Although some studies have shown an increase in ROS production in aged tissue arising from ETC dysfunction (35) and others have shown the activation of p38 MAPK and JNK pathways by inhibitors of ETC (36), the coupling of specific sites of ETC ROS to stress response pathway activation remains unclear. Using liver cells in culture, we have shown that the mechanism of activation of the p38 MAPK pathway by mitochondrial ETC ROS production involves the regulation of the level of Trx-ASK1 complex formation and dissociation.

Our proposed mechanism is supported by the response of AML12 cells to ROT and ROT + NAC treatment. For example, ROT, an established inhibitor that generates ROS from ETC CI also activates p38 MAPK in cells in culture (36). Furthermore, the changes in Trx-ASK1 pool levels, activation of MKK5/6 and p38 MAPK, are consistent with our proposed mechanism that ROS from CI dysfunction activates the p38 MAPK pathway via the dissociation of the Trx-ASK1 complex. Our proposed mechanism is further supported by the NAC-mediated prevention of Trx-ASK1 dissociation and activation of the downstream components of the p38 MAPK pathway, i.e., MKK3, p38 MAPK, and ATF2.

Our past studies have shown that aging tissues develop a state of chronic stress and that this occurs in the absence of extrinsic challenges (4, 7, 8). This is indicated by the increased basal levels of (a) stress response genes, e.g., the acute phase response genes; (b) the transcription factors that target these genes in aged tissues (7, 8); and (c) the demonstration that the basal levels of the p38 MAPK signaling pathways are also up-regulated with age (9, 10). Thus, we proposed that the mechanism of the age-associated increase in activity of stress response genes involves the stabilization of the elevated basal level of activity of the p38 MAPK pathway and that the sustained and increased level of activity of these stress response pathways may be due to the alteration of the ASK1/Trx-ASK1 ratio in response to chronic increase in ROS production (Fig. 11). Our present studies with cells in culture thus suggest that ROS generated by ETC dysfunction may play a role in

**Figure 10.** Analysis of the composition of the MK2 p38 MAPK·MKP-1 complex in young vs. aged control and Snell dwarf mice. A) Levels of phosphorylated Thr334–MK2 and B) of Thr71–ATF-2 communoprecipitated by anti-p38 MAPK. C) Levels of MKP1 communoprecipitated by anti-p38 MAPK. Western blots of the proteins communoprecipitated by anti-p38 MAPK are shown beneath each bar graph. IP, immunoprecipitation with anti-p38 MAPK; WB, Western blot analysis of communoprecipitated A) p-MK2; B) P-ATF-2; C) MKP1 with their respective antibodies. The same 4 groups of mouse livers were used as in Fig. 3, except each lane represents pooled samples of 4–6 livers.

**Figure 11.** Regulation of ROS-mediated activation of the p38 MAPK stress response signaling pathway. The pathway shows the mechanism that links the rotenone-mediated production of ROS by mitochondrial ETC Complex 1 to the activation of the p38 MAPK and SAPK/JNK stress response pathways. The model suggests that the Trx-ASK1 complex plays a key role in regulation of the p38 MAPK and SAPK/JNK stress response signaling pathways.
the mechanism of increased endogenous activity of p38 MAPK stress signaling pathway in the aged liver. We attribute this to the regulation of the ratio of ASK1/Trx-ASK1. Our studies suggest that the level of the Trx-ASK1 complex, which inhibits ASK1 activity, decreases with age and that this may be a mechanism by which the elevated level of uncomplexed ASK1 responds to increased endogenous levels of ROS production in aged tissue. We propose this as a basic mechanism that causes the increased basal level of activity of p38 MAPK pathway (Fig. 11; ref 9).

Our hypothesis is also supported by the results with the Snell and Ames dwarf mutants, which have been reported to be resistant to oxidative stress (28, 30). The combined decrease in ASK1 pool levels and corresponding increase in the level of Trx-ASK1 complex may play a role in their resistance to oxidative stress and account for the decreased activity of downstream targets. For example, decreased MKK3 kinase activity, nuclear P-p38, p38 kinase activity, and ATF-2 suggest a sustained lower level of activity associated with the response to oxidative stress in both young and aged dwarf mice.

Recent studies have shown that ASK1 is selectively required for sustained activation of the p38 MAPK and SAPK/JNK pathways induced by oxidative stress (15). This was demonstrated in ASK1(-/-) embryonic fibroblasts in which the TNF- and H2O2-mediated sustained activation of p38 MAPK and JNK is lost. These cells are also resistant to TNF and H2O2-induced apoptosis (15). Thus, the constitutively elevated and sustained p38 MAPK activity in the aged livers may be due to the increased pool level of the uncomplexed ASK1.

The development of resistance to oxidative stress has been proposed to be a factor in development of the longevity of Snell and Ames dwarf mice (28, 30, 49). In this study we identified several characteristics of components of the p38 MAPK pathway that suggest that the stress response signaling pathway is attenuated in the long-lived Snell dwarf mouse. We propose that this may be an indication of their resistance to oxidative stress. Since the p38 MAPK can be activated by ROS generated by ETC dysfunction, the decreased state of oxidative stress in young and aged long-lived Snell dwarf mouse would explain their lower level of basal activity of the p38 MAPK pathway. Thus, the ratio of oxidized and reduced Trx, which determines the activity of both ASK1 and the downstream components of the p38 MAPK pathway suggests a lower level of endogenous oxidative stress in the dwarf mouse.

Our studies raise the question of whether the regulation or elevation of Trx-ASK1 levels due to the redox status of Trx may be a part of the mechanism of resistance to oxidative stress. For example, activation of p38 MAPK in ASK1(-/-) embryonic fibroblasts by TNF and H2O2 is abolished in these cells, which exhibit resistance to TNF- and H2O2-induced apoptosis (15). These results suggest that ASK1 activity is required for the sustained activation of p38 and SAPK/JNK by TNF-induced ROS. Thus, the decreased ASK1 pool level of dwarf mice as well as the elevated level of Trx-ASK1 complex may contribute to the resistance to oxidative stress by this long-lived mouse. Our hypothesis is supported by the observation that Snell dwarf fibroblasts are resistant to oxidative stress generated by UV light, heavy metal (Cd), H2O2, paraquat and heat (30).

Trx can exert its protective functions either directly as an antioxidant by reducing the ability of oxidized Trx peroxidase to scavenge ROS, such as H2O2, or indirectly by binding to signaling components and modulating their functions (50). The complexing of Trx with the N terminus of ASK1 determines the ROS-mediated regulation of p38 MAPK and SAPK/JNK stress response pathways (14). Since the levels of Trx bound to ASK1 are much higher in both young and aged dwarf livers than in their age-matched controls, we speculate that the dwarf livers are in a more reduced state than their age-matched control livers. Furthermore, the ability to maintain a lower level of stress activated p38 MAPK activity, which occurs throughout the life span of the dwarf mutant, suggests that longevity of this mutant may be associated with its redox state. Our studies provide further support of the hypothesis that the age-associated increase in ROS generated by mitochondrial dysfunction may be a major factor in the sustained increase in stress response activity in aged tissue.

This publication was supported by U.S.P.H.S. grant IP01 AG 021830-01 awarded by the National Institute on Aging, and the Sealy Center on Aging, University of Texas Medical Branch. We would like to thank Diane M. Strain for clerical assistance in the preparation of this manuscript.

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