Altered oxidative stress response of the long-lived Snell dwarf mouse

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Abstract

Several single gene mutations in mice that increase the murine life span have been identified, including the Pit-1 mutation which results in the Snell dwarf (Pit1dw/dw), however, the biological mechanism of this life-span extension is still unclear. Based on studies that show oxidative stress plays an important role in the aging process, we hypothesized that the increased longevity seen in Snell dwarf mice may result from a resistance to oxidative stress. We report that Snell dwarf mice respond to oxidative stress induced by 3-NPA differently than their wild type littermates. This altered response results in diminished activation of the MEK–ERK kinase cascade and virtually no phosphorylation of c-Jun at Ser63 in dwarf mice after 3-NPA treatment, despite a robust phosphorylation of Ser63 in wild type mice. We propose that this altered management of oxidative stress in dwarf mice is partially responsible for the increased longevity in Snell dwarf mice.

Keywords: Snell dwarf; Longevity; AP-1; Oxidative stress; 3-NPA; MAP kinase

The ~40% increase in life span of Snell dwarf (Pit1dw/dw) mice is attributed to a mutation in the Pit1 locus that prevents development of anterior pituitary cells that produce growth hormone, thyroid stimulating hormone, and prolactin [1]. In addition, life span in nematodes [2–5], Drosophila [6–9], and rodents [10] has been attributed to increased resistance to oxidative stress. Based on these reports, we initiated these studies to determine whether the long-lived Snell dwarf exhibits characteristics of resistance to oxidative stress induced by 3-nitropropionic acid (3-NPA). 3-NPA is an irreversible inhibitor of succinate dehydrogenase (mitochondrial complex II) that generates free radicals in the liver and other tissues [11,12]. Since 3-NPA initiates reactive oxygen species (ROS) production we used sublethal doses of this inhibitor to generate a state of oxidative stress and to investigate the response of the dwarf’s stress response signaling pathways to 3-NPA. Initial microarray studies on 3-NPA treated dwarf and wild type livers suggested that the AP-1 transcription family might play a role in this differential response to 3-NPA (in preparation).

Regulation of AP-1 activity involves both translational and post-translational control of its various members including c-Jun, the most potent transcriptional activator in the Jun family, and c-Fos [13]. AP-1 activity is regulated by a variety of stimuli, including oxidative stress that signals through the mitogen-activated protein kinase (MAPK) cascades leading to ERK, JNK, and p38 (Fig. 1) [14,15]. The regulation of c-Jun is of particular interest due to the role of phosphorylation at Ser63 and Ser73 on regulation of stress-induced apoptosis and cellular proliferation [16]. It has been shown that phosphorylation of these serine residues may be required for the enhancement of transcriptional activity of c-Jun mediated by the JNK 1/2, p38, and ERK 1/2 MAP kinase cascades [17–20]. More recent reports indicate that in response to oxidative stress, phosphorylation of Ser63, but not Ser73, is required for induction of apoptosis [21]. These data show the diversity of signaling activity conferred by specific phosphorylations, and that specific regulation of c-Jun phosphorylation may effect discrete biological functions.
In this report, we investigate the response of the MAP kinase cascades that target c-Fos and c-Jun, as well as the response of c-Fos and c-Jun themselves to oxidative stress induced by 3-NPA. Notably, we found that wild type mice have a rapid activation of MEK and ERK that is diminished in the dwarf. In addition, we show a marked absence of Elk-1 and MKK7 protein in dwarf mice. Finally, we demonstrate a lack of c-Jun Ser^{53} phosphorylation in dwarf mice following 3-NPA treatment, in sharp contrast to the robust phosphorylation in wild type mice at this particular phosphorylation site.

Materials and methods

Mice. Female B6DWF1 (Pit1^{+/+}) dwarf mice and their normal-sized littermates (B6DFWF1 Pit1^{+/−}), 3–6 months old, bred and housed at the Jackson Laboratories (Bar Harbor, ME), were used for this study. The mice were reared in a limited access, specific pathogen-free colony. Mating B6DW Pit1^{+/+} with DW/J Pit1^{+/+} mice produced B6DFWF1 dwarf mice (Pit1^{+/−}) and their normal-sized controls (Pit1^{+/+}). Although mice could have either a B6DW or a DW/J sire, for convenience, F1 mice will be designated as B6DFWF1 in this paper. Heterozygotes are phenotypically indistinguishable from normal wild type homozygotes [1], therefore, we used both as controls which we designate B6DFWF1 Pit1^{+/−}, and refer to them as wild type. Mice were kept on a 14:10 light cycle at 27 ± 1°C and 45 ± 5% relative humidity. Mice were given autoclaved NIH-31 diet (4% fat) (PMI Nutrition Int., Brentwood, MO) and acidified tap water (pH 3.6) supplemented with vitamin K. Dwarf mice were housed with at least one normal-sized littermate (B6DWF1) and acidified tap water (pH 3.6) supplemented with 0.05% bovine serum albumin (BSA, Sigma), as per the primary antibody manufacturers’ recommendation. Primary antibodies were obtained from Santa Cruz (M KK6, M KK4, M KK7, c-Fos) and Cell Signaling Technology (phospho-MKK3/6, p38 MAPK, phospho-p38, phospho-MKK4, SAPK/JNK, phospho-SAPK/JNK, MEK 1/2, phospho-MEK 1/2, ERK 1/2 (p44/42), phospho-ERK 1/2, Elk-1, c-Jun, and phospho-c-Jun). The membranes were then incubated for 1 h or overnight in accordance with the manufacturers’ recommendation, with the appropriate primary antibody in fresh blocking solution, washed in TBS-T, and incubated for 1 h with the corresponding HRP-conjugated secondary antibody (Amersham) in fresh blocking solution. The membrane was washed and the bands were detected by chemiluminescence with SuperSignal West Pico Chemiluminescent Substrate (Pierce), and Kodak X-OMAT film. The resulting bands were quantified using a ChemiImager 4400 (Alpha Innotech). All dwarf time course samples, 0–20 h post-injection (PI), were run on the same 18-well gel to minimize variability for the time course comparison, and wild type samples were treated in the same fashion on a separate gel; therefore, visual dwarf to wild type comparisons should not be made based on the time course figures because they have independent exposure times. The nontreated control mice were ran together on a third gel to make dwarf to wild type comparisons. The protein samples were loaded in the same order for all gels so that trends for individuals can be followed through a pathway. Western blot results were confirmed by a second Western blot on a subset of the individuals. Microsoft Excel was then used to calculate the average intensity and 2-tailed heteroscedastic Student’s t test. All fold changes discussed hereafter reached a statistical significance of $p \leq 0.05$ unless otherwise noted. After blotting was complete each membrane was stained with Coomassie brilliant blue G-250 (Fisher) to assure equal loading and transfer of proteins. Due to space considerations, one representative stained membrane is shown with each figure.

Results

Altered cytosolic pool levels and stress response of the MEK–ERK (p44/42) kinase cascade in dwarf mice

To investigate the response of dwarf mice to 3-NPA induced oxidative stress, we measured the effect of 3-NPA on three kinase cascades in the MAP kinase pathway. The first cascade we investigated was the MEK–ERK kinase cascade. Basal cytosolic levels of MEK 1/2 and ERK 1/2 are higher in wild type mice than dwarf mice by 2-fold and 1.3-fold, respectively; however, the active phosphorylated forms of MEK 1/2 and ERK 1/2 are higher in the dwarf by 1.7-fold and 3-fold ($p = 0.07$), respectively (Fig. 2A). This suggests an accumulation of inactive MEK and ERK in the cytosol of wild type mice compared to the long-lived dwarf mice. At 15 min PI, wild type mice have a small increase in the level of MEK 1/2 along with a substantial 7.7-fold increase in phosphorylated MEK 1/2 (Fig. 2B). This rapid
phosphorylation partially recovers at 1 h, e.g., a 2.6-fold increase over basal phosphorylation levels, and by 20 h PI the phosphorylated pool level has recovered completely. In contrast to the wild type response, dwarf mice do not have a statistically significant change in MEK pool level or phosphorylation level in response to 3-NPA (Fig. 2B). The ERK response to 3-NPA is similar to that of MEK, with a rapid 3-fold induction of phosphorylated ERK in wild type mice at 15 min and recovery by 1 h, but dwarf mice fail to show a statistically significant change in ERK phosphorylation levels (Fig. 2C).

**Lack of c-Jun modification in dwarf mice after 3-NPA treatment**

Although basal liver nuclear protein levels of c-Jun differ between dwarf and wild type mice, their response to 3-NPA is similar. Wild type mice have a 2.6-fold higher basal c-Jun protein pool level than dwarf mice (Fig. 3A), and in response to 3-NPA, wild type mice have a 2.8-fold increase at 15 min PI and remain elevated by 3.3-fold at 1 h; however, since only two of the three wild type animals showed elevated protein levels at this time this increase is not statistically significant (p < 0.10) (Fig. 3B). In dwarf mice there is a tendency toward increased c-Jun levels in response to 3-NPA, but these increases are not statistically significant due to a small sample size, but it is apparent that the c-Jun response in dwarf and wild type mice is similar.

While transcriptional regulation of AP-1 components plays a role in their activation, phosphorylation of c-Jun at Ser63 and Ser73 control specific action of the AP-1 complex. In response to 3-NPA, wild type c-Jun phospho-Ser73 levels increase 4.4-fold at 15 min PI, and two of the three mice show further elevation of phospho-Ser73 at 1 h, with the average for all three mice being 6.6-fold higher (p < 0.1) than non-treated wild type mice (Fig. 3B). Dwarf mice show similar increases in c-Jun phospho-Ser73 levels with 3.8- and 6.6-fold increases (p < 0.1) at 15 min and 1 h, respectively. Thus, both c-Jun protein pool and c-Jun phospho-Ser73 levels show the same pattern of response to 3-NPA.
The response of c-Jun phospho-Ser63 in the nucleus is strikingly different between dwarf and wild type mice, where wild type mice show a rapid 5.6-fold increase at 15 min, and an average increase of 6.4-fold (p < 0.05) at 1 h, while dwarf mice show no increase of phospho-Ser63 (Fig. 3B). Since no c-Jun phospho-Ser63 was detectable at 0 h for either genotype, a comparison of treated dwarf and wild type animals run on the same gel was used to normalize and compare the response (Fig. 3D). At 15 min and 1 h PI wild type mice have well over 10 times as much c-Jun phospho-Ser63 than dwarf mice. This represents a unique difference between the oxidative stress responses of the long-lived Snell dwarf mice versus their age-matched controls.

C-Fos, a major component of the AP-1 transcription factor, has a slightly higher basal nuclear protein pool in wild type mice. The response of c-Fos to 3-NPA was similar for dwarf and wild type mice, with ~1.6-fold inductions at 15 min that remained elevated at 20 h in both dwarf and wild type mice (Fig. 3B).

**Dwarf mice have much lower levels of Elk-1 protein**

Since Elk-1 is a downstream target of the MAP kinase signaling pathway (Fig. 1), we investigated whether 3-NPA generated oxidative stress affects its pool level. Elk-1 is of particular interest since our data show wild type mice have over 10 times more Elk-1 protein than dwarf mice (Fig. 4A). Elk-1 may play a key role in the differential response to 3-NPA in dwarf mice due to its role as a transcriptional regulator and its involvement in the activity of key transcriptional complexes such as the ternary complex and serum response element [24]. Our data show that 3-NPA treatment results in a 59% decrease in Elk-1 levels in wild type mice by 20 h PI but there is no measurable change in the small amount of Elk-1 in the dwarf (Fig. 4B). Interestingly, the greatly
decreased level of Elk-1 in dwarf mice may necessitate alternate signaling pathways in response to stress.

Subtle difference of the MKK 3/6-p38 and MKK4/7-JNK 1/2 signaling cascades in dwarf mice

We also investigated whether other main kinase cascades of the MAPK pathway respond to 3-NPA to determine if they might also play a role in the alternative stress response of dwarf mice. Non-treated dwarf and wild type mice have similar levels of MKK6 and phospho-MKK3/6 (Fig. 4A), but in response to 3-NPA, dwarf mice show a small but rapid 1.3-fold and 1.5-fold increase in active MKK 3/6 levels at 15 min and 1 h, respectively, and a recovery by 20 h PI (Fig. 4B). In contrast, wild type mice have a 46% decrease in active MKK3/6 levels at 1 h and a 62% decrease at 20 h PI (Fig. 4B). A differential response is also seen in MKK 6 protein pool levels, where wild type mice have a 63% decrease in pool level at 20 h PI, but dwarf mice show no change in their MKK 6 protein pool level after 3-NPA treatment. However, this response is not transduced to p38 MAPK which is one of MKK3/6’s main downstream targets. The MAPK response to 3-NPA is likely strain and/or sex dependent since previous reports (using male mice from an inbred background (C57BL6) vs female F1 mice in this study) have shown p38 and JNK responding to 3-NPA [25].

Levels of p38 are 2.5 times higher in wild type mice than in dwarf mice and there is also a small and not quite significant elevated phospho-p38 level (1.7-fold \((p = 0.056)\)) in wild type mice (Fig. 4A). Interestingly, in response to 3-NPA there are no statistically significant changes in either the pool level or the phosphorylated pool levels of p38 for dwarf or wild type mice. Therefore, we do not feel the MKK 3/6-p38 pathway is a major contributor to the differential oxidative stress response of dwarf mice.

Basal MKK 7 levels are more than three times greater in wild type mice than their dwarf littermates (Fig. 5A).
Unlike the MEK–ERK pathway, MKK7 shows a statistically significant change in the dwarf with a 1.7-fold increase of its pool levels at 15 min and 1 h PI while the protein pool levels of wild type mice do not change. MKK4 and phosphorylated MKK4 levels remain fairly steady after 3-NPA treatment, with no statistically significant changes at any time point (Fig. 5B). Protein levels of JNK1/2, which are downstream of MKK4/7, do not change in response to 3-NPA, nor does the phosphorylated form of JNK1; the phosphorylated form of JNK2 was not detectable. Thus, the MKK4/7-JNK1/2 kinase cascade does not show a significant difference between dwarf and wild type mice in response to 3-NPA.

**Discussion**

Our studies show that long-lived Snell dwarf mice manage oxidative stress differently than their wild type siblings through altered MEK–ERK kinase cascade signaling and differential phosphorylation of c-Jun. We
suggest that this difference in MEK 1/2 and ERK 1/2 activation in response to 3-NPA indicates that dwarf mice manage oxidative stress differently than wild type mice by utilizing different signaling pathways. These observations also suggest that wild type mice are more sensitive to mitochondrial generated ROS than dwarf mice, which is consistent with previous reports that correlate decreased sensitivity of ROS activated signaling with life span extension [4,10].

We show major differences between dwarf and wild type mice in the MEK 1/2–ERK 1/2 kinase cascade’s response to oxidative stress. The fact that wild type mice show an extremely strong and fast response to 3-NPA by increasing active MEK 1/2 levels over 7-fold just 15 min after injection versus virtually no response by dwarf mice suggests that the long-lived dwarfs are resistant to the oxidative stress induced by 3-NPA. In addition, wild type mice have a 3-fold increase in phospho-ERK 1/2 at 15 min, but dwarf mice show a very limited response to 3-NPA. Thus, the MEK–ERK kinase cascade is likely responsible, at least in part, for the differential transcriptional stress response, and/or the alternate regulation of c-Jun phosphorylation seen in dwarf mice in response to oxidative stress induced by 3-NPA. Furthermore, the differences in the basal levels of MEK 1/2, where wild type mice have nearly twice the level of MEK 1/2 as dwarf mice, but dwarf mice have 1.8 times more active/phosphorylated MEK 1/2 are consistent with a mechanism of resistance.

The fact that MEK 1/2 and ERK 1/2 are phosphorylated to a higher degree in non-treated dwarf mice indicates their targeted genes may be constitutively activated, resulting in a higher basal level of those genes in dwarf mice. In contrast, wild type mice only activate these genes momentarily in response to stress and quickly recover to basal levels. Thus, we should see certain targets of the MEK–ERK pathway activated in dwarf mice, and we are in the process of identifying these genes. These results point to the importance of differences in pool sizes, i.e., a larger pool of inactive MEK 1/2 and ERK 1/2 in wild type mice than dwarf mice, shown by wild type mice having higher protein pool levels, but lower phosphoprotein levels than dwarf mice, which suggests this may be part of the mechanism of resistance to further response to oxidative stress. Further studies are needed to define the molecular characteristics of signaling pathways such as MEK and ERK in resistance to oxidative stress, including the possible phosphorylation of c-Jun at serine 63 by ERK.

The notable lack of Elk-1, a member of the Serum Response Factor family, in dwarf mice is a major difference that likely plays a role in the transcriptional response to 3-NPA. The fact that three separate MAP kinase signaling pathways interact with the same protein, Elk-1 [14], indicates that it is an integration point for several signaling pathways and that it likely has multiple independent functions dependent upon the origin of the upstream signal. Elk-1 is a known activator of AP-1 member c-Fos, yet protein levels of c-Fos do not seem to be affected by the lack of Elk-1 protein. This lack of Elk-1 may be a result of the GH deficiency in dwarf mice, since GH has been shown to stimulate Elk-1 activation/phosphorylation through the MEK–ERK kinase cascade [24].

Finally, one of the most striking differences between dwarf and control mice involves the lack of c-Jun Ser63 phosphorylation in dwarf mice in response to 3-NPA treatment (Fig. 3B). The lack of c-Jun Ser63 phosphorylation in dwarf mice is especially interesting due to the role that c-Jun plays in many key biological processes such as transcriptional regulation, cell activation, and regulation of apoptosis, and it is important to note that phosphorylation at Ser63 and Ser73 is directly involved in regulating the diverse activities of c-Jun [16,21]. This lack of c-Jun Ser63 phosphorylation in dwarf mice, which results in over a 10-fold difference in phosphorylated Ser63 levels between dwarf and wild type mice would have a significant impact on the function of c-Jun and the genes regulated by AP-1. In fact, a recent report has shown that regulation of Ser63 and Ser73 is controlled independently, and that failure to phosphorylate only Ser63 results in a resistance to apoptosis when treated with nitric oxide [21]; thus, c-Jun Ser63 phosphorylation appears to be required in oxidative stress induced apoptosis. This lack of Ser63 phosphorylation in dwarf mice after an oxidative insult shows that there is no activation of a necessary step in the processes of apoptosis in dwarf mice, and therefore may not be as damaged as wild type mice.

We have shown that the management of 3-NPA induced oxidative stress is altered in long-lived Snell Dwarf mice, specifically, a significantly lower response in the MEK–ERK kinase cascade and a lack of c-Jun Ser63 phosphorylation, which is possibly a result of the reduced level of phosphorylated MEK–ERK. Therefore, we propose that phosphorylation of c-Jun at Ser63 plays an important role in oxidative stress response/stress management, and is likely partially responsible for the altered dwarf response to 3-NPA. This altered management of oxidative stress may also be a basal factor in the extended longevity in dwarf mice, due to the role of oxidative stress in aging.

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References


