Phase 2 Protein Inducers in the Diet
Promote Healthier Aging

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Oxidative stress drives many aging-associated problems. Because oxidative stress can be decreased by induction of phase 2 proteins, we hypothesized that incorporating the phase 2 protein inducer 2(3)-tert-butyl-4-hydroxyanisole (tBHA) into the diet would result in healthier aging. C57BL/6 mice were placed either on control mouse chow diet or on chow containing tBHA and were examined at 6, 12, and 18 months. Dietary tBHA resulted in the antioxidant response activation, decreased both oxidative stress and pro-inflammatory gene expression in tissues examined, counteracted the decrease in the transcription factors peroxisome proliferator-activated receptor-γ and increase in CCAAT/enhancer binding protein-α levels seen in liver with aging, and was associated with mice having less weight gain, despite having no differences in food consumption, and better locomotor function. We conclude that simple changes in the diet such as incorporation of phase 2 protein inducers can have a profound influence on health and, thereby, the aging process.

Key Words: Antioxidant response—Oxidative stress—Inflammation—Diet—Healthy aging.
increase the expression of phase 2 protein genes in this strain of mouse (34) and to increase GSH levels (35) in mice.

**Materials and Methods**

**Dietary Intervention**

Animals were treated in accordance with the Canadian Council on Animal Care Guidelines with the research approved by the University Committee on Animal Care and Supply. Female C57BL/6 mice (C57BL/6Ncri), obtained from Charles River Canada (St. Constant, PQ), were housed five to a cage. They were fed with a control diet or a diet containing 40 mmol tBHA/kg mouse chow, a dose previously used (32). For the experimental diet, 1 kg of mouse chow (Purina Mills LabDiet, ProLab RMH 2000; Richmond, IN) was soaked in 100 mL of 70% ethanol solution containing 7.5 g of tBHA. The ethanol was allowed to soak into the chow and evaporate in the dark. The chow was then stored in the dark. To mask the flavor of the tBHA, the ethanol also contained 0.6 g/100 mL of a cherry flavoring (Superstore’s No Name brand containing sugar, gelatin, adipic acid, and artificial flavor and color). For the control diet, mice were fed chow with only the cherry flavoring incorporated. Mice were placed on these diets at five to a cage. They were fed with a control diet or a diet containing 40 mmol tBHA/kg mouse chow, a dose previously used (32). For the experimental diet, 1 kg of mouse chow (Purina Mills LabDiet, ProLab RMH 2000; Richmond, IN) was soaked in 100 mL of 70% ethanol solution containing 7.5 g of tBHA. The ethanol was allowed to soak into the chow and evaporate in the dark. The chow was then stored in the dark. To mask the flavor of the tBHA, the ethanol also contained 0.6 g/100 mL of a cherry flavoring (Superstore’s No Name brand containing sugar, gelatin, adipic acid, and artificial flavor and color). For the control diet, mice were fed chow with only the cherry flavoring incorporated. Mice were placed on these diets at 5 weeks of age, and livers and spinal cords were examined at 6, 12, and 18 months using immunocytochemistry and western blotting.

**Immunohistochemistry and Western Blots**

Mice used for immunohistochemical studies were perfused with heparinized phosphate-buffered saline and then continued with the same solution containing 4% paraformaldehyde as previously described (29). Sections were cut and immunostained for Nrf2 using a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), for inducible nitric oxide synthase (iNOS; StressGen; Victoria, BC, Canada) or for intercellular adhesion molecule-1 (ICAM1; Santa Cruz Biotechnology) with an avidin–biotin–peroxidase detection system (Vector Laboratories, Burlingame, CA) or Cy3-conjugated secondary antibody and a Chemiluminescence Substrate Kit (Amersham Biosciences, Baie d’Urfe, PQ, Canada) and quantified using Scion image analysis. The membrane was reprobed, with the final reprobe being an anti-actin antibody (Sigma Chemical Co., St. Louis, MO). Quantification was relative to the actin.

Mouse monoclonal antibodies raised against iNOS and ICAM1 were obtained from Transduction Laboratories (supplier is BD Biosciences, Mississauga, ON, Canada) and Santa Cruz Biotechnology, respectively. Rabbit polyclonal antibodies raised against nuclear factor-κB (NF-κB) p65, IκBα, angiotensin II AT1 receptor, as well as a goat polyclonal antibody raised against leukemia inhibitory factor (LIF) were obtained from Santa Cruz Biotechnology. The following rabbit antibodies were also used: anti-superoxide dismutase 1 (SOD1; StressGen), and anti-Nrf2 (Santa Cruz Biotechnology. Tissue lysates from five animals per group were examined.

**Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry**

Identification of a 27-kd band that was greatly upregulated was done by using matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS) with peptide mass fingerprinting (PMF) as previously described (36). All reagents were supplied by Sigma unless otherwise noted. Coomassie Blue-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)-separated proteins were excised using a ProteomeWorks 2-D spot cutter (Bio-Rad, Hercules, CA) and placed in a 96-well microtiter plate (Sigma). Proteins in the excised gel pieces were automatically de-stained, reduced with dithiothreitol, alkylated with iodoacetamide, and digested with porcine trypsin (sequencing grade; Promega, Madison, WI) using a MassPREP protein digest station (Micromass, Manchester, UK). The resulting tryptic peptides were then extracted from the gel and analyzed by MALDI-TOF MS on a Voyager-DE STR instrument (Applied Biosystems, Framingham, MA) operating in the positive ion and reflectron modes (36). Five microliters of each digest were applied to a MALDI target plate and allowed to dry to a volume of approximately 1 μL. One microliter of α-cyano-4-hydroxy-cinnamic acid matrix solution (5 mg/mL in 0.1:24:9:75.0 vol/vol/vol trifluoroacetic acid/water/acetoni-trile) was then added to each sample and allowed to air dry. The instrument was calibrated using trypsin autoyolysis products (m/z 842.51 and 2211.10) as internal standards, where present, or a mixture of des-Arg bradykinin (m/z 904.4681) and adrenocorticotrophic hormone clip 18–39 (m/z 2465.1989) for close external calibration. Proteins were identified by PMF using MASCOT (Matrix Science, Boston, MA) to search the National Center for Biotechnology Information (NCBI) nonredundant sequence database. Searches were performed using carbamidomethylation of cysteine as the fixed modification and oxidation of methionine as the variable modification, allowing for one missed cleavage during trypsin digestion. Protein identity was considered unambiguous if the experimentally determined peptide (MH+ masses matched at least 10% of the protein sequence, with a mass deviation of <50 ppm, using at least four different peptides.

**Locomotory Testing**

The procedure followed is outlined in Modo and colleagues (37). Mice were placed at one edge of a square open field, enclosed in Plexiglas (40 × 40 × 40 cm) that was
elevated 3 cm above the table top. The floor was a wire grid with 1 cm spaces between the rods that comprised the grid. Mice were allowed to explore the grid for 2 minutes. Footfaults are defined as the mouse misplacing a fore or hind limb with a paw falling completely between the bars. As the mouse may not remain active for the entire 2-minute period, the time that the mouse was active was also measured.

**Statistical Analyses**

Unless otherwise indicated, the western blot and immunocytochemical data presented are from the tissues of five animals per group. All numerical values given are means ± standard errors of the mean. Statistical analysis was performed either using a one-way analysis of variance with a Student–Newman–Keuls post hoc test or a two-tailed Student’s *t* test when only two values were compared.

**RESULTS**

**Activation of the Antioxidant Response**

Aging is associated with a decline in total as well as transcriptionally active Nrf2 (26). Eighteen-month-old mice on the tBHA diet had higher levels of total liver Nrf2 (Figure 1A: 66.1 ± 9.3 arbitrary units [AU] vs 38.0 ± 8.0, *p* < .05, *n* = 5) as well as higher levels of nuclearly localized Nrf2 compared to mice on control diet (Figure 1B). Figure 1C illustrates representative western blots of nuclearly localized Nrf2 in the spinal cord of 18-month-old animals on control diet (Figure 1A) and tBHA diet (Figure 1B). The abundance of nuclearly localized Nrf2 relative to the general transcription factor TFIIe2 is increased in 18-month-old animals on the tBHA diet.

Nrf2 in the nucleus, whereas animals on the tBHA diet exhibited 123.6 ± 6.4 AU (*p* < .05, *n* = 5).

Higher levels of nuclearly localized Nrf2 suggests increased transcriptionally active Nrf2 and, thus, increased expression of phase 2 protein genes. Separation of liver proteins using SDS–PAGE demonstrated a marked increase in a ~27-kd protein band (Figure 2A) in both young (6 month) as well as old (18 month) mice on the tBHA diet. The major protein in this band, using MALDI-TOF MS and PMF, was identified as glutathione *S*-transferase M1, a phase 2 protein that has been previously shown to be markedly induced by tBHA (38,39).

If phase 2 protein gene expression were induced, one would predict that tissues are experiencing less oxidative stress. We examined the level of cytosolic SOD1 because oxidative stress increases expression of this antioxidant enzyme (40). As hypothesized, the tBHA diet significantly (*p* = .0077) decreased the level of SOD1 protein to 83.5 ± 11.3 AU as compared to 129.8 ± 6.6 AU in mice on the control diet (Figure 2B).

**Decrease in Inflammation in Liver and Spinal Cord**

Aging is associated with increased tissue inflammation with 40% of the genes that are upregulated being pro-inflammatory in nature (14). Inflammation is driven by activation of the NF-κB transcription factor, whereas oxidative stress promotes activation of NF-κB (41); thus,
it is not surprising that aging is associated with increased activation of NF-κB (42). We reasoned that decreasing oxidative stress should also decrease NF-κB activation and pro-inflammatory gene expression. An increase in the ratio of IκBα to NF-κB p65, indicative of less NF-κB activation, was seen in livers of 12-month-old animals fed a tBHA-containing diet (Figure 3A), with control mice having a ratio of 0.34 ± 0.07 and tBHA-fed mice having a ratio of 2.20 ± 0.19 (p < .0001).

Decreased activation of NF-κB was associated with a significant (p = .0066) decrease in the expression in liver of the proinflammatory iNOS (Figure 3B) from 71.0 ± 7.2 AU in control mice to 41.4 ± 3.7 AU in mice on the tBHA-containing diet. Spinal cords from aged mice on the tBHA-containing diet also had significantly lower levels (p < .05, n = 5) of iNOS protein than did mice on the control diet (Figure 4A). Immunocytochemistry demonstrated that most of the iNOS signal was present in neurons and vascular cells and that this signal increased markedly between 6 and 18 months of age in the mice on the control diet. Aged mice on the tBHA-containing diet also had significantly lower levels (p < .05, n = 5) of ICAM1 in spinal cord compared to mice on the control diet. Immunocytochemistry demonstrated that ICAM1 was restricted to the vasculature and the signal increased with age in the mice on the control diet (data not presented).

Fibrosis is also a characteristic of the aging liver (43). The tBHA-containing diet caused a modest, but significant (p = .0157, n = 5) decrease in the level of the angiotensin II AT1 receptor from 17.3 ± 4.8 AU to 15.4 ± 3.7 AU (Figure 3C). Activation of AT1 receptors, present in liver Kupffer cells, is thought to play an important role in activating hepatic stellate cells to differentiate into myofibroblasts that drive the fibrotic process (44). Myofibroblasts produce LIF.
(45); hence, LIF levels should be a more direct index of fibrosis. A diet containing tBHA significantly \((p = 0.0071, n = 3)\) decreased LIF content from 79.4 ± 12.5 AU to 30.7 ± 3.1 AU in livers of aged mice (Figure 3C).

**Metabolic Indices**

There were no significant differences in food consumption between the mice on the control diet or on the diet containing tBHA. At 6 and 18 months, respectively, mice on the control diet consumed 5.69 ± 0.25 and 4.83 ± 0.71 g chow/day \((n = 20/group)\), whereas mice fed the tBHA diet consumed 5.55 ± 0.48 and 4.51 ± 0.28 g chow/day \((n = 20/group)\). Mice on both diets gained weight (Figure 5A). Mice on the control diet had a steady increase in weight gain, with 18-month-old mice \((n = 18)\) weighing 42.6 ± 1.9 g, and had an abundance of intra-abdominal and pericardial fat. Mice on the tBHA-containing diet had a decreased weight gain that began to plateau between 12 and 18 months, with 18-month-old mice \((n = 18)\) weighing 28.9 ± 0.7 g with little visceral fat present. We examined two transcription factors that modulate expression of genes related to metabolism (peroxisome proliferator-activated receptor \(\gamma\) [PPAR\(\gamma\)] as well as CCAAT/enhancer binding protein \(\alpha\) [C/EBP\(\alpha\)]).

Incorporating tBHA into the diet was associated with a significantly higher \((p = 0.0009, n = 5)\) level \((54.2 ± 3.5 AU)\) of PPAR\(\gamma\) content in the liver of aged mice (Figure 3D) compared to the control diet \((29.5 ± 3.2 AU)\). Dietary tBHA was associated with a significantly \((p = 0.0008, n = 5)\) lower \((13.9 ± 0.9 AU)\) C/EBP\(\alpha\) content compared to the control diet \((19.3 ± 0.4 AU)\) in livers of aged mice (Figure 3E).

**Motor Function**

Motor functions that require coordinated control decline with age in both rodents (46) and humans (47). This decline in motor function in both rodents and humans is correlated with increases in inflammation (48,49). Because tBHA consumption not only decreased inflammation in both liver and spinal cord but also decreased the extent of weight gain, we hypothesized that mice on the experimental diet would exhibit better motor control. To examine this, 12- and 18-month-old mice were allowed to walk over an open field with a wire mesh floor for 2-minute periods, and the fraction of a 2-minute period occupied by movement as well as number of foot slippages per second of activity was recorded. Both groups of mice engaged in continuous exploration during the 2-minute time period at 12 months of age. Control 18-month-old mice were significantly less active \((p < .05)\) than were 12-month-old mice on the control diet (Figure 5B). There was no significant change in exploratory behavior in 18-month-old mice on the tBHA-containing diet compared to 12-month-old mice on either diet.

The older mice on the control diet also had an increased foot slippage rate compared to 12-month-old mice, although this did not reach significance (Figure 5C). The 18-month-old mice on the tBHA diet had significantly fewer \((p < .05)\) foot slippages per second of locomotory activity than did the 18-month-old mice on the control diet (Figure 5C).

**DISCUSSION**

Aging in the C57BL/6 mouse resembles human aging in at least the following ways: increasing inflammation in tissues, decreasing physical activity, decreasing motor control, and increasing weight. These features are likely interrelated.

Aging is associated with a decline in total as well as transcriptionally active Nrf2 (26). This age-related decline in Nrf2 protein is caused by decreased Nrf2 messenger RNA levels (50). Several reports have shown that administration of phase 2 protein inducers results in increased Nrf2 transcription (51,52). In agreement with these findings, we
show that incorporating tBHA into the diet increased both total as well as nuclear Nrf2.

We noted that dietary tBHA was associated with a marked increase of a protein band of ~27 kd when liver proteins were separated on a polyacrylamide gel. MALDI-TOF MS analysis demonstrated that the dominant protein in this band was glutathione S-transferase (GST) M1. MS analysis did not demonstrate the presence of other members of the GST \( \mu \) family or members of the GST \( \alpha \) and \( \pi \) families. It is known that constitutive and inducible levels of GST M1–4 and GST \( \alpha \)1 and \( \alpha \)2 is dependent on Nrf2 (39). McLellan and Hayes (38) have shown that, although there is induction of the \( \pi \) family of GST and GST \( \alpha \)-1 is markedly induced by tBHA, the dominating GST family induced is the \( \mu \) family. Similarly, Chaubey and colleagues (53) have shown that, although tBHA induces many GST family and members, there is 6 times more GST M1 protein than any other protein in the livers of female mice administered tBHA. In the present study it is likely that other members of the \( \mu \) family as well as members of the \( \alpha \) and \( \pi \) family were induced in the livers of female mice placed on the tBHA-containing diet, but the analytical approach chosen (MALDI-TOF MS) is not sensitive enough to identify proteins that do not dominate the mixture analyzed. Nevertheless, we have shown that there is a major induction of at least one phase 2 protein, GSTM1, as has been previously demonstrated by others.

Because oxidative stress increases expression of SOD1 (40), this is a good marker to examine for changes in oxidative stress. An increase in phase 2 protein expression should result in a decrease in oxidative stress. Indeed, consumption of tBHA was associated with a decrease in SOD1 protein levels. Oxidative stress is known to promote liver inflammation and fibrosis (54). In the present study, dietary tBHA not only decreased liver oxidative stress but, as hypothesized, reduced liver NF-\( \kappa \)B activation and liver inflammation as indicated by lower iNOS, AT1 receptor, and LIF protein expression. Thus, dietary tBHA, like caloric restriction (14), decreases aging-associated liver inflammation. Inflammation is also associated with aging in the CNS (55). We noted an increase in iNOS levels, mostly in neuronal somas and in the vasculature, in the spinal cord as mice aged. We have previously shown a similar pattern of spinal cord iNOS immunostaining in 6-month-old SHRsp, a model of accelerated aging, and in this strain of rat, dietary intake of phase 2 protein inducer was associated with decreased iNOS expression (30). In the present study, dietary intake of tBHA also resulted in significantly lower iNOS protein levels in the spinal cord of the aged mouse. Furthermore, dietary tBHA was also associated with a significantly lower expression of ICAM1, an adhesion molecule necessary for infiltration of activated leukocytes across the endothelium (56).

tBHA is used as a food additive because the isomers have direct antioxidant activity. Hence, the question arises whether the decreases in oxidative stress and associated inflammation are caused by the direct antioxidant properties of tBHA or to its induction of phase 2 proteins. A number of observations suggest that the decrease in oxidative stress is likely caused by tBHA’s ability to induce phase 2 proteins. Activation of the cap ‘n’ collar C (CcnC) Nrf2 homologue in Drosophila melanogaster induces phase 2 protein gene expression and increases resistance to oxidative stress that is associated with prolonged life span (57). Similarly, in Caenorhabditis elegans, activation of the transcription factor SKN-1, an Nrf2 homologue, results in induction of phase 2 protein expression that increases the resistance of the organism to oxidative stress and is associated with increased life span (58). These observations provide indirect support to the notion that phase 2 protein induction is responsible for healthier aging. Possibly a more direct piece of evidence is that dietary tBHA was associated with less weight gain even though food consumption was statistically the same in both diet groups. The lower weight was associated with less visceral fat accumulation; however, we found no evidence of fat accumulation in liver, and there were no obvious differences in liver histology between the two groups. It is known that Nrf2 is necessary to inhibit oxidative stress and lipid accumulation in mice fed a high-fat diet (59). Furthermore, activation of Nrf2 represses expression of a number of genes involved in cholesterol and lipid synthesis including sterol regulatory element-binding protein-1 (SREB1) (60). SREB1 is a key regulator of the expression of lipogenic genes (61).

In addition to decreased expression of SREB1, less fat accumulation may be related to the lower oxidative stress and inflammation that is a result of phase 2 protein induction in the tBHA group. Human clinical studies have shown that increased blood oxidative stress parameters are correlated with obesity (62). We also noted that 18-month-old mice on the control diet were significantly less active than 18-month-old mice on the tBHA diet as well as both 12-month-old mice diet groups. This increased physical activity associated with tBHA likely plays a role in less fat accumulation, although in the test used no differences in physical activity were noted at 12 months of age, a time when mice on the control diet were already significantly (\( p < .001 \)) heavier (36.4 ± 1.9 g) than mice on the tBHA-containing diet (27.1 ± 0.3 g). Increased activity of mice on the tBHA-containing diet may be related to a lower level of oxidative stress and inflammation in tissues including the CNS. Studies have shown that older human adults that are more physically active have lower levels of inflammatory markers in the blood (63). It is likely that there is a causal relationship among oxidative stress, inflammation, and obesity (64).

Possible factors that link oxidative stress, inflammation, and weight gain include transcription factors such as PPAR\( \gamma \). We noted that PPAR\( \gamma \) protein levels were higher in livers of mice on the tBHA-containing diet compared to mice on the control diet. The role of PPAR\( \gamma \) is complex. Decreased activity of the transcription factor PPAR\( \gamma \) is associated with inflammation (65). Steatosis is characterized by increased expression of PPAR\( \gamma \) (65), whereas specifically knocking out PPAR\( \gamma \) expression in liver results in hyperlipidemia (66). Our results show that increased expression of PPAR\( \gamma \) does not necessarily result in steatosis. It is known that expression of PPAR\( \gamma \) declines during aging (67); this decline may be associated with increasing weight gain and increasing inflammation that is associated with aging. PPAR\( \gamma \) activation also has been shown to decrease
inflammation in the cardiovascular system (68); furthermore, downregulation of PPARγ expression both in vivo and in vitro results in liver fibrosis, whereas increasing PPARγ expression decreased fibrosis (69). Like many things in biology, the effect of decreasing or increasing PPARγ expression is likely dependent on the presence of the specific constellation of other signaling pathways that influence gene expression. In our study we have shown that dietary intake of tBHA is correlated with increased hepatic PPARγ protein expression, decreased tissue inflammation, and decreases in indicators of liver fibrosis (AT1 receptor and LIF).

Another transcription factor that plays a role in fat metabolism is C/EBPα (70). Decreasing C/EBPα in mouse liver using a small interfering RNA approach has been shown to inhibit hepatic lipid accumulation (71). Our study has shown that incorporating tBHA into the diet decreased liver C/EBPα levels; this decrease was associated with less fat accumulation. Thus, dietary tBHA results in changes in the protein levels of two transcription factors (that play major roles in lipid metabolism) in directions that promote health. How reduction in oxidative stress is related to changes in C/EBPα and PPARγ expression is not clear at the moment and is the subject for future research. Our research has led to more questions than answers. It is clear that tBHA has a profound effect in ameliorating problems associated with aging. It is likely that many of the changes we have seen are the consequences of activation of Nrf2. Clearly more research is warranted using other known activators of Nrf2.

Conclusion

Aging is inevitable. What is dreaded most is the increased disease burden that generally accompanies aging. As noted at the beginning of this article, much of this disease burden is driven by oxidative stress. Our previous data obtained from spontaneously hypertensive stroke-prone rats indicated that incorporation of glucoraphanin, which is metabolized to the phase 2 protein inducer sulforaphane, into the diet decreased oxidative stress and associated problems such as hypertension and tissue inflammation in this rat strain characterized by accelerated aging. In the present study we demonstrate in a rodent model of normal aging, the C57BL/6 mouse, that the phase 2 protein inducer tBHA also decreases oxidative stress and associated inflammatory problems leading to healthier aging. The profound effects seen using phase 2 protein inducers are likely caused by the increases of multiple aspects of the endogenous cellular machinery for scavenging oxidants (19). The use of phase 2 protein inducers is, thus, a much more effective means of dealing with oxidative stress than is simple administration of antioxidants.

We are not suggesting that people begin to consume large quantities of tBHA. Certain of the foods we eat contain high levels of phase 2 protein inducers (19). Our findings, therefore, suggest that consuming adequate amounts of such foods that contain phase 2 protein inducers may well lead to healthier aging. Further research using other animal models of aging is required to determine if this dietary approach is a viable means of attaining healthier aging. In addition, other phase 2 protein inducers need to be examined for healthier aging effects. If our findings in rodents translate to humans, then a simple modification of diet may ameliorate many of the aging-related health problems, thereby resulting in decreased social and health care costs associated with an aging population.

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