Blueberry supplemented diet reverses age-related decline in hippocampal HSP70 neuroprotection

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Abstract

Dietary supplementation with antioxidant rich foods can decrease the level of oxidative stress in brain regions and can ameliorate age-related deficits in neuronal and behavioral functions. We examined whether short-term supplementation with blueberries might enhance the brain’s ability to generate a heat shock protein 70 (HSP70) mediated neuroprotective response to stress. Hippocampal (HC) regions from young and old rats fed either a control or a supplemented diet for 10 weeks were subjected to an in vitro inflammatory challenge (LPS) and then examined for levels of HSP70 at various times post LPS (30, 90 and 240 min). While baseline levels of HSP70 did not differ among the various groups compared to young control diet rats, increases in HSP70 protein levels in response to an in vitro LPS challenge were significantly less in old as compared to young control diet rats at the 30, 90 and 240 min time points. However, it appeared that the blueberry diet completely restored the HSP70 response to LPS in the old rats at the 90 and 240 min times. This suggests that a short-term blueberry (BB) intervention may result in improved HSP70-mediated protection against a number of neurodegenerative processes in the brain. Results are discussed in terms of the multiplicity of the effects of the BB supplementation which appear to range from antioxidant/anti-inflammatory activity to signaling.

Keywords: Heat shock protein 70; Polyphenolics; Phytochemicals; Phytonutrients; Free radicals; Oxidative stress; Inflammation; LPS; Aging; Rats

1. Introduction

There is a great deal of research suggesting that one of the most important factors mediating the deleterious effects of aging on behavior and neuronal function is a decreased antioxidant/oxidative/inflammatory stress balance (see [17] for review). The CNS appears to be especially vulnerable to OS effects, partially as a result of additional factors such as increases in the ratio of oxidized to total glutathione [50], significant lipofuscin accumulation [22] with bcl-2 increases [54], increases in membrane lipid peroxidation [72], decreased glutamine synthetase [10], reductions in redox active iron [22,55] and alterations in membrane lipids [14]. Importantly, in addition to these considerations, it has been shown that not only is the CNS particularly vulnerable to OS but this vulnerability increases during aging (see [31,32] for review).

With respect to aging, we [8] have shown that senescent rats exhibited significantly greater motor behavioral deficits and decreased tyrosine hydroxylase immunoreactivity (in pars compacta) than young rats following intra-nigral applications of dopamine (DA). Research has also shown that besides the factors discussed above (e.g., reductions in glutathione levels; [50]) OS vulnerability in aging may be the result of several additional factors, such as: (a) changes in the microvasculature [18], (b) increases in oxidized proteins and lipids [18], (c) alterations in the membrane microenvironment, (d) alterations in calcium buffering ability, and (e) differential vulnerability of neurotransmitter receptors [33,34].

Also paralleling the results seen with respect to oxidative stress are increases in sensitivity to inflammatory mediators with aging. For example, Manev and Uz [41] showed that
In this respect, studies indicate that oxidative stress and inflammation in inducible HSP70, possibly along with the other parameters be neuroprotective. It appears that the multiple bioactive compounds present in fruits such as BBs and their impact on central nervous system function may stem from a combination of factors including anti-inflammatory, anti-viral, anti-microbial, anti-adhesion, and membrane-modulating properties. However, we believed that there may be effects on more than one OS/inflammatory parameter. Since it appears, that, as discussed above, inducible HSP70 is a potent antioxidant/anti-inflammatory that is reduced in aging, it may be that BB supplementation may restore the activation of this protein during periods of stress and reduce the vulnerability to these insults and contribute to the beneficial effects observed previously. Therefore, the present study was conducted to determine if BB supplementation in senescent rats would reverse the decrements in LPS-induced HSP70. Note that while hippocampal (HC) responses in LPS-induced HSP70 were assessed in young animals as well, a young BB-supplemented group was not included, since the major focus of this study was to assess effects of BBs on this parameter in old rats as a partial explanation for the beneficial effects of BB supplementation on motor and cognitive function that we had seen previously.

2. Methods

2.1. Subjects and diet

Thirty male Fischer 344 (F344) rats from the NIA/Harlan colony were housed in pairs on a 12 h light/dark schedule with ad libitum access to food and water. Young rats (n = 7) received the control diet, from 4 to 6 months of age. Old rats were randomly assigned to either the control (n = 7) or the blueberry supplemented diet (n = 7) group and received the diet from ages 19 to 21 months. All subjects were maintained on the diets for 10 weeks prior to in vitro LPS treatment. The rats were weighed weekly. The animals were used in compliance with all applicable laws and regulations as well as principles expressed in the National Institutes of Health, United States Public Health Service Guide for the Care and Use of Laboratory Animals. This study was approved by the Institutional Animal Care and Use Committees of Simmons College and the Human Nutrition Research Center on Aging at Tufts University. The diet was prepared as previously.
described [71]. In brief, blueberries were mixed 1:1 with wa-
ter, homogenized in a blender for 3 min, and centrifuged at
13,000 × g for 15 min at 4 °C. The supernatant was frozen and
then freeze dried. The resultant crude blueberry extract
was assayed for antioxidant capacity [63] and incor-
porated into standard nutritionally complete rat diet at 2% by
weight as in Youdim et al. [71] and Joseph et al. [28]. An
equivalent amount of corn was added to the control diet.

2.2. In vitro LPS treatment

Brains were dissected on ice and hippocampal regions
were individually crosscut (300 μm, McIlwain tissue chop-
per) and transferred to glass vials containing cold modi-

dified Krebs–Ringer buffer (21 mM NaHCO 3 , 3.4 mM glucose,
1.3 mM NaH 2 PO 4 , 1 mM EGTA, 0.93 mM MgCl 2 , 127 mM
NaCl, 2.5 mM KCl, bubbled with 95% O 2 /5% CO 2 ). Tissue
samples were washed and aliquoted into fresh buffer with es-

ther 0 or 10 μg/ml LPS (lipopolysaccharide, Sigma, E. coli,
serotype 055:B5, TCA extraction), shaken in a 37 °C water
bath for 0, 30, 90 and 240 min with fresh buffer s−/− LPS
replaced at 30-min intervals. Vials were then put on ice,
and samples transferred to centrifuge tubes, spun at 10,000 × g,
10 min, 4 °C, and dry tissue pellets frozen at −80 °C. This
method was adapted from one we have used successfully to
assess the effect of oxidative stress on signal transduction in
freshly dissected brain tissue [30,35,36].

2.3. Protein preparation

Processing to total cytosolic lysates for western blots be-
gan with tissue pellets being resuspended in cold RIPA buffer
(1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS,
ph 7.4) plus protease inhibitors (40 μM PMSF, 2 μg/ml le-
upeptin, 1 μg/ml pepstatin, 0.1 nM Na vanadate, 1 μM NaF,
5 mM DTT). Samples were vortexed, placed in ice for 30 min,
centrifuged at 10,000 × g and 4 °C for 10 min, and the su-

pernatant assayed for protein concentration. The resulting
total cytosolic lysates were stored at −80 °C for western im-
munoblots.

2.4. Western immunoblot and statistics

For Western blots, protein samples (60 μg) were run on a
10% SDS-PAGE gel [44]. Control samples included HeLa
cell lysate (heat shocked and control), rat brain lysate and
HSP70 (SPP-758E, Stressgen). The gel was soaked in pro-
tein transfer buffer (TB: 200 mM glycine, 25 mM Tris base,
20% methanol) for 10 min at room temperature, and then
blotted onto nitrocellulose using a Hoeffer Transphor Unit.
Blots were blocked with TBST (137 mM NaCl, 20 mM Tris,
0.1% Tween-20, pH 7.5) with 5% non-fat dried milk and
incubated with primary antibodies (HSC/HSP70, SC1060,
chHSC70, SC1059, Santa Cruz; iHSP70, SPA810, Stressgen)
in TBST with 5% milk, followed by washing with TBST,
and incubation with a horseradish peroxidase-conjugated sec-
ondary antibody in TBST with 5% milk. After final washing
in TBST, signal was detected using the Renaissance West-
ern Blot Chemiluminescence kit (DuPont NEN). The amount
of protein per lane was confirmed using a primary antibody
against actin (SC-8432, Santa Cruz). Autoradiograms of
molecular dynamics densitometer and expressed in units of
optical density.

Three dependent measures of inducible HSP70 were
analyzed: baseline levels (0 μg/ml LPS), stimulated levels
(10 μg/ml LPS) and percent change from baseline, which
was calculated as the difference from baseline with LPS
stimulation expressed as a percent of baseline (10 μg/ml
LPS − 0 μg/ml LPS × 100). For each HSP70 measure, and for HSC70 results and actin, two-way between-
subjects analysis of variance (ANOVA) models comparing
the three groups at the various times (0, 30 and 90 min post
LPS) were performed using Systat (SPSS Inc., Chicago, IL)
to test for statistical significance at the p < 0.05 level. Post-
hoc comparisons, to determine differences among the groups,
were performed using Fisher’s LSD test.

Note that for the 240-min time point a pooled sample was
analyzed, since the tissue was used across all the times and
was limited for this time. Therefore, the 240 time point was
analyzed separately.

3. Results

3.1. Body weights

Analysis of the body weights indicated that the young animals weighed less (388.1 ± 13.8 g) than the BB-
supplemented (467.3 ± 12.6 g) or control (469.8 ± 12.7 g)
senescent animals contributing to an overall significant differ-
ence in the analysis of variance (F(2,26) = 124.16, p < 0.000).
However, as can be seen from the data, there were no dif-

erences between the two groups of aged animals (p > 0.05
post-hoc Fisher’s LSD test).

3.2. Baseline rat HC HSP70 protein levels

As shown in Fig. 1, baseline levels of HSP70 in hippocam-
apal regions across the various times did not differ among the
various groups [F(4,32) = 2.48, p = 0.064]. However, it ap-
ppeared that the HSP70 was higher at the 30 and 90 min times
than at the 0 time [F(2,32) = 11.73, p < 0.001].

3.3. Time course of HSP70 induction in rat HC
following LPS challenge

The results of the putative age by diet-related differences
in the time course of the hippocampal HSP70 response to
LPS indicated that these differences did not appear
until 90 min following LPS exposure (Fig. 2) [group
effect, F(2,32) = 8.47, p < 0.01; time effect, F(2,32) = 48.52,
Fig. 1. Baseline levels of heat shock protein 70 (HSP70) at various incubation times (0, 30, 90 and 240 min) in hippocampal regions of young rats fed a control diet, old rats fed a control diet, and old rats fed a blueberry supplemented diet for 10 weeks.

Post-hoc testing showed that following 90 min of in vitro exposure to 10 ug/ml LPS, increases in HSP70 levels were significantly lower in old control diet rats as compared to young control diet rats in brain hippocampal regions (p < 0.001). Hippocampal regions from BB-supplemented old rats produced HSP70 levels that were higher than old control diet rats (p < 0.05) and no different from the young rats (p > 0.05). These group differences were maintained at 240 min after LPS treatment [F(2,6) = 8.95, p < 0.05] (Fig. 2).

When examining percent change from baseline, i.e., no LPS induced HSP70 versus LPS induced HSP70 at each time, the group differences were primarily seen at 90 min (F(2,12) = 4.17, p < 0.05) (Fig. 3). Again, the percent change in senescent rats fed the control diet was lower than that of young control diet rats (p < 0.05), while the BB-supplemented old rats had a percent increase that was not different than the young rats (p = 0.71), yet higher than age-matched controls (p < 0.05). However, at the 240 min time point, there were no differences between the groups on the effects of LPS on HSP70 induction [F(2,6) = 0.33, p = 0.73] (Fig. 3). As is also shown in Fig. 3, the non-significant differences among the groups at 240 min appears to be the result of reductions in LPS-induced HSP70 in the young and BB-supplemented groups and a slight but non-significant increase in the old control group, rather than a slower rise in the induction of HSP70 in this group.

Finally, there were no differences in HSC70 or actin as a function of age or diet as a function of the length of the LPS exposure.

4. Discussion

As mentioned in the introduction, with age there are increased levels of, and reduced protective mechanisms against, cellular stresses such as inflammation and oxidative stress [5,46,48]. One of the most important of these is HSP70. The induction of HSP70 in response to heat stress has been demonstrated in a wide range of studies to protect cells from subsequent damage. HSP70 has been described as a molecular chaperone that works in part to restore damaged proteins to their original structure and function [57,65]. The amount of protection afforded has been shown to be proportional to the level of HSP70 induction [1–3,68]. Within the brain, HSP70 mediated cell survival is also affected by cell type, brain region, and age-dependent differences in the time course of the response to hyperthermia [47,51]. In general, mammals become less tolerant to heat stress with old age, as evidenced by the higher rate of death by hyperthermia in old rats [28] and humans [16]. A reduction in the size of the HSP70 response [49,27] may play a role in the increased vulnerability to stressors that occurs with age, and as mentioned in the Introduction, both oxidative stress [58] and inflammation [25,26] are contributing factors to the behavioral decrements seen in aging.
In this regard, the present findings show that there were no differences among the groups in basal HSP70. However, it also appeared that acute LPS-induced HSP70 production was significantly decreased at the 90 and 240 min post LPS times in the senescent control rats as compared to the aged BB-supplemented and young control rats. The finding showing that there were age differences in the response at both of these later time points suggest that the differences observed were not the result of age differences in amount of time required to mount a full HSP70 response as compared to the age differences in overall size of response.

Instead the results showed that short-term nutritional intervention with BB supplementation increased HSP70 mediated protection in the hippocampal regions of old rats against a stressor such as LPS, and this difference is maximized at the 90 and 240 post LPS time points (Fig. 2). These findings suggested that short-term BB supplementation restored the ability of the aged hippocampal cells to respond to an inflammatory challenge with a large production of inducible HSP70.

Thus, it might be postulated that the increases in stress induced HSP70 activity in the BB-supplemented aged rats could play a role in shifting the balance between pro- and anti-oxidant forces [5] to a response that was similar to those seen in the young animals. As HSP70 is synthesized in response to a host of damaging stimuli, these findings suggest that the blueberry supplemented diet may result in improved protection against a number of age-related neurodegenerative processes in the brain.

Previous studies have suggested that although age-related changes in hippocampal function impair spatial learning and memory in rats and humans [20,39,42,53], the improved protection may combine with other factors such as increases in hippocampal neurogenesis and signaling [12] that ultimately translate into improved behavioral performance. In this regard, as mentioned in Section 1, BB supplementation significantly improved performance on tests of spatial learning and memory [8,35,36,71] in the senescent rats.

The mechanisms involved in the enhanced HSP70 response to LPS in the hippocampal tissue also may involve altered signaling, since we have recently shown that BB supplementation enhanced hippocampal ERK activity in APP/PS-1 mice [29]. Recent work also indicates that hyperthermic-induced stress decreased hippocampal ERK activity and increased HSP70 activity. There is also work to show that curcumin, a potent spice with antioxidant/anti-inflammatory activity, antagonizes the effects of LPS by down-regulating the Janus (JAK) kinase-STAT inflammatory pathway [37]. Results from several experiments suggest that the LPS involves activation of microglia and subsequent induction of glutamate. These would ultimately affect neuronal cells in the hippocampus (e.g., see [40]). Further analyses of the putative role of BB supplementation in altering signaling in LPS-induced HSP70 activity will be the subject of future experiments, as will determination of the particular cell type involved in these activations.

Finally, it should be mentioned that although the BB-supplemented animals did not show any loss of weight as compared to the rats fed the control diet, the effects seen here with respect to LPS-induced enhancement of HSP70 are similar to those seen previously with long-term caloric restriction (CR) (see [11] for review). It should also be noted that transgenic mouse models of Alzheimer disease (AD) respond to food restriction with increased resistance to AD-related synaptic changes [74]. This protection correlates with enhanced HSP70 production in cortical synaptosomes and with HSP70’s effect on calcium regulation, membrane potential, and resistance to oxidative stress [24,62,73]. This neuroprotection is postulated to be a critical factor in the beneficial effect of caloric restriction on both life expectancy and animal models of Alzheimer disease [24,73].

5. Conflict of interest statement

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References


[53] Rosenwweg ES, Barnes CA. Impact of aging on hippocampal func-

[54] Sadoul R. Bcl-2 family members in the development and de-

hippocampal changes in Bcl-2:Bax ratio, oxidative stress, redox-
active iron and apoptosis associated with aluminum-induced neu-

[56] Schipper HM. Astrocytes, brain aging, and neurodegeneration. Neu-


[58] Shukitt-Hale B. The effects of aging and oxidative stress on psy-

[59] Steffen B, Breier G, Butcher E, Schulz M, Gregoraiti B. VCAM-1, and
MAdCAM-1 are expressed on choroid plexus epithelium but
not endothelium and mediate binding of lymphocytes in vitro. Am

[60] Stella N, Estelles A, Siciliano J, Tence M, Desagher S, Pi-
omelli D, et al. Interleukin-1 enhances the
ATP-evoked release of

KT. Feeding rats diets enriched in lowbush blueberries for six

[62] Walton TJ, Ryan KL, Mason PA. Regional distribution of Hsp70 in
the CNS of young and old food-restricted rats following hyperther-


[64] Welch WJ. Mammalian stress response: cell physiology, struc-
ture/function of stress proteins, and implications for medicine and

[65] Welch WJ. Heat shock proteins functioning as molecular chaper-
ones: their roles in normal stressed cells. Phil Trans R Soc Lond
1993;339:327–33.

[66] Woodroffe M. Cytokine production in the central nervous system.

[67] Wu X, Brecher GR, Holden JM, Hayotowitz DB, Gebhardt SE, Prior
RL. Lipophilic and hydrophilic antioxidant capacities of common

[68] Yunari MA, Fink SL, Sun GJ, Chang LK, Patel MK, Kunis DM,
et al. Gene therapy with HSP72 in neuroprotective in rat models of

[69] Youdim KA, Joseph JA. A possible emerging role of phytochemicals
in improving age-related neurological dysfunction: a multiplicity of

[70] Youdim KA, Martin A, Joseph JA. Essential fatty acids and the brain:

[71] Youdim KA, Shukitt-Hale B, Martin A, Wang H, Demirors N, Brick-
ford PC, et al. Short-term dietary supplementation of blueberry
polyphenolics: beneficial effects on aging brain, performance and

[72] Xu BP. Cellular defenses against damage from reactive oxygen

[73] Yu ZF, Mattson MP. Dietary restriction and 2-deoxyglucose admin-
istration reduce focal ischemic brain damage and improve behavioral
outcome: evidence for a preconditioning mechanism. J Neurosci Res

[74] Zhu H, Guo Q, Mattson MP. Dietary restriction protects hippocampal
neurons against the death-promoting action of a presenilin-1 muta-