JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Apple Polyphenols Extend the Mean Lifespan of *Drosophila melanogaster*

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ABSTRACT: Apple polyphenols (AP) are an excellent source of dietary antioxidants. The present study investigated the effect of AP on the lifespan of fruit flies and their interaction with gene expressions of superoxide dismutase (SOD), catalase (CAT), methuselah (MTH), Rpn11, and cytochrome *c* oxidase (CcO) subunits III and VIb. Results showed the mean lifespan was significantly extended by 10% in fruit flies fed the AP diet. This was accompanied by up-regulation of genes SOD1, SOD2, and CAT and down-regulation of MTH in the aged fruit flies. Paraquat and H_2O_2 challenge tests demonstrated that AP prolonged the survival time only for Oregon R wild type flies but not for SOD^{n108} or Cat^{n1} mutants, in which either SOD or CAT was knocked out. Chronic paraquat exposure could shorten the maximum lifespan from 68 to 31 days and reduce the climbing ability by 60%, whereas AP could partially reverse the paraquat-induced mortality and decline in climbing ability. AP could up-regulate Rpn11 at day 30, whereas it appeared to have no significant effect on gene expression of ubiquitinated protein, CcO subunits III and VIb. These AP-induced changes were unlikely associated with caloric restriction as the gustatory assay found no difference in average body weight and stomach redness index between the control and AP fruit flies. It was therefore concluded that the antiaging activity of AP was, at least in part, mediated by its interaction with genes SOD, CAT, MTH, and Rpn11.

KEYWORDS: apple polyphenols, lifespan, fruit fly, antioxidant, paraquat, climbing ability

INTRODUCTION

Organisms become aged partially due to accumulation of free radical damage in their cells over time. Reactive oxygen species (ROS), namely, hydroxyl radical, superoxide anion, and hydrogen peroxide, are produced as natural byproducts of the normal cellular metabolism of oxygen. The oxidative damage initiated by ROS is believed to be a major contributor to the functional decline associated with aging. In this regard, organisms have two antioxidant systems to scavenge ROS and minimize their associated adverse effect. First, they endogenously produce a group of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), which serves as a first line of defense against ROS. Second, exogenous antioxidants such as vitamins E and C may build a secondary defense base to terminate the propagation of ROS reactions and slow the aging process.[1]

Various predictive biomarkers have been suggested for oxidative stress, physiological aging, and age-linked diseases. First, it is widely accepted that mitochondrial respiratory capacity declines during the aging process. Cytochrome *c* oxidase (CcO), the terminal component of the mitochondrial electron transport chain (ETC), has been reported to have its subunits III and VIb significantly reduced in the aging flies.[2] It has been also reported that CcO deficiency would lead to reduction of ETC activity as a whole due to increased production of either superoxide anion radicals or hydrogen peroxide in mitochondria.[3] Second, Rpn11 has been recognized as a suppressor of progressive neurodegeneration. Knocking down of Rpn11 can lead to the accumulation of ubiquitinated proteins, shorten life span, and reduce 26S proteasome activity.[4] Third, the *methuselah* (MTH) gene has been shown to be one of the longevity-determining genes. It has been reported that MTH mutant flies can live 35% longer than wild type flies and exhibit greater resistance to oxidative stress,[5] even though the underlying mechanism remains poorly understood.

Fruit flies (*Drosophila melanogaster*) have been employed as a model in aging research because they share many conserved biological pathways, and >70% of known disease-causing genes in humans are conserved.[6] In addition, the benefits of using fruit flies as an aging model are associated with their short lifespan, tiny body size, easy maintenance, and known sequence of the full *Drosophila* genome. Dietary modification is thought to be able to prolong the lifespan and slow age-related diseases. On the one hand, calorie restriction has been shown to extend lifespan in various animal models;[7−10] on the other hand, supplementation of different nutraceuticals into the daily diet has been also recognized as a potential way to slow the aging process.[11] Apple is an excellent source of polyphenol antioxidants and has been regarded as a healthy fruit in many cultures.[12−15] The present study was to investigate (i) the lifespan-prolonging activity of apple polyphenols (AP) and (ii) the interaction between dietary AP and gene expressions of endogenous antioxidant enzymes, CcO subunits III and VIb, Rpn11, and MTH in fruit flies.

Received: December 1, 2010
Accepted: January 25, 2011
Revised: January 19, 2011
Published: February 14, 2011

dx.doi.org/10.1021/jf1046267 | *J. Agric. Food Chem.* 2011, 59, 2097–2106

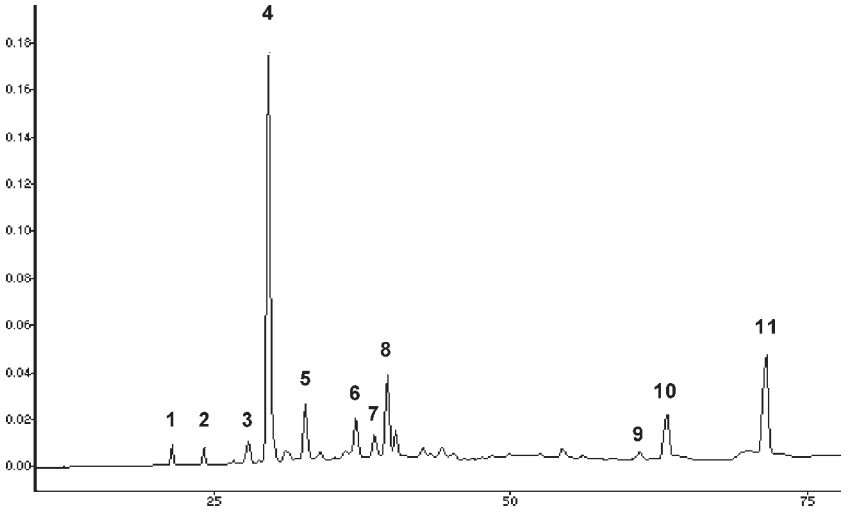

Figure 1. HPLC chromatogram of apple polyphenols (AP). Peaks: 3, catechin; 4, chlorogenic acid; 5, proanthocyanidin B2; 6, epicatechin; 9, rutin; 11, phloridzin; 1, 2, 7, 8, 10, unknown.

MATERIALS AND METHODS

Fly Strains. Fly strains used in this study were Oregon-R-C (OR), SOD^{n108}/TM3 (SOD^{n108}), and OE^{-}/SM5 × Cat^{n1}/TM3 (Cat^{n1}) (Bloomington Drosophila Stock Center, Department of Biology, Indiana University, Bloomington, IN). OR is a wild type fly used in all experiments unless specified otherwise. SOD^{n108} is a mutant with one pair of single SOD gene on chromosome 3 L being knocked out, whereas Cat^{n1} is a mutant with CAT gene on chromosome 3 L being knocked out by a point mutation.

Diet. A control diet was prepared according to the standard formulation described previously.[16,17] In brief, 1000 mL of diet contained 105 g of cornmeal, 21 g of yeast, 105 g of glucose, and 13 g of agar. Ethyl 4-hydroxybenzoate (0.4%) was added to the diet to prevent mold growth. The two AP diets were prepared by adding 2 mg (AP2) and 10 mg (AP10) in the control diet per milliliter, respectively. For rearing the stocks, 15 mL of the basal diet was poured and set into a vial. For the experimental flies, 5 mL of the basal or experimental diets was prepared per vial. If humans have 2000 kcal per day, the amount of AP in the diet of 2000 kcal was equivalent to the consumption of six apples per day.

Isolation of AP and HPLC Analysis. AP was isolated from the pomace of Red Fuji as we previously described.[18] In brief, AP was extracted into ethanol and then concentrated, followed by purification through adsorption on an AB-8 macroporous resin column. The yield of AP was about 0.4 kg of AP/100 kg of apple pomace. Individual polyphenols in AP were separated on a Luna C_{18} column (100A, 250 × 4.6 mm i.d.) and quantified on a Shimadzu LC-10AT HPLC system equipped with a UV detector at 280 nm. The flow rate was set at 1 mL/min, whereas the gradient mobile phase consisted of 2% acetic acid (solvent A) and 0.4% acetic acid with 80% acetonitrile (solvent B). The ratio of A to B was programmed from 10:1 to 2:8 in 80 min and then back to 10:1 in 3 min and then was held for another 22 min (Figure 1). The peaks were identified according to the retention times of standards. AP contained chlorogenic acid (16.4%) followed by phloretin (6.2%), proanthocyanidin B2 (4.3%), epicatechin (2.6%), catechin (1.2%), rutin (0.2%), and other unidentified proanthocyanidins and phenolics (69.0%).

Effect of AP on Longevity of OR Flies Fed the Basal Diet. Newly eclosed male flies were divided into three groups (n = 200 flies each) and housed in 10 vials (20 flies per vial). The first group was maintained on the basal diet, whereas the two AP groups were fed one of the two diets containing 2 or 10 mg AP/mL, respectively. Dead flies were counted every 2−3 days, and the remaining live flies were transferred to a new vial containing the same diet. The feeding lasted 74 days (Figure 2A). The same experiments described above were similarly repeated, and the fruit flies were killed at selected time points to quantify the expression of SOD, CAT, and MTH.

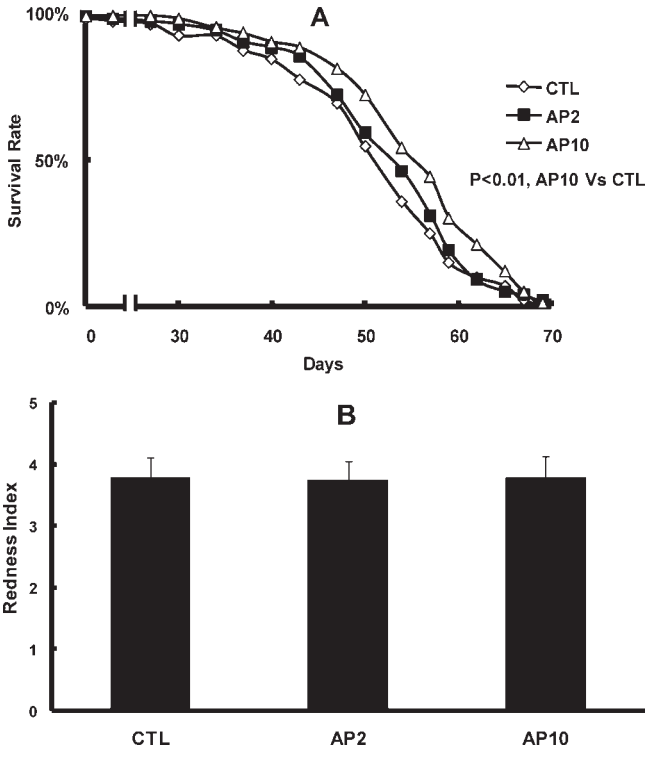

Figure 2. (A) Lifespan curve of wild type flies (OR) fed diets containing 0 mg/mL (control, CTL), 2 mg (AP2), and 10 mg AP (AP10) per milliliter of diet. Data are expressed as the maximum lifespan of last fly, 50% survival time, and mean lifespan (n = 200 flies) for each group (Table 1). The Kaplan−Meier test found AP10 could significantly extend the mean lifespan of fruit flies (P < 0.01). (B) Gustatory assay compared food intake on the basis of the differences in the degree of abdomen redness among CTL, AP2, and AP10 groups. Data are expressed as the mean ± SD.

Table 1. Lifespan of OR Wild Type Flies Fed the Control Diet and the Two Experimental Diet Containing 2 and 10 mg of AP/mL

	max lifespan of last fly (days)	50% survival (days)	mean lifespan[a] (days)
control	72	50	50 ± 2 a
2 mg AP	74	53	52 ± 2 a
10 mg AP	74	55	55 ± 2 b

[a] Means with different letters differ significantly at $p < 0.01$.

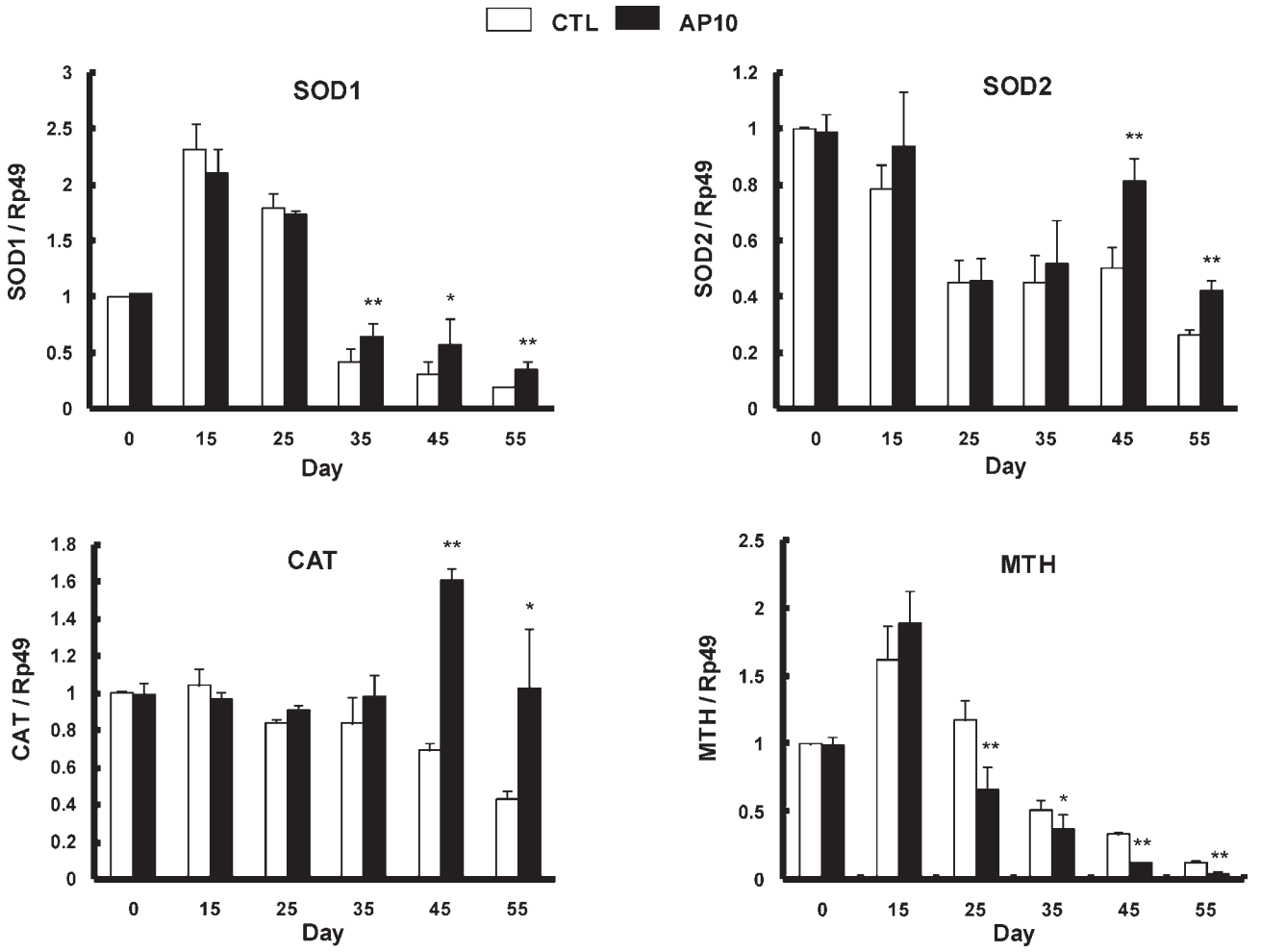

Figure 3. Effect of apple polyphenols (AP) supplementation (10 mg/mL diet) on mRNA of copper−zinc-containing superoxide dismutase (SOD1), manganese-containing superoxide dismutase (SOD2), catalase (CAT), and methuselah (MTH) compared with those in the control diet (CTL). The wild type (OR) flies (n = 300/group, n = 20/vial) were incubated at 25 °C for 0, 15, 25, 35, 45, and 55 days. Data are expressed as the mean ± SD. *, $p < 0.05$, and **, $p < 0.01$ compared with the control value.

Gustatory Assay. To exclude the possibility that lifespan extension in survival assay might be induced by dietary restriction, a gustatory assay was carried out.[19] In brief, 60 newly eclosed male flies were collected (20 flies per vial) and reared on a standard diet for 5 days and then starved for 24 h on Kimwipes paper soaked with distilled water. Afterward, flies were maintained on the basal or AP-supplemented diets containing 0.2% sulforhodamine B sodium salt (Acid-Red) for 2 h. Then the degree of fly abdomen redness was blind-scored using a grading scale ranging from grade 0 (colorless abdomen) to grade 5 (fully red abdomen). Food intake was compared on the basis of the difference in the degree of abdomen redness between the control and AP-fed group (Figure 2B).

Intensive Paraquat Challenge Experiment. Paraquat, chemically named 1,1′-dimethyl-4,4′-bipyridinium dichloride (Sigma, St. Louis, MO), is able to generate superoxide anion radicals.[20] To examine the resistance of flies against superoxide-induced stress, both OR flies (n = 400 in 20 vials) and SOD^{n108} mutant flies (n = 400 in 20 vials) were maintained on their corresponding control diet and experimental diet containing 10 mg of AP/mL and incubated at 25 °C. At day 25, the fruit flies in the two groups were first starved for 2 h and then transferred into new vials containing a filter paper saturated with 1 mL of 20 mM paraquat in a 6% glucose solution. Every 4−6 h, dead flies were counted until all flies had died.

Hydrogen Peroxide (H_2O_2) Challenge Test. H_2O_2 is able to generate a hydroxyl radical and, therefore, it was used to examine the resistance of flies against OH-induced oxidative stress. OR flies (n = 400) and Cat^{n1} mutant flies (n = 400) were maintained on their corresponding control diet or experimental diet containing 10 mg of AP/mL and incubated at 25 °C. Similarly, the fruit flies in the two groups were first

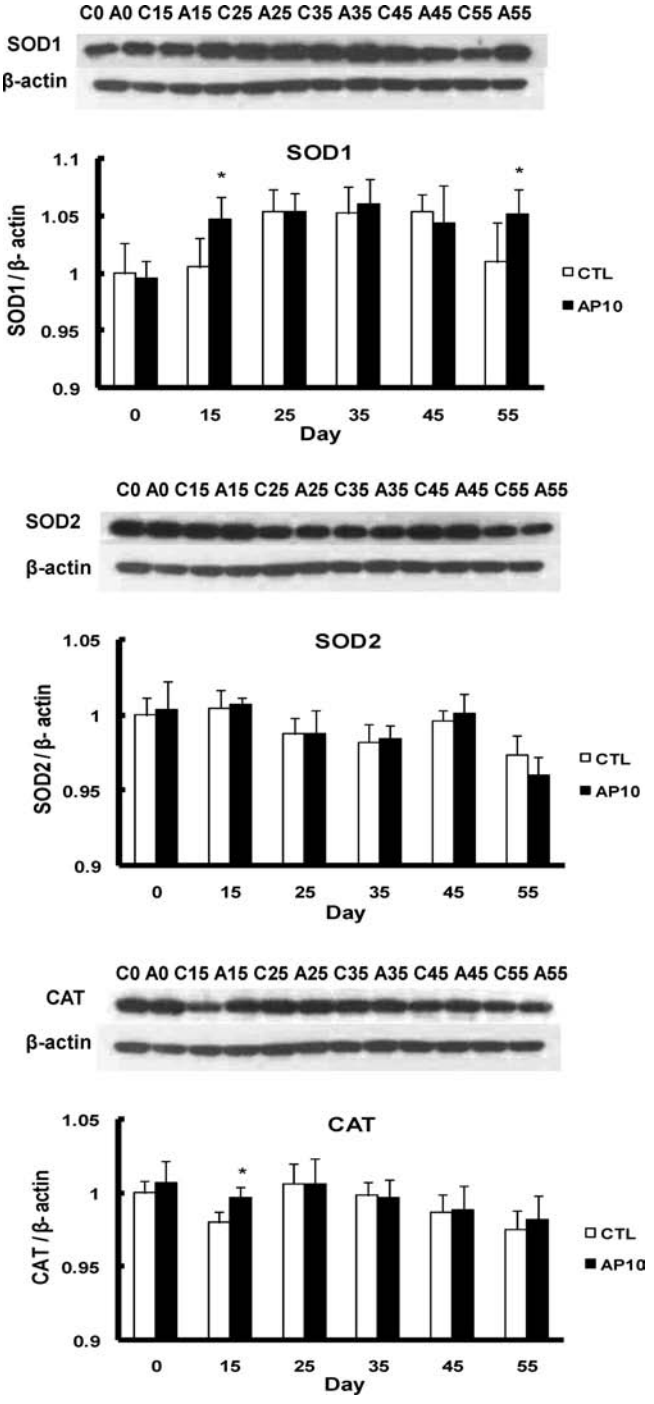

Figure 4. Effect of apple polyphenols (AP) supplementation (10 mg/mL diet) on the relative protein mass of copper−zinc-containing superoxide dismutase (SOD1), manganese-containing superoxide dismutase (SOD2), and catalase (CAT) compared with those in the control diet (CTL). The wild type (OR) flies (n = 300/group, n = 20/vial) were incubated at 25 °C for 0, 15, 25, 35, 45, and 55 days. C0−C55 represent the protein bands for the control at days 0−55, whereas A0−A55 represent the protein bands for AP10 at days 0−55. Data are expressed as the mean ± SD. ∗, $p < 0.05$, compared with the control value.

starved for 2 h and then were transferred into new vials containing a filter paper saturated with 1 mL of 30% H_2O_2 in a 6% glucose solution at day 25. Every 4−6 h, dead flies were counted until all flies had died.

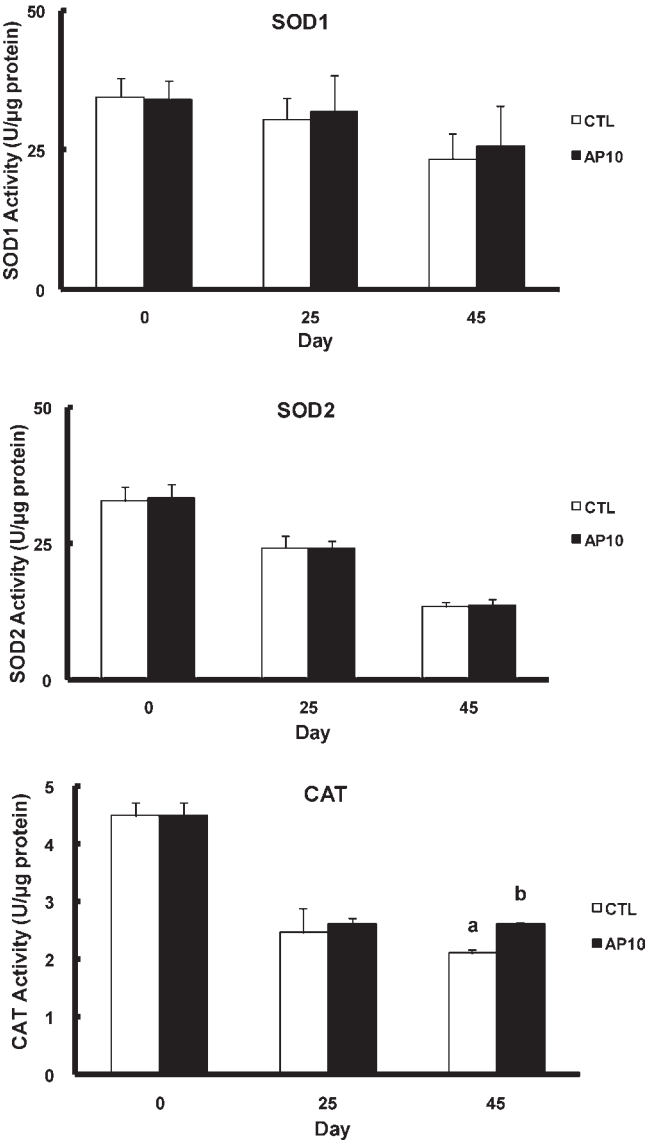

Figure 5. Effect of apple polyphenols (AP) supplementation (10 mg/mL diet) on enzymatic activity of copper−zinc-containing superoxide dismutase (SOD1), manganese-containing superoxide dismutase (SOD2), and catalase (CAT) compared with those in the control diet (CTL). The wild type (OR) flies (n = 300/group, n = 20/vial) were incubated at 25 °C for 0, 25, and 45 days. Data are expressed as the mean ± SD. Means with different letters differ significantly at $p < 0.05$.

Chronic Paraquat Challenge. A long-term exposure to paraquat has been recognized as a potential risk factor for the risk of having neurodegenerative diseases such as Parkinson's disease. To examine the resistance of flies against paraquat-induced mortality and locomotor deficiency, 800 newly eclosed male OR flies were randomly divided into three groups, namely, blank control group (BCTL), control group (CTL), and the experiment diet containing 10 mg of AP/mL (AP10). Every 3 days, flies, after 2 h of starvation, were transferred into vials containing a filter paper that was saturated with 1 mL of 20 mM paraquat in a 6% glucose solution. After 24 h, flies were moved to new vials containing only a water-saturated filter paper for 2 h before they were transferred back into vials containing respective diets. The experiment lasted 42 days until all of the fruit flies in the AP10 group had died. Another set of the experiment described above was similarly repeated, and the fruit flies were killed at days 0, 10, 20, and 30 to quantify the

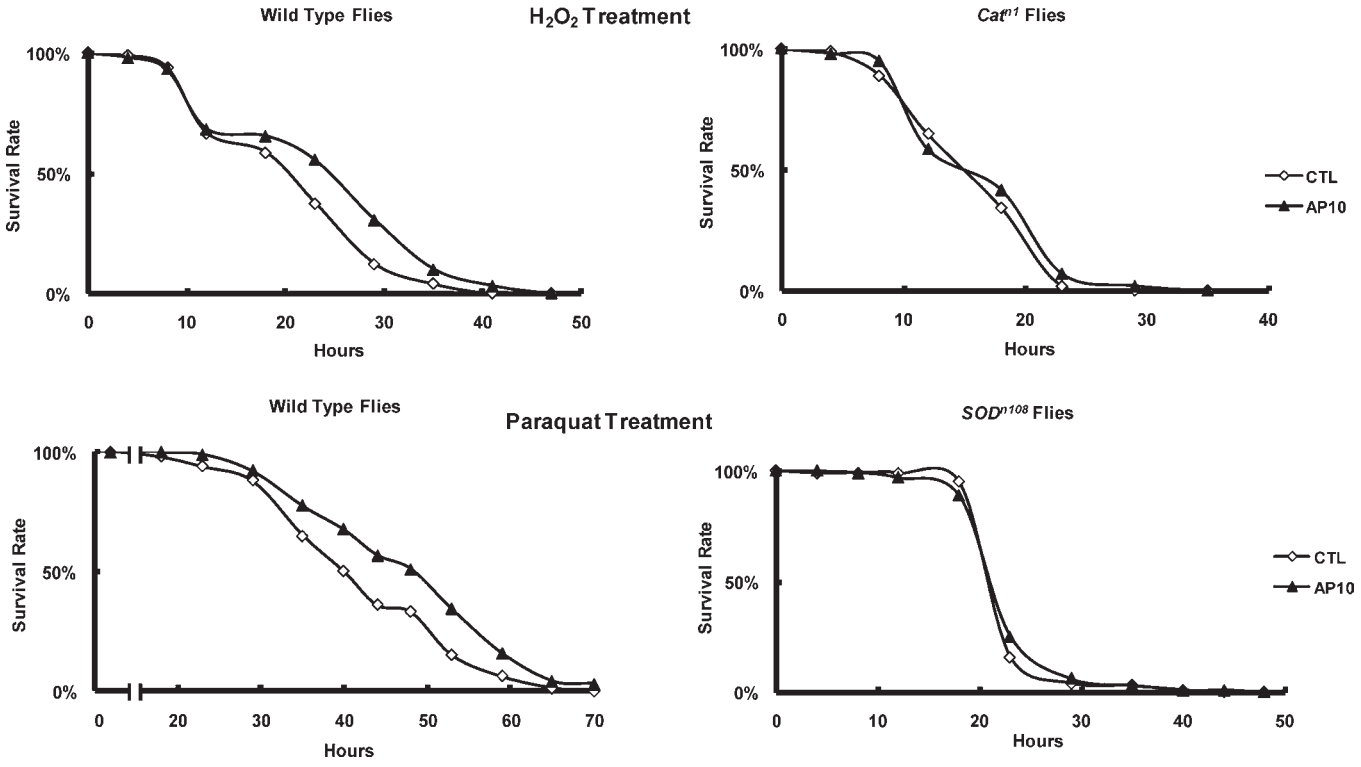

Figure 6. Effect of paraquat treatment or hydrogen peroxide treatment on the survival time of the mutant flies (SOD^{n108}) or mutant flies (Cat^{n1}) fed the diets containing 0 mg/mL (CTL) or 10 mg AP/mL compared with the wild type (OR) flies. The Kaplan−Meier test found both that the AP-fed OR group survived better than its corresponding OR control ($p < 0.05$) and that the survival rates of AP-fed SOD^{n108} and Cat^{n1} groups were not different from their corresponding control groups.

expression of SOD, CAT, MTH, CcO subunits, ubiquitinated proteins, and Rpn11.

Climbing Assay. Locomotor function of fruit flies was assessed using a climbing assay as previously reported with some slight modifications.[21] In brief, 10 male flies were placed in a plastic vial, and given 20 s to climb up. At the end of each trial, the number of flies that climbed up to a vertical distance of 15 cm or above was recorded. Each trial was performed three times. Flies were tested at selected time points during the chronic paraquat challenge survival assay.

SOD Activity. An assay kit (Cayman Chemical, Ann Arbor, MI) was used to quantify the SOD activity in fruit flies.[16] The principle is that a tetrazolium salt can detect superoxide anion radicals generated by xanthine oxidase and hypoxanthine, whereas SOD is able to remove the superoxide anion. In general, one unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radicals. The fruit flies ($n = 100$) were homogenized in 1 mL of cold 20 mM HEPES buffer (pH 7.2, with 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose) followed by centrifugation at a speed of 1500*g* for 5 min at 4 °C. The supernatant was transferred into a new tube on ice and then subjected to centrifugation at 10000*g* for 15 min at 4 °C. The supernatant contained the cytosolic CuZnSOD (SOD1), and the pellet contained mitochondrial MnSOD (SOD2). The supernatant was removed into a new tube, and the mitochondrial pellet was suspended in 0.5 mL of cold HEPES buffer. The sample (10 μL) in triplicates was used for each test. The diluted radical detector containing tetrazolium salt (200 μL) was added onto 96-well plates together with 10 μL of sample. The reaction was initiated by adding 20 μL of diluted xanthine oxidase followed by shaking of the plate for 20 min at room temperature. After incubation, the absorbance was recorded at 450 nm in a microplate reader.

CAT Activity. CAT was measured using a catalase assay kit (Sigma, St. Louis, MO).[16] The principle is based on the measurement of the hydrogen peroxide substrate remaining after the action of catalase present in the sample. In brief, flies ($n = 100$) were homogenized in 1 mL of enzyme dilution buffer followed by centrifugation at a speed of 1500*g* for 5 min at 4 °C. The supernatant was moved into a new tube and diluted 15 times by 1× assay buffer (5 mM potassium phosphate buffer, pH 7.0) in triplicates. The resultant sample (10 μL) was diluted again with 65 μL of 1× assay buffer. Then, 25 μL of 200 mM hydrogen peroxide solution was added to initiate the reaction. At exactly 1 min, 900 μL of stop solution (15 mM sodium azide) was added. The reaction mixture (10 μL) was mixed with 1 mL of color reagent containing 0.25 mM 4-aminoantipyrine, 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, and freshly added peroxidase (0.8−1.2 U/mg). After incubation at room temperature for 15 min, the absorbance of each sample was measured in a spectrometer at 520 nm.

Real-Time PCR. Gene expression of SOD1, SOD2, CAT, MTH, and Rpn11 was measured as we previously described.[16] Total RNA was extracted using the commercial extraction agent TRIzol (Invitrogen, Carlsbad, CA). Fruit flies ($n = 15$) were homogenized in 800 μL of TRIzol solution and then centrifuged at 12000*g* at 4 °C for 10 min, and the supernatant was transferred to another new tube containing 160 μL of chloroform. The mixture was then subjected to centrifugation at 12000*g* at 4 °C for 15 min. The upper layer was mixed with 400 μL of isopropanol. After 10 min of incubation at room temperature, the samples were centrifuged at 12000*g* at 4 °C for 10 min, and the pellet was saved and washed in 1 mL of 75% ethanol followed by recentrifugation. Finally, 25 μL of DEPC water was employed to resuspend the RNA pellet. The concentration and purity of RNA obtained were determined by measuring their absorbance at 260 and 280 nm. A High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to construct cDNA. RNA (2 μg) was used for each reaction together with $MgCl_2$, 10× RT buffer, dNTP, random hexamers, RNase

dx.doi.org/10.1021/jf1046267 |J. Agric. Food Chem. 2011, 59, 2097–2106

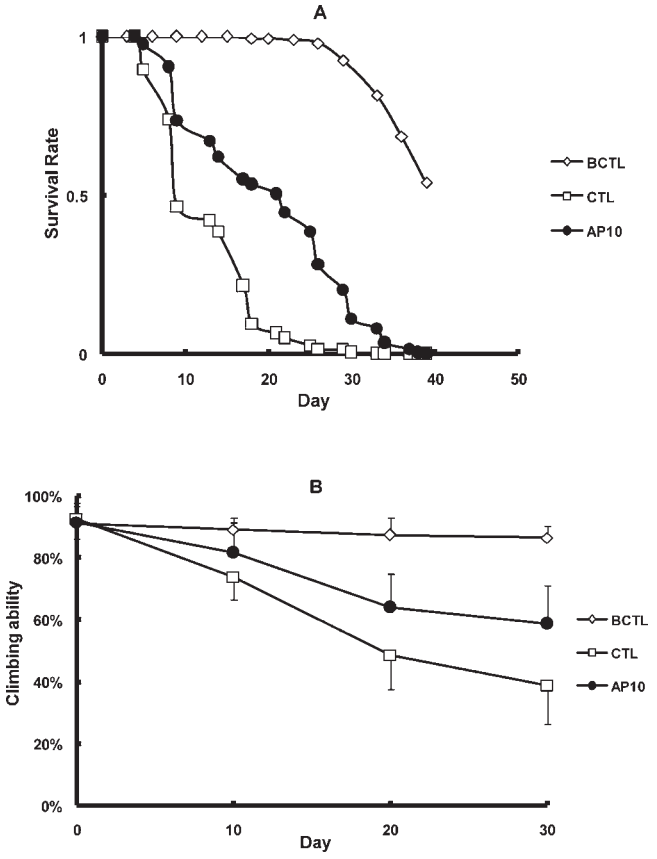

Figure 7. (A) Lifespan of OR fruit flies fed either a basal diet without exposure to paraquat (BCTL) or a basal diet with paraquat chronic exposure (CTL) with the addition of 10 mg/mL AP (AP10) at 25 °C. The Kaplan−Meier test found the AP10 group survived better than the CTL ($p < 0.01$). (B) Effect of AP on the climbing ability in BCTL, CTL, and AP10 groups. The AP10 group could significantly alleviate locomotor deficiency in the CTL group. C0−C30 represent the protein bands for CTL group at days 0−30; B10−B30 represent the protein bands for BCTL groups at days 10−30; and A10−A30 represent the protein bands for AP10 at days 10−30.

inhibitor, and MultiScribe Transcriptase. The final volume was adjusted to 10 μL. cDNA was synthesized in the thermocycler GeneAmp PCR system 9700 (Applied Biosystems) and stored at −20 °C.

Real-time PCR amplification was carried out on a Fast Real-time PCR System 7500 (Applied Biosystems). Five target genes included SOD1 (NCBI Reference Sequence NM_057387.3), SOD2 (NCBI Reference Sequence NM_057577.2), CAT (NCBI Reference Sequence NM_080483.2), MTH (NCBI Reference Sequence NM_079147.2), and Rpn11 (NCBI Reference Sequence NM_135061.2). The expressions of target genes were normalized with that of rp49 (NCBI Reference Sequence NM_079843.2), a housekeeping gene used as the internal control. Gene expressions were calculated on the basis of the comparative threshold cycle (C_T) value. Levels of gene expressions in all groups are shown as a ratio of the day 0 control group.

Western Blot Analysis. Total proteins or mitochondria protein were extracted and subjected to Western blot analysis. For total proteins, 50 flies were homogenized in a 1.5 mL tube containing 500 μL of homogenizing buffer (20 mM Tris-HCl, 2 mM $MgCl_2$, 0.2 M sucrose, and protease inhibitor cocktail (Roche, Mannheim, Germany)). The extracts were centrifuged at 13000*g* for 5 min at 4 °C, and the supernatant were collected. To isolate the mitochondria, approximately 50 flies per sample (4 samples/group) were homogenized in mitochondrial isolation medium (MIM; 250 mM sucrose, 10 mM Tris, pH 7.4, 0.15 mM $MgCl_2$). The samples were centrifuged twice at 1000*g* for 5 min to remove debris. The supernatant was centrifuged at 13000*g* to obtain a mitochondria-enriched pellet, which was washed with 1 mL of MIM. This pellet was resuspended in 50 μL of MIM, and the membranes were disrupted by two freeze−thaw cycles. Protein concentration was determined using a protein concentration assay kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA). After adding 6× loading dye and homogenizing buffer to adjust the volume, the protein was boiled at 95 °C for 5 min and then stored at −80 °C. Mitochondria protein was subjected to Western blot analysis specifically for CcO subunit III, VIb, and porin.

For the measurement of CAT and β-actin, 20 μg of total protein was size-fractionated on 7% SDS-PAGE gel at 130 V for 70 min, whereas the same amount of total protein was loaded to measure SOD1 and SOD2 on a 15% SDS-PAGE gel at 130 V for 180 min. To measure Cco subunit III, VIb, and porin, 10 μg of mitochondria protein was size-fractionated on a 10% SDS-PAGE gel at 110 V for 100 min. The proteins were then transferred to a Hybond-P PVDF membrane (Millipore, Billerica, MA). The membranes with total proteins were incubated for 1 h in blocking solution (5% nonfat milk) at room temperature and then in the same solution containing diluted anti-catalase/anti-actin/anti-CuZnSOD/anti-MnSOD/anti-Ub antibodies, respectively, at 4 °C overnight. For immunodetection of mitochondria proteins, membranes were incubated with the anti-CcO subunits III, VIb, and porin at 37 °C for 1 h. The membrane was washed in 1× TBST and was then incubated for 1 h at 4 °C in diluted horseradish peroxidase-linked goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-mouse IgG (Santa Cruz Biotechnology). The washes were repeated before the membranes were developed with ECL enhanced chemiluminescence agent (Santa Cruz Biotechnology) and subjected to autoradiography for 1 s−5 min on SuperRX medical X-ray film (Fuji, Tokyo, Japan). Densitometry was quantified using the computer software Quantity one (Bio-Rad).

Statistics. Data are expressed as the mean ± standard deviation. The Kaplan−Meier test was employed to compare the difference between the survival curves using SPSS 15.0 (Statistical Package for the Social Sciences software, SPSS Inc., Chicago, IL). The significance of difference between means was assessed using *t* test and one-way ANOVA. Differences were considered to be significant when $p < 0.05$.

RESULTS

Effect of AP on Longevity of OR Flies Fed the Basal Diet and AP Diets. The AP10 group had the longest lifespan among the three groups of OR wild type male flies (Figure 2A). The maximum lifespan increased about 3% in the AP10 group, whereas 50% survival time increased from 50 days to 55 days compared with the control flies. To be specific, the mean lifespans for the control and the two AP groups were 50, 52, and 55 days, respectively (Figure 2A; Table 1). However, a significant difference in the mean lifespan was found only between AP10 and the control ($p < 0.01$).

The SOD1 gene was significantly up-regulated in the AP10 group compared with the control at days 35, 45, and 55, whereas gene expressions of SOD2 and CAT in the AP10 group were greater than those in the control at days 45 and 55 (Figure 3). In contrast, the MTH gene was significantly down-regulated in the AP10 group compared with the control at day 25 (Figure 3).

Western blot data showed that, at days 15 and 55, the AP10 group had protein mass of SOD1 greater than that in the control, whereas at day 15, a significant difference in CAT protein mass was observed between the AP10 group and the control. Otherwise, no significant differences in protein mass were seen

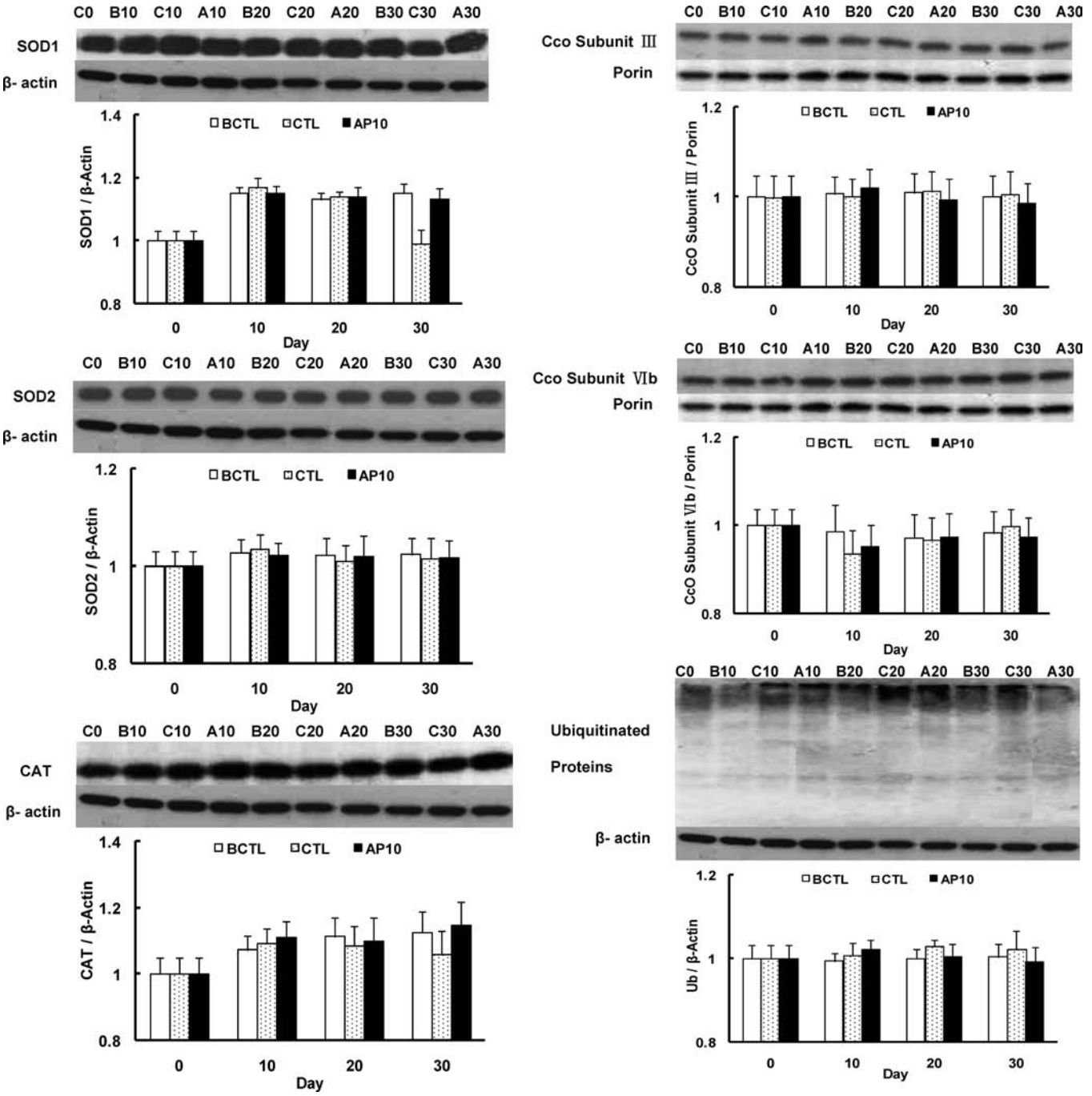

Figure 8. Relative immunoreactive mass of copper−zinc-containing superoxide dismutase (SOD1), manganese-containing superoxide dismutase (SOD2), catalase (CAT), cytochrome *c* oxidase subunit III (CcO subunit III), subunit VIb (CcO subunit VIb), and ubiquitinated proteins (Ub) in OR fruit flies fed either a basal diet without going through the paraquat challenge cycle (BCTL) or a basal diet with paraquat chronic exposure (CTL) with the addition of 10 mg/mL AP (AP10). C0−C30 represent the protein bands for the CTL group at days 0−30; B10−B30 represent the protein bands for BCTL groups at days 10−30; and A10−30 represent the protein bands for AP10 at days 10−30.

between AP10 and the control (Figure 4). The present study also investigated the effect of AP on the activity of SOD1, SOD2, and CAT in OR wild type male flies at days 0, 25, and 45. In general, SOD1, SOD2, and CAT activity decreased with aging. Compared with the control group, AP10 could not prevent the decreasing trend in SOD1, SOD2, and CAT activity at all time points except at day 45, when AP10 showed a greater CAT activity than the control (Figure 5).

Effect of AP on Paraquat and H_2O_2-Challenged OR, Cat^{n1}, and SOD^{n108} Flies. Results from an intensive paraquat challenge test showed that the AP10 OR group had a longer survival time than the corresponding control ($p < 0.05$). To be specific, for OR wild type flies the maximum survival time increased from 70 h in the control to 98 h in the AP10 group, with mean survival time being prolonged by 14% ($p < 0.05$). However, no difference was found in SOD^{n108} flies fed the control and AP10 diets. Similar

dx.doi.org/10.1021/jf1046267 |*J. Agric. Food Chem.* 2011, 59, 2097–2106

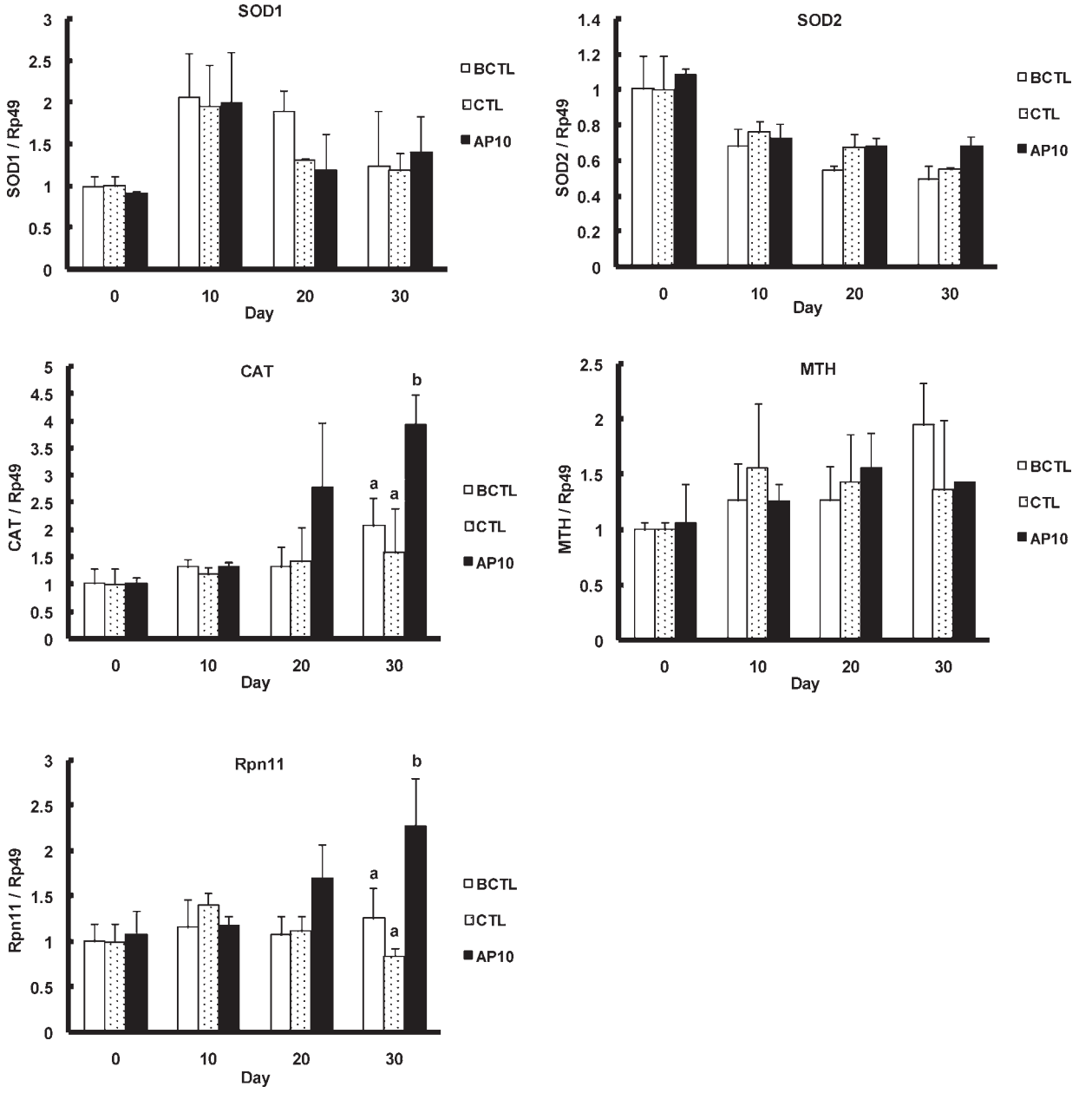

Figure 9. mRNA of copper−zinc-containing superoxide dismutase (SOD1), manganese-containing superoxide dismutase (SOD2), catalase (CAT), methuselah (MTH), and Rpn11 in OR fruit flies fed either a basal diet without going through paraquat exposure (BCTL) or a basal diet with the paraquat challenge cycle (CTL) with the addition of 10 mg/mL AP (AP10) at 25 °C. Means at the same time point with different letters differ significantly at $p < 0.05$.

results were obtained in the H_2O_2 challenge assay. Supplementation of AP prolonged the maximum survival time, 50% survival time, and mean survival time only in OR wild type but not in the Cat^{n1} fly strain (Figure 6).

Effect of AP on Chronic Paraquat Challenge in OR Flies. Long-term exposure to 20 mM paraquat could induce a high mortality rate in fruit flies, shorten their maximum lifespan to 31 days, and reduce their climbing ability by >60% (Figure 7). Supplementation of AP in the diet could partially reverse the paraquat-induced mortality and decline in climbing ability. Results demonstrated that the maximum lifespan was 31 days in the paraquat-control group, whereas it was 39 days in the paraquat-AP10 group (Figure 7A). At the same time, the climbing ability was <40% in the paraquat-control groups, whereas it was partially recovered to >60% in the paraquat-AP10 flies at day 30 (Figure 7B).

Western blot analysis data did not found any significant difference in protein abundance of SOD1, SOD2, CAT, ubiquitinated protein, and CcO subunits III and VIb among the blank control, paraquat-control, and paraquat-AP10 groups (Figure 8). mRNA analysis revealed that AP could up-regulate the gene expression of only CAT and Rpn11 at day 30, with no effect on SDO1, SOD2, ubiquitinated protein, or CcO subunits III and VIb (Figure 9). With regard to the enzyme activity, SOD1 did not change over time and no difference could be seen among the three groups. In contrast, CAT had a decreasing trend over time

dx.doi.org/10.1021/jf1046267 |J. Agric. Food Chem. 2011, 59, 2097–2106

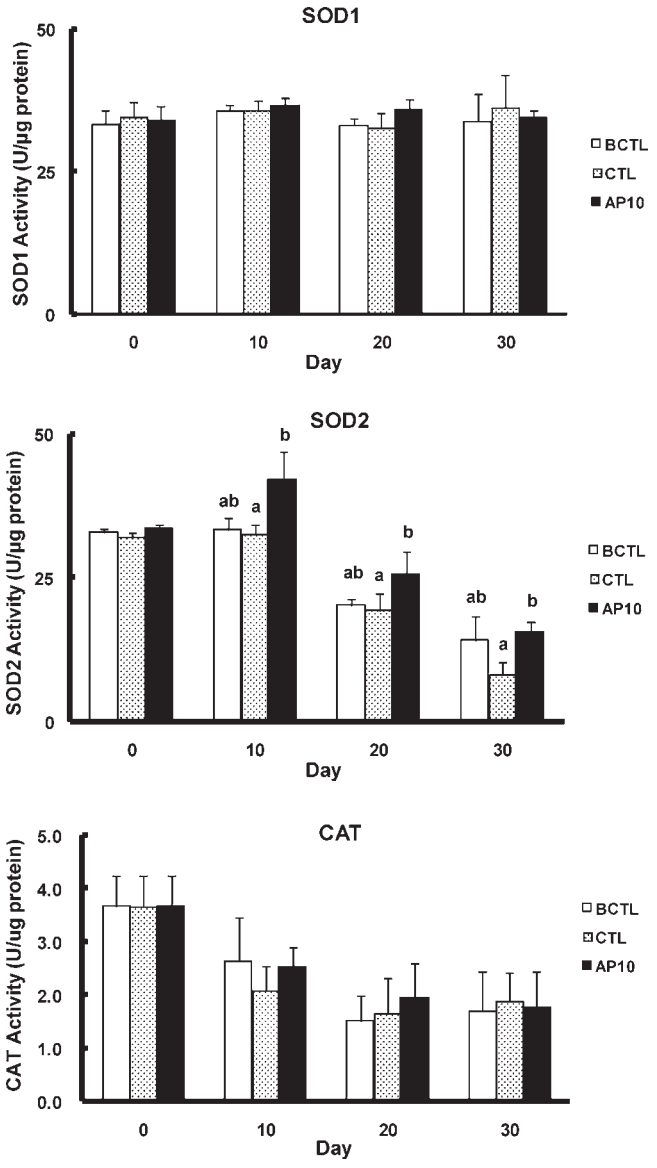

Figure 10. Enzymatic activity of copper−zinc-containing superoxide dismutase (SOD1), manganese-containing superoxide dismutase (SOD2), and catalase (CAT) in OR fruit flies fed either a basal diet without paraquat exposure (BCTL) or a basal diet with paraquat challenge cycle (CTL) with the addition of 10 mg/mL AP (AP10) at 25 °C. Means at the same time point with different letters differ significantly at $p < 0.05$.

and, similarly, no difference was found among the three groups. After day 10, SOD2 activity showed a decreasing trend over time in the paraquat-control flies, and supplementation of AP could partially restore the SOD2 activity (Figure 10).

DISCUSSION

The present study is the first to show AP was effective against aging in fruit flies. AP supplementation at 10 mg/mL prolonged the mean lifespan by 10% compared with the control (50 versus 55 days, Figure 2). However, AP had little or no significant effect on the maximum lifespan of fruit flies (Table 1). This is in agreement with the study of Sunagawa et al.,[22] who found that apple procyanidins could extend the mean lifespan of *Caenorhabditis elegans* by 8−12%. No similar studies on AP lifespan extensions have been conducted in either animals or humans. However, apple juice concentrate, administered ad libitum in drinking water, can compensate for the increased ROS and decline in cognitive performance in mice deprived of folate and vitamin E.[23] In humans, it has been demonstrated that plasma ROS generation, within 30 min after apple juice consumption, could be effectively suppressed in plasma, and this radical scavenging effect was maintained for around 2 h postconsumption.[24] Results in a women's health study found that women who often ingested apples had a 13−22% decrease in cardiovascular disease risk.[25]

The present study found that supplementation of AP was associated with greater mRNA of SOD1, SOD2, and CAT with lesser MTH mRNA only in the aged but not in young fruit flies, implying the antiaging activity of AP was mediated at least in part by up-regulation of endogenous antioxidant enzymes with down-regulation of the longevity MTH gene (Figure 3). However, up-regulation of mRNA of SOD1, SOD2, and CAT was not accompanied by greater protein masses or increased activity of these antioxidant enzymes (Figures 4 and 5). Perhaps this discrepancy is due to the insensitivity of the Western blot assay compared with the RT-PCR technique. The involvement of SOD and CAT associated with AP's antiaging activity can be further reaffirmed in both paraquat and H_2O_2 challenge tests, which demonstrated that AP could prolong the survival time only in OR wild type flies but did not affect that of SOD^{n108} or Cat^{n1} mutants, in which either SOD or CAT was knocked out (Figure 6), supporting the hypothesis that the antiaging activity of AP was mediated in part by interaction with genes of SOD and CAT. Results from an intensive paraquat/H_2O_2 challenge assay were in agreement with the previous report of Jimenez-Del-Rio et al.,[26] who found that AP in the diet could significantly reduce the mortality rate of OR wild type flies. In this regard, SOD activity in the hippocampus of the aged rats fed an apple-rich diet could be maintained at the level of the young animals.[27]

Supplementation of AP in the diet could partially reverse the chronic paraquat exposure-induced mortality and decline in climbing ability (Figure 7), demonstrating the superoxide anion could accelerate, while dietary antioxidants could delay, the aging. It is suggested that chronic paraquat exposure may be one factor contributing to neurodegenerative disorders such as Parkinsonian syndrome.[28] Data from survival and locomotor activity in this assay implied that AP could help ameliorate the neurodegenerative disorder.

Administration of AP significantly down-regulated gene expression of MTH in the wild type fruit flies (Figure 3). However, the effect was not evident in the chronic paraquat challenge test. It was reported that MTH mutant flies could reach a 35% longer lifespan and acquire higher resistance to oxidative stress, compared with the wild type peers.[5] With chronic paraquat exposure, gene expression of Rpn11 was down-regulated from day 10. Rpn11 is a suppressor of progressive neurodegeneration, and knocking out Rpn11 could lead to formation of ubiquitinated proteins and shorten the lifespan. This result together with data on MTH suggests an additional mechanism by which AP prolonged the mean lifespan of fruit flies exists in addition to its regulating effect on genes of SOD and CAT.

In conclusion, the present study demonstrated that AP in the diet could prolong the mean lifespan, attenuate the paraquat-induced mortality rate, and partially reverse the decline of locomotor deficiency in fruit flies. The antiaging and

antineurodegenerative disorder activity was at least in part mediated by its interaction with gene expressions of MTH, Rpn11, SOD, and CAT. It was unlikely that the lifespan-prolonging activity of AP in the fruit flies was associated with any changes in food intake, as the gustatory assay found no difference in average body weight and stomach redness index between the control and AP fruit flies.

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ABBREVIATIONS USED

CAT, catalase; CcO, cytochrome *c* oxidase; CuZnSOD or SOD1, copper−zinc-containing superoxide dismutase; AP, apple polyphenols; MTH, methuselah; MnSOD or SOD2, manganese-containing superoxide dismutase; SOD, superoxide dismutase; ROS, reactive oxygen species.

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