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Genetic Mechanisms of Coffee Extract Protection in a *Caenorhabditis elegans* Model of β-Amyloid Peptide Toxicity

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ABSTRACT

Epidemiological studies have reported that coffee and/or caffeine consumption may reduce Alzheimer's disease (AD) risk. We found that coffee extracts can similarly protect against β-amyloid peptide (Aβ) toxicity in a transgenic *Caenorhabditis elegans* Alzheimer's disease model. The primary protective component(s) in this model is not caffeine, although caffeine by itself can show moderate protection. Coffee exposure did not decrease Aβ transgene expression and did not need to be present during Aβ induction to convey protection, suggesting that coffee exposure protection might act by activating a protective pathway. By screening the effects of coffee on a series of transgenic *C. elegans* stress reporter strains, we identified activation of the *skn-1* (Nrf2 in mammals) transcription factor as a potential mechanism of coffee extract protection. Inactivation of *skn-1* genetically or by RNAi strongly blocked the protective effects of coffee extract, indicating that activation of the *skn-1* pathway was the primary mechanism of coffee protection. Coffee also protected against toxicity resulting from an aggregating form of green fluorescent protein (GFP) in a *skn-1*–dependent manner. These results suggest that the reported protective effects of coffee in multiple neurodegenerative diseases may result from a general activation of the Nrf2 phase II detoxification pathway.

EPIDEMIOLOGICAL studies have indicated that coffee consumption may be protective in Alzheimer's (AD) (Barranco Quintana *et al.* 2007) and Parkinson's diseases (PD) (Hu *et al.* 2007). Although a universal consensus on the cause(s) of Alzheimer's disease has not been reached, the best-supported hypotheses posit that accumulation of the β-amyloid peptide (Aβ) is central to the induction of Alzheimer's disease pathology. The strongest evidence for this claim is the identification of germ line mutations in the gene encoding amyloid precursor protein (APP), (Chartier-Harlin *et al.* 1991; Murrell *et al.* 1991), or in the presenilin genes involved in cleaving Aβ from APP (reviewed in Cruts *et al.* 1996) in familial cases of early-onset AD. The pathological similarities between familial and sporadic AD, along with the results of many studies of transgenic mice engineered to overexpress Aβ in the brain, argue for a causal role of Aβ accumulation in all forms of AD. Intramuscular accumulation of Aβ is also observed in inclusion body myositis (IBM), a severe myopathy that can be phenocopied by overexpression of APP in transgenic mice (Sugarman *et al.* 2002).

Models of Aβ toxicity can potentially be used to investigate the biological mechanisms underlying the protective effects of coffee consumption in Alzheimer's disease. In this context, caffeine administration has been reported to reverse cognitive impairment and amyloid β (Aβ) levels in transgenic Alzheimer's disease model mice (Arendash *et al.* 2009). To examine the protective effects of coffee in a genetically tractable model, we investigated the effects of aqueous coffee extracts in a *C. elegans* model of Aβ toxicity (Link *et al.* 2003). In this model, temperature upshift induces expression of human $A\beta_{42}$ in body wall muscle. This induction of $A\beta_{42}$ leads to a highly reproducible paralysis phenotype, in which all induced animals become paralyzed within 28 hr of temperature upshift. This model has previously been used to investigate gene expression changes in response to Aβ accumulation (Link *et al.* 2003) and the role of specific genes (Fonte *et al.* 2008; Hassan *et al.* 2009) and autophagy (Florez- McClure *et al.* 2007) in countering Aβ toxicity. Here we employ this model to investigate candidate genes and pathways that might play a significant role in the protective effects of coffee.

MATERIALS AND METHODS

***C. elegans* strains used in this study:**

CB1301 unc-54(e1301) I
CB66 unc-22(e66) IV
DA465 eat-2(ad465) II
CL4176 smg-1(cc546) I; dvIs27 [pAF29 (Pmyo-3::$A\beta_{42}$) + pRF4] X
CL437 ced-7(n1892) III; ced-5 (n1815) IV; mec-4 (u231) X
CL2166 dvIs19(Pgst-4::GFP) III

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CL2070 dvIs70[pCL25(Phsp-16.2::GFP) + pRF4) V
SJ4006 zcIs4(Phsp-4::GFP) V
TJ356 zIs356(daf-16::GFP)
CL685 ldIs001(skn-1::GFP)
CL6176 smg-1(cc546) I; dvIs19(Pgst-4::GFP) III; +/nT1(unc d;let-? IV;V); dvIs27 [pAF29 (Pmyo-3::Aβ_{42}) + pRF4] X
CL6180 smg-1(cc546) I; dvIs19(Pgst-4::GFP) III; skn-1(zu67) IV/nT1(unc d;let-? IV;V); dvIs27 [pAF29 (Pmyo-3::Aβ_{42}) + pRF4] X
CL6222 smg-1(cc546) I; dvIs19(Pgst-4::GFP) III; skn-1(zu169) IV/nT1(unc d;let? IV;V); dvIs27 [pAF29 (Pmyo-3::Aβ_{42}) + pRF4] X
CL2337 smg-1(cc546) I; dvIs38 [pCL60 (Pmyo-3::GFP::degron/long 3′ UTR) + pRF4].

Coffee extract preparation: We employed an aqueous extraction protocol originally developed in the Pallanck lab (Trinh *et al.* 2010). Coffee beans (Starbucks House Blend, caffeinated or decaffeinated) were ground for 3 min in a standard coffee grinder, and then an 18.4% (w/v) slurry of grounds in deionized water was boiled for 30 min before removing grounds with a French press. Extracts were filter sterilized by passage through a Nalgene Fast PES Filter Unit, a 75-mm diameter membrane (0.2 μm pore size), and stored a 2°.

Media preparation: Small (5.7 cm) Petri plates containing 10 ml nematode growth media agar (Wood 1988) were supplemented with coffee extract or purified caffeine (Sigma) after agar was solidified and allowed to diffuse throughout the agar before addition of bacteria. Caffeine concentrations were based on the caffeine concentration reported for Starbucks caffeinated drip coffee or from direct mass spectroscopy measurements of decaffeinated coffee extracts (Trinh *et al.* 2010). Plates were spotted with *Escherichia coli* strain OP50 or, for feeding RNAi, strain HT115 transformed with a *skn-1* dsRNA-expressing plasmid or vector-only control (Kamath *et al.* 2003). In all experiments worms were exposed from hatching to the coffee extracts or caffeine.

Quantification of paralysis kinetics: Staged populations of CL4176, CL6176, CL6180, CL6222, or CL2337 transgenic worms were prepared by synchronous egg lay and induced to express Aβ as third-stage larvae by upshift from 16° to 25° as previously described (Link *et al.* 2003). All paralysis plots were done in triplicate with an average of 129 worms per strain and condition. Plots shown in Figures 1 and 4–7 are representative; all experiments were independently replicated (see supporting information, Table S1). Statistical analysis of paralysis curves was performed using a paired log rank survival test (Peto and Peto 1972) implemented in Statistica.

Feeding RNAi interference knockdown of *skn-1*: *skn-1* expression was knocked down by feeding RNAi interference using the corresponding *E. coli* from the Ahringer RNAi feeding library (clone verified by DNA sequencing). Using standard RNAi feeding protocols (Kamath *et al.* 2003) we observed that one generation of exposure to the *skn-1* RNAi clone was insufficient to induce the 100% embryonic lethality phenotype observed for *skn-1*(*zu169*) homozygotes. We therefore developed a two-generation RNAi exposure protocol in which third larval stage worms were placed on the *skn-1* (or control vector only, VO) RNAi plates and allowed to grow until the second day of adulthood at 16°. These treated adults were then used for a synchronous egg lay on RNAi plates to generate populations for paralysis quantitation after upshift. Under these conditions, control populations that were not upshifted grew to adulthood but laid 100% dead eggs.

Microscopy: DIC and epifluorescence images were acquired on a Zeiss Axiophot compound microscope equipped with a computer-controlled Z-drive and software from Intelligent Imaging Innovations. Photoshop software (Adobe) was used to globally adjust brightness and contrast of digital images and to fuse DIC and epifluorescence images (Figures 4C and 7B).

Quantification of P*gst-4*::GFP expression: Eggs of CL2166 were collected by hypochlorite, hatched overnight at 16°, and first-stage larvae (L1s) were placed onto NGM or 10% decaffeinated coffee plates and allowed to grow to L4 stage at 16°. Worms were harvested 1000 worms/ml in 1X S-Basal, and sorted using COPAS Biosort 250 Worm Sorter (Union Biometrica Inc., Harvard Biosciences). Length, optical absorbance, and integrated fluorescence intensity at 488 nm (GFP) were factors in the worm sorting. A total of 100 worms were sorted per condition. All sorting was done at room temperature.

Quantitative RT–PCR: Aβ mRNA levels were quantified as previously described (Link *et al.* 2003) using an ABI Prism 7000 thermocycler using a TM of 55° and the following primers: forward primer 5′ CTTTCTGGCACCAGCAGGTAC and reverse primer 5′ CTTGCAGACTTCTCGCTGCTAG.

Aβ mRNA levels were normalized to reference genes *cdc-42*, *pmp-3*, and Y45F10D.4 as described in Hoogewijs *et al.* (2008).

Immunoblotting: Worms were harvested in deionized water with protease inhibitor cocktail (Sigma, P 2714) added, and then snap frozen. Worms were boiled in sample buffer (1X protease inhibitor cocktail, 62 mm Tris pH 6.8, 2% SDS, 10% glycerol, 4% BME) for 10 min, put on ice, and then centrifuged 1 min at 14,000 *g*. Supernatant was quantitated via Bradford assay (Pierce, 23238).

Just before loading, samples were boiled for 5 min in sample buffer with dye (62 mm Tris pH 6.8, 2% SDS, 10% glycerol, 4% BME, 0.0005% BPB). Samples were run at 180 V on Nu PAGE 4–12% Bis-Tris Gel (Invitrogen, NP0321) using MES SDS Running Buffer (Invitrogen NP0002). ECL DualVue Western markers (GE Healthcare, RPN810) were used as size reference. Gel was transferred to 0.45 μm supported nitrocellulose (GE Osmonics, WP4HY00010) using 20% methanol, 39 mm glycine, and 48 mm Tris base. Transfer conditions were 21 V, 108 min.

Blots were visualized by Pouceau stain and then boiled for 3 min in PBS. Blots were blocked in TBS-Tween + 5% milk (100 mm Tris 7.5, 150 mm NaCl, 0.1% Tween-20). Aβ was detected with 6E10 (Covance, SIG-39320) at 1 μg/ml; secondary anti-mouse IgG peroxidase conjugate (Sigma, A5906). GFP was probed with 11E5 (Quantum Biotechnologies, AFP5001) at 0.5 μg/ml; secondary anti-mouse IgG (same as above). After stripping, blots were probed with CTSF64 (gift of Blumenthal Lab) at 1/7500; secondary antibody anti-rabbit IgG peroxidase conjugate (Jackson Immunoresearch Laboratories, 211-032-171). Secondary HRP-conjugated antibodies were developed in ECL Plus (Amersham, RPN2132). CstF-64 immunoreactivity was used as an internal control for immunoblots because preliminary experiments indicated that accumulation of actin, a more commonly used internal control, was actually altered by loss of *skn-1* activity.

RESULTS

Coffee extracts delay paralysis induced by Aβ expression: We found that addition of coffee extract to the agar media (10% extract, vol/vol) used to propagate *C. elegans* significantly delayed the onset of paralysis in worms induced to express Aβ_{42} (Figure 1A). Suppression of paralysis was observed with extracts from both caffeinated and decaffeinated coffee, with caffeinated coffee having a slightly stronger effect ($P < 0.02$, paired log rank survival test). Addition of pure caffeine at a concentration equivalent to that found in caffeinated coffee had a moderate effect, and addition of caffeine at the concen-

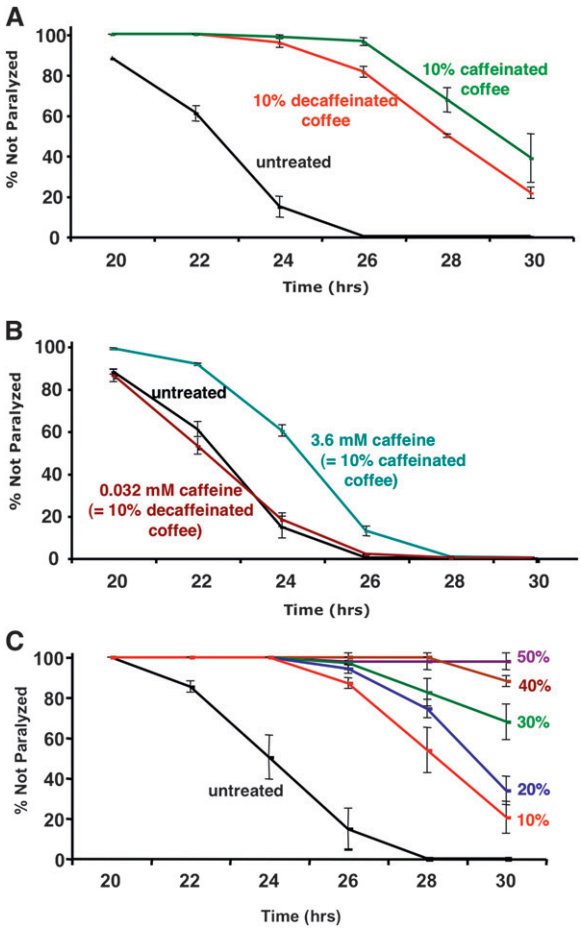

FIGURE 1.—Effects of coffee extracts and caffeine on paralysis in inducible Aβ expression strain CL4176 (P*myo-3*::$A\beta_{42}$). Time refers to hours after Aβ expression was induced by temperature upshift. (A) Incorporation of 10% caffeinated or decaffeinated coffee extract into agar media plates suppresses paralysis (control *vs.* caffeinated or decaffeinated coffee, $P < 0.001$; decaffeinated coffee *vs.* caffeinated coffee, $P < 0.02$, paired log rank survival test). (B) Incorporation of pure caffeine into agar media plates can also slow induced paralysis, but this effect is weaker than that of decaffeinated coffee. (This experiment was done in parallel with that shown in A; separate plots are presented for clarity.) (C) Dose response paralysis curves for agar media containing 0–50% coffee extract. Error bars = SEM.

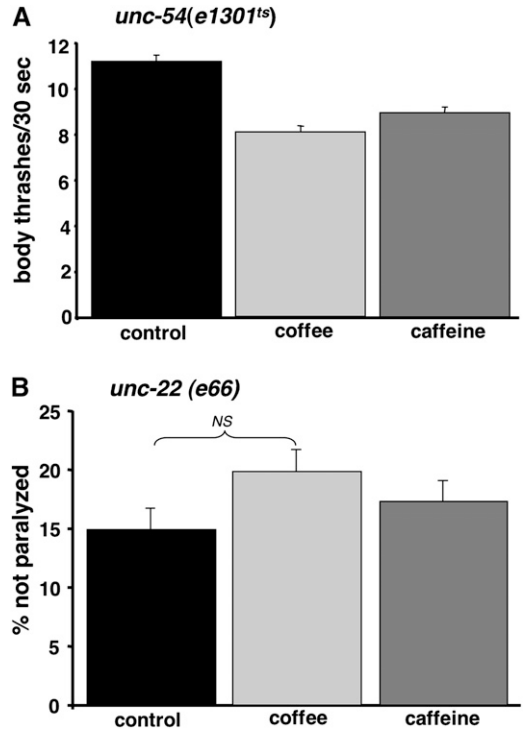

FIGURE 2.—Effect of coffee extracts and caffeine on control mutants with movement defects. (A) Rate of body thrashing in liquid for *unc-54*(*e1301*^{ts}) mutant raised at 25° on agar media plates. Decaffeinated coffee extract (10%) and caffeine (3.6 mM) do not improve movement of this mutant and actually caused small but statistically significant reductions in thrashing (control plates, 11.2 thrashes/30 sec; coffee plates, 8.1 thrashes/30 sec; caffeine plates, 8.9 thrashes/30 sec; $P < 0.0001$ calculated by one-way ANOVA followed by Dunnett's multiple comparisons test). (B) Fraction unparalyzed *unc-22*(*e66*) mutant worms after propagation at 25° on control, 10% coffee extract, or 3.6 mM caffeine agar media plates. Neither coffee extract nor caffeine significantly increase the fraction of unparalyzed *unc-22* mutant worms (χ^2, $P = 0.5$, treatment *vs.* paralyzed/nonparalyzed). Error bars = SEM.

tration present in the decaffeinated coffee extract showed no protection (Figure 1B). We conclude that most of the protective effect is from coffee components other than caffeine, although caffeine may also contribute. The protective effects of decaffeinated coffee was also dose dependent (Figure 1C).

To investigate whether the reduced paralysis rates resulting from coffee extract or caffeine exposure might be due to a general effect on *C. elegans* movement or muscle function, we assayed extract effects on two well-characterized mutants with a partial paralysis phenotype, *unc-54*(*e1301*^{ts}) and *unc-22*(*e66*). (*unc-54* encodes a myosin protein expressed in body wall muscle, while *unc-22* encodes twitchin, a large sarcomere-associated protein homologous to mammalian titin.) Movement of *unc-54*(*e1301*^{ts}) was assayed by counting body thrashes in liquid for 30-sec intervals. At the nonpermissive temperature (25°), the thrashing rate of *unc-54*(*e1301*^{ts}) was ~10% of wild-type animals (11.2 thrashes/30 sec for *unc-54*(*e1301*^{ts}) *vs.* 105.0 thrashes/30 sec for wild-type strain N2) when these worms were propagated on standard media. As shown in Figure 2A, thrashing rates of *unc-54*(*e1301*^{ts}) were actually slightly reduced by propagation on 10% decaffeinated coffee or 3.6 mM caffeine, indicating that these treatments do not improve the movement of this strain. *unc-22* mutants have a spontaneous "twitcher" phenotype; at 25° the majority of the *unc-22*(*e66*) mutant worms have a more severe paralysis phenotype. As shown in Figure 2B, propagation on 10% decaffeinated coffee or 3.6 mM caffeine did not significantly reduce the fraction of paralyzed *unc-22*(*e66*) worms.

We also examined whether decaffeinated coffee could reduce neuronal cell loss induced by a dominant mutation [*mec-4*(*u231*)] in the MEC-4 sodium channel.

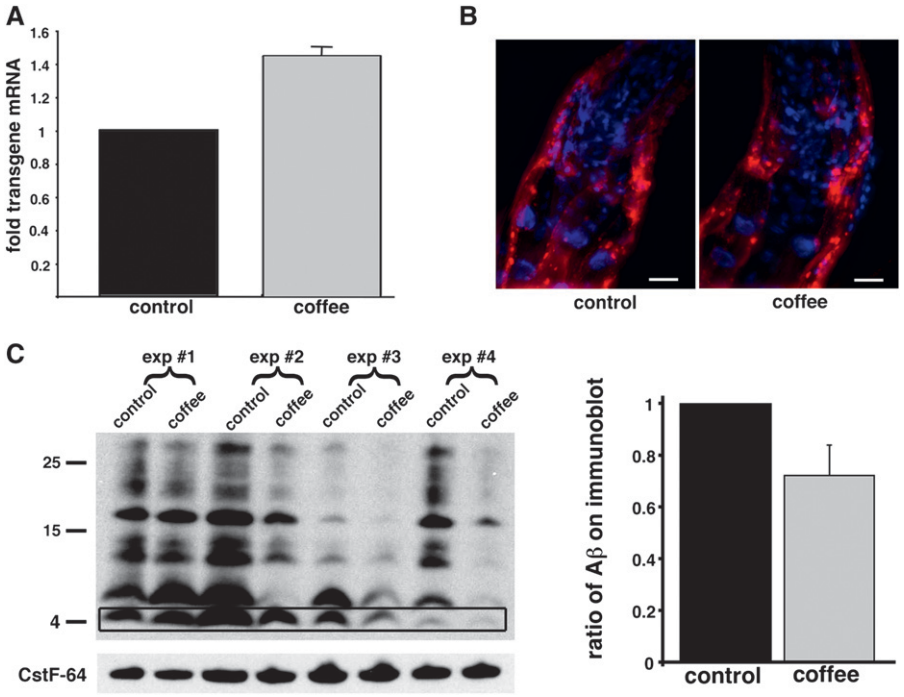

FIGURE 3.—Effect of coffee extract treatment on Aβ transgene expression and Aβ accumulation. (A) Quantitative RT–PCR of measurement of Aβ mRNA levels 24 hr after induction of CL4176 transgenic worms propagated on control or 10% coffee extract plates, average of three biologically independent experiments. (B) Anti-Aβ immunohistochemistry of anterior region of CL4176 worms induced for 24 hr after propagation on control or 10% decaffeinated coffee plates. Aβ immunoreactivity, red; DAPI counterstain, blue. Bar, 10 μM. (C) Immunoblot assay of Aβ accumulation 24 hr after upshift of CL4176 grown on control or 10% coffee extract plates. Left, visualization of monomeric (4 kDa band, boxed) and multimeric (higher molecular weight species) Aβ recovered from CL4176 worms induced for 24 hr after propagation on control or 10% decaffeinated coffee plates. Shown are total protein preparations (20 μg/lane) from four biologically independent experiments fractionated on a 4–12% polyacrylamide SDS gel and probed with anti-Aβ monoclonal antibody 6E10. The gel blot was reprobed with antibody against CstF-64 (EVANS *et al.* 2001) to demonstrate equal lane loading. Right shows quantitation of average Aβ levels in coffee extract-treated CL4176 worms relative to untreated worms, obtained from the four independent experiments shown on the left. Total Aβ immunoreactivity (monomeric and multimeric bands) in each lane was quantified. Error bars = SEM.

This mutation induces necrotic neuronal cell death by causing excessive ion influx, resulting in vacuoles visible by DIC microscopy. To enhance scoring of *mec-4*(*u231*)-induced vacuoles, we used a strain containing additional mutations [*ced-5*(*n1815*) and *ced-7*(*n1892*)] that block cell corpse removal and increase the persistence of vacuoles (Chung *et al.* 2000). Propagation on 10% decaffeinated coffee did not significantly reduce the fraction of staged *ced-7*(*n1892*); *ced-5*(*n1815*); *mec-4*(*u231*) worms displaying vacuoles (88/100 on control media, 85/100 on 10% decaffeinated coffee, exact χ^2, $P = 0.34$).

Coffee extracts do not reduce Aβ transgene expression: To determine whether coffee extract might trivially slow Aβ-induced paralysis by reducing expression of the Aβ transgene, quantitative RT–PCR was used to assay Aβ mRNA levels and anti-Aβ antibody (monoclonal 6E10) was used to monitor Aβ peptide accumulation. Both coffee-treated and control worms were harvested 24 hr after Aβ induction, and parallel populations were processed for quantitative RT–PCR, immunohistochemistry, and immunoblots. As shown in Figure 3A, Aβ transcript levels were actually slightly higher (~1.4-fold) in coffee-treated worms than in the controls. (This increase in Aβ transcript levels in coffee-treated worms may be due to the improved robustness of these animals at time of harvest.) No qualitative difference in the accumulation or distribution of large Aβ deposits was observed between treated and control worms as assayed by whole-animal immunohistochemistry (Figure 3B). However, coffee treatment did result in a variable reduction in total Aβ levels as assayed by immunoblot (Figure 3C). The average reduction in Aβ accumulation induced by coffee exposure was ~25% when averaged over four biologically independent experiments.

Coffee extracts induce the *skn-1*/Nrf2 pathway: To determine whether coffee extract needed to be present in the media to convey protection, Aβ transgenic worms were relocated at the third larval (L3) stage (48 hr after hatching) from coffee to control plates and upshifted to induce Aβ expression. As shown in Figure 4A, paralysis rates in worms that were shifted to control plates from coffee plates were still significantly slowed relative to worms maintained solely on control plates (although the protection was less than that observed with continual coffee exposure). This result suggested that coffee extract components do not need to be present at the time of Aβ accumulation to reduce Aβ toxicity. We hypothesized that the protective compounds in coffee extract might be acting by induction of a previously identified stress response pathway.

To test this hypothesis, we used a suite of transgenic GFP-reporter strains to determine whether 10% decaffeinated coffee could induce any of the major stress response pathways identified in *C. elegans*. The reporter strains and their previously characterized response patterns are listed in Table 1. No induction was observed for general heat shock response (P*hsp-16*::GFP), the unfolded protein response (P*hsp-4*::GFP), or reduced insulin-like signaling (nuclear localization of *daf-16*::GFP) (data not shown). However, significant induction was

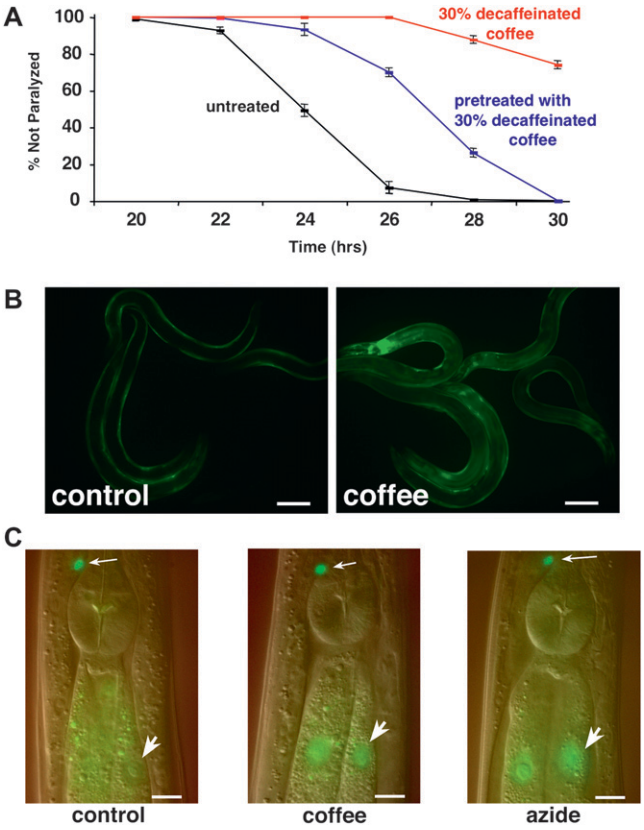

Figure 4.—Coffee extract exposure induces the *skn-1* detoxification pathway. (A) CL4176 (P*myo-3*::Aβ_{42}) worms were propagated from hatching to third larval stage (48 hr) on agar media plates containing 30% decaffeinated coffee, either moved to control media or maintained on 30% decaffeinated coffee plates, and then induced to express Aβ_{42}. Note that significant protection against paralysis is observed even in worms shifted to control plates before induction of Aβ_{42} expression ($P < 0.001$, paired log rank survival test). (B) CL2166 (P*gst-4*::GFP) worms were propagated from hatching to adulthood on control or 10% decaffeinated coffee plates. Coffee extract exposure induces strong upregulation of the *gst-4*::GFP reporter, particularly in the intestine. This induction requires the *skn-1* transcription factor (data not shown). Bar, 100 μM. (C) Worms containing transgene *ldIs001*(*skn-1*::GFP) were propagated from hatching on control and 10% decaffeinated coffee plates. The translational fusion protein expressed by this transgene is expressed in the ASI neurons (small arrows) and the intestine. Propagation on coffee extract plates causes nuclear localization of the fusion protein (large arrow) similar to what is observed if this reporter strain is exposed to 100 mM sodium azide for 1 hr (right). Digitally fused DIC/epifluorescence images. Bar, 10 μM.

observed for a P*gst-4*::GFP reporter (Figure 4B). To quantify this induction, staged populations of the P*gst-4*::GFP reporter strain (CL2166) were propagated on 10% decaffeinated coffee extract or control plates, and individual whole-animal GFP fluorescence was measured using a COPAS worm sorter. Coffee exposure resulted in a 77% increase in induction of GFP fluorescence for this reporter [control: 219.8 ± 9.0 arbitrary fluorescence units; 10% coffee: 389.7 ± 18.1 arbitrary fluorescence units (± SEM)]. *gst-4* encodes a glutathione-s-transferase and is a downstream effector of the conserved *skn-1* (Nrf2 in mammals) phase II detoxification pathway (Kahn *et al.* 2008).

Like Nrf2, activation of SKN-1 requires a redistribution of this protein from the cytoplasm to the nucleus (An and Blackwell 2003). We therefore tested the ability of coffee extract to cause nuclear localization of a *skn-1*::GFP translation fusion reporter gene. As shown in Figure 4C, propagation on 10% coffee extract induced nuclear localization of SKN-1::GFP in intestinal nuclei, qualitatively similar to the effect of sodium azide exposure, a known inducer of *skn-1* activation (Kell *et al.* 2007).

Coffee extract protection requires the SKN-1 transcription factor: To determine whether induction of the *skn-1* pathway was required for coffee protection in this model, we first knocked down expression of the SKN-1 master transcription factor by feeding RNA interference. SKN-1 has an essential (but maternally rescuable) role in *C. elegans* embryonic development, but we were able to establish RNAi conditions (see materials and methods) that resulted in treated worms that reached adulthood normally but had 100% inviable embryos. Under these conditions, *skn-1* RNAi reversed the protective effects of coffee extracts (Figure 5A).

To test the role of *skn-1* genetically, we constructed a strain (CL6222) with inducible Aβ expression and a strong loss-of-function allele of *skn-1*(*zu169*) balanced in *trans* with a translocation (nT1) marked with a dominant uncoordinated (Unc) mutation and a recessive lethal mutation. This strain segregates non-Unc *skn-1*(*zu169*) homozygous worms that are wild type in movement but become self-sterile adults. These *skn-1* homozygotes show no protection from coffee exposure (Figure 5B), confirming the *skn-1* requirement for coffee protection.

We also tested whether *skn-1* activity was required for the reduced accumulation of Aβ peptide. Knockdown of *skn-1* resulted in a significant increase in Aβ accumulation in transgenic worms on both coffee extract and control plates (Figure 5C), suggesting that the *skn-1* pathway may generally play a role in modulating Aβ accumulation.

Coffee exposure does not reduce feeding: Expression of the SKN-1 transcription factor specifically in the two C. *elegans* ASI neurons is necessary for life-span extension in response to dietary restriction (Bishop and Guarente 2007). Dietary restriction has also been shown to reduce the toxicity of aggregating proteins in *C. elegans*, including toxicity due to Aβ accumulation (Steinkraus *et al.* 2008). We considered the possibility that coffee extract might trivially provide protection by inhibiting *C. elegans* feeding and secondarily inducing a protective dietary restriction response. Mutations in *eat-2* slow pharyngeal pumping (Raizen *et al.* 1995) thereby producing a genetic dietary restriction model that shows SKN-1–dependent life-span extension (Park *et al.* 2010). We directly measured pharyngeal pumping in response to coffee exposure but found no effect (Figure 6A). We also observed that coffee exposure had no effect on the developmental rate of wild-type worms, while in

TABLE 1

Strain	Genotype	Pathway	Responsive to	Reference
CL2166	*dvIs19* (P*gst-4*::GFP)	*skn-1*	Oxidative stress	Link and Johnson (2002)
CL2070	*dvIs70* (P*hsp-16.2*::GFP)	*hsf-1*	Heat shock	Link *et al.* (1999)
SJ4005	*zcIs4* (P*hsp-4*::GFP)	*xbp-1*	ER stress	Calfon *et al.* (2002)
TJ356	*zIs356* (*daf-16*::GFP)	*daf-16*	Starvation, heat shock	Henderson and Johnson (2001)

Transgenic stress-responsive GFP reporter strains used to assay the ability of coffee extracts to induce a stress response.

comparison *eat-2* mutants grown on standard media show a day delay in reaching adulthood. To independently assess the possibility that coffee exposure was inducing dietary restriction, we made use of the observation that *skn-1* loss-of-function mutations that preserve expression of a *skn-1* isoform in the ASI neurons, such as *zu67*, can still show (attenuated) life-span extension in response to dietary restriction (Bishop and Guarente 2007). This result predicts that introduction of *zu67* would not block coffee protection if the extract was acting by directly inducing dietary restriction. As shown in Figure 6B, transgenic worms homozygous for *skn-1*(*zu67*) are not protected by coffee exposure, consistent with the view that this protection was not through the ASI signaling underlying dietary restriction life-span extension.

Coffee extract protects against toxicity from model aggregating protein: We have previously demonstrated that induced expression of GFP::degron, a model aggregating protein, in *C. elegans* muscle can lead to a paralysis phenotype grossly similar to that caused by induction of $A\beta_{42}$ (Link *et al.* 2006). To determine whether coffee extract exposure might be generally protective against aggregating protein toxicity, we assayed the effects of coffee extract exposure on a transgenic strain (CL2337) with inducible GFP::degron expression. As shown in Figure 7A, coffee exposure strongly blocked the paralysis induced by GFP::degron, and this protective effect was reversed by RNAi knockdown of SKN-1. Coffee exposure did not result in any qualitative change in GFP::degron deposits (Figure 7B), but did cause a small reduction in the accumulation of GFP::degron as assayed by immunoblot (Figure 7C).

DISCUSSION

We have shown that exposure to aqueous coffee extracts strongly reduces the paralysis resulting from induction of expression of $A\beta_{42}$ in a transgenic *C. elegans* model. Coffee extract exposure-induced expression of a reporter transgene (*gst-4* promoter::GFP) controlled by the SKN-1 transcription factor, and protection against $A\beta_{42}$ toxicity was reversed if SKN-1 is knocked down by RNAi or removed by genetic mutation. These results support the model that coffee protection against $A\beta_{42}$ toxicity results from activation of the *skn-1* pathway. SKN-1 is a member of the Cap'n'collar family of transcription factors that includes the Drosophila Cnc protein and the vertebrate Nrf1, Nrf2, and Nrf3 proteins (Sykiotis and Bohmann 2010). Although initially identified due to its role in embryonic development (Bowerman *et al.* 1992), subsequent studies demonstrated SKN-1 plays a role in the response to oxidative stress (An and Blackwell 2003). Thus, SKN-1 appears functionally analogous to Nrf2, the best-studied member of the Nrf family, which controls phase II detoxification response genes and is centrally involved in cellular responses to oxidative stress (reviewed in Osburn and Kensler 2008). Nrf2 activation, either by tert-butylhydroquinone treatment or viral Nrf2 gene transfection, has been shown to be protective in an APP/PS1 transgenic mouse model (Kanninen *et al.* 2008, 2009), as well as in neuronal cells exposed to exogenous Aβ (Wruck *et al.* 2008). Interestingly, a polymorphism in the human Nrf2 gene, NFE2L2, has recently been associated with earlier AD onset (Von Otter *et al.* 2010).

Studies have suggested that Nrf2 activation may be protective in a range of neurodegenerative conditions. For example, Nrf2 activation has been reported to be protective in a mouse MPTP model of PD (Chen *et al.* 2009), as well as a transgenic mouse model of familial amyotrophic lateral sclerosis (ALS) (Vargas *et al.* 2008). Similarly, chemical or genetic activation of the Nrf2 pathway is protective in multiple Drosophila models of PD (Trinh *et al.* 2008). Of perhaps greatest relevance to our *C. elegans* studies, coffee and tobacco extracts have recently been shown to be protective in Drosophila neurodegeneration models, and this protection is Nrf2 dependent (Trinh *et al.* 2010). Our demonstration that coffee exposure protects against GFP::degron, a model toxic aggregating protein not associated with a specific disease, further supports the generality of protection by Nrf2 activation.

What are the molecular mechanisms underlying *skn-1*–dependent coffee protection in the *C. elegans* Aβ toxicity model? We found that coffee exposure variably reduced the accumulation of Aβ (and GFP::degron) as assayed by immunoblot, without reducing transgene expression. These results suggest that coffee extract activation of *skn-1* may increase the degradation of Aβ. Nrf2 activation is known to upregulate expression of proteasomal subunits and proteasome activity (Arlt *et al.* 2009; Kapeta *et al.* 2010), and Nrf2-dependent upregulation of proteasomal activity has been directly implicated in protection

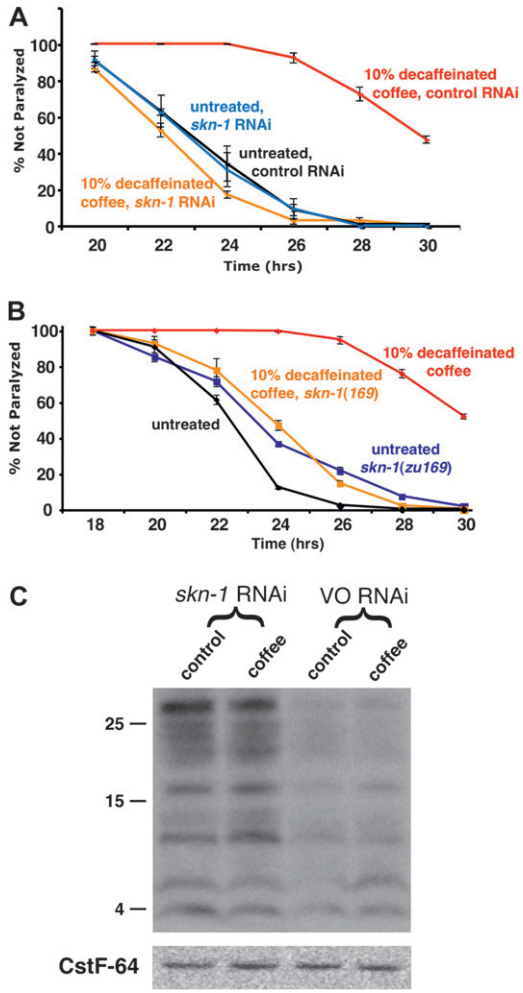

FIGURE 5.—SKN-1 is required for coffee extract protection against $A\beta_{42}$-induced paralysis. (A) CL4176 worms were propagated on control or 10% decaffeinated coffee plates seeded with *E. coli* expressing control (vector only) or *skn-1* dsRNA. Coffee extract exposure produces strong protection in worms propagated on the control RNAi strain (black *vs.* red lines), but not in worms propagated on skn-1 RNAi (blue *vs.* orange lines). (B) $A\beta_{42}$ transgenic worms homozygous for the *skn-1*(*zu169*) (segregated from CL6222) allele show no coffee extract protection (blue *vs.* orange lines), while +/+ worms (segregated from control strain CL6176) also containing the nT1 balancer chromosome) are strongly protected (black *vs.* red lines). (C) CL4176 (P*myo-3*/$A\beta_{42}$) worms propagated control or 10% coffee plates exposed to *skn-1* or control (VO, vector only) RNAi were induced for 24 hr and 20 μg total protein was run on an immunoblot and probed with anti-Aβ monoclonal antibody 6E10. Note increased accumulation of Aβ on both control and coffee plates in worms treated with *skn-1* RNAi. (Blot reprobed with anti-CstF-64 antibody to demonstrate equal lane loading.)

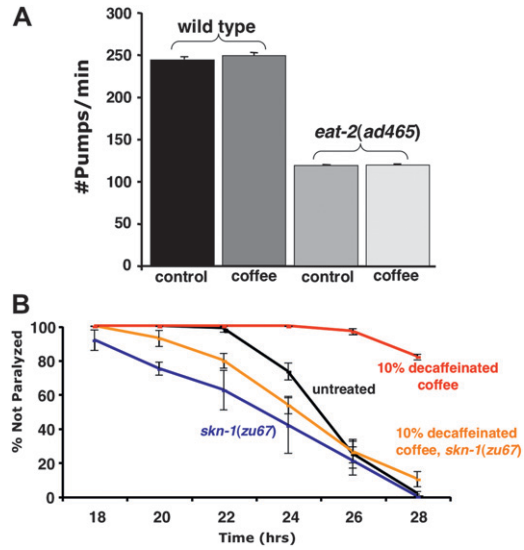

FIGURE 6.—Coffee extract protection is not explained by reduction in feeding and subsequent induction of a dietary restriction response. (A) Pharyngeal pumps per minute were determined for fourth larval stage wild-type and *eat-2*(*ad465*) worms propagated on control or 10% decaffeinated coffee plates. Coffee extract exposure does not affect pharyngeal pumping rates under a Student's *t*-test analysis ($P = 0.806$). Furthermore, coffee extract has no effect on pumping rates while controlling for strain ($N = 30$ for each strain/condition), as per a two-way ANOVA ($P = 0.304$). (B) Coffee extract protection is blocked by a *skn-1* mutation that does not block the effects of dietary restriction on life span. $A\beta_{42}$ transgenic worms homozygous for the *skn-1*(*zu67*) allele show no coffee extract protection (blue *vs.* orange lines), while +/+ worms (segregated from control strain CL6176 also containing the nT1 balancer chromosome) are strongly protected (black *vs.* red lines). Error bars = SEM.

against Aβ toxicity in cell culture models (PARK *et al.* 2009a). However, in some experiments we observed coffee-induced protection against Aβ toxicity without a detectable decrease in accumulation (*e.g.*, first two immunoblot lanes in Figure 3C). Thus, although loss of *skn-1* leads to significant increase in the accumulation of Aβ (Figure 5C), *skn-1* activation by coffee extract exposure leads to a smaller and more variable decrease in Aβ accumulation (Figure 3C). Therefore, protection in *C. elegans* by *skn-1* activation may primarily result from the upregulation of other effectors of this pathway, particularly the numerous antioxidant genes that have been identified by gene expression analysis (PARK *et al.* 2009b). Supporting this model, Nrf2 activation is protective in PD models that do not involve accumulation of a toxic protein: MPTP treatment in mice (CHEN *et al.* 2009) and parkin loss-of-function in flies (TRINH *et al.* 2008). It may be possible to identify additional protective processes induced by coffee exposure by RNAi knockdown of individual *skn-1*–modulated genes.

What are the specific compounds in coffee extract responsible for activation of the *skn-1*/Nrf2 pathway? Our results indicate that caffeine is not the primary protective compound in aqueous coffee extracts, in agreement with the Drosophila studies examining the protective effects of coffee in PD models (TRINH *et al.* 2010). We cannot exclude the possibility that the protective effects of coffee are due to changes in the *E. coli* food source used in these experiments, but this seems highly unlikely given the effects of coffee extracts in other systems, such as flies,

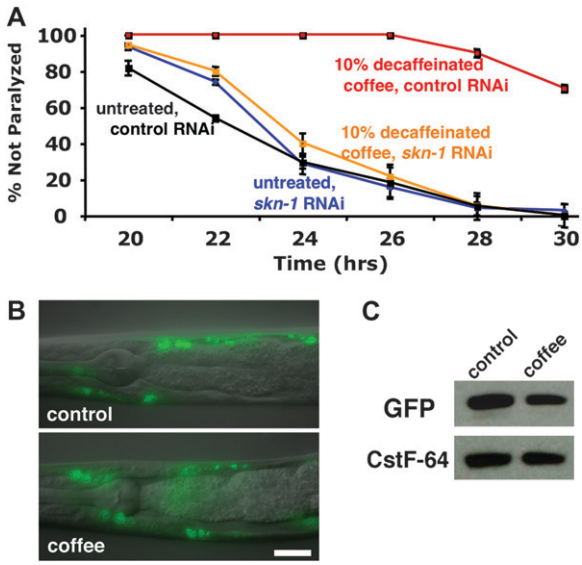

FIGURE 7.—Coffee extract exposure protects against GFP::degron toxicity in a *skn-1*–dependent manner. (A) CL2337 (P*myo-3*::GFP::degron) worms were propagated on control or 10% decaffeinated coffee plates seeded with *E. coli* expressing control (vector only) or *skn-1* dsRNA. Induction of GFP::degron expression also results in paralysis (LINK *et al.* 2006). Coffee extract exposure produces strong protection in worms propagated on the control RNAi strain (black *vs.* red lines, $P < 0.001$, paired log rank survival test), but not in worms propagated on *skn-1* RNAi (blue *vs.* orange lines). (B) Fused DIC/epifluorescence image of GFP::degron worms propagated on control or 10% decaffeinated coffee plates. Distribution or size of the GFP::degron deposits was not detectably altered by coffee exposure. Bar, 20 μM. (C) Immunoblot assaying GFP::degron accumulation in worms propagated on control or 10% decaffeinated coffee plates. Equal gel loading was confirmed by reprobing the blot with antibody against CstF64, a slicing factor unlikely to be influenced by coffee exposure.

that do not use a bacterial food source. Multiple components of coffee have been shown to induce Nrf2 activation, including cafestol and kahweol (HIGGINS *et al.* 2008), and cafestol is protective in the Drosophila PD models (TRINH *et al.* 2010). A number of natural polyphenols, including hydroxytyrosol (MARTIN *et al.* 2010), quercetin (KIMURA *et al.* 2009), and epigallocatechin-3-gallate (NA *et al.* 2008), have also been shown to activate the Nrf2 pathway. These observations suggest that the *skn-1*/Nrf2 pathway has evolved to be broadly responsive to xenobiotic compounds, and that there may be multiple compounds in coffee extracts that contribute to activation of this pathway. Given that invertebrate neurodegeneration models are actively being used to screen for protective compounds, it may be wise to test all initial positive compounds for *skn-1*/Nrf2 activation, as this may be a common molecular mechanism underlying protective responses.

It is currently unclear whether the protective effects of coffee in this *C. elegans* $A\beta_{42}$ toxicity model are due to cell autonomous activation of the *skn-1* pathway or more global physiological changes initiated by *skn-1*–activated intercellular signaling. This latter possibility is supported by reports that *skn-1* expression in *C. elegans* is restricted to the intestine and the two ASI neurons, while $A\beta_{42}$ accumulation and toxicity occur in body wall muscle cells (LINK *et al.* 2001; AN and BLACKWELL 2003). [We note that low-level expression of *skn-1* in other cell types, such as muscle, may have been missed by the standard transgenic techniques used in the AN and BLACKWELL (2003) study]. The role of *skn-1* in dietary restriction-induced life-span extension depends solely on expression of SKN-1 in the ASI neurons (BISHOP and GUARENTE 2007), implying that it is likely there are intercellular signaling events downstream of *skn-1* activation modulating life span. Indeed, a neuropeptide-like hormone, NLP-7, has been recently shown to act downstream of *skn-1* to promote life-span extension by dietary restriction (PARK *et al.* 2010). It is also interesting to note that astrocyte activation of Nrf2 is sufficient to convey neuronal protection in mouse models of PD and ALS (VARGAS *et al.* 2008; CHEN *et al.* 2009), demonstrating that Nrf2-dependent neuroprotection in mammals can also be non-cell autonomous. The genetic tools available in *C. elegans* should allow us to determine to what degree cell autonomous activation of *skn-1* contributes to protection against $A\beta_{42}$ toxicity.

Our studies in *C. elegans* provide a plausible explanation of why epidemiological studies indicate consumption of coffee (and other plant extracts such as tea) may be protective against Alzheimer's disease and other neuropathologies. However, given the differences in human and nematode physiology, more study is needed to determine whether coffee activation of Nrf2 is actually the relevant mechanism in people. Of particular relevance are the questions of whether Nrf2-activating components of coffee actually cross the blood/brain barrier and whether Nrf2 activation in the periphery can lead to physiological changes that impact the brain. Nevertheless, our studies add to the growing body of literature indicating that Nrf2 activation is neuroprotective and accessible to dietary modulation.

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Genetic Mechanisms of Coffee Extract Protection in a *Caenorhabditis elegans* Model of β-Amyloid Peptide Toxicity

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TABLE S1

Statistical analysis of repeat paralysis experiments

Table S1 is available for download as an Excel file at http://www.genetics.org/cgi/content/full/genetics.110.120436/DC1.