Aspirin extends the lifespan of *Caenorhabditis elegans* via AMPK and DAF-16/FOXO in dietary restriction pathway

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**A B S T R A C T**

Aspirin has been revealed to have many beneficial effects for health since it was discovered as a nonsteroidal anti-inflammatory drug (NSAID) to treat pain and inflammation. Here, we investigated the molecular mechanism of aspirin on the lifespan extension of *Caenorhabditis elegans*. Our results showed that aspirin could extend the lifespan of *C. elegans*, and increase its health span and stress resistance. The extension of lifespan by aspirin requires DAF-16/FOXO, AMPK, and LKB1, but not SIR-2.1. Aspirin could not extend the lifespan of the mutants of eat-2, clk-1, and isp-1. Aspirin could marginally extend the lifespan of long-live insulin-like receptor mutant *daf-2(e1370) III*. Taken together, aspirin might act through a dietary restriction-like mechanism, via increasing the AMP:ATP ratio and activating LKB1, subsequently activating AMPK, which stimulates DAF-16 to induce downstream effects through a DAF-16 translocation independent manner.

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1. Introduction

Since the discovery of aspirin as one of the nonsteroidal anti-inflammatory drugs (NSAIDs) more than a century ago, aspirin has become the most widely administered medicine in the treatment of conditions such as pain, fever, and inflammation. In addition to its anti-inflammatory effects, it was reported that long-term use of aspirin could improve many aspects of health status. Several epidemiological, preclinical, and clinical studies showed that the chronic use of aspirin could significantly reduce the risk of numerous cancers, such as colorectal, lung, and breast cancer (Deutsch, 1992; Luciani et al., 2007; Schreinemachers and Everson, 1994). In addition, aspirin could exert an anti-diabetic effect (Hundal et al., 2002), inhibit atherosclerotic plaque formation (Mehta et al., 2004), and increase its health span and stress resistance of *C. elegans* by aspirin, since many genetic pathways regulating the longevity of *C. elegans* have been shown to be evolutionarily conserved (Bishop and Guarente, 2007; Guarente and Kenyon, 2000; Henderson and Johnson, 2001; Kenyon, 2005, 2010; Lakowski, 1998; Mair and Dillin, 2008). We found that aspirin could increase the mean lifespan of *C. elegans* by mediating solid dietary restriction (sDR)-like response (Greer and Brunet, 2009). Aspirin could decrease the mitochondrial respiration efficiency, resulted in an increased AMP:ATP ratio, and activate kinase LKB1/PAR-4 and AMPK, which subsequently activate DAF-16, resulted in the lifespan extension and enhanced stress resistance of *C. elegans*.

2. Methods

2.1. Chemicals and strains

All strains were from *Caenorhabditis* Genetics Center (CGC) and maintained at 20 °C as described previously (Brenner, 1974), unless otherwise stated. The strains used in this study were: wild type N2, DA1116 *eat-2(ad1116) II*, VC199 *sir-2.1(ok434) IV*, MQ887 *isp-1(qm150) IV*,...
CB4876 clk-1(e2519) III, CB1370daf-2(e1370)III, T(j356daf-16(zs356))/V, CF1038daf-16(mu86)L, RB754 aak-2(ok524)X, and KK184par-4(irt47)V. All strains grew and were maintained on NGM plates seeded with *Escherichia coli* OP50 at 20 °C, except for par-4(irt47)V. Strain par-4(irt47)V was temperature-sensitive maternal effect lethal (Morton et al., 1992), and was maintained at 15 °C and shifted to 20 °C at the L4 stage in lifespan experiments.

Aspirin was purchased from Sigma, and resolved in PBS. NGM plates containing aspirin were equilibrated overnight before use. For each experiment, at least 60 worms were used. Late L4 larvae or young adults were transferred to NGM plates (9 cm, diameter) containing inactivated OP50 (65 °C for 30 min) and 40 μM of 5-fluoro-2′-deoxyuridine (FUDR, Sigma) to prevent self-fertilization. The L4 molt was set as a start point for lifespan analyses. To ensure that the drugs retained their potency throughout the entire experiment, animals were transferred before being used for lifespan analysis. For each experiment, at least 2.5. qRT-PCR assay

Pharynx-pumping rate of animals growing on a plate with (100 μM) or without aspirin, and cultured at 20 °C for 24 h. Then, worms were pooled and washed twice with M9 buffer. After that, worm samples were suspended with 2 mM of boiling MgSO4 and incubated in boiling water for 10 min. The samples were then sonicated for 10 min, and briefly centrifuged. The supernatants were collected and filtered through a 0.22 μm filter and analyzed by Reverse-phase HPLC. Samples were separated in a Zorbax SB C18 250 × 4.6 mm 5 μm column by a flowing solution containing 5% of buffer A (100% MeOH) and 95% of buffer B (0.043 mol/L ammonium acetate). Nucleotide was detected at 254 nm with a Varian Pro Star detector. Peak areas were measured with Varian Star Workstation software. Nucleotide identities were confirmed by co-migration with AMP and ATP standards (Sigma).

3. Results

3.1. Aspirin extended the lifespan of *C. elegans*, delayed age-related decline of phenotypes, and increased stress-resistance

To address the molecular mechanism of health-span benefits conferred by aspirin, we first asked whether aspirin could induce the physiological effects in *C. elegans* similar to what were reported (Ayyadevara et al., 2012; Phillips and Leeuwenburgh, 2004; Strong et al., 2008). Dose–response analyses indicated that animals raised at 20 °C on NGM plates containing 100 μM of aspirin displayed the largest lifespan extension by up to 15.5% (Fig. 1C, D). Animals exposed to either higher or lower 100 μM of aspirin exhibited a smaller but significant lifespan extension (Fig. 1B, C).

The body movement and pharyngeal pumping rate of *C. elegans* decreased accompanying nematode aging (Huang et al., 2004). To investigate whether aspirin could delay the age-related decline of phenotypes, we analyzed the effect of aspirin on body movement. In both treated and non-treated animals, body movement declined progressively during aging. However, aspirin treated animals exhibited significantly lower decline of body movement than non-treated controls (Fig. 2B).

*C. elegans* with extended lifespan resulted from genetic and non-genetic manipulations often present increased stress resistance (Finkel and Holbrook, 2000). Therefore, we examined the effect of aspirin on the lethality of heat stress. As shown in Fig. 2A, aspirin treatment suppressed the lethality of heat stress in wild-type *C. elegans*.

3.2. Aspirin extends the lifespan of *C. elegans* through FOXO Transcription Factor DAF-16

The *C. elegans* Forkhead box O transcription factor (FOXO) homolog DAF-16 plays a central role in mediating stress resistance, longevity,
development, fat storage, and reproduction (Henderson and Johnson, 2001; Oh et al., 2006; Yen et al., 2011). We tested if DAF-16 played a role in lifespan extension by aspirin. Our result showed that aspirin did not further extend the lifespan of daf-16 null mutant (Fig. 3B). To further examine that aspirin did act on DAF-16, we examined the effect of aspirin on the expression of sod-3,
which is a known DAF-16 target gene involved in both stress resistance and longevity, by qRT-PCR (Honda and Honda, 1999; Murphy et al., 2003). The expression of sod-3 was significantly increased when worms were exposed in 100 μM of aspirin for 72 h (Fig. 3D). When DAF-16 was activated, it would translocate to nuclei from cytoplasm (Henderson and Johnson, 2001). Thus, we examined whether aspirin could trigger the nuclear localization of DAF-16. But we failed to observe the nucleus accumulation of DAF-16 after stimulated by aspirin (Fig. 3C). Taken together, these results indicated that aspirin-induced lifespan extension is mediated by DAF-16.

DAF-16 is downstream to several signaling pathways, such as the IGF pathway, and executes downstream effects (Puig and Mattila, 2011). Treatment with aspirin resulted in a modest extension (3.2%) of the lifespan of the long-lived insulin-like receptor mutant daf-2(e1370)III (Fig. 3A) much less than the extension of wild-type (15.5%). Since daf-2 (e1370)III was not a null mutant, lifespan extension by aspirin might depend or partly depend on IIS pathway.

3.3. AMPK and kinase LKB1 are required for aspirin to extend the lifespan of C. elegans

It was reported that salicylate could directly activate AMP-activated protein kinase (AMPK) (Hawley et al., 2012). To determine whether AMPK mediated the ability of aspirin to extend lifespan in worms, we used a worm strain carrying a deletion in the gene encoding one of the catalytic subunits of AMPK (AMPKα2 or aak-2) (Apfeld et al., 2004). As shown in Fig. 5A, aspirin treated animals didn’t significantly increase the mean lifespan of aak-2 mutant, suggesting that aspirin-induced lifespan extension requires AMPK.

Because AMPK could be activated by low energy levels, (Carling et al., 1987; Hawley et al., 1996), we asked whether aspirin could affect the energy level of C. elegans. We measured the AMP:ATP ratio in both aspirin-treated and non-treated worms by using HPLC. The AMP:ATP ratio was significantly increased in worms treated with aspirin than in control worms (Fig. 5C). AMP could stimulate AMPK by two mechanisms: first, AMP binding could allosterically activate AMPK; and second, and likely more importantly, binding of AMP to AMPK could promote the phosphorylation of AMPK by LKB1 (Dale et al., 1995; Hawley et al., 2010; Sanders et al., 2007; Suter et al., 2006). We therefore tested whether C. elegans LKB1 homolog PAR-4 is required for aspirin-induced lifespan extension. There was no significant difference between the lifespans of aspirin-treated and non-treated LKB1 mutant par-4(it47)V (Fig. 5B), suggesting that LKB1 was required for aspirin-induced lifespan extension.

3.4. Effects of aspirin on lifespan extension may be conferred by DR-like mechanisms

The lower energy level of worms caused by aspirin was reminiscent of the similar effect as dietary restriction (DR), which was known to extend lifespan in a wide range of species (Masoro, 2005). The pharyngeal pumping defective mutant eat-2(ad1116)II was considered DR-constitutive for its reduced food intake and extended lifespan (Lakowski, 1998). Treatment with 100 μM of aspirin at 20 °C did not

Fig. 3. Aspirin extended adult lifespan in a DAF-16-dependent manner. (A) Survival curves of daf-2 mutants in control (black) or treated with 100 μM of aspirin (red), aspirin extended the mean lifespan of daf-2 mutant by up to 3.2%. (B) Survival curves of daf-16 mutants grown on control plates (black) and plates with 100 μM of aspirin (red) at 20 °C, aspirin could not further extend the mean lifespan of daf-16 mutants. In all cases, these data represented the results of a single trial. Repeats of these experiments and statistical details were summarized in Table S1 (supplementary information). (C) Aspirin could not cause DAF-16 nuclear localization. DAF16::GFP-expressing worms were placed on plates with 100 μM of aspirin and control plates at 20 °C for 24 h. (D) sod-3 mRNA level of worm treated with 100 μM of aspirin and controls. The results represent the mean and SEM of two independent experiments: * P = 0.008281 in t test.
further increase the lifespan of eat-2(ad1116)II, suggesting that aspirin might act through a DR-like mechanism (Fig. 4A).

One mechanism by which compound could trigger DR-like effect was to lower the pharyngeal pumping rate. To rule out this possibility, we measured pharyngeal pumping, fast pharyngeal pumping span and pharyngeal pumping span in wild-type animals further increase the lifespan of eat-2(ad1116)II, suggesting that aspirin might act through a DR-like mechanism (Fig. 4A).

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Fig. 4. Lifespan extension by aspirin was mediated by DR-like mechanism, but sir-2.1-independent. Survival curves of eat-2(ad1116)II (A) and sir-2.1(ok434)IV (B) at 20 °C. Control was in black, red was treated with 100 μM of aspirin. All drug treatments were initiated from the first day of adulthood and continued until death. In all cases, these data represent the results of a single trial. Repeats of these experiments and statistical details are summarized in Table S1 (supplementary information). (C) Effects of aspirin on pharyngeal pumping. Pharyngeal pumping rate declined with adult aging. Significance of differences by t test: *P < 0.05 for aspirin vs. control at 8 days and 10 days. Mean pumping rates (pumps per minute) with SEM are shown for each time point (n = 20–30 animals, there were fewer animals on later days).

Fig. 5. Aspirin-related lifespan extension required AAK-2 and LKB1. Survival curves of aak-2(ok524)X (A) and par-4(it47)V (B), aspirin could not further extend the mean lifespan of aak-2(ok524)X and par-4(it47)V. Control was in black, aspirin treatment was in red. In all cases, these data represented the results of a single trial. Repeats of these experiments and statistical details were summarized in Table S1 (supplementary information). (C) The AMP:ATP ratio, measured by reverse-phase HPLC, increased when worms were exposed to 100 μM aspirin. The results represented the mean and SEM of two independent experiments. *P = 0.04644 by t test.
treated with aspirin and a non-treated control. We found no differences in fast pharyngeal pumping span and pharyngeal pumping span between animals treated with aspirin and non-treated controls (Table S4). In addition, the pharyngeal pumping rate in worms treated with aspirin decreased with age more slowly than in control worms (Fig. 4C). These results indicated that aspirin-induced lifespan extension was not by limiting the feeding capacity of animals, but by a conserved metabolic pathway to extend lifespan.

Mitochondrial respiration which plays the major role in energy production, has been reported to mediate the DR response and aging process (Lakowski, 1998; Dillin et al., 2002). The mutation of mitochondrial respiration components, such as clk-1, the enzyme in ubiquinone synthesis and isp-1, a component of respiratory chain complex III, were shown to reduce the oxygen consumption and ATP synthesis of worms. The clk-1 mutant clk-1(e2519)III and isp-1 mutant isp-1(qm150)IV have the defect of mitochondrial respiration and were long-lived (Feng and Hekimi, 2001; Lakowski and Hekimi, 1996). Aspirin treatment could not further extend the lifespan of mutants clk-1(e2519)III and isp-1(qm150)IV, indicating that aspirin might extend lifespan by reducing mitochondrial respiration (Fig. 6).

The silent information regulator 2 (Sir2), which encodes a nicotinamide adenine dinucleotide (NAD)-dependent deacetylase, also could bind to DAF-16 and extend the lifespan of C. elegans upon stress in a 14–3–3 protein dependent manner (Anderson et al., 2003; Berdichevsky et al., 2006; Lin et al., 2004). We investigated if aspirin could act on Sir2 to extend the lifespan of C. elegans with a null mutant strain sir-2.1(ok434)IV (Wang and Tissenbaum, 2006). Aspirin increased the lifespan of sir-2.1 mutant (Fig. 4B), indicating that Sir-2.1 was not necessary for aspirin-mediated lifespan extension.

4. Discussion

In addition to anti-inflammatory effects as a NSAID, it was reported that long-term use of aspirin could ameliorate the onset of various age-related diseases, such as thrombotic and ulcerogenic disorders (Kalugturk, 1998), cancers (Fukutake et al., 1998; Schreinemachers and Everson, 1994; Thun et al., 1992), Alzheimer’s disease, and other neurodegenerative disease (Esposito et al., 2007; Gasparini et al., 2004). Consistent with the above observations, aspirin was recently reported to extend the lifespan of C. elegans and mice (Ayyadevara et al., 2012; Phillips and Leeuwenburgh, 2004; Strong et al., 2008), but the molecular mechanisms remain unclear. Here, we confirmed that aspirin treatment could increase the mean lifespan of C. elegans, delay the age-related decline of body movement, and increase heat stress resistance.

Since DAF-16/FOXO plays a central role in the regulation of aging and stress resistance, we first investigated if the observed effects of aspirin on C. elegans were acting through DAF-16. As expected, aspirin increased the expression level of DAF-16 regulated gene sod-3 and failed to extend the lifespan of the DAF-16 null mutant daf-16(mu86)I. But we failed to observe the difference in the nucleus accumulation of DAF-16 between worms treated with aspirin and untreated controls, although the possibility of insensitivity of our method could not be excluded.

Subsequently, we looked for what pathways or molecule upstream of DAF-16 might be needed to regulate the effect of aspirin on C. elegans. We found that aspirin could marginally extend the lifespan of long-live insulin-like receptor mutant daf-2(e1370)III (3.2%). Since daf-2(e1370)III was not a null mutant, aspirin might at least partially depend on IIS/insulin pathway to extend the lifespan of C. elegans. AMPK was also required for aspirin to extend the lifespan of C. elegans. AMPK could phosphorylate and activate DAF-16 upon sensing the AMP:ATP level (Greer et al., 2007). Our results showed that aspirin could lower the energy level of worms, which mimicked the effect of calorie restriction on C. elegans. Consistent with this observation, aspirin failed to extend the lifespan of DR-constitutive mutant eat-2(ad1116)/II (Fig. 4A). Sir-2.1 could also sense the energy level under DR and activate DAF-16 and its nuclear localization (Canto and Auwerx, 2009). But Sir-2.1 was not required for the action of aspirin in our results. Together, these results suggested that aspirin could extend the lifespan of C. elegans by sDR-like mechanism, which depends on activating AMPK to regulate DAF-16 independently of its subcellular localization (Greer and Brunet, 2009; Greer et al., 2007).

Our results indicated that aspirin-induced lifespan extension requires LKB1 (Fig. 5B), the upstream kinase of AAK-2, suggesting that aspirin could activate AMPK by increasing the AMP:ATP ratio (Fig. 5C) and the activation of LKB1, which is a mechanism conserved from nematodes to humans (Hawley et al., 2010). However, how aspirin activated LKB1 remains unclear.

Mitochondria are the major energy producers and they have been reported that they mediate DR-like response and organism aging (Dillin et al., 2002; Lakowski, 1998). The mitochondrial respiration defective mutants of isp-1 and clk-1 had higher AMP:ATP ratios and extended lifespan with an enhanced AAK-2 activity (Curtis et al., 2006; Greer and Brunet, 2008). Our results showed that aspirin could not further extend the lifespan of the long-live mutants of isp-1 and clk-1, suggesting that aspirin might act through a similar mechanism of reducing mitochondrial respiration. DR is not a uniform condition triggering universal and linear genetic pathways. For example, solid DR (sDR) is mediated by AMPK, FOXO, and CLK-1, whereas eat-2 is mediated by FOXA, SIR-2.1 and CLK-1, but...
FOXO-independent (Greer and Brunet, 2009). However, our results showed that aspirin induced eat-2 mimetic DR response requiring AMPK, FOXO and CLK-1, but not SIR-2.1. One possibility was that different DR-induced longevity pathways cross-talk and co-exist, and aspirin may simultaneously evoke two independent pathways, in which the sDR DR-like pathway initiated by aspirin might trigger eat-2 DR-like pathway. Another possibility was that a 'novel' pathway, triggered by aspirin to extend lifespan, which requires AMPK, FOXO, and CLK-1, does not further increase the lifespan of eat-2, but fully depend on DAF-16. This aspirin longevity pathway could be conserved in other organisms, including humans. Clear genetic pathway regulated by aspirin should be disclosed in subsequent studies.

Conflict of interest

There is no conflict of interests exists in the submission of this manuscript, and the manuscript is approved by all authors for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jexger.2013.02.020.

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