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The natural phytochemical dehydroabietic acid is an anti-aging reagent that mediates the direct activation of SIRT1

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ABSTRACT

Dehydroabietic acid (DAA) is a naturally occurring diterpene resin acid of confers, such as pinus species (*P. densiflora*, *P. sylvestris*) and grand fir (*Abies grandis*), and it induces various biological actions including antimicrobial, antiulcer, and cardiovascular activities. The cellular targets that mediate these actions are largely unknown yet. In this report, we suggest that DAA is an anti-aging reagent. DAA has lifespan extension effects in *Caenorhabditis elegans*, prevents lipofuscin accumulation, and prevents collagen secretion in human dermal fibroblasts. We found that these anti-aging effects are primarily mediated by SIRT1 activation. Lifespan extension effects by DAA were ameliorated in *sir-2.1* mutants and SIRT1 protein expression was increased, resulting in the deacetylation of SIRT1 target protein PGC-1α. Moreover, DAA binds directly to the SIRT1 protein independent of the SIRT1 substrate NAD^+ levels. Through a molecular docking study, we also propose a binding model for DAA-SIRT1. Taken together, our results demonstrate that the anti-aging effects are the first identified biological property of DAA and that the direct activation of SIRT1 enzymatic activity suggests the potential use of this natural diterpene, or related compounds, in age-related diseases or as a preventive reagent against the aging process.

1. Introduction

Sirtuins have been identified in yeast as essential factors for lifespan extension in the model organisms *Caenorhabditis elegans* (*C. elegans*) (Rizki et al., 2011; Tissenbaum and Guarente, 2001; Viswanathan and Guarente, 2011) and *Drosophila melanogaster* (*D. melanogaster*) (Banerjee et al., 2012; Rogina and IIelfand, 2004) when their expression levels are increased. However, other reports found no effect on the longevity of either of these organisms (Burnett et al., 2011). SIRT1 has been implicated as a key regulator of the pathways downstream of calorie restriction that have been shown to delay the onset and reduce the incidence of age-related disease (Moynihan et al., 2005; Strai et al., 2002).

Therefore, SIRT1-activating compounds (STACs) that produce anti-aging effects consistent with direct SIRT1 activation have been developed (Dai et al., 2010). STACs, including resveratrol and more target-specific, chemically distinct molecules, have been developed over the past several years (Bemis et al., 2009; Milne et al., 2007; Vu et al., 2009). Polyphenol resveratrol found in grape berry skins is the only STAC from natural compounds up to now, and it extends the lifespan of the model organism *C. elegans* and *Drosophila* (Baur and Sinclair, 2006). Sirtuins represent a distinct class of trichostatin A-insensitive lysyl-deacetylases (class III HDACs) and catalyze a reaction that couples lysine deacetylation to the formation of nicotinamide and *O*-acetyl-ADP-ribose from NAD^+ and the free acetyl group (Imai et al., 2000; Tanner et al., 2000; Tanny and Moazed, 2001). Together, these results have significant implications regarding an important role of the SIRT1 in modulating the sensitivity of cells in apoptotic response and in anti-aging process.

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Dehydroabietic acid (DAA), a naturally occurring diterpene resin acid, is contained in many dietary and herbal plants. DAA and its derivatives are major component of the rosin fraction of oleoresin of conifers, such as pinus (*P. densiflora*, *P. sylvestris*) and grand fir (*Abies grandis*). DAA and its derivatives have a variety of biological actions. DAA improves diabetes and hyperlipidemia in obese diabetic mice (Kang et al., 2009), activates peroxisome proliferator-activated receptor-γ (PPAR-γ) and stimulates insulin-dependent glucose uptake into 3T3-L1 adipocytes (Takahashi et al., 2011). DAA has also shown antiulcer, antimicrobial, anxiolytic, antiviral, and antitumor activities (Savluchinske-Feio et al., 2007; Tagat et al., 1994; Tanaka et al., 2008; Tolmacheva et al., 2006; Wada et al., 1985). Recent studies have confirmed that DAA is a chemical modulator that opens large-conductance calcium-activated BK channels (Ohwada et al., 2003). Together, these reports suggest that DAA could be a possible treatment for acute stroke, epilepsy, asthma, hypertension, gastric hypermotility and psychoses. However, so far, the molecular mechanism underlying the age-related effects of DAA has not been uncovered.

The purpose of this work is to discover SIRT1 activating compounds originated from oriental medicinal plants and to provide scientific evidence of the anti-aging effect of the activators. We suggest that DAA could be a novel anti-aging compound that is derived from food, and we provide a clue to elucidating the molecular mechanism. We investigated the anti-aging effects of DAA in *C. elegans* through lifespan estimation and by using a *sir-2.1* deletion mutant. Significantly, we found that the lifespan extension effect of DAA is due to DAA directly binding to and activating SIRT1. We suggest that DAA exhibits anti-aging effects in *C. elegans*, a model organism of aging, and human dermal fibroblasts through the direct activation of SIRT1. Finally, we propose a direct binding model for DAA and SIRT1.

2. Materials and methods

2.1. C. elegans *strains and growth conditions*

The wild-type N2 and *sir-2.1* (*ok434*) strains were provided by the *Caenorhabditis* Genetic Center (University of Minnesota). The strains were maintained at 20 °C. Age-synchronous populations were prepared as previously described (Kim et al., 2008). Hatched worms (L1-stage larvae) were transferred to fresh agar plates and cultured at 20 °C with *Escherichia coli* OP50 as a food source until they reached the L4 larval stage.

2.2. Antibodies and reagents

Antibodies against SIRT1 (#8469), PGC-1α (#2178), and acetylated lysine (#9441) were obtained from Cell Signaling Technology (Beverly, MA). Secondary antibodies for western blots were obtained from Cell Signaling Technology. Resveratrol (R5010) and 1, 1′-dimethyl-4, 4′-bipyridinium dichloride (paraquat) were purchased from Sigma-Aldrich. Pine needle-purified dehydroabietic acid was used (Supplementary Methods and Fig. S1).

2.3. Lifespan analysis

For the lifespan assays, L4 larvae were transferred to S-medium (S-basal medium supplemented with 3 mM $CaCl_2$, 3 mM $MgSO_4$, 50 μM EDTA, 25 μM $FeSO_4$, 10 μM $MnCl_2$, 10 μM $ZnSO_4$, 1 μM $CuSO_4$, and 10 mM KH_2PO_4 (pH 6.0)) with OP50. Reagents were added to the medium. This transfer day was designated as "age of 0 day". We transferred the worms to fresh culture medium every second day and their survival were recorded at the time of transfer. The worms that died from vulval bursting were excluded from the analysis. All the assays were repeated more than three times.

2.4. Stress resistance

We started treating the N2 worms with DAA or resveratrol on day 5 for the oxidative stress resistance assay. After treating for 2 days, the worms were washed three times with M9 buffer (22 mM Na_2HPO_4, 22 mM KH_2PO_4, 85 mM NaCl, 1 mM $MgSO_4$ and 0.02% gelatin) and then exposed to 0.4 M paraquat in S-medium at 20 °C. Surviving worms were counted at 3 hrs after paraquat treatment by prodding them with pipette tips. Thermotolerance assays were performed with N2 animals on day 5. The animals were transferred to S-medium and incubated at 35 °C for 16 hrs and their survival was determined afterwards (Kim et al., 2008). The significance of differences between control and treated groups were determined by two-way Student's t-test.

2.5. Cell culture and growth activity assay

Dermal fibroblasts from adult skin were obtained from Lonza (Walkersville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA). The fibroblasts were cultured until they were 90% confluent before being passaged. Cell proliferation and cytotoxicity were measured with Cell Counting Kit 8 (Dojindo, Japan).

2.6. Measuring lipofuscin accumulation

Dermal fibroblasts from adult skin were treated with each reagent (DAA and resveratrol) for 10 passages (3–4 days per 1 passage), washed with PBS, fixed for 30 min in 4% paraformaldehyde, washed again, and incubated for 10 min in 0.1% Triton X-100. After washing, the cover slips were mounted onto glass slides and visualized under a confocal laser scanning microscope (LSM7, Carl Zeiss) (with excitation at 350 nm and emission at 420 nm). The fluorescence intensities of the cells were calculated densitometrically with ZEN Lite software (Carl Zeiss) by measuring the average pixel intensity in each cell. The significance of differences between the control and treated groups were determined by one-way ANOVA.

2.7. Western blotting

Human dermal fibroblasts were lysed in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.5 mM PMSF, and a protease inhibitor cocktail (Protease Inhibitor, Sigma) and incubated with the appropriate antibodies overnight at 4 °C. Western blotting was performed by following standard protocols. The cell lysates (20 μg protein) were boiled in SDS sample buffer and resolved by 4–12% SDS–PAGE. The proteins were then transferred to a PVDF membrane (Invitrogen) and probed with specific antibodies.

2.8. RT-qPCR

The human dermal fibroblasts were homogenized in TRIzol reagent (Gibco), and total RNA was extracted according to the standard protocol. Total RNA (4 μg) was reverse-transcribed by using random hexamers and SuperScript III (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed by using the ABI 7500 Fast Real-Time PCR System with Taqman Universal Master Mix II (Applied Biosystems) and TaqMan site-specific primers and probes (Applied Biosystems). We used the $\Delta\Delta C_T$ analysis method; the efficiency of the target and the reference amplification were approximately equal. Reactions were performed in triplicate, and mRNA expression levels were quantified by using the relative C_T method and normalized to the *GAPDH* level.

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