Cortisol promotes stress tolerance via DAF-16 in Caenorhabditis elegans

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ABSTRACT
In this study, we studied the effects of cortisol and cortisone on the age-related decrease in locomotion in the nematode Caenorhabditis elegans and on the tolerance to heat stress at 35 °C and to oxidative stress induced by the exposure to 0.1% H2O2. Changes in mRNA expression levels of C. elegans genes related to stress tolerance were also analyzed. Cortisol treatment restored nematode movement following heat stress and increased viability under oxidative stress, but also shortened worm lifespan. Cortisone, a cortisol precursor, also restored movement after heat stress. Additionally, cortisol treatment increased mRNA expression of the hsp-12.6 and sod-3 genes. Furthermore, cortisol treatment failed to restore movement of daf-16-deficient mutants after heat stress, whereas cortisone failed to restore the movement of dhs-30-deficient mutants after heat stress. In conclusion, the results suggested that cortisol promoted stress tolerance via DAF-16 but shortened the lifespan, whereas cortisone promoted stress tolerance via DHS-30.

1. Introduction
Cortisol is a human steroid hormone classified as a glucocorticoid [1]. Mammalian glucocorticoids mainly act to maintain energy homeostasis, and cortisol has the strongest activity among this family of hormones. Cortisol secretion is regulated by the adrenocorticotropic hormone (ACTH). Cortisone, a precursor of cortisol, is an inactive form of cortisol. Cortisol synthesis from cortisone is mediated by 11β-hydroxysteroid dehydrogenase [1–4]. Normally, glucocorticoid receptors (GRs) are not active when they are bound to heat shock protein (HSP)-90. When cortisol binds GR, HSP-90 dissociates from GR, allowing the latter to translocate into the nucleus and act as a transcription factor [2–4]. In addition, cortisol suppresses inflammatory processes and is used as a medical drug [5]. Furthermore, cortisol positively regulates gluconeogenesis and increases blood pressure to prevent a reduction in movement under stress conditions. Therefore, cortisol is known as a stress hormone. Based on these facts, the cortisol level in the saliva has been used as a stress marker [6–8]. It is known that effects of cortisol may differ depending on the duration of treatment. For example, high concentrations of cortisol in the blood have been suggested to affect the central nervous system [9]. Furthermore, long-term treatment with corticosteroids as medical drugs provokes several adverse effects, such as steroid-induced glaucoma and diabetes [10,11].

To date, many studies have been performed to determine the physiological functions of cortisol. In addition to the well-described roles of cortisol, other physiological effects of this hormone are likely to exist and need to be clarified to understand the overall impact of cortisol on metabolism. Such hitherto unknown physiological functions may be uncovered by experiments in relatively simple model organisms, such as Caenorhabditis elegans. For example, previous studies have shown that estrogen plays an important role in sensing temperature and that oxytocin promotes stress tolerance in C. elegans, revealing the existence of functions that had not been described for the homologous human hormones [12,13].

Although C. elegans possesses some steroid hormone signaling molecules, such as estrogen and dafachronic acid, the full spectrum of cortisol effects in C. elegans is unclear [13–15]. Furthermore, a previous molecular evolution study suggested that invertebrates do not have GRs [16]. Nevertheless, C. elegans has been reported to possess DHS-30, an ortholog of human dehydrogenase/reductase SDR family member 7B (DHRS7B), which affects steroid hormone synthesis, and the ACTH-releasing hormone receptor SEB-3 [17,18]. DHS-30 is implicated in cortisone reductase deficiency 2 [17]. Thus, cortisol possesses several functions in C. elegans that are similar to those in higher animals but may also regulate other physiological processes. In this study, we describe antiaging actions of cortisol and cortisone and their protective effects against heat stress in C. elegans.

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2. Materials and methods

Experimental methods used in this study have been described in detail by us previously [12].

2.1. Strain and culture

Wild-type *C. elegans* Bristol N2 and *daf-16*-deficient mutants (mgD50) were obtained from the Caenorhabditis Genetics Center (GCG; University of Minnesota, Minneapolis, MN, USA). The mutants deficient in dhs-30 (tm6889) were obtained from the National BioResource Project (NBRP; Shizuoka, Japan). Each strain was cultured on the nematode growth medium (NGM) agar plates seeded with *Escherichia coli* OP50 (CGC).

2.2. Age synchronization

To synchronize the growth of *C. elegans*, adult worms were treated with a NaClO solution (5:1 ratio of NaClO [Haiter; KAO, Tokyo, Japan] and distilled water). Eggs were cultured in S-basal (0.1 M NaCl, 50 mM potassium phosphate buffer [pH 6.0]) at 20 °C until hatching.

2.3. Assessment of the thrashing movements sensitive to aging

Age-synchronized L1 larvae were transferred onto NGM plates seeded with OP50 and cultured at 20 °C for 3 days. Subsequently, the worms were exposed to 0.5 mg/mL 5-fluoro-2-deoxyuridine (FUDr; Wako, Osaka, Japan) to prevent generation of progenies and cultured at 20 °C. After 1 day, the worms were transferred onto NGM plates dripped with 200 μL of the OP50 suspension containing 7.5 or 15 mM cortisol (Fig. 1) or cortisone (Sigma, Tokyo, Japan) and cultured at 20 °C. This time point was defined as day 0, and worm movements were measured on days 0, 3, 6, and 9. To prevent contamination by the next generation of progeny worms, 200 μL of 0.5 mg/mL FUDr was added into the plate every 3 days, at each plate change.

2.4. Lifespan assay

Age-synchronized L1 larvae were transferred onto NGM plates seeded with OP50 and cultured at 20 °C for 3 days. The worms were exposed to 0.5 mg/mL FUDr to prevent generation of progenies and cultured at 20 °C for 1 day. Subsequently, 60 worms were transferred onto NGM plates (20 worms/plate) dripped with 100 μL of the OP50 suspension containing cortisol or cortisone and cultured at 20 °C. The worms were further exposed to 0.5 mg/mL FUDr every 2 days from day 0 to 8. Surviving worms were counted every 2 days. To evaluate worm viability, worms were prodded with a platinum wire, and those not responding were deemed dead.

2.5. Assessment of the thrashing movements after heat stress

Age-synchronized L1 larvae were transferred onto NGM plates seeded with the OP50 suspension containing cortisol or cortisone and cultured at 20 °C for 4 days. Subsequently, adult worms were transferred to NGM plates without food and heat stressed at 35 °C for 4 h. After heat stress application, the worms were transferred to a solution dripped with 200 μL of the OP50 suspension and incubated at 20 °C for 12 h. The worms were then transferred to the S-basal medium, and their movement was measured for 15 s. Recovery of the whole-body movement was calculated by dividing the movement count of the heat treatment group by that of the control group, which did not receive heat treatment.

2.6. Assessment of worm viability after heat stress

Age-synchronized L1 larvae were transferred onto NGM plates seeded with the OP50 suspension containing cortisol or cortisone and cultured at 20 °C for 4 days. Adult worms were then transferred to an OP plate containing ampicillin and maintained at a constant temperature of 35 °C. Survival rate was assessed 10 h after the initiation of heat treatment and every 2 h thereafter.

2.7. Assessment of worm viability under hydrogen peroxide-induced stress

Age-synchronized L1 larvae were transferred onto NGM plates seeded with the OP50 suspension containing cortisol or cortisone and cultured at 20 °C for 4 days. Subsequently, 500 μL of 0.1% H$_2$O$_2$ (Sigma, Tokyo, Japan) was added to each well of a 24-well plate, and one nematode was placed into each well. Worm survival was assessed every hour as described above.

2.8. Measurement of reactive oxygen species levels

Age-synchronized L1 larvae were transferred onto NGM plates seeded with the OP50 suspension containing cortisol or cortisone and cultured at 20 °C for 4 days. Subsequently, the worms were treated with 50 μM 2,7-dichlorofluorescein (Wako). The worms were then washed, fixed with 4% paraformaldehyde, and observed and photographed by using a BZ8000 fluorescence microscope (Keyence, Osaka, Japan). Fluorescence intensity was determined by using ImageJ software (National Institute of Health, Bethesda, MD, USA).

2.9. Reverse transcriptase-quantitative PCR (RT-qPCR)

Age-synchronized L1 larvae were transferred onto NGM plates seeded with the OP50 suspension containing cortisol or cortisone and cultured at 20 °C for 4 days. RNA was purified from whole-cell extracts of the worms by using RNAiso PLUS (Takara, Kusatsu, Japan). cDNA was synthesized by using a PrimeScript RT reagent kit with gDNA Eraser.
following default cycling conditions: 50 °C for 2 min, 95 °C for 10 min, (95 °C/15 s, 60 °C/min) × 40. Expression level of actin mRNA was used as internal control. The primers used for RT-qPCR are listed below.

actin.

Fw TCGGTATGGGGACGAGAGGAC.
Rv CATCCAGTTGGTGACGATA

hs-12.60.

Fw TGAGGTGTCAATGTGCTCG.
Rv GACTCTAATCTTTTGGGAGG

sod-3.

Fw TATTAAGCGGAGACTTGGG.
Rv CTGGTTTGCAGCCTCGG.

2.10. Statistical analysis

Statistical significance of differences was analyzed by using the log-rank test for the survival curves and one-way ANOVA for all other data. Differences were considered to be significant if \( P < 0.05 \).

3. Results

3.1. Physiological role of cortisol and cortisone in stress tolerance

First, the effects of cortisol and cortisone on the health and lifespan of *C. elegans* were evaluated. Typically, the average lifespan of nematodes is 1 month, and whole-body movements are known to decrease with aging [19]. Thus, as expected, the frequency of worm movements decreased with time (3, 6, and 9 days) when the worms were kept at 20 °C. Cortisol and cortisone appeared to significantly prevent the decrease in the locomotion in aging worms (Fig. 1A). However, cortisol, but not cortisone, at a concentration of 7.5 mM shortened worm lifespan (Fig. 1B).

We subsequently evaluated whole-body movements of the worms after heat stress and examined the effect of cortisol and cortisone on the resistance to heat stress as described in previous studies [12,20]. After culturing the nematodes at 35 °C for 4 h, the worms displayed a significantly lower number of movements than that in the control group, although the locomotion gradually recovered with time (0, 12, and 24 h). Treatment with cortisol or cortisone significantly improved the recovery of worm thrashing movements after heat stress (Fig. 2A and B). We also evaluated worm survival under heat or oxidative stress in the absence or presence of cortisol or cortisone. Neither cortisol nor cortisone significantly increased the worm survival rate under heat stress (Fig. 2C). Then, we analyzed oxidative stress tolerance by using exposure to 0.1% H2O2 [12]. Treatment with cortisol, but not cortisone, increased the rate of worm survival under oxidative stress (Fig. 2D).

3.2. Mechanism of physiological action of cortisol in *C. elegans*

Reactive oxygen species (ROS) have been previously shown to be generated when cortisol is synthesized from cortisone, indicating that ROS levels can be a marker of cortisol synthesis [21]. In this study, cortisone, but not cortisol, significantly increased ROS level relative to that in the control group (Fig. 3). Thus, we evaluated the role of DHS-30. We found that cortisone did not increase ROS levels in *dhs-30*-deficient worms (Fig. 3). We also evaluated the effect of cortisone on the recovery from heat stress in *dhs-30*-deficient worms. The movements of *dhs-30*-deficient worms decreased substantially after culturing at 35 °C for 4 h and they failed to recover with time (24 h). Cortisol, but not cortisone, restored the number of movements in *dhs-30*-deficient worms (Fig. 4).

3.3. Cortisol promotes stress tolerance via DAF-16

The recovery of thrashing movements after heat stress depends on FOXO/DAF-16 [22]. Thus, we cultured *daf-16*-deficient worms at 35 °C for 4 h and observed a noticeable decrease in the number of the movements. In contrast to wild-type N2 worms, *daf-16*-deficient worms showed reduced movement recovery with time (12 and 24 h), and transferred to a 24-well plate containing 0.1% H2O2. Worm viability was measured every 2 h. Viability and survival time are shown in the y-axis and x-axis, respectively; \( n = 24 \). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
cortisol did not restore the movement of daf-16-deficient worms after heat stress (Fig. 5).

Since we showed that stress tolerance afforded by cortisol was dependent on DAF-16 (Fig. 5), we further evaluated mRNA expression of DAF-16 target genes (hsp-12.6 and sod-3) by using RT-qPCR [23]. The expression of these genes was increased by cortisol treatment, whereas cortisol did not restore the movement of daf-16-deficient worms after heat stress (Fig. 5).

Fig. 3. Cortisone increases reactive oxygen species level via DHS-30. Age-synchronized wild-type N2 (solid bars) or dhs-30-deficient L1 larvae (patterned bars) were transferred onto NGM plates seeded with the OP50 suspension containing DMSO only (control; CT), cortisol, or cortisone, and cultured at 20 °C for 4 days. The worms were then treated with 50 μM 2′,7′-dichlorofluorescein, washed, and fixed with 4% paraformaldehyde. (A) Representative fluorescent images of the worms; scale bars = 600 μm. (B) Relative fluorescence intensity values (the value for CT group is set as 1) presented as the mean ± standard error of the mean. Statistical significance of differences from the values in CT group is indicated as follows: **P < 0.01.

Fig. 4. Cortisone promotes heat stress tolerance via DHS-30. Age-synchronized wild-type N2 or dhs-30-deficient L1 larvae were transferred onto NGM plates seeded with the OP50 suspension containing DMSO only (control; CT), cortisol, or cortisone, and cultured at 20 °C for 4 days. The worms were then heat stressed at 35 °C for 4 h. After recovery at 20 °C, lateral swimming movements (thrashing) were measured for 15 s in the S-basal medium. The y-axis shows movement recovery ([movement at 35 °C/movement at 20 °C] × 100%). Data are presented as the mean ± standard error of the mean (n = 10). Statistical significance of differences from values in CT group is indicated as follows: **P < 0.01.

Fig. 5. Cortisol promotes stress tolerance via DAF-16. Age-synchronized wild-type N2 (solid bars or lines) or daf-16-deficient L1 larvae (patterned bars or dashed lines) were transferred onto NGM plates seeded with the OP50 suspension containing DMSO only control (CT) (black) or cortisol (blue) and cultured at 20 °C for 4 days. The worms were then heat stressed at 35 °C for 4 h. After recovery at 20 °C, lateral swimming movements (thrashing) were measured for 15 s in the S-basal medium. The y-axis shows movement recovery ([movement at 35 °C/movement at 20 °C] × 100%) and the x-axis shows the measurement time post recovery. Data are presented as the mean ± standard error of the mean (n = 10). Statistical significance of differences from the values in CT group is indicated as follows: **P < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
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the exposure to cortisol had qualitatively similar positive effects on hsp-12.6 and sod-3 mRNA expression, which however did not reach statistical significance (Fig. 6).

4. Discussion

C. elegans worms share hormones such as estrogen and oxytocin with humans but do not possess cortisol [12,13,16,24]. However, pregnenolone, a precursor of many steroid hormones, is present in C. elegans and has been found to prolong C. elegans lifespan [15]. Steroid hormones that derive from pregnenolone metabolism have been suggested to play an important role in the lifespan of C. elegans. Generally, chronic high cortisol has a negative effect on human health [9–11]. However, in this study, cortisol treatment remarkably prevented age-related decrease in locomotor activity and enhanced tolerance to stress, although this also shortened lifespan (Fig. 1). Indeed, these and other potentially novel properties of cortisol can be easily revealed by using C. elegans worms that do not have complicated biological structures such as blood circulation system [25]. For example, novel, cholesterol-independent effects of the lipid-lowering drugs statins on aging-associated phenotypes have been demonstrated in C. elegans, which lacks a complete cholesterol synthesis pathway such as the one present in humans [26]. Similar to the results of this study, our present data will contribute to the understanding of the mechanisms regulating human lifespan.

Our experiments demonstrated that cortisone had to be converted to cortisol to promote heat stress tolerance via DHS-30 (Figs. 2–4). Furthermore, the increase in stress tolerance induced by cortisol depended on DAF-16 (Figs. 5 and 6). DAF-16, a homolog of human FOXO, positively regulates stress tolerance of nematodes [23]. Notably, microarray and RNA-seq studies have shown that dhs-30 expression was affected by daf-16 levels [17,27]. Moreover, DAF-16 was activated by heat (35 °C) stimulation, whereupon it translocated to the nucleus [22]. Taken together, feedback of DHS-30 may be caused by the activation of DAF-16, and we have shown here that cortisone promoted heat stress tolerance via DHS-30. For these reasons, the effect of cortisone may appear later than that of cortisol. Generally, the stress response to cortisol is mediated by blood pressure changes in higher animals [28]. In this study, cortisol promoted stress tolerance via DAF-16 and DAF-16-associated target genes (Figs. 5 and 6). These results are similar to the mechanism of heat stress response in C. elegans reported in several previous studies [22,29]. Although a previous study suggested that invertebrates, including C. elegans, do not possess GR, it is possible that cortisol affected C. elegans via another kind of nuclear receptors. A recent study reported that among HSP-90-client nuclear receptors, such as human GR, the ligand-binding domains of NHR-25, NHR-47, DAF-12, and FAX-1 have high homology with human GR [30], suggesting that there are nuclear receptors and signaling pathways functionally similar to GR in nematodes. Thus, the corresponding receptor and the pathways that involve DAF-16 could be identified in future studies.

5. Conclusions

Our experiments revealed novel physiological effects of cortisol in C. elegans. Incubation with either cortisol or cortisone reversed the age-associated decrease in locomotion, although cortisol treatment also shortened the lifespan. In addition, cortisol and cortisone promoted heat stress tolerance via FOXO/DAF-16 and DHS-30, respectively. The present findings open up a possibility of new pharmacological applications of corticosteroid hormones in mammals, including humans.

Declaration of competing Interest

There are no conflicts of interest to declare.

Author statement

This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. We have read and understood your journal’s policies, and we believe that neither the manuscript nor the study violates any of these. There are no conflicts of interest to declare.

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