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RESEARCH ARTICLES

Suppressive effect of quercetin on acute stress-induced hypothalamic-pituitary-adrenal axis response in Wistar rats☆

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Abstract

The flavonoid quercetin is considered to have beneficial effects on human health. We recently have shown that quercetin-enriched foods reduced the duration of immobility time in a rat forced swimming test, indicating that dietary quercetin is promising as an antidepressant-like factor, whereas its mechanism of action is poorly understood. The aim of this study is to investigate the effects of quercetin on water immersion-restraint (WIR), stress-induced hypothalamic-pituitary-adrenal (HPA) axis activation, which is a major component of stress response and plays an important role in the pathology of depression. Quercetin administration to rats significantly suppressed WIR stress-induced increase of plasma corticosterone and adrenocorticotropic hormone levels as well as the mRNA expression of *corticotropin-releasing factor* (*CRF*) in the hypothalamic region. In addition, quercetin modulated the DNA binding activities of glucocorticoid receptor and phosphorylated cyclic adenosine 3′,5′-monophosphate (cAMP) response element binding protein as well as the phosphorylation of extracellular signal-regulated kinase 1/2 in the hypothalamic region, all of which are known to regulate the expression of *CRF* mRNA. Taken together, these results suggest that dietary quercetin attenuates the HPA axis activation by the suppression of the *CRF* mRNA expression.

Keywords: Quercetin; Hypothalamic-pituitary-adrenal axis; Corticotropin-releasing factor; Glucocorticoid receptor; cAMP response element binding protein

1. Introduction

The flavonoid quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a typical polyphenolic compound found in a variety of fruits and vegetables, such as onion, apple and broccoli. A number of studies showing its biological properties including antioxidative, anti-inflammatory and apoptosis-inducing activities indicate that quercetin is potentially beneficial for human health [1]. It should be noted that recent studies have demonstrated the permeability of quercetin across the blood–brain barrier in situ and in vivo [2,3]. Thus, this evidence provides the possibility that quercetin may exert modulating effects on the central nervous system. EGb 761, which is a standardized extract from the leaves of the herbal medicine *Ginkgo biloba* and contains a high amount of quercetin glycosides and other flavonoids, exerted neuroprotective effects against oxidative damage induced by β-amyloid and 6-hydroxydopamine in in vivo studies [4–7]. Also, quercetin and rutin (quercetin-3-rutinoside) were found to attenuate neuronal damage in the hippocampus of rodents with cerebral ischemia treatment, resulting in the improvement of spatial memory impairment [8,9]. In addition, our previous studies have shown that both freeze-dried onion powder and an extract from *G. biloba* leaves (Ginkgolon-24) possess the antidepressant-like activity in a rat forced swimming test, an animal model for assessing the efficacy of antidepressant drugs [10,11]. Of importance, quercetin glycosides, such as isoquercitrin (quercetin-3-glucoside) and hyperoside (quercetin-3-galactoside), were active in the forced swimming test [12].

The hypothalamic-pituitary-adrenal (HPA) axis is a major component of the stress response and is vital for survival, whereas its abnormal activation by chronic and severe stressful conditions is included as an important risk factor for depression, one of the most prevalent psychiatric disorders [13,14]. In response to stress, corticotropin-releasing factor (CRF) is released from the hypothalamus, which in turn stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland. Increase in plasma ACTH levels finally leads to the secretion of cortisol (CORT, corticosterone in rodents) from the adrenal cortex. CORT then induces not only the catabolic process to deal with stressors but also negative-feedback regulation of the HPA axis via the glucocorticoid receptor (GR) in the hypothalamus, the pituitary gland and the hippocampus. However, excessive release of these hormones impairs the function and integrity of the hippocampus by causing neuronal cell loss, resulting in vulnerability to depressive disorders [15]. Therefore, the HPA axis components are important targets for preventing and treating this psychiatric disease.

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Quercetin and its enriched foods have shown antidepressant-like activity [10–12]; however, its mechanisms of action remain unclear. There is one study evaluating the effects of quercetin glycosides on the basal levels of CORT and ACTH in rat plasma for periods of 2- and 8-week administration [16]. However, to our knowledge, no reports have shown their effects on stress response. Therefore, we examined the effects of quercetin on the activation of the HPA axis induced by water immersion-restraint (WIR), an acute stress model, and addressed its underlying molecular mechanisms. As a result, oral administration of quercetin suppressed the WIR-induced increase in the plasma levels of stress hormones including CORT and ACTH as well as the *CRF* mRNA levels in the dissected hypothalamic region. Also, quercetin modulated the DNA activities of the hypothalamic transcription factors GR and cyclic adenosine 3′,5′-monophosphate (cAMP) response element binding protein (CREB) and suppressed extracellular signal-regulated kinase (ERK) 1/2 phosphorylation.

2. Materials and methods

2.1. Reagents

Quercetin was purchased from Sigma-Aldrich. TRIzol Reagent and anti-lamin B1 antibody (clone L-5) were from Invitrogen. Complete protease inhibitor and PhoSTOP phosphatase inhibitor were from Roche Diagnostics. NE-PER Nuclear and Cytoplasmic Extraction Reagents, SuperBlock Blocking Buffer and Restore Western Blot Stripping Buffer were purchased from PIERCE. Enzyme-linked immunosorbent assay (ELISA) kits for CORT and rat ACTH were from Cayman Chemical and Phoenix Pharmaceuticals, respectively. Antibodies directed against ERK1/2 (9102), Pi-ERK1/2 (9101), CREB (9197), Pi-CREB (9198), as well as horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (7074), were obtained from Cell Signaling Technology. The anti-GR antibody (clone BuGR2) was purchased from Affinity BioReagents. HRP-conjugated anti-goat (P0449) and anti-mouse (P0447) antibodies came from Dako, and anti-β-actin antibody (C-11) was from Santa Cruz Biotechnology. ECL Western blotting detection reagents was obtained from GE Healthcare UK. All other reagents used were purchased from Wako Pure Chemicals, unless otherwise specified.

2.2. Animals

Wistar rats (male, 9 weeks old) were purchased from Japan SLC. All animals were housed individually in a controlled environment of 23±1°C with a relative humidity of 45–50% and a 12-h light/dark cycle (08:00-20:00) and had free access to commercial rodent MF pellets (Oriental Yeast) and tap water. The rats were kept for 1 week before starting the experiments. All experimental protocols in this study were approved by The Institutional Animal Care and Oversight Committee, and carried out according to the guidelines for the care and use of laboratory animals of The University of Tokushima Graduate School, Institute of Health Biosciences.

2.3. Water immersion and restraint stress exposure

The rats were restrained in a wire net cage (5×4.5×18.5 cm), and their entire body, except their head, was immersed for 3 h (for ELISA) or 30–60 min [for real-time reverse transcriptase-polymerase chain reaction (RT-PCR), Western blotting and transcription factor activity assay] in water maintained at 24±1°C. In other studies, this type of stress was used for inducing gastric ulcer [17], whereas such aberrations were not observed under our experimental conditions (data not shown). Quercetin was dissolved in propylene glycol and orally administrated in a volume of 1 ml/kg body weight (BW). Control rats received the same volume of vehicle. After 30 min of administration (set as “0 min”), rats were subjected to the stress session. At the end of the experimental period, all animals were killed by decapitation, and then blood was collected in heparin-coated tubes and centrifuged to separate plasma at 4°C for 15 min at 2000×*g*. The dissected hypothalamic regions and the pituitary glands were treated with RNA*later* (Ambion). All of samples were stored until analysis at −80°C.

2.4. ELISA

The levels of ACTH and CORT in plasma were measured using a commercial experimental kit, according to the manufacturer's instructions. The plasma was diluted by twofold (for ACTH) or 1000-fold (for CORT) with assay buffers in each kit and then was subjected to the assay.

2.5. Real-time RT-PCR

Total RNAs from the hypothalamic region and the pituitary gland were extracted using a TRIzol Reagent, and then 2 μg of RNA was reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), as described by the manufacturer. Real-time amplification and detection were performed using the TaqMan Gene Expression Master Mix (Applied Biosystems) on an Applied Biosystems 7500 Real-Time PCR System under the following conditions: one cycle at 95°C for 10 min, then 40 cycles at 95°C for 15 s and at 60°C for 1 min. TaqMan Gene Expression Assays (*CRF*, Rn01462137_m1, Applied Biosystems) and TaqMan Ribosomal RNA Control Reagents (18S ribosomal RNA, Applied Biosystems) were used for detection with manufacture-recommended concentrations. The expression levels were normalized with 18S ribosomal RNA as an internal standard and calculated by $\Delta\Delta C_t$ method.

2.6. Nuclear and cytoplasmic protein extraction

The dissected hypothalamus regions were homogenized and fractionated using NE-PER Nuclear and Cytoplasmic Extraction Reagents, according to the recommendation of the supplier. Extraction buffers were supplemented with inhibitors of proteases and phosphatases.

2.7. Western blotting

Aliquots of nuclear (40 μg) and cytoplasmic (80 μg) proteins were separated on 10% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). After blocking for 30 min, the membranes were incubated with anti-GR antibody or each phosphospecific antibody overnight at 4°C and then with the corresponding HRP-conjugated secondary antibody for 1 h at room temperature. The blots were developed using ECL Western blotting detection reagents, followed by stripping and reprobing with anti-lamin B1 antibody anti-β-actin antibody or each non-phosphospecific antibody. Lamin B1 and β-actin were used as the internal standard for nuclear and cytoplasmic fractions, respectively. Band intensities were quantified using Scion image software.

2.8. Transcription factor activity assay

Both activities of GR and Pi-CREB were determined using TransAM kits (Active Motif), according to the manufacturer's instructions. Briefly, 20 μg of nuclear extracts were incubated within a 96-well plate precoated with oligonucleotides for either GR (1 h) or Pi-CREB (3 h) with mild agitation. Then, the wells were incubated with a specific primary antibody followed by a HRP-conjugated secondary antibody. The absorbance was read at 450 nm with a reference wavelength of 655 nm.

2.9. Protein determination

The protein concentrations in tissue and plasma were determined using the Bradford method (Protein Assay Kit, Bio-Rad Laboratories), with γ-globulin as the standard.

2.10. Statistical analysis

Data are shown as mean±S.D. (n=6). Statistical analysis was performed using two-way analysis of variance, followed by the Tukey–Kramer test with StatView 5.0 (SAS Institute) on untransformed data. P values below .05 were considered significant.

3. Results

3.1. Oral administration of quercetin suppressed the increase in the levels of CORT and ACTH in rat plasma by WIR treatment

To evaluate the effect of quercetin on acute stress response, we measured the level of CORT in plasma in WIR-exposed rats with or without quercetin administration. Three hours of WIR stress increased the plasma CORT level by 10-fold (P<.05) as compared with that of the vehicle nonstress group (Fig. 1A). Interestingly, quercetin administration showed dose-dependent suppression of CORT release. Quercetin at 10 mg/kg BW had a tendency, but not significantly, to reduce the concentration of plasma CORT by 23% and, at 50 mg/kg BW, exhibited a marked reduction by 88% (P<.05) as compared with the vehicle stress group. Although the basal CORT levels in rat administrated quercetin showed slight increases, the difference was not significant.

Next, we investigated whether quercetin could suppress a WIR-induced increase in plasma ACTH levels, as it is well known as an inducer of CORT release from the adrenal cortex in both circadian regulation and stress response. Exposure to WIR led to a substantial increase in the level of ACTH by 3.5-fold (P<.05) as compared with

that of the vehicle nonstress group (Fig. 1B), while quercetin at 50 mg/kg BW substantially suppressed it by 71% ($P<.05$).

3.2. WIR-induced expression of CRF mRNA in the hypothalamus was blocked in quercetin-administrated rats

Since the hypothalamic peptide hormone CRF is a major regulator of ACTH secretion from the pituitary gland, we examined the effect of quercetin on the expression of the *CRF* gene in the hypothalamic region in response to WIR stress. The level of *CRF* mRNA showed a time-dependent increase during the stress session (1.6-fold at 30 min; no statistical difference, 1.9-fold at 60 min; $P<.05$) as compared with the basal level of the vehicle group (Fig. 2). It is notable that, in the quercetin group, the expression of *CRF* mRNA increased by 1.4-fold at 30 min, whereas it was decreased to the basal level at 60 min ($P<.05$).

3.3. The activities of GR and CREB in the hypothalamus were altered by quercetin administration

The transcription factors CREB and GR are considered to be essential as positive and negative regulators of the hypothalamic *CRF* mRNA expression, respectively [18]. Therefore, we next investigated using the nuclear extract from the hypothalamic region whether quercetin modulates the activities of these factors. In the vehicle group, the phosphorylation of CREB at Ser133, an active form, significantly increased by at 30 min of WIR treatment, but tended to decrease at 60 min (2.0–2.5-fold, $P<.05$) (Fig. 3A), while its DNA binding activity showed marked and sustained increase by 1.5- to 1.6-fold during the period of 30–60 min ($P<.05$) (Fig. 3C). It is notable that quercetin completely suppressed the phosphorylation of CREB during the experimental period ($P<.05$). In addition, its DNA binding activity was decreased to the basal level during the 30–60-min period in the quercetin group ($P<.05$). On the other hand, the nuclear localization and DNA binding activity of GR in the vehicle group did not change during the stress session (Fig. 3B). Most intriguingly, quercetin induced GR nuclear translocation in a time-dependent manner (no statistical difference), which was associated with an increase in its DNA binding activity at 60 min by 1.5-fold ($P<.05$), as compared with that of the vehicle group (Fig. 3C).

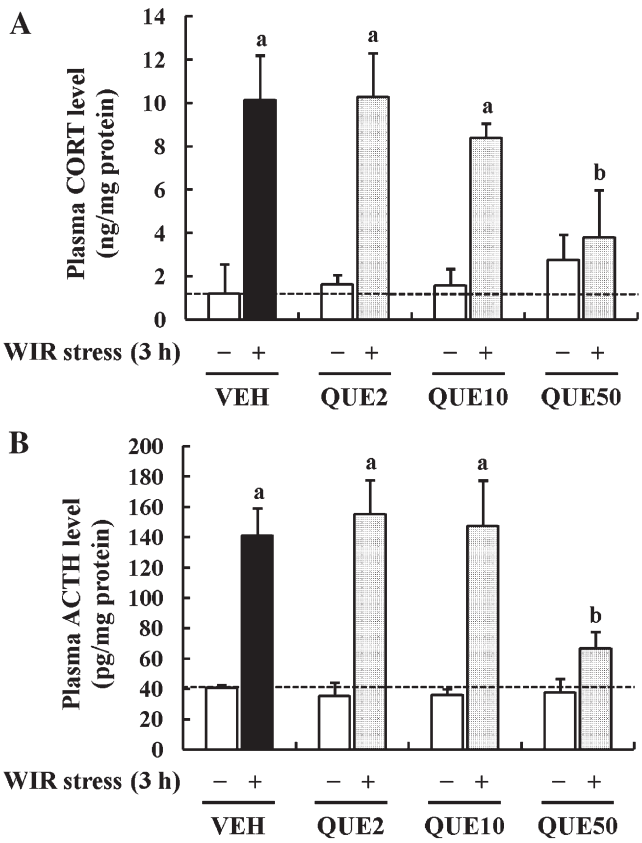

Fig. 1. Quercetin suppressed WIR stress-induced increase of the levels of CORT and ACTH in rat plasma. After 30 min of oral administration of vehicle (VEH) or quercetin (QUE; 2, 10 or 50 mg/kg BW), the animals were subjected to a WIR stress session for 3 h. The levels of CORT (A) and ACTH (B) in the plasma were measured using commercial ELISA kits. Each column represents mean±S.D. of six rats per group. Open bar indicates nonstress; closed bar, stress. Lowercase letters indicate a statistically significant difference ($P<.05$). a, versus nonstress in each group; b, versus stress in the VEH group.

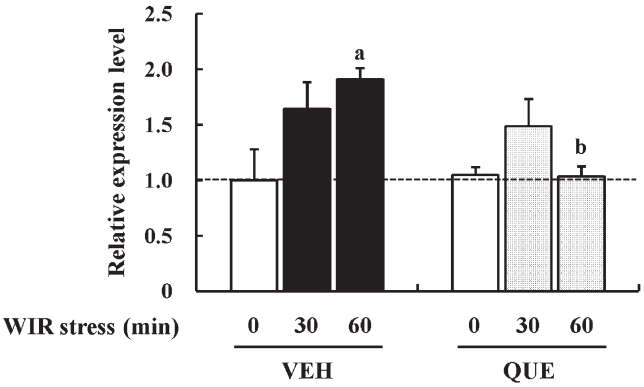

Fig. 2. Quercetin decreased the expression of the CRF gene in the hypothalamus in the pituitary gland in the WIR stress-treated rat. After the administration of vehicle or quercetin (50 mg/kg BW), rats were treated with WIR stress for 0, 30 and 60 min. Total RNA in the dissected hypothalamus region and the pituitary gland was isolated using TRIzol® reagents. Then, the level of CRF mRNA was analyzed by real-time RT-PCR. Each column represents mean ± SD of six rats per group. The relative expression level was normalized using the value of the VEH at 0 min. Small capital letters indicate a statistically significant difference ($P < .05$). a, versus 0 min in the VEH group; b, versus 60 min in the VEH group.

3.4. Quercetin inhibited WIR-induced phosphorylation of ERK1/2 in the hypothalamus

ERK1/2, a member of mitogen-activated protein kinase, is a well-known upstream factor of CREB. The hypothalamic ERK1/2 was found to be activated by several stressors, such as restraint and forced swimming, and involved in *CRF* mRNA expression [19–21]. Hence, we also investigated the effect of quercetin on ERK1/2 phosphorylation. Its phosphorylation state was analyzed by Western blotting using the cytoplasmic fraction of the hypothalamic region (Fig. 4). An active form of ERK1/2 was detected (weak band) in the cytoplasm at 0 min in all groups. In the vehicle group, the levels of phosphorylated ERK1 and 2 increased by sevenfold and 12-fold ($P<.05$), respectively, at 30 min, and decreased at 60 min during the WIR stress session. Meanwhile, quercetin significantly suppressed the increase in the phosphorylation levels of ERK1 and 2 by 75% and 70% ($P<.05$), respectively, at 30 min but had no effect on the phosphorylation state at 60 min.

4. Discussion

Since abnormal activation of the HPA axis is closely related to the pathology of depression, stress control through the dietary habit is an

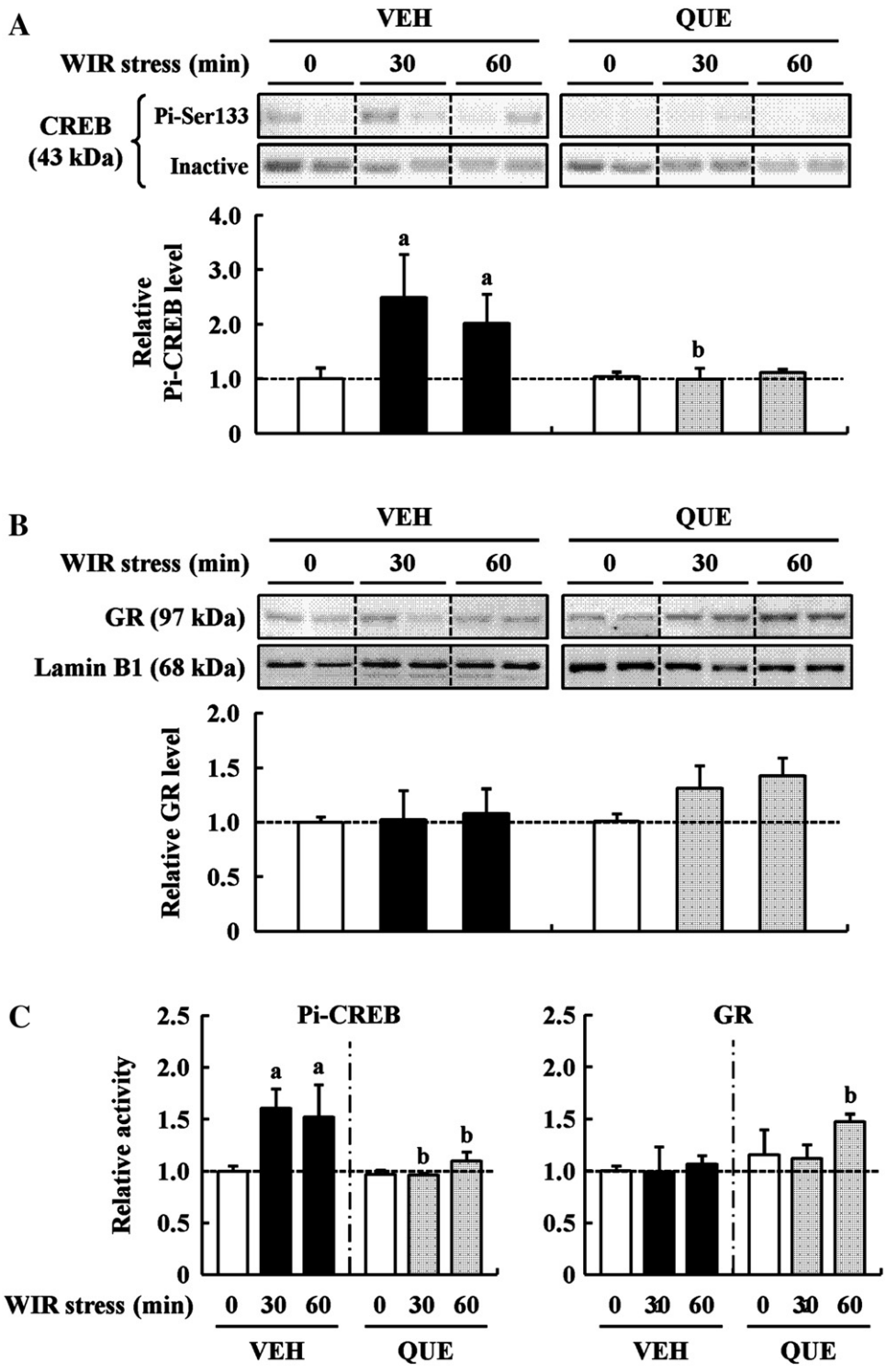

Fig. 3. Quercetin modulated the activities of hypothalamic CREB and GR in the WIR stress-treated rat. Following oral administration of the vehicle or quercetin (50 mg/kg BW), the animals were treated with WIR stress for 0, 30 and 60 min. In nuclear extracts from the hypothalamus regions, the levels of CREB (active and inactive) (A), GR and lamin B1 (B) were analyzed by Western blotting using specific antibodies for each, and the transcriptional activities of Pi-CREB and GR were measured using TransAM™ kit (C). The level of Pi-CREB and GR were corrected with those of CREB and lamin B1, respectively, as the internal standards. Data were normalized using the value of the VEH group at 0 min and are shown as mean ± SD of six rats per group. Small capital letters indicate a statistically significant difference ($P < .05$). a, versus 0 min in the VEH group; b, versus the VEH group at corresponding time points. The images shown are representative bands from two animals of each group.

important strategy for prevention and treatment of the disease. So far, few studies have observed the modulating effects of dietary components and herbal medicines on HPA axis activation (epigallocatechin-3-gallate [22], curcumin [23], apigenin [24], Koso-san [25] and icariin [26]), all of which showed an improvement of stress-related behavioral responses. Recent studies demonstrated the antidepressant-like activity of quercetin, although its mechanism of action is still poorly understood. Our present study attempted to

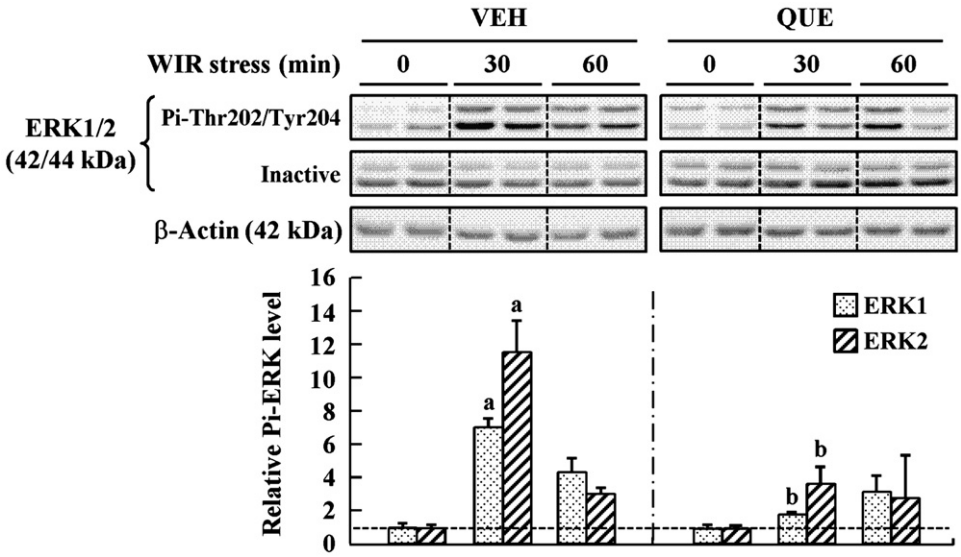

Fig. 4. Quercetin suppressed the phosphorylation of ERK1/2 in the hypothalamus in WIR stress-treated rat. The animals were orally administrated vehicle or quercetin (50 mg/kg) and then subjected to a WIR stress session for 0, 30 and 60 min. Cytoplasmic extracts from the hypothalamus regions were analyzed by Western blotting using specific antibodies. The images shown are representative bands from two animals of each group. The level of Pi-ERK/ERK was corrected with that of β-actin as the internal standard and normalized using the value of the VEH at 0 min. Data are shown as mean±S.D. of six rats per group. Lowercase letters indicate a statistically significant difference ($P<.05$). a, versus 0 min in the VEH group; b, versus 30 min in the VEH group.

obtain insights into the effects of quercetin on acute stress-induced HPA axis activation and clarified for the first time that it produced a marked suppression of the increase in stress hormone levels, including CORT and ACTH in the plasma and *CRF* mRNA in the hypothalamic region in the WIR stress-treated rat. From the results shown here, a mechanism by which quercetin attenuates stress responses are proposed in Fig. 5.

The HPA axis is a hormone cascade comprised of CRF, ACTH and CORT. Since the hypothalamic CRF is the primary factor of this cascade, the suppression of its expression by quercetin might be essential for attenuating HPA axis activation. Thus, the suppression of the *CRF* mRNA expression (Fig. 2) by quercetin might lead to the down-regulation of secretions of ACTH (Fig. 1B) and, in turn, CORT (Fig. 1A) into the plasma.

The expression of *CRF* mRNA in the hypothalamic neurons is known to be regulated by some transcription factors including GR, CREB, activator protein-1 and nerve growth factor-induced gene B, in response to stressors [18]. Among them, CREB is considered to be an essential transcriptional factor for increasing *CRF* mRNA expression. In response to stressful stimuli, CREB is rapidly phosphorylated, and its DNA-binding activity is increased [27–29]. In the present study, both the phosphorylation and DNA binding activity of CREB have been inhibited in the hypothalamic region in the quercetin-administrated rat (Fig. 3). Correspondingly, the activation of ERK1/2 has been decreased in such rats (Fig. 4), implying that quercetin suppressed the CREB activation via an ERK1/2-dependent manner. It is also noteworthy that quercetin induced the activation of GR in the hypothalamic region (Fig. 3), which is known to play a critical role in the negative feedback regulation of both the expression and release of CRF during a stress response. The GR is considered to exert its inhibitory actions on the expression of the *CRF* gene through DNA binding to the putative glucocorticoid response element (GRE) site, direct interactions with transcription factors and nuclear cofactors or via mRNA destabilization [18]. An inverse correlation between the *CRF* mRNA level and the GR DNA binding activity might indicate the involvement of GR in the down-regulation of *CRF* expression. The mechanisms of action by which quercetin induces the GR activation and GR decreases the expression level of the *CRF* gene remain to be elucidated.

Several reports suggest that quercetin passes through the blood–brain barrier and directly influences neuronal function. After quercetin is ingested, it is known to circulate in the bloodstream as glucuronidated, sulfated and/or methylated metabolites, but not as an aglycone in rat and human [30,31]. The blood-brain barrier is a biological membrane formed by the brain capillary endothelium and some associated cells, which restricts the entry of xenobiotics into the brain from the blood to maintain the central nervous system microenvironment. However, a study has reported that

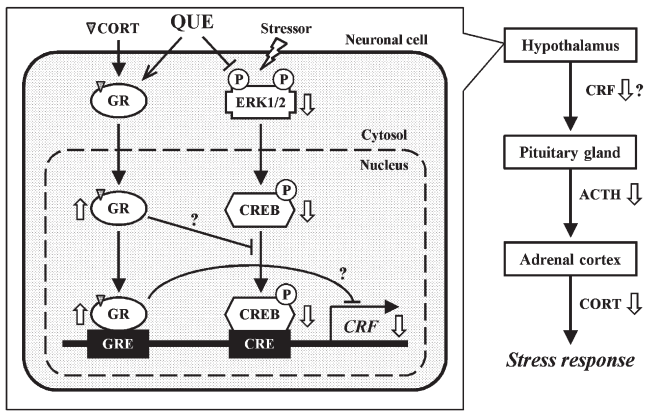

Fig. 5. Proposed scheme for the action of quercetin on HPA axis activation. The HPA axis is a major component of the stress response and a characteristic cascade of hormones. Hypothalamic CRF induced by stressful stimuli induces the release of ACTH from the pituitary gland, which finally incites the synthesis of CORT in the adrenal cortex. Secreted CORT into the plasma causes physiological and behavioral changes for adaptation to stressors and exerts the negative feedback action to suppress HPA axis activity via a GR-dependent mechanism. Quercetin suppressed a WIR stress-induced increase in the levels of stress hormones, including CORT and ACTH in the plasma and *CRF* mRNA in the hypothalamic region. In addition, it regulated the DNA binding activities of GR and Pi-CREB and the phosphorylation level of ERK1/2 in the hypothalamic region, all of which are involved in the expression of *CRF* mRNA.

quercetin can permeate across an in vitro (ECV304 endothelial cell/C6 glioma cell coculture) and in situ (rat brain) blood–brain barrier model, but to a lesser extent than the flavone naringenin, but quercetin metabolites were not examined [3]. In addition, Paulke et al. [2] demonstrated the presence of quercetin metabolites in the brain from rats who received isoquercitrin or St. John's wort (*Hypericum perforatum* L.) extract, which is a herbal medicine used in the treatment of depression and contains quercetin glycosides at a high level. A total of 4 h after feeding, quercetin and its methylated metabolites (isorhamnetin or tamarixetin) were detected at 35–119 ng/g and 7–72 g/g, respectively, in acid-hydrolyzed brain homogenate. Furthermore, recent studies have demonstrated that quercetin metabolites possess a variety of biological effects, such as anti-oxidative, anti-inflammatory and anti-atherosclerotic actions [32–35]. In addition, we have found that quercetin metabolites accumulate in rat brain 30 min after oral administration of quercetin at 50 mg/kg BW (Y. Kawai, A. Ishisaka, S. Saito and J. Terao, unpublished data). Thus, these observations led us to deduce that quercetin metabolites may have biological activities on the HPA axis and other central nervous system functions, such as neurotransmission and neurogenesis.

The absorption of oral administrated quercetin aglycone was estimated to be about 20% in rats and human, while quercetin glucosides were absorbed by 50% in human [36,37]. Considering that yellow onions cultivated in Japan were reported to contain around 40 mg of quercetin glucosides (20 mg of quercetin aglycone equivalent) per 100 g fresh weight [38], rats were administrated with a single and high dose of quercetin aglycone in this study. Additionally, we previously reported that onion powder showed antidepressant-like activity in the forced swimming test but did not affect the plasma CORT level [10], suggesting a possibility of an unknown active compound(s) other than quercetin in onion power. The levels of quercetin metabolites in the brain after long-term administration at the range of normal dietary intake and its contribution to control the physiological and behavioral responses to stressful stimuli still needs to be addressed in the near future.

In conclusion, we demonstrated for the first time that oral administration of quercetin attenuates the activation of the HPA axis in response to acute rat WIR stress. The underlying molecular mechanism might involve, at least in part, the suppression of *CRF* mRNA expression via GR- and CREB-dependent manners. These findings suggest that quercetin and quercetin-enriched diets are useful for preventing and treating stress-related diseases.

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