Behavioural Pharmacology

Flavonoid quercetin protects against swimming stress-induced changes in oxidative biomarkers in the hypothalamus of rats

Nagaraja Haleagrahara a,⁎, Ammu Radhakrishnan b, Nagarajah Lee c, Ponnusamy Kumar a

a Division of Human Biology, Faculty of Medicine and Health, International Medical University, Kuala Lumpur, 57000, Malaysia
b Department of Pathology, Faculty of Medicine and Health, International Medical University, Kuala Lumpur, 57000, Malaysia
c Department of Community Medicine, Faculty of Medicine and Health, International Medical University, Kuala Lumpur, 57000, Malaysia

ABSTRACT

Quercetin is a bioflavonoid abundant in onions, apples, tea and red wine and one of the most studied flavonoids. Dietary quercetin intake is suggested to be health promoting, but this assumption is mainly based on mechanistic studies performed in vitro. The objective of this study was to investigate the effect of quercetin on stress-induced changes in oxidative biomarkers in the hypothalamus of rats. Adult male Sprague Dawley rats were subjected to forced swimming stress for 45 min daily for 14 days. Effect of quercetin at three different doses (10, 20 and 30 mg/kg body weight) on serum corticosterone and oxidative biomarkers (lipid hydroperoxides, antioxidant enzymes and total antioxidants) was estimated. Swimming stress significantly increased the serum corticosterone and lipid hydroperoxide levels. A significant decrease in total antioxidant levels and superoxide dismutase, glutathione peroxidase and catalase levels was seen in the hypothalamus after stress and treatment with quercetin significantly increased these oxidative parameters and there was a significant decrease in lipid hydroperoxide levels. These data demonstrate that forced swimming stress produced a severe oxidative damage in the hypothalamus and treatment with quercetin markedly attenuated these stress-induced changes. Antioxidant action of quercetin may be beneficial for the prevention and treatment of stress-induced oxidative damage in the brain.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Stress is any internal or external stimulus that disturbs the physiological homeostasis and it elicits neurobehavioral alterations (Chakraborti et al., 2007). The central nervous system plays an important role in the regulation of stress responses and complex neurochemical pathways are involved in the activation of these processes (Carrasco and VandeKar, 2003). Stressors activate sympato-adrenomedullary system and hypothalamic-pituitary-adrenal (HPA) axis (Cohen and Hamrick, 2003). The hypothalamus is the primary locus for integration of the stress signals originating from different parts of the body. Reactive oxygen species are generated by a variety of physiological and pathological conditions and despite their vital importance to normal cell function including proliferation, growth, signaling and apoptosis (Reid, 1997) they cause continuous damage to lipids, proteins and DNA (Goto and Nakamura, 1997). Intense stress response results in the release of reactive oxygen species, which cause lipid peroxidation and play an important role in tissue injury (Saito et al., 2005). Brain is the target for different stressors because of its high sensitivity to stress-induced degenerative conditions. There is a link between stressful events and production of free radicals in the brain (Emel and Saadet, 2004). Brain is known to be susceptible for oxidative damage because of its high utilization of oxygen, and its high levels of unsaturated lipids and transition metals (Hyang et al., 2003). Brain is also relatively deficient of anti-oxidative defense mechanisms (Reiter, 1995; Halliwell and Gutteridge, 1985).

Flavonoids are a class of polyphenolic compounds which display a variety of biologic activities. Quercetin is a flavonoid abundant in onions, apples, tea and red wine (Maria et al., 2003). The best described action of Quercetin is its ability to act as antioxidant. It is the most powerful flavonoids for protecting the body against oxidative stress caused by reactive oxygen species (De Groot, 1994). Quercetin directly scavenges the superoxide anion and inhibits several superoxide-generating enzymes such as xanthine oxidase (Chan et al., 1993; Sanhueza et al., 1992) or the neutrophil membrane NADPH oxidase complex (Tauber et al., 1984). Quercetin seems to be the most powerful flavonoids for protecting the body against reactive oxygen species, produced during the normal oxygen metabolism or are induced by exogenous damage (De Groot, 1994).

Forced swimming stress is a continuous stressor and has both psychological and physiological components. Swimming in small laboratory animals has been widely used for studying the physiological changes and the capacity of the organism in response to stress (Tan et al., 2005).
and given food and water.  

2.1. Animals and treatments

All experimental protocols conducted on rats were performed in accordance with the standards established by the Institutional Animal Care and Use Committee and the study obtained approval from the Ethics Committee. Male Sprague–Dawley rats aged three months, weighing 200–300 g were housed in polypropylene cages in groups of two per cage and given food and water ad libitum. The animals were maintained at 12 h light–dark cycle and a constant temperature of 24 ± 1 °C at all times. Rats were randomly divided into following experimental groups with 8 animals in each group. Group 1: control; Group 2: rats exposed to swimming stress; Group 3: rats treated with quercetin – rats in three subgroups received quercetin (Sigma–Aldrich) injection in three different doses (10, 20 and 30 mg/kg body weight) (Indap et al., 2006; Daniel, 1998) Group 4: rats exposed to swimming stress and treated with quercetin. Rats in three subgroups received quercetin injection daily (10, 20 and 30 mg/kg body weight) and these groups were exposed to forced swimming stress for 14 days. All experiments were performed between 9:00 a.m. and 11:00 a.m. daily.

A stock solution of quercetin was prepared in dimethyl sulphoxide (DMSO) and 0.5 ml of DMSO stock solution of quercetin was taken in Tween 80 (1 ml) and diluted with normal saline to make 10 ml injectable suspension of quercetin and injected in a volume of 500 µl/animal/day (Indap et al., 2006). Fresh quercetin solution was prepared daily a few minutes prior to injection and administered intraperitoneally (i.p). Group 4 received quercetin daily, 1 h before the swimming stress for a period of 14 days. Rats in the control and swimming stress groups received 500 µl of vehicle in a similar manner throughout the duration of the experiment.

2.1.1. Swimming stress

Rats were submitted to forced swimming individually in a transparent, cylindrical polypropylene tank (height—40 cm; diameter—24 cm) containing water to a level of 30 cm at 25 °C for 45 min daily (Yan et al., 2000), for 14 days. The swimming stress experiments were carried out in the mornings between 0900 and 1100 hours to prevent the effect of variation in plasma corticosterone levels.

Following each swimming stress session, the rats were towel dried, then returned to their home cages and able to access food and water for the remaining time of the day. At the end of the 14 day experimental period, animals were sacrificed 24 h after the last quercetin administration and swimming stress. The rats were anaesthetized with diethyl ether and blood was collected by cardiac puncture and serum was separated. The brain was removed and the hypothalamus was dissected. The tissue was rinsed with phosphate buffered saline (PBS) solution, pH 7.4, homogenized in six volumes of ice cold potassium phosphate buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA) using glass homogenizer (Fisher Scientific) and centrifuged at 10,000 × g for 15 min at 4 °C (Fournie-Zaluski et al., 2004). Supernatant was collected, aliquoted, and frozen at −80 °C till further assay. From the serum and hypothalamus homogenate, lipid hydroperoxides (LPO), total antioxidants, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase levels were assayed using ELISA kits (Cayman Chemicals and Pierce Biotechnology USA).

2.2. Serum corticosterone measurement

Post-stress corticosterone was measured in serum obtained from blood samples obtained by cardiac puncture after stress by ACE™ Competitive Enzyme Immunoassay (Maclouf et al., 1987) using EIA kits (Cayman Chemicals and Pierce Biotechnology USA.). The assay is based on the competition between corticosterone and corticosterone-acetyl cholinesterase (AChE) conjugate (corticosterone tracer) for a limited number of corticosterone-specific rabbit antisemium binding sites. The amount of corticosterone tracer that is able to bind to the rabbit antisemium is inversely proportional to the concentration of corticosterone in the well. The rabbit antisemium–corticosterone complex binds to the mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate was washed to remove any unbound reagents and then Ellman’s Reagent (which contains the substrate to AChE) is added to the well. The intensity of the product of this enzymatic reaction was determined spectrophotometrically at 412 nm to determine the amount of free corticosterone present in the well.

2.3. Lipid hydroperoxide (LPO) assay

A quantitative extraction method as provided in the kit method for lipid hydroperoxide assay was used to extract lipid hydroperoxides into chloroform and the extract was directly used. This procedure eliminates any interference caused by hydrogen peroxide or endogenous ferric ions in the sample and provides a sensitive and reliable assay for lipid peroxidation. The absorbance was read at 500 nm using a 96 well plate spectrophotometric reader and a dose response curve of the absorbance unit vs. concentration in nmol was generated. Lipid hydroperoxide levels were expressed as nmol/mg of protein.

2.4. Superoxide dismutase (SOD) assay

This assay kit utilizes a tetrazolium salt for the detection of superoxide radicals (O2−) generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme necessary to exhibit 50% dismutation of the superoxide radical. Oxidation rate of tetrazolium salt to Formazan dye by O2− is inversely proportional to the endogenous activity of SOD. The formazan dye stains the wells and its staining intensity was detected by absorbance spectrophotometry at 450 nm using a plate reader. Superoxide dismutase levels were determined from a standard curve and expressed as U/mg of protein.

2.5. Glutathione peroxidase (GPx) assay

This assay kit measures GPx levels indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione, produced upon reduction of an organic hydroperoxide by GPx, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the absorbance at 340 nm was directly proportional to the GPx levels in the sample. Glutathione peroxidase enzyme levels were expressed as nmol/mg of protein.

2.6. Catalase assay

The assay kit utilizes the peroxidatic function of catalase for determination of enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H2O2. The formaldehyde produced is measured spectrophotometrically with purpald as the chromogen. One unit is defined as the amount of enzyme that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 °C. The catalase levels were expressed as nmol/min/mg of protein.
2.7. Total antioxidant assay

Using the total antioxidant assay kit, aqueous and lipid soluble antioxidants were not separated and thus combined antioxidant activities of all its constituents were assessed. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2',2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS®+ by metmyoglobin. The amount of ABTS produced was monitored by reading the absorbance at 405 nm. Total antioxidant levels were calculated from a standard curve and expressed as µmol/mg of protein.

2.8. Statistical analysis

Data were presented as mean ± S.D. Statistical analysis was done by SPSS v 14.0 software package. Differences among the groups and the significance were calculated by non-parametric Kruskal Wallis and Mann–Whitney Test. P value less than 0.05 was considered statistically significant.

3. Results

3.1. Serum corticosterone

Forced swimming stress significantly increased the serum corticosterone levels (P < 0.001). Quercetin alone also increased the serum corticosterone levels at 20 and 30 mg/kg body weight dose (P < 0.05). Treatment with quercetin before stress exposure resulted in a significant decrease in corticosterone levels compared to stressed groups (P < 0.01). More significant reversal of corticosterone change was observed in the 30 mg/kg group (P < 0.001) (Fig. 1). Even though quercetin treatment decreased the corticosterone, it was significantly higher than control corticosterone levels.

3.2. Lipid hydroperoxides

Serum lipid hydroperoxide levels increased significantly in the stressed group (P < 0.001). A significant decrease serum LPO was seen with quercetin treatment along with stress (P < 0.001), where 30 mg/kg quercetin reversed the effect completely (P < 0.001) and the LPO level was found to be lower than control. Treatment with 10 mg/kg body weight quercetin was able to reduce the serum LPO levels significantly (P < 0.01), but the level was significantly higher (P < 0.05) than control rats (Fig. 2). A significant increase in LPO levels was seen in stressed rat hypothalamus (P < 0.001) and quercetin treatment along with stress, reduced the LPO levels (P < 0.001). Treatment with 30 mg quercetin significantly reduced the hypothalamus LPO levels almost near to normal (Fig. 2).

3.3. Superoxide dismutase

Serum superoxide dismutase decreased significantly after swimming stress for 14 days (P < 0.001), but treatment with quercetin significantly increased the SOD levels after 14 days (P < 0.001). Quercetin at 10 mg/kg, increased the SOD levels more than stressed group (P < 0.05) but this level was significantly lower than control and quercetin alone groups (P < 0.05). More significant changes were seen with 30 mg quercetin (P < 0.001). Similar changes were noticed in SOD levels in the hypothalamus homogenates. More significant decrease in the hypothalamus SOD level was seen after swimming stress (P < 0.001) and concurrent treatment with quercetin significantly elevated the SOD levels (Fig. 3).
3.4. Glutathione peroxidase

Quercetin alone when injected at a dose of 30 mg/kg significantly increased the serum glutathione peroxidase levels. But swimming stress decreased the glutathione peroxidase levels significantly \((P<0.001)\) and treatment with quercetin along with stress was able to increase the serum glutathione peroxidase levels. Serum GPx level was brought to near normal in swimming stressed group with 30 mg/kg quercetin \((P<0.001)\) (Fig. 4). A similar trend was observed in the glutathione peroxidase level in the hypothalamus. Stress decreased the GPx levels to more than 60% of the normal \((P<0.001)\) and treatment with quercetin was able to increase the glutathione peroxidase levels to near normal with 30 mg/kg dose \((P<0.001)\) (Fig. 4).

3.5. Catalase

Serum catalase levels decreased more than 50% after swimming stress \((P<0.001)\). Quercetin alone did not bring about any change in serum catalase levels. But quercetin given at three different dosages during swimming stress was able to increase the catalase levels more than stressed groups \((P<0.01)\). More significant increase \((P<0.001)\) in catalase was seen in groups with 30 mg/kg quercetin and the level reached more than the control group (Fig. 5). Hypothalamus catalase level also significantly decreased after swimming stress for 14 days \((P<0.001)\). Quercetin reversed this change caused by stress as catalase level was found to be significantly higher in the hypothalamus in all the three groups with quercetin \((P<0.05)\) (Fig. 5).

3.6. Total antioxidants

There was a significant decrease in serum total antioxidants after forced swimming stress \((P<0.001)\). Quercetin alone treatment at 30 mg significantly increased the total antioxidant levels \((P<0.05)\). Concurrent treatment with quercetin significantly increased serum total antioxidant levels \((P<0.001)\) and there was more significant
increase in total antioxidants after 30 mg/kg body weight quercetin (Fig. 6). Forced swimming stress for 14 days significantly decreased the hypothalamus total antioxidants (P < 0.001) and quercetin treatment during stress was able to increase the hypothalamus antioxidant contents after 14 days (P < 0.01). However, there was no significant change in the hypothalamus antioxidant levels when rats were treated with quercetin alone in three doses (Fig. 6).

4. Discussion

In the present study, serum corticosterone levels of all stress groups were found to be significantly elevated after 14 days with respect to the control group which indicates the activation of hypothalamic-pituitary-adrenal axis. Our results are in accordance with the previous researchers, that corticosterone has been one of the most useful indicators of stress (Sahin and Gümüslü, 2004; Chrousos and Gold, 1992). There are numerous reports showing the elevated serum corticosterone depending on stress (Lehmann et al., 2002; Ricart-Jan et al., 2002). Several parameters have been evaluated after stress exposure in inbred and outbred animals (plasma levels of ACTH, corticosterone, prolactin, progesterone, homocysteine) (Delle et al., 2002; Asai et al., 2004). Estimation of serum levels of corticosterone is an easy and specific method to predict oxidative/inflammatory consequences in the brain after stress (Pérez-Nieves et al., 2007). Glucocorticoids are largely responsible for the brain damage after stress exposure by different ways: (i) inhibition of glucose uptake in neurons; (ii) potentiation of the excitatory amino acid-induced toxicity; (iii) inhibition of excitatory amino acid uptake (Virgin et al., 1991; Goodman et al., 1996). Interestingly, the loss of dendrites and reduction of their length in rat brain neurons seen after stress, first described as a marker of brain damage, is nowadays considered as a compensatory reaction (Magarinos and McEwen, 1995). Diethyl ether has been used in the present study for euthanizing the animals prior to blood and tissue collection. Observed level of corticosterone could be partially influenced by ether stress on rats. Exposure of rats to ether anaesthetics during laboratory procedures are known to activate hypothalamic-pituitary-adrenal axis and produce a significant rise in serum corticosterone levels (Cui et al., 1990; Manev and Pericic, 1983). But in majority of the experiments on ether stress it has been proved that a significant neuroendocrine activation and marked hypothalamic-pituitary-adrenal response was seen with exposure duration of more than 20–30 min (Glowa, 1993; Cook et al., 1973), but in our study, the rats from all the groups, including control group, were sacrificed after an exposure period of 5–7 min.

Swimming stress for 14 days resulted in an increase in lipid hydroperoxide levels and decreased the total antioxidants and the three important antioxidant enzymes, super oxide dismutase, glutathione peroxidase and catalase, both in serum and hypothalamus. Several studies have investigated the stress-induced oxidative modification in the rat brain (Kaushik and Kaur, 2003; Sahin and Gümüslü, 2004; Dembele et al., 2006). Intensive stress has been shown to bring about changes in antioxidant defense system in rats (Al-Qirim et al., 2002; Klotz and Sies, 2003). Increased production of reactive oxygen species, e.g. hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO•) and superoxide anion radical (O$_2^{−}$) in the tissues cause lipid peroxidation, especially in membranes and can play an important role in tissue injury (Kovacs et al., 1996). One of the possible causes for the production of free radicals during swimming stress is increased catecholamine metabolism such as dopamine and norepinephrine. Increased catecholamine as a result of stress-induced activation of sympathetic-adrenal system may cause auto-oxidation, in which electrons are generated that in turn can produce reactive oxygen species (Venaricci et al., 1999; McIntosh and Szapolsky, 1986). Oxidative stress initiated by imbalance in oxidants and antioxidants in the hypothalamus might mediate cell damage and may be responsible for the neuronal disorders during stress (Halliwell and Gutteridge, 1985; Klotz and Sies, 2003).

After forced swimming stress, the antioxidant enzyme levels decreased both in serum and hypothalamus. Fall in super oxide dismutase and catalase activity could be the result of inactivation of these enzymes by interaction with oxygen radicals (Yuksel, and Asma, 2006; Zaidi and Banu, 2004). Glutathione peroxidase is an extremely important antioxidant enzyme, with respect to cellular protection. Change in the concentration of glutathione peroxidase is one of the earliest signs of oxidative injury (Liu et al., 1994). Increased free radical scavenging activity under swimming stress condition might have caused the decrease in glutathione peroxidase levels in the hypothalamus. Increased level of hydroperoxides may arise from the reaction of super oxide with super oxide dismutase, the activity of several enzymes, or the oxidation of endogenous substances during stress (Yuksel, and Asma, 2006; Chakrabarti et al., 2007). Significant elevation of lipid hydroperoxide levels in the hypothalamus after stress confirms the previous researcher’s observations, which are related to stress-induced lipid peroxidation (Kovacs et al., 1996; Hu et al., 2000). Our study indicates that swimming stress might elevate the formation of O$_2^{•}$ and H$_2$O$_2$ in the hypothalamus, and as a consequence, the main detoxifier of free radicals, the antioxidants (Cu, Zn, SOD, GPx) may work effectively to remove them.

Flavonoids are polyphenolic compounds widely distributed in fruits and vegetables. The average daily intake of flavonoids in the diet is about 25 mg, of which quercetin is one of the most abundant representing more than 60% of the average polyphenol ingestion (Goldberg et al., 1995; Sampson et al., 2002; Boots et al., 2008). It has been shown that quercetin scavenges reactive oxygen species and reduces oxidative DNA damage (Cai et al., 1997). Quercetin treatment at three different dosages has decreased the stress-induced elevation of corticosterone. Various reports suggest that quercetin passes through the blood-brain-barrier and influences the neuronal cells directly. A higher concentration of quercetin metabolites appears in the brain after several hours of administration of quercetin (Day et al., 2007;
2001; Paulke et al. 2006). These quercetin metabolites might have influenced the swimming stress-induced activation of hypothalamic-pituitary-adrenal axis. Observed decrease in corticosterone levels after quercetin treatment in stressed rats could be the result of quercetin action in the hypothalamus to decrease corticotropin releasing factor (CRF). Most recently, Kawabata et al. (2009) reported that quercetin suppresses CRF mRNA in the hypothalamus. Since hypothalamic CRF is the most important component of hypothalamic-pituitary-adrenal hormonal cascade, there may be decrease in CRF and ACTH after quercetin treatment, and in turn, it decreased the serum corticosterone levels.

In the present study, pretreatment with quercetin was found to reverse the oxidative stress in the hypothalamus caused by swimming stress. Quercetin inhibits lipid peroxidation and preserves the serum and hypothalamic levels of super oxide dismutase, catalase and glutathione peroxidase hence there is significant elevation in the antioxidant enzymes in the quercetin alone groups. This proves the two important actions of quercetin in oxidative stress i.e., quenching of reactive oxygen species and enhancing the cellular antioxidant defense system. The antioxidant efficacy of quercetin is reported to be due to its higher diffusion into the membranes (Moridani et al. 2003) allowing it to scavenge oxygen radicals at several sites throughout the lipid bilayer; and its pentahydroxy flavone structure allowing it to chelate metal ions via the ortho dihydroxy phenolic structure, thereby scavenging lipid alkoxyl and peroxyl radicals (Lien et al., 1999; Cao et al., 1997). Quercetin treatment caused the decrease in lipid hydroperoxides levels in the study as it can neutralize the reactive oxygen species by directly reacting with O2−, NO and peroxynitrite (Muthukumaran et al., 2008; Bongiovanni et al., 2007). One of the possible mechanisms for decrease in lipid peroxidative indices after stress is the chain-breaking action of quercetin in the free radical process of the oxidation of membrane lipids. Most recently, a novel mechanism for quercetin induced cytoprotection has been described involving the sterol regulatory element binding protein-2 (SREBP-2) mediated sterol synthesis that decreases lipid peroxidation by maintaining membrane integrity during oxidative stress (Bischoff, 2008). It has also been reported that quercetin metabolites can also inhibit peroxynitrite-mediated oxidation, similar to free quercetin and confirming that flavonoids can protect against reactive oxygen species (Klotz and Sies, 2003). In the present study, there was more significant antioxidant effect of quercetin at 30 mg/kg body weight dose compared to 10 and 20 mg/kg confirming that flavonoid quercetin reduced the oxidative stress in the hypothalamus at higher dose (>20mg/kg). The observation that quercetin treated animals had increased total antioxidants and decreased lipid hydroperoxides in blood and hypothalamus supports the antioxidant properties of this flavonoid and demonstrates the protective effect of quercetin on swimming stress-induced oxidative stress.

5. Conclusion

In summary, the findings of the present study suggest that forced swimming stress induces the oxidative damage in the hypothalamus due to increased lipid hydroperoxides and depletion of antioxidant enzymes. There is a highly reduced capacity to scavenge free radicals produced in the hypothalamus in response to stress. Flavonoid quercetin inhibits lipid peroxidation and preserves the blood and hypothalamic antioxidant enzymes. Acting as an antioxidant quercetin alleviates the oxidative damage in the hypothalamus. Pharmacological agents like flavonoid quercetin, capable of scavenging free radicals and/or inhibiting lipid hydroperoxides, and thereby protecting neurons from oxidative injuries may provide useful therapeutic potentials for the prevention or treatment for the neurodegenerative disorders caused by stress. Neuroprotective action of quercetin may be beneficial for the prevention and treatment of stress-induced oxidative damage in the brain.

Acknowledgement

The authors would like to thank Mrs Sheba D Mani for her help in preparing the manuscript. Ms Parvin provided excellent laboratory assistance. This project received financial support from International Medical University, research grant.

References
