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Research Article

Profiling of Hypothalamic *phoenixin* and *nesfatin* Gene Expression in Stressed Rats Treated with Chrysin

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Abstract

Stress is one of the most prevalent mental health disorders. Chrysin, a phytochemical compound, is known for its anti-stress effects; however, the molecular mechanisms underlying its anxiolytic properties are not well understood. The present study aimed to investigate the effects of chrysin on the gene expression of hypothalamic *phoenixin* and *nesfatin-1* in a rat model of acute restraint stress. In the study, twenty male Wistar rats weighing 200 ± 10 g were split up into four groups (n=5). A cannula was surgically implanted into the third cerebral ventricle. Following a one-week recovery period, the rats were exposed to a two-hour acute restraint stress protocol. While the control and stress groups received saline, two additional experimental groups subjected to stress were administered either 20 µg or 40 µg of chrysin via the third cerebral ventricle. Hypothalamic samples were removed. Then, RNA was extracted. In the next step, cDNA was synthesized. Finally, relative gene expression was assessed by a real-time polymerase chain reaction (PCR). The findings revealed a significant upregulation of *nesfatin-1* mRNA in the stressed rat relative to the control group. The mRNA level of *nesfatin-1* in the chrysin-treated group was significantly reduced compared to the stressed group. Furthermore, the stressed rats showed a significant decrease in mRNA levels of *phoenixin* in comparison to the control group. There was no significant increase in the mRNA level of *phoenixin* between the stressed group and the group receiving chrysin. In conclusion, downregulation of the hypothalamic nesfatin may be involved in mediating the anti-anxiety effects of chrysin.

***Keywords*:** Chrysin, Stress, Nesfatin, Phoenixin

Introduction

Stress is a state that is caused by a variety of internal or external stressful factors. Various methods, including drugs and herbal remedies, are used to manage stress (Agorastos and Chrousos, 2022). The benzodiazepine anxiolytics are ineffective against immunity, behavior, cognition, peptic ulcers, or hypertension, despite having strong anti-stress activity against acute models of stress. Moreover, these medications have negative effects on the newborn while nursing and the fetus during pregnancy. An efficient anti-stress is required, since it is becoming more widely acknowledged that stress, especially when an individual is unable to manage the stressor, may be the main cause of the rising prevalence of stress-related physical and mental diseases (Bhattacharya and Muruganandam, 2003). Plant-based compounds offer a promising alternative for managing stress, as they are often associated with fewer adverse effects. Furthermore, investigating these novel treatments can help uncover the molecular mechanisms that underlie stress-related disorders.

Nesfatin-1 is a neuropeptide derived from the precursor protein nucleobinding2 (NUCB2), and is encoded by the nucb2 gene (Pałasz et al., 2021). It is primarily expressed in the hypothalamus and other brain regions involved in stress regulation. In addition, nesfatin-1 is expressed in peripheral tissues such as adipose tissue, pancreatic beta cells, and the testis. Nelcleobindig2 is a protein containing 396 amino acids, of which amino acids 1-82 belong to nesfatin-1. Studies have revealed that nesfatin-1 mediates anxiety-inducing behavior, so that blocking endogenous nesfatin-1 reduces anxiety-like behavior in rats (Friedrich and Stengel, 2021; Schalla et al., 2020).

Phoenixin (PNX) is a neuropeptide that is derived from the precursor protein, small integral membrane protein 20 (CIMM20) (McIlwraith et al., 2022) and is coded by the smim20 gene (Pałasz et

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al., 2021). It is predominantly expressed in the hypothalamus and other regions, including the heart, ovaries, adipose tissue, pituitary, gastrointestinal tract, and pancreatic islets. Activation of the GPR173 receptor mediates the biological actions of PNX. Moreover, PNX has anti-inflammatory, cell-protective, and anxiolytic properties, and it has an impact on behavior, appetite, memory, sensory perception, and energy metabolism (Billert et al., 2020; Kalamon et al., 2020). Emerging evidence suggests that PNX may play a crucial role in decline anxiety and could serve as a promising target for stress management (Schalla et al., 2020).

Chrysin is one of the bioflavonoids that is present in high concentrations in plants such as Passiflora incarnata, Passiflora coerulea, and Oroxylum indicum (Talebi et al., 2021). Chrysin exhibits several biological activities, including anti-pain, anti-inflammatory, immune-regulatory, antioxidant, and neuroprotective (Stompor-Gorący et al., 2021). Moreover, the use of chrysin has been approved as a potential treatment for anxiety and depression. Research shows that chrysin has GABArgic activity, and it seems that the GABArgic system is one of the pathways involved in the effect of chrysin on stress reduction (Rayiti et al., 2020). It has never been thought of before how the chrysin molecular mechanism helps to reduce stress. Consequently, the purpose of this research was to determine whether chrysin can reduce stress by means of hypothalamic neuropeptides.

Materials and Methods

Animal

In this study, male Wistar rats weighing 200 ± 10 g were used. The animals were housed in the laboratory for two weeks under a 12-hour light/dark cycle at a constant temperature of 22 ± 2°C. The study was approved by the Research Ethics Committee of the University of Mohaghegh Ardabili (code: IR.UMA.REC.1400.029).

Surgical procedure

Animals were anesthetized with injection of xylazine (10 mg kg^{-1}) and ketamine (80 mg kg^{-1}). The head was then secured in a stereotactic apparatus, and the Bregma and Lambda points were located. Based on the Paxinos and Watson Atlas coordinates (AP=0.84 mm, ML= 00, DV= 6.5 mm), the cannula was placed in the skull. For one week, in order to recover, the animals were housed in the laboratory. A Hamilton syringe fitted to a polyethylene tube 20 was used to provide the injection (Mahmoudi et al., 2014).

Design and treatment

Chrysin was supplied by Sigma-Aldrich (Cas No. 480-40-0, Co. USA). There were twenty male rats (n = 5) split up into four groups. Group I (Control) and Group II (Stress) were administered only saline. Groups III and IV consisted of stressed rat that were injected with 20 µg or 40 µg of chrysin, respectively (Medina et al., 1990), 30 minutes before stress induction. Each injection was administered via the intracerebroventricular (ICV) route in a volume of 3 µL.

Acute restraint stress

Following a week of recuperation, the rats were subjected to acute restraint stress in an 18 cm long and 5 cm wide plastic tube that was well-ventilated. Then, animals were detained in a quiet room for two hours. The rats were given chrysin 30 min before stress was applied (Bahari et al., 2023).

Hypothalamic sample dissection

First, the animals were euthanized. The skull was broken to remove the brain. The hypothalamus region was determined according to the Watson-Paxinos atlas. Then the brain was placed in the position of the ventral surface, and a 4 mm thick slice containing the hypothalamus was dissected (from the front near the optic chiasma, from the back to the vicinity of the mammillothalamic system, and laterally to the hypothalamic sulcus). The hypothalamus was extracted and immediately stored at -80 °C for RNA extraction (Neghaddadgar et al., 2024).

Microdissections and real-time polymerase chain reaction (PCR)

A TRIzol kit to extract the RNA and a cDNA synthesis kit (Biotech rabbit, Germany) to convert the RNA to cDNA were used. Then, gene amplification was carried out with a PCR instrument and SYBR Green I (Takara Bio Inc., Japan). The thermal cycling conditions were as follows: an initial cycle of 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 20 secs, annealing at 60 °C for 15 secs, and extension at 72 °C for 10 secs. The sequences used to generate the forward and reverse primers are listed in Table 1. The phoenixin, nesfatin-1, and GAPDH amplified products were 71, 204, and 120 base pairs, respectively. The $2^{-\Delta\Delta CT}$ method was utilized to evaluate the relative changes

in gene expression after normalizing the relative expression of *phoenixin* and *nesfatin-1* to GAPDH.

Statistical analysis

The SPSS software was used to conduct the statistical analysis. Mean ± SEM was used to express the obtained results. Tukey's post hoc test was used after One-way ANOVA analysis to compare the significance between the groups. The level of significance was set at $P \leq 0.05$.

Table. Sequence of sense and antisense primers

genes	primers sequences
nesfatin-1	
sense	5′- TGCAGAGAAGAACGCACCAG -3′
antisense	5′- ACAGTACCGTGCTTGGATGG -3′
phoenixin	
sense	5′- GGAGCCGCCTTCTACCCTAT -3′
antisense	5′- ACAGCCTGCTCCTTCTGGTA -3′
GAPDH	
sense	5′- AAGTTCAACGGCACAGTCAAG -3′
antisense	5′- CATACTCAGCACCAGCATCAC -3′.

Results

To explore the molecular mechanisms by which chrysin reduced stress in rats, the expression of *nesfatin-1* and *phoenixin* was examined. Hypothalamic *nesfatin-1* gene expression was significantly increased in stressed rats in comparison to the control group and significantly decreased in chrysin (20 or 40 μg) administered rats in comparison to the stressed group ($p \leq 0.05$) (Figure 1). Additionally, hypothalamic *phoenixin* gene expression significantly decreased in stressed ($p \leq 0.05$) in comparison to the control group. Chrysin (20 or 40 μg) administration to stressed rats increased *phoenixin* gene expression. The increase was not significant in both groups receiving 20 or 40 μg of chrysin compared to the stressed group (Figure 2).

Discussion

The present study reveals that stress triggers changes in the hypothalamic *phoenixin* and *nesfatin-1* gene expression. Present results showed that the expression of the *nesfatin-1* gene increased in the stress group. Previous studies have also shown that nesfatin-1 administration increases stress-induced behaviors in rats subjected to various stress paradigms (Merali et al., 2008). Nesfatin-1 has been distributed in stress-related regions, including the lateral hypothalamus (LH), paraventricular nucleus (PVN), and arcuate nucleus (ARC). Nesfatin-1 interacts with CRH in the PVN. CRH released from the paraventricular nucleus plays a key role in the stress response by activating the hypothalamic-pituitary-adrenal (HPA) axis, which in turn induces the secretion of ACTH and corticosterone. This is exemplified by research showing that nesfatin-1 stimulates CRH release from the hypothalamus, thereby enhancing HPA axis activity (Yoshida et al., 2010). On the other hand, the researchers found that CRH stimulated the secretion of nesfatin-1 in rats, so that sympathetic responses to nesfatin-1 are inhibited by pharmacological suppression of CRH signaling (Tanida et al., 2015). There is some evidence to suggest that chrysin may modulate the HPA axis and CRH activity. One study conducted in rats found that chrysin administration reduced expression of CRH in the hypothalamus (Haghighat et al., 2024).

In response to stress, a number of hypothalamic neuropeptides modulate the HPA axis by either activating or inhibiting CRH neurons. Nevertheless, research on the molecular processes in the hypothalamus responsible for chrysin's downregulation of nesfatin-1 is unknown. The current study examined the effects of chrysin on hypothalamic *nesfatin-1* mRNA levels in rat stress model in order to identify some mechanisms upstream of the CRH neurons. The glutamatergic and GABArgic system can influence nesfatin-1 and other stress-inducing variables. The possible involvement of GABA-nesfatin-1 or glutamate-nesfatin-1 interaction in anxiety processes is worth considering. The NUCB2/nesfatin-1 neurons can be innervated by both excitatory and inhibitory neurons. The glutamatergic system is beneficial to excitatory innervation, while the GABAergic system contributes to the inhibitory process (Aghayeva et al., 2024). Furthermore, there is interaction between CRH neurons and the GABArgic system in the lateral hypothalamus. According to studies, injecting a $GABA_A$ receptor antagonist causes stress induction and an increase in corticosterone levels in the plasma (Keim and Shekhar, 1996). Additionally, the synthesis and release of CRH are mediated, either directly or indirectly, by the glutamatergic system. This neurotransmitter is essential for the activation of neurons and the production of stress because it stimulates neurons. According to several studies, neurons that have higher glutamate levels are more susceptible to stress (Zhou et al., 2018).

Some studies have explored the potential interaction between chrysin and GABA receptors in the rat brain. The researchers found that chrysin enhanced GABArgic system activity, which could contribute to its anxiolytic effects. Glutamate is the main excitatory neurotransmitter in the central nervous system. The study showed that chrysin

significantly reduced glutamate (Bortolotto et al., 2020). In this study, we observed a decrease in *nesfatin-1* gene expression with ICV injection of chrysin. Therefore, chrysin may, by inhibiting the activity of the glutamatergic system and stimulating the activity of the GABArgic system that interacts with CRH neurons, reduce the expression of the *nesfatin-1* gene in the stressed rats.

Furthermore, our research revealed that the expression of the phoenixin gene decreased in stressed rats compared to the control group. This finding is consistent with previous research demonstrating that phoenixin levels are downregulated in a rat restraint stress model (Liang et al., 2022). Additionally, the outcomes of recent investigations support the idea that phoenixin may be involved in the pathophysiology of stress-related mental illnesses. Animal research has indicated that phoenixin deficiency is associated with heightened anxiety-related behaviors (Friedrich and Stengel, 2021). Recent studies have focused on the relationship between phoenixin and the HPA axis. Exposure to stress leads to increased activation of the HPA axis (Grover et al., 2020). It is likely that HPA axis hyperactivity causes a reduction in *phoenixin* gene expression in rat under stress. However, ICV injection of chrysin did not significantly increase *phoenixin* gene expression.

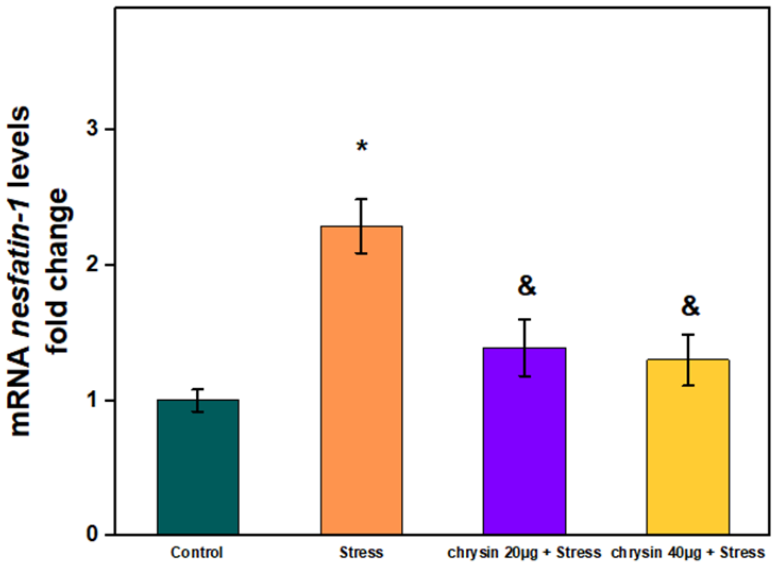

Figure1. The effect of chrysin (20 or 40 µg) on the hypothalamic *nesfatin-1* gene in a rat stress model. The results are expressed as mean ± SEM, and significance was defined by $p \leq 0.05$. *: compared with control; &: compared to the stress group.

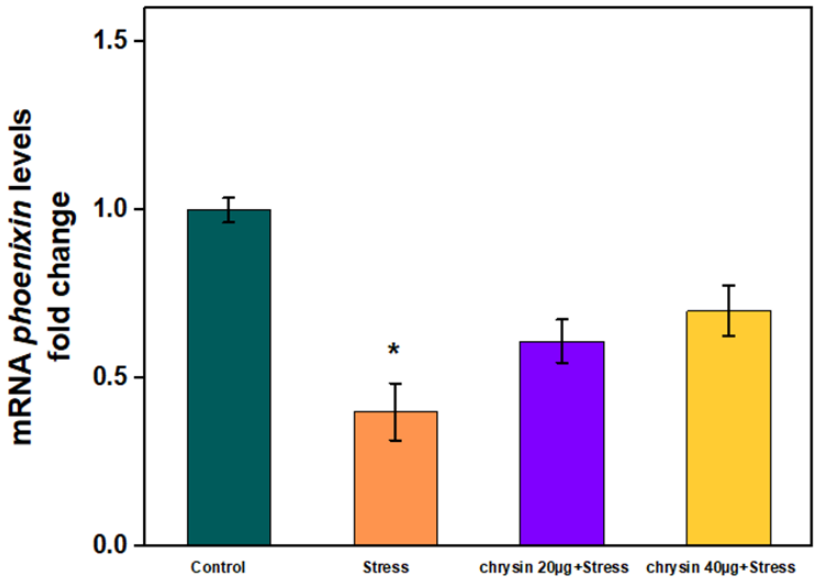

Figure 2. The effect of chrysin (20 or 40 µg) on the hypothalamic *phoenixin* gene in a rat stress model. The results are expressed as mean ± SEM, and significance was defined by $p \leq 0.05$. *: compared with control.

Animal research has indicated that phoenixin deficiency is associated with heightened anxiety-related behaviors (Friedrich and Stengel, 2021). Recent studies have focused on the relationship between phoenixin and the HPA axis. Exposure to stress leads to increased activation of the HPA axis (Grover et al., 2020). It is likely that HPA axis hyperactivity causes a reduction in *phoenixin* gene expression in rat under stress. However, ICV injection of chrysin did not significantly increase *phoenixin* gene expression.

Conclusion

Briefly, changes in *nesfatin-1* and *phoenixin* gene expression were observed in the hypothalamus of stressed rat. Chrysin can regulate the HPA axis via modulating the expression of *nesfatin-1* and *phoenixin* genes, thereby alleviating stress-related symptoms. Treatment with chrysin significantly downregulated *nesfatin-1* gene expression. Chrysin may be a promising therapeutic target for anxiety-related conditions by controlling the expression of hypothalamic neuropeptides upstream of CRH neurons.

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Conflict of interests
There is no conflict of interest in this article.

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