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Pain relieving impacts of chrysin via down-regulation of hypothalamic *Tac1* and *CGRP* in a Rat model of formalin-induced pain

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Abstract

Background: Chrysin is a natural bioactive compound belonging to the flavonoid group. The pain-relieving effects of chrysin have been reported in several studies; however, the molecular mechanism underlying its analgesic properties remains unknown. In the present study, the analgesic effect of chrysin was investigated on the hypothalamic Calcitonin Gene-Related Peptide (*CGRP*) and Tachykinin 1 (*Tac1*) gene expression in a formalin-induced pain model.

Methods: Twenty male rats weighing 200 ± 10 g were divided into four groups. Pain was induced by injecting 50 µl of formalin into the hind paw. The control and formalin groups received saline. In addition, 20 or 40 µg of chrysin was injected into pain-induced rats via the third cerebral ventricle. After 30 minutes, a behavioral test was conducted. Hypothalamus samples were then dissected, and real-time polymerase chain reaction (PCR) was performed to measure gene expression.

Results: The mRNA levels of *CGRP* and *Tac1* significantly increased in the formalin-treated rats compared to the control group. In contrast, the mRNA levels of *CGRP* and *Tac1* were significantly reduced in the chrysin-treated groups compared to the formalin group. Furthermore, the pain score was significantly lower in the chrysin-treated groups compared to the formalin group.

Conclusion: The pain-relieving effects of chrysin were mediated through the downregulation of hypothalamic *CGRP* and *Tac1* in the pain model rats.

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Highlights

What is current knowledge?

The molecular mechanism of the analgesic effects of chrysin remains unknown.

What is new here?

Chrysin can exert its analgesic effects by inhibiting central neuron signaling in the rat hypothalamus.

Introduction

Pain is an unpleasant sensation caused by the stimulation of nerve endings due to tissue damage. It is one of the most debilitating symptoms of medical conditions, capable of reducing quality of life and making daily tasks more difficult (1).

Current management strategies for pain include non-steroidal anti-inflammatory drugs and opioids. Although these drugs are commonly used in therapeutic settings, only a small percentage of individuals who take them experience satisfactory pain relief. Furthermore, their use is associated with dose-limiting adverse effects. Therefore, it is necessary to understand the molecular mechanisms of pain and identify safe and efficient analgesic treatments (2).

The *Tac1* gene encodes substance P (SP), an 11-amino-acid neuropeptide. It is the most effective tachykinin ligand for the neurokinin-1 receptor (NK-1R) (3,4). The SP concentration in different brain regions changes in response to painful stimuli. Furthermore, SP affects other brain areas, such as the thalamus, lateral hypothalamus, and amygdala, which are known to be involved in pain mechanisms (5,3). A study has also shown that the injection of an SP antagonist leads to pain inhibition (6).

CGRP consists of 37 amino acids and belongs to the calcitonin (CT) peptide family. *CGRP* receptors in the central nervous system are distributed across various areas associated with pain, particularly the insula, amygdala, and lateral hypothalamus (7). Acute and chronic pain cause sensory nerve endings and central terminals to release varying amounts of *CGRP*. Evidence shows that pain is reduced following the administration of a *CGRP* receptor antagonist in rats (8). Therefore, research on intra-cerebral molecular mechanisms to control pain is crucial for improving and selecting appropriate treatments for individuals with pain syndromes.

Chrysin is a phytochemical compound found in several plants, such as Passiflora *incarnata*, Passiflora coerulea, and Oroxylum indicum (9). Chrysin also exhibits potent pharmacological properties, including anti-stress, anti-pain, anti-inflammatory, immune-regulatory, antioxidant, anticancer, neuroprotective, and antiviral activities (10). Studies reveal that chrysin's anti-pain benefits may be due to its GABAergic activity and interactions with specific neurotransmitter systems (11). While the analgesic effects of chrysin have been reported, it has also been observed that neuropeptides in the hypothalamus participate in analgesic activities, yet the molecular mechanism of chrysin's effects remains unclear. Therefore, in the present study, the analgesic effect of chrysin was investigated on hypothalamic *CGRP* and *Tac1* gene expression in a formalin-induced pain model.

Methods

Material

Chrysin (CAS No. 480-40-0, Co, USA) and formaldehyde solution (37%) were purchased from Sigma-Aldrich. The kits used included TRIzol (Biotech Rabbit, Germany), cDNA (Vivantis Co., Malaysia), and SYBR Green I (Takara Bio Inc., Japan).

Animal

Male Wistar rats weighing 200 ± 10 g were used. The rats were housed in the laboratory for two weeks and had free access to food and water. The temperature was maintained at 23 ± 2ºC with a 12-hour light/dark cycle.

Surgical procedure

First, the rats were anesthetized with an intraperitoneal administration of ketamine (80 mg/kg) and xylazine. The coordinates of the third cerebral ventricle were determined to implant the cannula in the skull (AP = 0.84 mm, ML = 0, DV = 6.5 mm) (12,13). The rats were kept in the laboratory to recover. After a one-week recovery period, chrysin (3 µl) was injected into the third cerebral ventricle using a Hamilton syringe.

Design and treatment

Twenty male rats were divided into four groups (n = 5). Chrysin was injected into the rats as follows: Group I and II: Control and formalin rats received only saline. Group III: The formalin group received chrysin (20 µg, I.C.V.). Group IV: The formalin group received chrysin (40 µg, I.C.V.) (14). After 30 minutes, pain was induced with formalin, and the animals' behavior was examined. Finally, at the end of the experiment, the animals were euthanized. The hypothalamic samples were removed and immediately stored at -80°C.

Pain induction and behavior test

To induce pain, 50 µl of formalin (5%) was injected subcutaneously into the plantar hind paw of the rat using a 30-gauge syringe. Immediately after the formalin injection, the animal was placed in a transparent compartment (30 × 30 × 30 cm). The behavioral test was conducted for 60 minutes. Then, the pain score (every 5 min) in response to the formalin injection was calculated as follows: 0T0

× 1T1 × 2T2 × 3T3/300. 0: the animal with equal weight places both feet on the floor; 1: the foot is placed a short distance from the floor, and the paw is not spread; 2: the foot is completely elevated; 3: when the foot is licked. Finally, the total score was obtained by summing the calculated scores (15).

Reverse transcriptase *PCR*

The hypothalamic sample was used with a TRIzol reagent kit to extract total RNA. The RNA concentration was measured using a NanoDrop. Next, cDNA was synthesized following the kit's instructions (Biotech Rabbit, Germany). Gene amplification was performed according to the kit's instructions using a PCR apparatus and SYBR Green I (Takara Bio Inc., Japan). The device was set up with the following time cycle: one cycle at 95 °C for 15 minutes, followed by 40 cycles consisting of denaturation at 95 °C for 20 seconds, annealing at 60 °C for 15 seconds, and extension at 72 °C for 10 seconds. The sequences used to produce the forward and reverse primers are listed in Table 1 (16,17). The *Tac1*, *CGRP*, and GAPDH amplified products were 195, 155, and 120 base pairs, respectively. The fold change in each gene expression was calculated using the equation $2^{-\Delta\Delta CT}$ (18).

Table 1. Sequences of sense and antisense primers

Primer Name	Primers sequences
Tac1: Sense antisense	5'-TGACCTCCTCAGACAGAAGTAGAA-3' 5'-TAAAAGCAACCAAGGGAAGC-3'
CGRP: Sense antisense	5'- TCTAAGCGGTGTGGGAATCT-3' 5'- TAGGGGTGGTGGTTTGTCTC-3'
GAPDH: Sense antisense	5′- AAGTTCAACGGCACAGTCAAG-3′ 5′- CATACTCAGCACCAGCATCAC-3′

Statistical analysis

Data analysis was performed using SPSS software (Version 16) and One-way ANOVA. Tukey's post-hoc test was conducted to determine the significant differences between the groups. A significance level of P-Value ≤ 0.05 was used. The results are expressed as mean ± SEM.

Results

Effects of chrysin on the modulation of pain responses

The pain score in the formalin group was significantly higher compared to the control group in phase 1 (0-5 min) and phase 2 (20-60 min). Investigating the pain score showed that the injection of 20 or 40 µg of chrysin decreased the pain score compared to the formalin group in phase 1. The decrease was significant only in the 40 µg group (P-Value ≤ 0.05). In addition, in both the 40 µg and 20 µg chrysin groups, pain scores were significantly reduced compared to the formalin group in phase 2 (P-Value ≤ 0.05) (Figure 1).

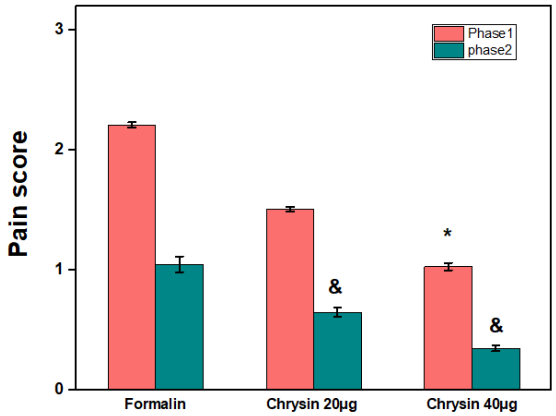

Figure 1. The effect of chrysin (20 or 40 µg) on pain score in rats. Four groups of rats received saline or chrysin during the induction of pain. The results showed that the pain score decreased in animals that received chrysin compared to the saline group in Phase 1 (0-5 min) and Phase 2 (20-60 min). The results are expressed as mean ± SEM, and significance was defined by P-Value ≤ 0.05. *: compared with control (Phase 1); and: compared with control (Phase 2).

Effect of chrysin on the expression of hypothalamic genes

Hypothalamic *Tac1* mRNA levels in the formalin group significantly increased compared to the control group (P-Value ≤ 0.05). Injection of 20 or 40 µg of chrysin in both groups, compared to the formalin group, caused a decrease in *Tac1* mRNA levels. The decrease was statistically significant (P-Value ≤ 0.05) (Figure 2). In addition, hypothalamic *CGRP* mRNA levels significantly increased in the formalin group compared to the control group (P-Value ≤ 0.05). In both groups receiving 20 or 40 µg of chrysin, compared to the formalin group, a significant decrease in *CGRP* mRNA levels was observed (P-Value ≤ 0.05) (Figure 3).

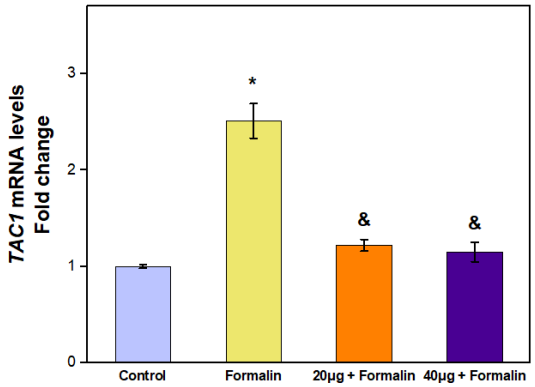

Figure 2. The effect of chrysin (20 or 40 µg) on hypothalamic Tac1 mRNA levels. Four groups of rats received saline or chrysin during the induction of pain. The results showed that Tac1 mRNA levels decreased in animals that received chrysin compared to the saline group. The results are expressed as mean ± SEM, and significance was defined by P-Value ≤ 0.05. *: compared with control; &: compared with formalin.

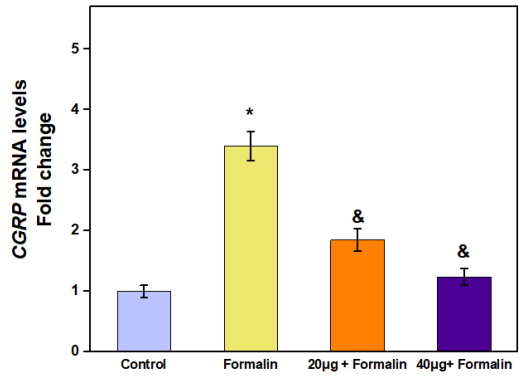

Figure 3. The effect of chrysin (20 or 40 µg) on hypothalamic CGRP mRNA levels. Four groups of rats received saline or chrysin during the induction of pain. The results showed that CGRP mRNA levels decreased in animals that received chrysin compared to the saline group. The results are expressed as mean ± SEM, and significance was defined by P-Value ≤ 0.05. *: compared with control; &: compared with formalin.

Discussion

In the present study, the effect of chrysin on the expression of *Tac1* and *CGRP* genes and pain-related behaviors in formalin-induced pain rats was investigated. It appears that pain behaviors in phases 1 and 2 are differentially regulated, as the pain behavior in the first phase is primarily caused by the direct stimulation of nociceptors, whereas the pain behavior in the second phase involves both inflammatory mechanisms and central sensitization (19). Our results indicated that chrysin is effective in relieving the pain score in both phases 1 and 2 in formalin-induced pain rats. Our findings are consistent with previous studies showing that chrysin decreased formalin-induced pain during both phases 1 and 2 (20). The effect of chrysin on both phases is likely due to its neuroprotective and anti-inflammatory properties.

Our results showed that *CGRP* gene expression increased significantly in the formalin group compared with the control group. This result is consistent with a previous report showing that *CGRP* neurons are activated following chronic pain (21). It has been shown that corticotropin-releasing hormone (CRH) in the hypothalamus plays an important role in pain and stress responses (22). In addition, in the hypothalamus, *CGRP* neurons are located upstream of CRH neurons, which indicates an interaction between CRH neurons and *CGRP*. As a result, the increase in the activity of CRH neurons leads to an enhancement in *CGRP* levels. On the other hand, it has been reported that *CGRP* modulates CRH signaling (23,24).

Different neurotransmitters are synthesized in the hypothalamus, which together control the pain signaling pathways. One of the most important neurotransmitters is GABA. The GABAergic system in the lateral hypothalamus is connected with *CGRP* neurons, so that stimulation of GABA neurons leads to inhibition of *CGRP* levels in response to pain (25). In addition, the GABAergic system interacts with CRH neurons in the lateral hypothalamus. Studies show that the injection of a GABA receptor antagonist leads to an increase in the plasma level of corticosterone and pain induction (26). Moreover, the importance of $GABA_B$ receptors in pain processing has been confirmed by injecting baclofen (A $GABA_B$ receptor agonist) into acute and chronic pain model rats (27). Some research shows that increased levels of glutamate make neurons more sensitive to pain (28). Furthermore, the glutamatergic system can also affect other factors involved in pain, such as *CGRP* (29).

Chrysin exerts stimulatory effects on the GABAergic system. In addition, this compound acts as an antagonist of glutamate receptors in the central nervous system (30). Therefore, chrysin may reduce the expression of the *CGRP* gene in formalin-treated rats by inhibiting the activity of the glutamatergic system and stimulating the activity of the GABAergic system, which interacts with CRH neurons.

There is also a close relationship between sympathetic activity and pain induction. For example, increasing the levels of adrenaline and norepinephrine hormones can strengthen pain signals (31). In addition, an increase in the activity of the sympathetic system may lead to an increase in the synthesis of *CGRP*, indicating its important role in pain regulation (32). On the other hand, it has been demonstrated that chrysin inhibits the activity of the sympathetic system (19). Therefore, another possible mechanism for the inhibitory effects of chrysin on *CGRP* gene expression and pain inhibition may involve suppressing the activity of the sympathetic system.

Our results also showed that in the formalin-model rats, the expression of *substance P (Tac1)* gene increased compared to the control group. Evidence also shows that inhibiting substance P and its receptors can help reduce pain and inflammation (33). Pain and increased levels of substance P in the hypothalamus lead to activation of the HPA axis and increased corticosteroid levels. On the other hand, corticosterone injection leads to an increase in substance P (34). In addition, studies show that substance P neurons of the lateral hypothalamus are under the control of the excitatory effects of glutamate and the inhibitory effects of GABA neurons (35). Therefore, intracerebral injection of chrysin, due to its GABAergic and anti-glutamatergic actions, may lead to inhibition of hypothalamic substance P (*Tac1*) gene expression in formalin model rats through suppression of HPA axis activity.

Basic studies suggest that the substance P receptor is effective in modulating pain through its effects on serotonin (5-HT) neurons. Pain induction in rodents is associated with an increase in substance P synthesis. On the other hand, it has been shown that an increase in the level of serotonin leads to a decrease in the level of substance P in rats (36). In addition, it has been reported that serotonin has analgesic effects and reduces pain sensitivity (37). Previous studies have shown that treatment with chrysin leads to an increase in serotonin levels in brain tissues in rats (30). Based on this, chrysin may decrease the expression of the *substance* P (*Tac1*) gene in formalin-induced pain rats by increasing the activity level of the serotonergic system.

Conclusion

In conclusion, the findings of the pain behavioral test showed that formalin-induced pain behaviors were improved following the third cerebral ventricular injection of chrysin. One of the possible intra-hypothalamic molecular mechanisms underlying chrysin's analgesic effects may be the down-regulation of SP (Tac1) and CGRP mRNA levels. Chrysin may be a potential target for the management of pain syndrome.

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Ethical statement

The University of Mohaghegh Ardabili's Research Ethics Committee oversaw the study's execution (Code: IR.UMA.REC.1400.029).

Conflicts of interest

The authors have nothing to disclose. There is no conflict of interest in this article.

Author contributions

Experimental design: Fariba Mahmoudi and Homayoun Khazali. Data curation: Fariba Mahmoudi and Khadijeh Haghighat. Formal analysis: Fariba Mahmoudi, Homayoun Khazali, and khadijeh Haghighat. Writing-review and editing: Fariba Mahmoudi, Homayoun Khazali, and Khadijeh Haghighat.

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