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Original Article

Protection against Morphine-Induced Inhibitory Avoidance Memory Impairment in Rat by Curcumin: Possible Role of Nitric Oxide/ cAMP-Response Element Binding Protein Pathway

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Abstract

Background: Although a substantial body of research suggests curcumin (CUR) has the preventive potential in memory impairment, the mechanism by which CUR prevents memory loss is still being investigated. This study employs an inhibitory avoidance (IA) model to investigate whether CUR can prevent morphine (Mor)-induced memory impairment as well as the possible role of cAMP-response element binding (CREB) protein, and nitric oxide (NO) signaling in this mechanism.

Methods: This experimental study was conducted at the Animal Lab of the Physiology Research Center, Kashan University of Medical Sciences (Kashan, Iran) in 2018. Forty rats were randomly divided into four groups: control, CUR (pretreatment gavage of CUR [10 mg/Kg] for 35 days), Mor (7.5 mg/Kg, i.p.), and CUR+Mor (n=10 per group). Following the evaluation of the IA memory and locomotor activity of the animals, the CREB protein expression in the hippocampus and NO metabolites (NOx) level in the brain tissue were also investigated. The data were analyzed using Sigmaplot software (version 14.0) by using the ANOVA, Kruskal–Wallis, Holm-Sidak, and Dunn's *post hoc* tests. $P<0.05$ was considered to be statistically significant.

Results: In the Mor group, the IA memory of the rats was significantly impaired ($P=0.001$). CUR prevented the Mor-induced IA memory impairment ($P=0.075$). While the Mor treatment decreased the phosphorylated CREB (p-CREB) expression, the CUR+Mor cotreatment increased p-CREB expression ($P=0.010$). Nevertheless, the Mor treatment increased the total CREB expression ($P=0.010$). The NOx concentration in the brain tissue was decreased following the Mor treatment ($P=0.500$) but increased after the CUR+Mor cotreatment ($P=0.001$).

Conclusion: The present findings suggest that CUR prevents the memory impairment of rats, possibly through NO and its downstream CREB signaling.

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Keywords ● Memory ● Morphine ● Curcuma ● Nitric oxide ● CREB-binding protein

What's Known

- Curcumin is a spice and the main active ingredient in the turmeric plant.
- Curcumin has been shown to have a beneficial effect on memory and has the potential to prevent memory impairment.

What's New

- For the first time, the current findings show that curcumin prevents morphine-induced impairment of inhibitory avoidance memory in rats.
- The mechanism possibly occurs through nitric oxide and its downstream cAMP-response element-binding protein signaling pathway.

Introduction

Curcumin (CUR) is a non-toxic natural flavor as well as a polyphenol with widespread neuroprotective and cognition-enhancing

properties.[1] Although the importance of the CUR in the treatment of memory disorders is well established due to its potential neuroprotective effects, its mechanisms of action are still being investigated. CUR supplements were shown to be beneficial in the treatment of Alzheimer's disease[2] and other central nervous system-related neurodegenerative disorders, such as Parkinson's diseases, and brain malignancies.[3]

The transcription factor cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) exerts its main role in long-term memory and plasticity by phosphorylation at Ser 133.[4] The phosphorylated form of CREB (p-CREB) is known as the synaptic plasticity molecular interface and the final phase of long-term potentiation.[4] A review of the literature reveals the role of CREB phosphorylation in the neuroprotective effects of phytochemicals such as CUR.[5] CREB signaling is involved in the CUR's neuroprotective effects against nicotine.[1] According to a substantial body of research, CUR protects against the impairment induced by scopolamine,[6] nicotine, acrylamide,[1] and alcohol-induced hippocampal neurotoxicity and neurodegeneration.[1, 5]

Morphine (Mor) were shown to impair memory in laboratory animals.[7] Mor application at different times of training and testing affects learning and memory.[8] Since the cellular and molecular correlates of drug dependence and memory for inducing neuronal plasticity were similar, Mor-conditioned response was also associated with an alteration in the p-CREB in the hippocampus.[9, 10]

Moreover, the importance of nitric oxide (NO) in regulating CREB phosphorylation in rats has been well documented. Furthermore, there is a functional association between NO and CREB in nervous system functions, and NO facilitates the regulation of CREB phosphorylation and expression,[11] and there is evidence that NO affects the CUR mediation.[12]

Emotional memory is an aversive type of memory in animals that has primarily been used in research on the mechanisms of the different phases of learning and memory. Among the existing aversive memory methods, the inhibitory avoidance (IA) method stands out.[13]

Taken together, the interaction between opioids and NO in memory, as well as opioid involvement in the modulation of NO function, is well established.[14] Considering that there is a functional association between NO and CREB in nervous system functions,[11] as well as the large body of evidence on the neuroprotective effects of CUR as a probable mechanism against numerous memory impairing agents (see above), the present study was designed to evaluate the novel mechanism of CUR against Mor-induced memory impairment (MMI) and its possible mediation through the CREB-NO pathway.

Materials and Methods

This experimental study was conducted at Kashan University of Medical Sciences (Kashan, Iran), in 2018. The study was approved by the Ethics Committee for Animal Studies (code: IR.KAUMS.MEDNT.REC.1396.26), and all the animal care and behavioral tests were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.[15]

Animals

Forty male Wistar rats (weighing 180–200 g) were housed in polycarbonate cages (four rats in each cage) in the standard living conditions including controlled room temperature (22±2 ºC) with a 12-hour light/dark cycle and relative humidity (40%-60%). During the study, the animals had free access to standard food and water. All the experiments were carried out between 08:00 a.m to 03:00 p.m.

Experimental Protocol

The sample size for the study was calculated using data from a previous study.[8] The rats were randomly assigned into four groups (n=10) and treated as follows: control (CTL), curcumin-pretreatment (CUR), morphine (Mor), and CUR-pretreatment+Mor (CUR+Mor) groups. The CTL group received saline. The rats in the CUR and CUR+Mor groups were pretreated with oral CUR (10 mg/Kg) once a day for 35 days. The Mor and CUR+Mor groups received a post-training intraperitoneal (i.p.) injection of Mor (7.5 mg/Kg/i.p.) immediately after training.[8] A single post-training injection of Mor was administered to the CUR+Mor group one day after the CUR pretreatment was terminated. Based on the findings of the previous study, the dosage and route of CUR administration were determined.[16]

When Mor was injected, the groups of rats that had not received Mor were injected with saline. Similarly, when rats were gavaged with CUR, those rats that had not received Mor were gavaged with saline. Following the termination of behavioral tests (IA memory and open field), the brain tissues of some rats and the hippocampi of the remaining animals in each group were removed for NO metabolites (NOx) assay and western blotting, in respective order (figure 1).

Drugs

The CUR was acquired from Sigma Aldrich

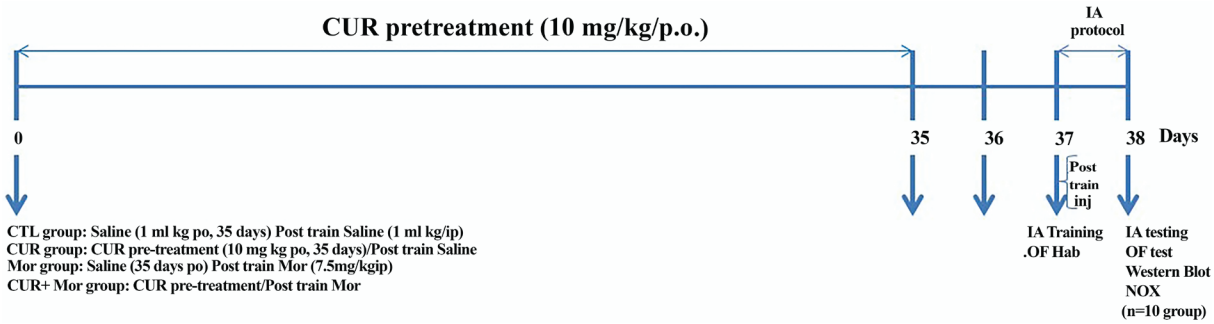

Figure 1: The figure depicts a schema of the experimental design. CTL: Control; CUR: Curcumin; IA: Inhibitory avoidance; OF: Open field; NOx: Nitric oxide metabolites; Hab: Habituation; Inj: Injection; ip: Intraperitoneal

(USA) and Mor from Temad Co. (Tehran, Iran). All of the drugs were dissolved in sterile saline 0.9% and were freshly prepared in the required concentration. Antibodies directed against phospho-CREB (9198), β-actin (4970), and secondary horse radish peroxidase (HRP)-conjugated (7074) were purchased from Cell Signaling Technology (The Netherlands). The total CREB antibody (sc-186) was obtained from Santa Cruz Biotechnology (USA). Amersham enhanced chemiluminescence (ECL)select™ (RPN2235) reagent kit and polyvinylidene fluoride (PVDF) membrane were purchased from GE Healthcare (USA).

Inhibitory Avoidance Setup

Given the findings of the researchers' previous study,[17] step-through IA was utilized for the memory assessment. This apparatus was a two-chambered black/white Plexiglass apparatus (30×30×40 cm) with a grid floor consisting of parallel stainless steel rods (0.3 cm diameter, spaced 1 cm apart). The black and white chambers were separated by a guillotine door. The test consisted of a training session followed by a memory retention session carried out 24 hours after the training. During the training session, each animal was gently placed in a white chamber, and its latency to step through the guillotine door and into the dark chamber with all four paws was measured.

After entering the dark chamber and placing their four paws on the grid floor, an isolated stimulator (Technique Azma, Tabriz, Iran) was used to deliver an electric shock (50 Hz, 3 s, 1 mA). The memory retention session was conducted in the same way as the training session, with the exception that no shock was administered. The latency time (s) to enter the dark chamber was taken as a criterion for measuring memory. For latency time, a cut-off time of 300 seconds was taken. Considering the effect of environmental conditions on plasticity,[18] all of the animals were subjected to the same experimental settings.

Open Field

For the locomotor activity, the animals were placed in an open-field apparatus after the completion of their training (on the first day) only for habituation purposes, and the data were collected five minutes after the termination of testing, on the second day, in an open-field chamber using a charge-coupled device video monitoring system (Technique Azma, Tabriz, Iran), as previously described.[19] The total horizontal distance traveled (cm) in the chamber was used to obtain the data on open-field locomotor activity.

Western Blotting

Some of the animals were deeply anesthetized by CO_2 inhalation and then decapitated immediately after the termination of the behavioral tests. Their hippocampi (n=3) were isolated on ice over a short period of time and then were stored at -80 °C, until they were ready for molecular experiments. The hippocampi of the rats were quickly weighed and homogenized on the ice at three times the volume/weight of cold radioimmunoprecipitation assay lysis buffer containing protease and phosphatase inhibitor cocktail (Sigma, USA). The lysates were centrifuged at 13,000 rpm for 35 min at 4 °C, and the protein containing the supernatants was collected. The protein concentration was determined using Bradford's method, and bovine serum albumin (BSA) as a reference standard.[20] The samples with equal protein concentrations (20 µg/well) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After that, the gel electrophoresis was transferred to PVDF membranes. The blots were subsequently blocked in 5% BSA-Tris Buffered Saline with Tween (TBST) and then were probed with primary antibodies (1/4000) overnight at 4 °C. The next day, after washing them with TBST, the blots were incubated at room temperature for 110 minutes with rabbit IgG conjugated to HRP (1/10,000) as the secondary antibody. The membranes were developed using ECL select™,

followed by autoradiography. A densitometric scan of the films was used to quantify the results, and the density of the bands was calculated using Image J software (National Institute of Health, USA).

Nitric Oxide Assay

Following the termination of the behavioral trials, some animals in each group (other than those remaining from the western blot analysis) were sacrificed, and their brains (five right and five left hemispheres) were extracted for NOx evaluation. The NOx assay was performed using the Griess reaction.[21] For this purpose, after preparing nitrite standard curves, the brain samples were homogenized using phosphate buffer. Then, 100 µL of the tissue suspensions were added to the Griess reagent, including 100 µL of vanadium (III) chloride (VCl^3), 50 µL of sulfanilamide, and 50 µL of N-(1-Naphthyl) ethylenediamine dihydrochloride. The nitrate was reduced to nitrite using VCl^3. The proteins were subsequently precipitated with the addition of 50 µL of trichloroacetic acid 10%. After a 45-minute incubation period, the contents were centrifuged. The supernatants were then transferred to a 96-well flat-bottom microplate. Absorbance was read at 540 nm using a spectrophotometer (NanoDrop Technologies, USA), and the final values were calculated using nitrite standard curves. NOx concentrations were measured in all the groups.

Statistical Analysis

After ensuring that the data distribution was normal, the collected data were analyzed using Kruskal–Wallis and analysis of variance (ANOVA). Paired group comparisons were performed using Holm-Sidak and Dunn's *post hoc* tests. The level of significance was 0.05. All analyses were conducted using Sigmaplot software (version 14.0, Systat Software, Inc. UK).

Results

The Effect of Curcumin and Curcumin+Morphine on Inhibitory Avoidance Memory

The effect of oral CUR pretreatment (10 mg/Kg for 35 days) alone and the effect of CUR+Mor coadministration on IA memory are illustrated in figure 2. The Kruskal–Wallis test showed that, while the post-training administration of Mor (7.5 mg/Kg, i.p) significantly impaired IA memory (P=0.001), CUR+Mor coadministration prevented the MMI (P=0.075).

Locomotor Activity of the Curcumin and Curcumin+Morphine Groups in the Open Field

Figure 3 shows the effect of oral CUR pretreatment (10 mg/Kg for 35 days) alone on locomotion in the open field, as well as the effect of CUR+Mor coadministration. In this regard, the Kruskal–Wallis test revealed no differences between the groups (P=0.203).

The Expression of CREB in the Curcumin and Curcumin+Morphine Groups

In order to determine whether there were differences in the expression of CREB and the phosphorylated isoform p-CREB, the western blot analysis of the hippocampus tissue from the CUR pretreatment (10 mg/Kg/po for 35 days) alone and the analysis of the tissue from the CUR+Mor coadministration was performed. The concentrations of p-CREB were normalized to the total CREB concentrations and expressed as arbitrary relative density units. Total protein concentrations were normalized to β-actin loading control. Moreover, the densitometric

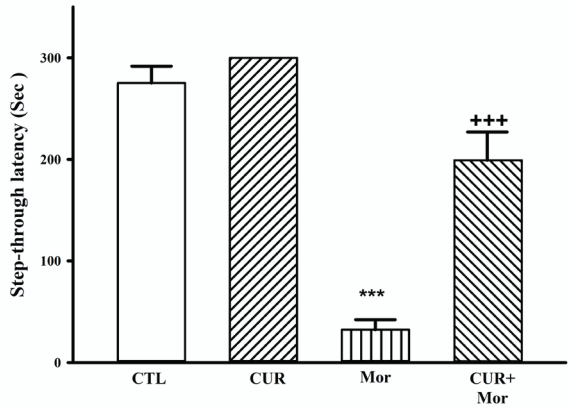

Figure 2: The figure shows the effect of curcumin (CUR), morphine (Mor), and CUR+Mor on IA latency (sec). Four groups of rats received CUR pretreatment (35 days p.o.) followed by post-training saline, morphine (7.5 mg/Kg, i.p.), and their coadministration. Data were shown as median±inter quartile, n=10 in all groups. ***P=0.001 compared with CTL (control) group; +++P=0.001 compared with Mor group.

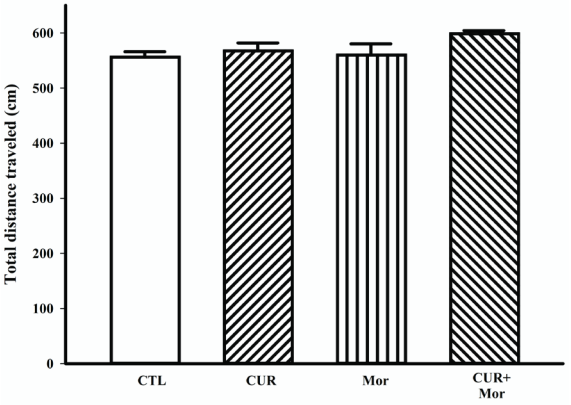

Figure 3: The figure reveals the effect of curcumin (CUR), morphine (Mor), and CUR+Mor on locomotion. The locomotion was performed on the second day in an open field after the completion of the memory testing. Data were shown as median±inter quartile, n=10 in all groups.

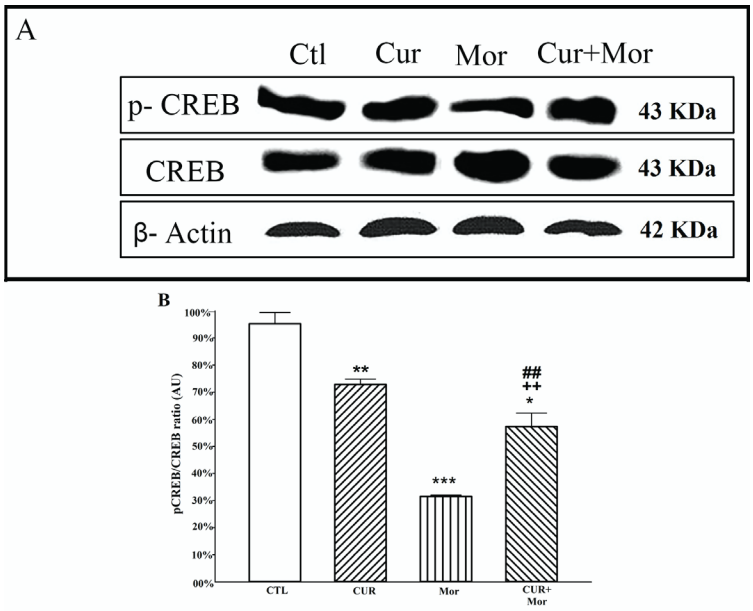

Figure 4: The figure reveals the changes in the hippocampal p-CREB/CREB ratio in curcumin (CUR), morphine (Mor), and CUR+Mor groups. Four groups of animals were used. Representative western blot for p-CREB/CREB ratio was shown in the upper panel (A). The mean hippocampal p-CREB/CREB ratio calculated from densitometric quantification of the corresponding bands was shown in the lower panel (B). Data were shown as mean±S.E.M for three rats. *P=0.050, **P=0.011 and ***P=0.001 compared with CTL group; $^{++}$P=0.010 compared to Mor group; $^{\#\#}$P=0.010 compared with CUR group.

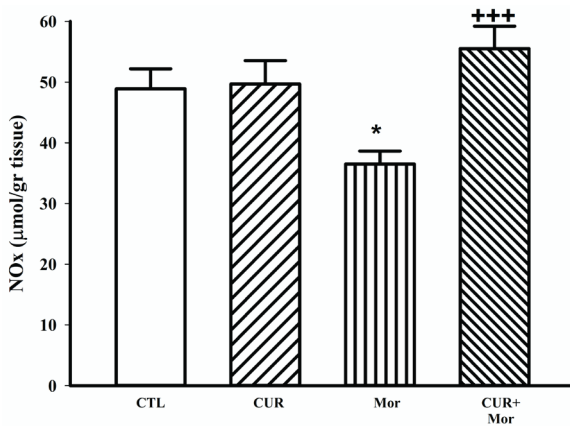

Figure 5: The figure reveals the effect of curcumin (CUR), morphine (Mor), and CUR+Mor on nitric oxide metabolites (NOx) level (µM/gr tissue) in the brain. Data were shown as median±inter quartile, n=10 for each group. *P=0.500 compared with CTL (control) group; $^{+++}$P=0.001 compared with Mor group.

analysis revealed that the p-CREB/CREB ratio was higher in the hippocampus (2.06 fold, P=0.012) of the CUR+Mor coadministration group than the Mor group (figure 4a). As shown in figure 4b, the one-way ANOVA revealed that the post-training Mor group (7.5 mg/Kg, i.p.) had a lower level of p-CREB (70.55 %, P=0.001) than the CTL group (P=0.001). However, the one-way ANOVA revealed a significant difference in total CREB concentration between the groups (P=0.011). Moreover, the Holm-Sidak *post hoc* test revealed that the concentration of total CREB in the Mor group was higher than in the CTL group (1.54 fold, P=0.012).

Alterations of Nitric Oxide in the Curcumin and Curcumin+Morphine Groups

Figure 5 shows the effect of oral CUR pretreatment (10 mg/Kg for 35 days) alone on NOx, as well as the effect of CUR+Mor coadministration. The Kruskal–Wallis test indicated a statistically significant difference between the groups (P=0.001). Furthermore, Dunn's *post hoc* analysis revealed that the post-training administration of Mor (7.5 mg/Kg, i.p) significantly reduced NOx (P≤0.500). Nonetheless, the coadministration of CUR+Mor increased NOx compared with Mor administered alone (P=0.001).

Discussion

The behavioral investigation results demonstrated that step-through latency decreased in the Mor-treated rats compared to the control group, which is indicative of MMI. The impairing effect of post-training Mor has been extensively studied in different memory paradigms, most notably in the researchers' previous study.[22]

Moreover, in the present study, while post-training Mor administration alone impaired IA memory, CUR+Mor coadministration prevented MMI. For the first time, the current findings demonstrated that CUR prevented IA memory in rats subjected to MMI. Liu and others administered the same dose of oral CUR over the same treatment period and reported a similar effect, which was consistent with the current findings.[16] Likewise, CUR were shown

to have a preventative effect in a large number of studies, both in the short-term and long-term doses.[23, 24] Sarlak and colleagues, on the other hand, used low to moderate doses of CUR (5 and 15 mg/Kg, i.p.) in an IA model and reported no significant effects on rats' memory.[25] Since the latter study used a single i.p. administration, and the current study used an oral pretreatment of CUR for 35 days, it seems that, besides the route of administration, the duration of CUR administration is also a factor in this discrepancy. Furthermore, in the current study, the non-significant result of open field locomotor activity ruled out the possibility that our observed effects of CUR and Mor or CUR+Mor could not be secondary to the effects of Mor on general motor behavior.

Considering the ceiling effect on memory performance[26] as a constraint to show the memory-reversing effect of some drugs, in the present study, memory impairment was induced by Mor to observe the preventive effect of CUR. Given the fact that no significant preventive effect of CUR has been reported in intact animals with the absence of memory impairing agents, evidence suggests that CUR-induced prevention mostly occurs when the intact memory were previously impaired by a memory impairing agent.[27, 28]

Furthermore, when comparing the Mor group to the control group, the western blot analyses in this study revealed a significant reduction in p-CREB/CREB ratios in the Mor group. CUR is thus found to play a regulatory role in the alteration of p-CREB expression.[1, 29] In contrast to the findings of the present study, Guitart and others found that the administration of Mor increased CREB phosphorylation.[30] This discrepancy in findings may be attributed to the different models, durations, and sites of administration.

In agreement with the current findings, Akbarabadi and others also found that Mor administration decreased the hippocampal p-CREB expression.[31] Following acute Mor administration, Gago and others reported a reduction in p-CREB in the medial part of the caudate.[32] Given that evidence signifies alterations in p-CREB in different brain regions following Mor administration, the findings of this study suggest that the unequal distribution of the proteins involved in the effective phosphorylation of CREB may have a role in this difference. There is some evidence that supports this suggestion. For instance, as the main upstream kinase of CREB that also plays a role in CREB phosphorylation, learning, and memory, calcium-calmodulin kinase II (CaMKII) is highly distributed in the hippocampus and comprises nearly 2% of the total proteins.[33]

To the best of the researchers' knowledge, few studies on the subject of learning reported the regulation of CREB phosphorylation associated with Mor. Few studies have been done on the effect of Mor on CREB in conditioning paradigms, e.g., conditioned place preference.[9, 10, 34]

Meanwhile, the present study suggests a potential link between MMI and CREB phosphorylation regulation as a possible preventive mechanism for CUR. Nevertheless, further studies are needed to clarify the role of CUR in the CREB signaling pathway. The present study found that CUR inhibits MMI-induced memory loss in rats in the IA test. These findings indicated that the CREB signaling pathway was involved in memory impairment prevention by CUR and that Mor down-regulation of CREB phosphorylation leaded to memory deficits. As a result of a CUR-induced increase in CREB, Nam and colleagues reported cognitive improvement in aged mice.[35]

Furthermore, the NOx assay results revealed decreased NOx concentrations only in the Mor group rather than the control group, and a significant increase was also observed in the CUR+Mor coadministration group compared to the Mor group. Mor-induced NOx and memory responses were also in the same direction. Accordingly, changes in NO production by different NO inducers and inhibitors affected the Mor response to memory,[36] and the interaction between Mor and NO modulated learning and memory in the brain.[36] In agreement with this finding, Farahmandfar and others found that the coadministration of L-arginine, as a NO precursor and pre-training Mor prevented MMI demonstrating the prevention of MMI through increased NO production.[36] Given the fact that Mor increases and decreases the GTPase- and cGMP-related protein kinases (e.g., CAMKII) upon binding to inhibitory G-proteins, one may hypothesize that Mor-induced inhibition of adenylate cyclase decreases the cAMP, and the reduced Ca^{+} entry into cells thus dissociates the Ca^{+}-calmodulin complex.[37] By this hypothetical pathway, Mor may inhibit the neuronal NO synthase activity, resulting in reduced NO production.[38] In support of these findings, some research demonstrated that NO mediates CUR effects.[12] According to Yu and others, CUR prevented the memory impairment induced by aging in mice by increasing NO concentrations.[39] Similarly, Zhu and others reported that increased NO production in the hippocampus by CUR resulted in impairment prevention via the cGMP/PKG pathway.[40]

The failure to work on the molecules involved

in the p-CREB upstream or downstream signaling pathway is one of the limitations of this study. Working on such molecules may help researchers better understand the mechanism of CUR in Mor-induced p-CREB alteration. In addition, while this study did not use some NO inducers and inhibitors to firmly confirm the role of NO signaling, it is a preliminary work that shows the initial contribution of signaling in the process. Obviously in the next phase, understanding the details of this mechanism requires more pharmacological and/or molecular confirmations.

Conclusion

The present findings suggested the existence of a CUR, CREB, and NO interaction that inhibited MMI in IA memory models. In addition, targeting the NO-CREB signaling pathway may represent an interesting approach for the development of new CUR-derived drugs to prevent memory impairments caused by Mor administration.

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Authors' Contribution

K.K: Performing behavioral, biochemical, and molecular experiments, artwork and data analysis; B.A: Study design, performing molecular experiments, artwork and data analysis, drafting the manuscript; A.H: Biochemical experiments; A.A: Study design, artwork and data analysis. All authors were involved in critically revising the present version and made a notable contribution to the final revision of the manuscript. All authors approved the present version of the manuscript. All authors agreed on being accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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