

Original Article

Morphine-induced analgesic tolerance is associated with alteration of protein kinase C γ and transient receptor potential vanilloid type 1 genes expression in rat lumbosacral cord and midbrain

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Abstract

Introduction: Transient receptor potential vanilloid type 1 (TRPV1) and protein kinase C γ (PKC γ) are involved in sensitization/desensitization to noxious stimuli. We aimed to examine the gene expression levels of TRPV1 and PKC γ in rat lumbosacral cord and midbrain on days 1, 4 and 8 of induction of morphine analgesic tolerance.

Methods: Two groups of male Wistar rats received twice daily saline (1 ml/kg) or morphine (10 mg/kg) for eight days and were monitored for analgesic tolerance with a hotplate test on days 1, 4 and 8 of the injections. Six independent groups in three sets were also treated with saline or morphine, decapitated on days 1, 4 or 8 of the schedule, respectively and their lumbosacral cord and midbrain were dissected.

Results: The result of the hotplate test revealed induction of analgesic tolerance on days 4 and 8 of morphine injections. The TRPV1 gene expression in the lumbosacral cord was significantly increased only on day 4 of morphine injections, but the PKC γ gene expression remained with no significant changes on days 1, 4 and 8. In the midbrain, the TRPV1 gene expression was significantly increased only on day 1; however, the PKC γ gene expression was significantly increased on days 4 and 8 of morphine injections.

Conclusion: It can be concluded that the TRPV1 gene expression changes in the lumbosacral cord and midbrain is associated with early phase of morphine-induced analgesic tolerance but the PKC γ gene expression is altered only in midbrain at the later phase of process.

Keywords:

Morphine;
Analgesic tolerance;
Gene expression;
Spinal cord;
Midbrain;
TRPV1;
PKC γ

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Introduction

Development of tolerance to the analgesic effect of morphine severely limits its efficacy in clinical uses (Cantrell and Catterall, 2001; Klecha et al., 2006). The exact underlying molecular mechanisms

responsible for tolerance has remained to be completely understood. A growing body of evidence shows that μ -opioid receptor desensitization and progressive loss of receptor function under continued exposure to the opioid may underlie morphine tolerance (Martini and Whistler, 2007; Koch and Holtt, 2008; Williams et al., 2013). Receptor

phosphorylation is thought to be a key initial event for acute μ -opioid receptor desensitization (Koch and Holtt, 2008). In addition, tolerance is associated with the development of abnormal pain sensitivity such as hyperalgesia (Chen et al., 2007; Chen et al., 2008). Phosphorylation of transient receptor potential vanilloid type 1 (TRPV1) has been considered as a major mechanism of the receptor sensitization, finally leading to hyperalgesia and tolerance to morphine (Mandadi et al., 2006; Chen et al., 2008).

TRPV1 is a member of a large family of ligand-gated ion channels that is expressed in primary nociceptors and is activated by capsaicin, heat, and low pH inducing pain sensation (Immke and Gavva, 2006; Kauer and Gibson, 2009). TRPV1 receptors are also expressed in the central nervous system especially in regions that regulate pain transmission and modulation including spinal cord and midbrain (Szallasi, 1995; Szallasi et al., 1995; Roberts et al., 2004). Accumulating data shows that TRPV1 is a molecular target in morphine-induced reward, dependence, anti-nociception, tolerance and hyperalgesia (Nguyen et al., 2010; Nguyen et al., 2014; Naziroglu and Demirdas, 2015). There are also several evidences regarding the existence of a functional interaction between μ -opioid and TRPV1 receptors. In particular, Chen et al. (2008) reported co-localization of TRPV1 and μ -opioid receptors in dorsal root ganglia (Chen et al., 2008). According to previous researches, the TRPV1 channels on nociceptor terminals in the dorsal horn of the spinal cord may play an important role in modulation of pain and transmission of nociceptive signals (Spicarova and Palecek, 2008).

On the other hand, protein kinase C (PKC) is a serine/threonine protein kinase that plays important roles in various intracellular processes such as neurotransmitter release, ion channel activity, and receptor desensitization (Igumenova, 2015). The gamma isoform of the PKC (PKC γ) shows a unique neuronal distribution and intracellular localization among at least 10 isoforms of the PKC family, which its mRNA is solely found in the brain and spinal cord (Newton, 2003). There are numerous reports that implicate the PKC γ in both acute and chronic tolerance to the analgesic effects of opioids (Narita et al., 1994a; Bilsky et al., 1996; Granados-Soto et al., 2000). In addition, the PKC has been associated with TRPV1 phosphorylation (Woo et al., 2008). It has

been reported that loss of TRPV1-expressing sensory neurons attenuates the development of morphine analgesic tolerance possibly by reducing μ -opioid receptor desensitization through PKC γ in the spinal cord. This suggests that TRPV1 channels by permitting Ca²⁺ influx may act as a mediator between PKC γ activation and μ -opioid receptor desensitization to induce opioid tolerance (Chen et al., 2007).

According to previous researches, repeated morphine exposure alters gene expression and subsequently induces stable changes in protein components (Ammon-Treiber and Holtt, 2005). Involvement of the TRPV1 receptors in morphine effects and its changes at molecular levels in some areas of nervous system including dorsal root ganglia, amygdala and hippocampus have been previously reported (Chen et al., 2007; Chen et al., 2008; Hakimizadeh et al., 2014). Widespread regional analysis on the expression of PKC γ and TRPV1 genes after chronic opioid treatment in the midbrain and spinal cord still remains to be performed (Bailey et al., 2006). Considering previous proposed roles for the PKC γ and TRPV1 in morphine tolerance and importance of lumbosacral cord and midbrain in pain transmission and modulation, we aimed to investigate possible changes in the PKC γ and TRPV1 gene expression in these areas during eight days morphine treatment to show their association with tolerance to analgesic effect of morphine in rats. Development of tolerance to the analgesic effect of morphine severely limits its efficacy in clinical uses (Cantrell and Catterall, 2001; Klecha et al., 2006). The exact underlying molecular mechanisms responsible for tolerance has remained to be completely understood. A growing body of evidence shows that μ -opioid receptor desensitization and progressive loss of receptor function under continued exposure to the opioid may underlie morphine tolerance (Martini and Whistler, 2007; Koch and Holtt, 2008; Williams et al., 2013). Receptor phosphorylation is thought to be a key initial event for acute μ -opioid receptor desensitization (Koch and Holtt, 2008). In addition, tolerance is associated with the development of abnormal pain sensitivity such as hyperalgesia (Chen et al., 2007; Chen et al., 2008). Phosphorylation of transient receptor potential vanilloid type 1 (TRPV1) has been considered as a major mechanism of the receptor sensitization, finally leading to hyperalgesia and tolerance to morphine (Mandadi et al., 2006; Chen et al., 2008).

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Materials and methods

Animals

Adult male Wistar rats weighing 280-320 g at the beginning of the experiments were used. They were housed 4 per cage under a 12-h light/dark cycle (lighting at 07:00-19:00) with food and water supplied *ad libitum*. Temperature of the animal house was maintained at 22 \pm 2 °C and relative humidity was set at 60%. Rats were handled for 5 days before injections. All procedures were performed in accordance with institutional guidelines approved by Research and Ethical Committee of Faculty of Science, University of Kurdistan (1011, 2013) in compliance with the Guideline for the Care and Use of Laboratory Animals (2011), prepared by the National Academy of Sciences' Institute for Laboratory Animal Research.

Experimental design

Morphine sulfate was purchased from Temad (Temad, Co. Tehran, Iran). For induction of morphine analgesic tolerance, two groups of rats received saline (1 ml/kg) or morphine (10 mg/kg) twice a day with six hours interval for 8 days (Ahmadi and Rashdi, 2016). The following experimental groups were used to complete the study. Four sets of animals (each set including two groups of control and

tolerant) were used. In the first set, two groups of control and tolerant ($n = 6$, in each group) were used to examine induction of tolerance to analgesic effect of morphine during a schedule of 8 days morphine treatment. In the second set, two groups of control and tolerant ($n = 4$, in each group) were used to study the TRPV1 and PKC γ genes expression in the lumbosacral cord and midbrain after a single injection of morphine (10 mg/kg). In the third set, two groups of control and tolerant rats ($n = 4$, in each) were used to examine the TRPV1 and PKC γ genes expression in the lumbosacral cord and midbrain after 4 days injection of morphine (10 mg/kg). In the fourth set, two groups of control and tolerant ($n = 4$, in each) were used to examine the TRPV1 and PKC γ genes expression in the lumbosacral cord and midbrain after 8 days injection of morphine (10 mg/kg).

Hotplate test of analgesia

A hotplate apparatus (Pooya-Armaghan Co., Tabriz, Iran) was used to assess pain behavior in rats according to our previous reports (Ahmadi et al., 2015a; Ahmadi et al., 2015b). Hotplate test of analgesia was done on days 1, 4 and 8 of the injection of saline or morphine. The animals were acclimated to the testing room 30 min before testing. Then, each animal was placed on the hotplate ($52 \pm 1^\circ\text{C}$) that was surrounded by a glass square (30 cm height) to prevent escaping of the animal. Hotplate latency was defined as the time interval between placement of the animals on the hotplate and the first observation of a jump or lick of a hind limb. First, "baseline latency" was recorded for each animal before any injection on the testing days. Second, each rat received injection of saline or morphine and then it was tested 30 min after the injection to measure "test latency". A cut-off time of 80 s was defined as complete analgesia to prevent tissue damage (Ahmadi et al., 2015a; Ahmadi et al., 2015b). Some other investigators have also reported the cut-off time of 80-120 sec in their studies (Dogrul et al., 2005; Jin and Yu, 2010; Hosseini et al., 2011). We also checked the paws of the animals and no tissue damage was observed. Finally, the recorded latencies were converted to percentage maximum possible effect (%MPE) based on the following formula: $\%MPE = [(test\ latency - baseline\ latency)/(cut-off\ time - baseline\ latency)] \times 100$ (Ossipov et al., 1990; Keil and Delander, 1995):

Tissue dissection and total RNA extraction

Six groups of rats were divided into three independent sets, and two groups in each set received saline (1 ml/kg) or morphine (10 mg/kg) treatments. The animals in the first set on day 1, the second set of the animals on day 4 and the last set of rats on day 8 were decapitated three hours after the morning session injection on the mentioned days. Then, their lumbosacral cord and midbrain were immediately dissected on an ice-chilled sterile surface and moved into a tube containing 400 μl RNAlater solution (RNA stabilization solution, Qiagen, USA). The tissues were incubated in the RNAlater solution overnight at 4°C , then the RNAlater solution was aspirated and the tissues were stored at -70°C until further analysis. Total RNA was isolated from 70 mg of each tissue using a highly pure tissue RNA extraction protocol (RNX-Plus, Sinaclon, Iran) according to our previous reports (Ahmadi et al., 2015a; Ahmadi et al., 2015b). Quality of the extracted RNAs were assessed by agarose gel electrophoresis and concentrations and purity were determined by spectrophotometry (Spicord210, Analytik Jena, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA samples converted to cDNA using a two-step RT-PCR kit according to the manufacture manual (Thermo Scientific, USA). PCR was used for amplification of PKC γ and TRPV1 genes along with β -actin as control gene (C1000 Thermal Cycler, BIO-RAD, USA). Primers for the genes had the following sequences: β -actin forward primer: 5'-CTGGGTATGGAATCCTGTGG-3'; β -actin reverse primer: 5'-AGGAGGAGCAATGATCTTGAT-3'; PKC γ forward primer: 5'-ACCGCCTGTATTTTGTGATG3'; PKC γ reverse primer: 5'-ACATCTCATACAGCAGGACTC-3'; TRPV1 forward primer: 5'-CAGGGGTCCATAGATTGCAC-3'; TRPV1 reverse primer: 5'-GAAGTGCCAGGAAACGGTC-3'. First, PCR parameters were optimized. Then, a multiplex PCR for cDNA amplification of PKC γ and β -actin was performed including first denaturation at 95°C for 5 min, followed by 30 cycles of thermal cycling (94°C for 30 sec, 63°C for 30 sec, 72°C for 45 sec), and then a 10 min of extension at 72°C . The PCR program for cDNA amplification of TRPV1 along with β -actin was initiated with a first denaturation step

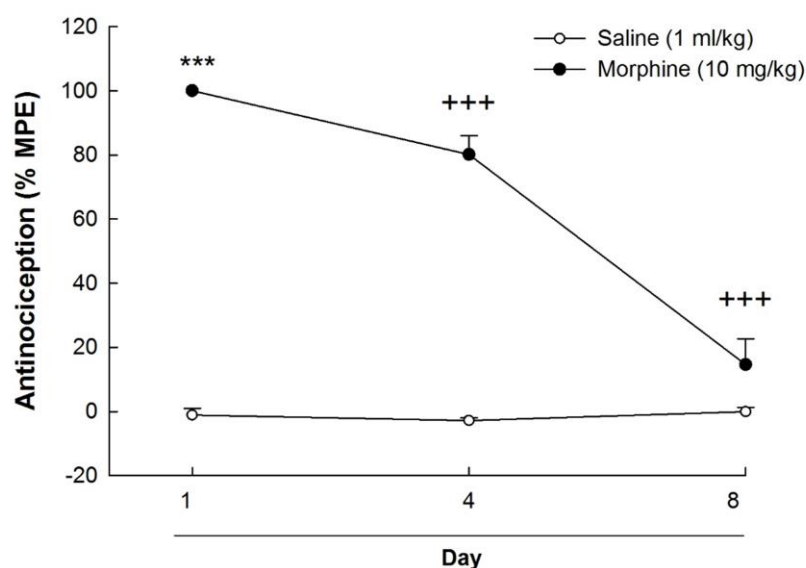


Fig.1. Induction of tolerance to analgesic effect of morphine. Two groups of rats received saline (1 ml/kg) or morphine (10 mg/kg) twice daily for eight days. Each point represents mean \pm S.E.M. of %MPE in six rats per each group on the indicated days. *** $P < 0.001$ compared to saline treated group on the same day, +++ $P < 0.001$ compared to morphine treated groups on days 1 and 4 of the injections.

of 95°C for 5 min, followed by 33 cycles of thermal cycling (94°C for 30 sec, 61°C for 30 sec, 72°C for 45 sec) followed by 10 min final extension step at 72°C. All reactions were terminated at 4°C. The PCR products were subsequently analyzed on 2% agarose gel electrophoresis. Densitometry was performed using an Image J program to quantify bands on gel electrophoresis.

Data analysis

Data from the hotplate test were analyzed with repeated measure two-way ANOVA and after a significant F value, post hoc Holm-Sidak's test was used for paired comparisons. The densitometry data for evaluating gene expression was analyzed using an independent t-test. $p < 0.05$ was considered statistically significant throughout.

Results

Tolerance to analgesic effect of morphine was induced during eight day of repeated injections of the drug

A mixed between-within subjects analysis of variance (two-way repeated measure ANOVA) was conducted to assess the impact of the drug as factor A with two

levels (saline or morphine) and time or days of hotplate test as factor B with three levels (days 1, 4 and 8) on antinociception. The results showed significant interaction between drug and time, Wilks' Lambda = 0.024, $F(2, 9) = 183.91$, $p < 0.001$, partial eta squared = 0.98, suggesting significant difference in the effectiveness of morphine analgesia during three days of hotplate test. There was a substantial main effect for time, Wilks' Lambda = 0.24, $F(2, 9) = 182.51$, $p < 0.001$, partial eta squared = 0.98. The main effect, comparing the drugs (saline or morphine as factor A) was also significant, $F(1, 10) = 1129.28$, $p < 0.001$, partial eta squared = 0.99. Post hoc test revealed that morphine induced a complete analgesia after first injection on day 1 but its analgesic effect decreased with repeated injections of the drug on day 4 compared to day 1 ($P < 0.001$) and on day 8 compared to days 1 and 4 ($P < 0.001$).

Gene expression profile of TRPV1 and PKC γ in the lumbosacral cord on days 1, 4 or 8 of morphine injections

Analysis of the gene expression data in the lumbosacral cord showed that the TRPV1 gene expression was significantly increased only on day 4 of morphine injection without significant changes on

days 1 and 8 (Fig. 2). On the other hand, the PKC γ gene expression was not significantly altered during 8 days of the injections (Fig 3).

Gene expression profile of TRPV1 and PKC γ in the lumbosacral cord on days 1, 4 or 8 of

morphine injections

Analysis of the gene expression data in the midbrain showed that the TRPV1 gene expression was significantly increased on day 1 of morphine injection without significant changes on days 4 and 8 (Fig. 4).

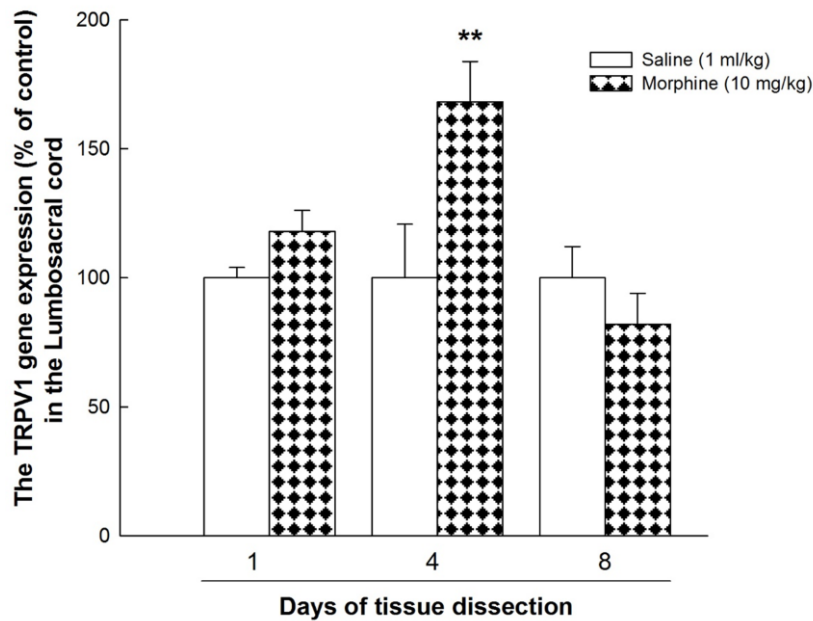


Fig.2. Gene expression profile of TRPV1 in the lumbosacral cord on days 1, 4 or 8 of morphine injections. Each bar represents mean \pm S.E.M. of the TRPV1 gene expression (n=4). Data were normalized to β -actin and set at 100% in control group. ** P<0.01 compared to the respective saline treated control group.

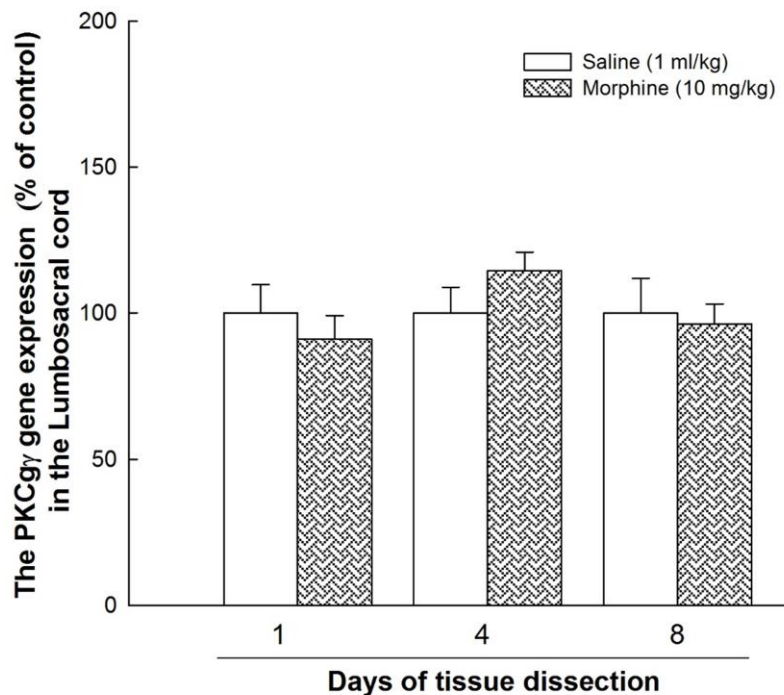


Fig.3. Gene expression profile of PKC γ in the lumbosacral cord on days 1, 4 or 8 of morphine injections. Each bar represents mean \pm S.E.M. of the PKC γ gene expression (n=4). Data were normalized to β -actin and set at 100% in control group.

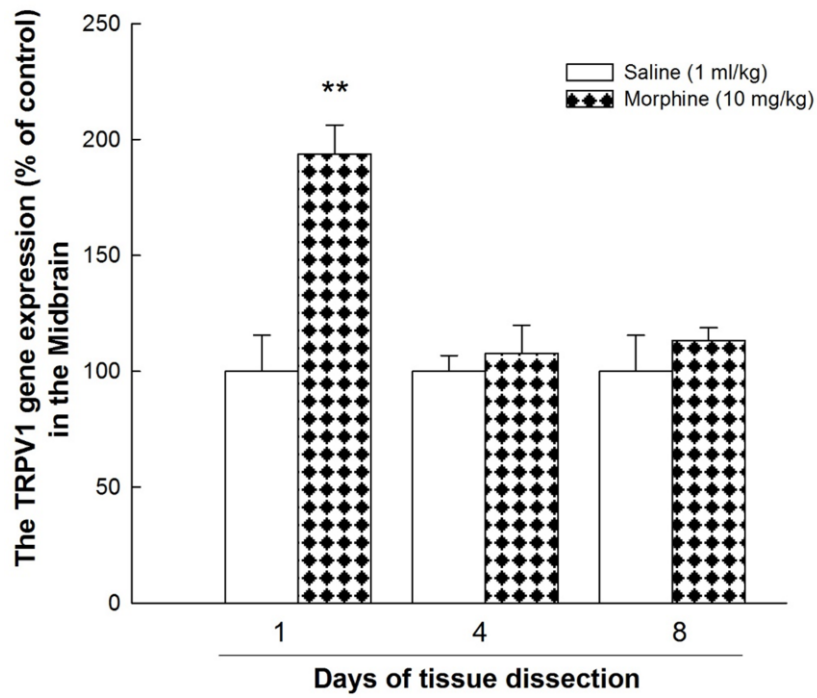


Fig.4. Gene expression profile of TRPV1 in the midbrain on days 1, 4 or 8 of morphine injections. Each bar represents mean± S.E.M. of the *TRPV1* gene expression (n=4). Data were normalized to β -actin and set at 100% in control group. ** P<0.01 compared to the respective saline treated control group.

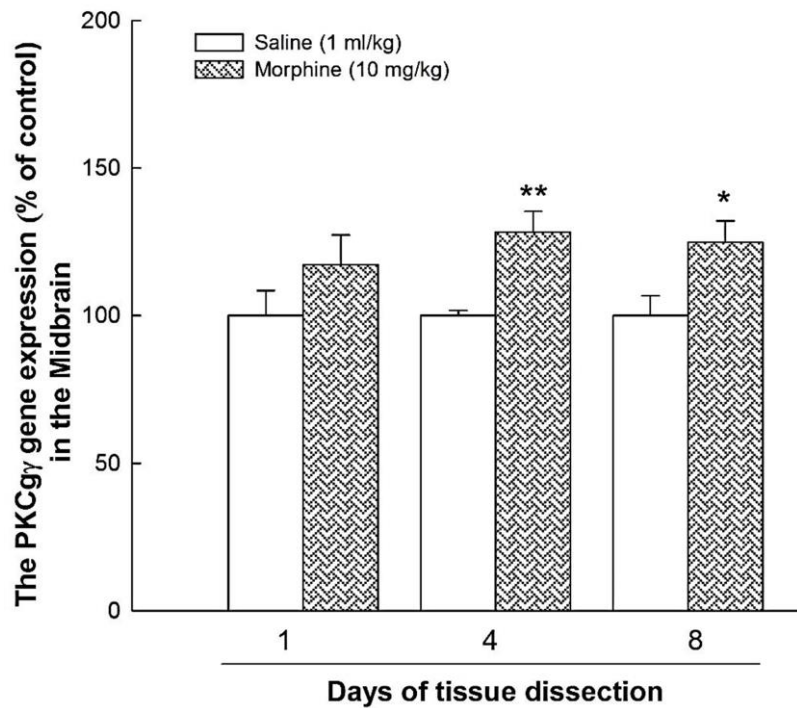


Fig.5. Gene expression profile of PKCγ in the midbrain on days 1, 4 or 8 of morphine injections. The PKCγ gene expression was also examined in the same rats used for evaluating the TRPV1 gene expression. Each bar represents mean± S.E.M. of the PKCγ gene expression (n=4). Data were normalized to β -actin and set at 100% in control group. * P<0.05 and **P<0.01 compared to the respective saline treated control group.

However, the PKC γ gene expression was significantly increased on days 4 and 8 of the injections (Fig 5).

Discussion

According to the present results, injections of morphine twice a day for 8 days induced tolerance to analgesic effect of the drug. There are many reports regarding development of tolerance to analgesic effect of morphine after chronic injection of the drug in patients and laboratory animals (Mao et al., 2002; Wang et al., 2011; Ahmadi et al., 2013; Lee et al., 2014); however, it remains as a major unresolved problem in the clinical management of pain. The major clinical manifestation of morphine-induced analgesic tolerance is that increased opioid doses would be necessary to achieve adequate analgesia (Mao, 2006; DuPen et al., 2007). It is well known that more effective pain treatment can be achieved when these conditions are recognized and managed (DuPen et al., 2007). Therefore, many researchers have focused on elucidating the molecular mechanisms underlying morphine-induced analgesic tolerance. With more knowledge about the process, more effective pain treatment could be achieved (Benyamin et al., 2008).

The results of the gene expression in the present study revealed that the TRPV1 gene expression in the lumbosacral cord was significantly increased on day 4 of morphine injection with no significant changes on days 1 and 8 of the injections. However, the PKC γ gene expression in the lumbosacral cord remained with no significant changes on days 1, 4 and 8 of morphine injections. Furthermore, the present data showed in rats receiving morphine, the TRPV1 gene expression in the midbrain was significantly increased on day 1 of morphine injections but returned to control level on days 4 and 8 of the injections. In addition, the PKC γ gene expression in the midbrain was altered during 8 days of morphine injections with significant increases on days 4 and 8 of the injections.

Based on such results, one could propose a link between the TRPV1 gene expressions in the lumbosacral cord and the beginning of analgesic tolerance on day 4 of morphine injection, though it may not be necessary for maintaining the process of analgesic tolerance. In addition, increased level of the

TRPV1 gene expression in the midbrain on day 1 of morphine injection suggests that this gene is more susceptible to transcriptional changes in the midbrain compared to the lumbosacral cord in response to acute morphine treatment. It has been reported that differential expression of PKC γ in neurons expressing μ opioid receptors may lead to a heterogeneous desensitization of μ opioid receptors in different populations of sensory neurons in the dorsal root ganglia and spinal cord (Chen et al., 2007). Therefore, a possible interpretation for the absence of any changes in the PKC γ gene expression in the lumbosacral cord may result from a complex decrease and increase in its expression in different population of neurons, which finally may counterbalance each other. In addition, the present results propose a site-specific and temporal pattern for gene expression of the TRPV1 and PKC γ genes in the lumbosacral cord and the midbrain after repeated injections of morphine.

According to previous researches, chronic morphine treatment enhanced cytosolic PKC activity in the brainstem of mice (Narita et al., 1994b). However, our results suggest that the PKC γ gene expression in the lumbosacral spinal cord is protected from significant changes during days 1, 4 and 8 of induction of analgesic tolerance to morphine. It can be suggested that changes in PKC γ protein level in the spinal cord reported in other studies might result from either post-transcriptional changes or trafficking between membrane-bound and cytoplasmic forms of the PKC γ protein (Mayer et al., 1995). In addition, increases in the PKC γ expression in the midbrain on days 4 and 8 of the injections may prevent an increase in the TRPV1 gene expression, suggesting an opposing effect of the PKC γ on the TRPV1 gene expression during eight days of morphine injections. However, the decrease in the TRPV1 gene expression on days 4 and 8 to near the basal level had no attenuating effect on induction of morphine tolerance. Therefore, according to the current results, it can be proposed that the alterations in the TRPV1 gene expression is not a key regulator of morphine tolerance but may affect μ -opioid receptors as central mediators of induction of morphine tolerance. The TRPV1 gene expression in the lumbosacral cord may also be independent from PKC γ since changes in the PKC γ did not affect the TRPV1 gene expression on days 4 or 8 of morphine injection. However, the present data

lack measurement of protein levels of PKC γ and TRPV1; therefore complementary and more experiments are necessary to further establish underlying mechanism.

Conclusion

According to the obtained results, it can be concluded that the TRPV1 gene expression is more sensitive to morphine treatment in the midbrain compared to the lumbosacral cord. In addition, an opposing effect of the PKC γ on the TRPV1 gene expression during eight days of morphine injections may exist in the midbrain but not in the lumbosacral cord. According to the present results, there is an association between induction of analgesic tolerance to morphine and changes in the TRPV1 and PKC γ at gene expression level in the lumbosacral cord and midbrain.

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Conflict of interest

The Authors declare that they do not have any conflict of interest.

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