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Original article

Simvastatin prevents morphine-induced tolerance and dependence in mice

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ABSTRACT

Background: Tolerance to analgesic effects of opioids and dependence to them are main concerns in the treatment of chronic pain conditions, limiting clinical application of these drugs. This study aimed to evaluate the effect of simvastatin on the morphine-induced tolerance and dependence in mice.
Material and methods: For this purpose, mice were treated with either daily morphine (20 mg/kg, s.c.) alone, or in combination with simvastatin (2.5, 5 and 10 mg/kg, i.p.), for 9 continuous days. Antinociceptive effect of morphine was assessed through measuring latency time withdrawal of paw exposed to thermal stimulus, in the hot plate test. Naloxone-precipitated morphine withdrawal (5 mg/kg, i.p.), was used for dependence evaluation. Changes in brain gene expression levels of induced nitric oxide synthase (iNOS), astroglia marker, glial fibrillary acidic protein (GFAP), ionized calcium-binding protein (Iba1) a microglia activation marker, a pro-inflammatory mediator and tumor necrosis alpha (TNF-α) were measured after withdrawal by real-time polymerase chain reaction (RT-PCR).
Results: Behavioral tests indicated that latency time increased after morphine treatment in the hot plate test. However, this effect decreased on day 7, demonstrating tolerance to antinociceptive effect of morphine. Reduced anti-nociceptive effect of morphine was returned in animals treated with simvastatin (5 and 10 mg/kg) in combination with morphine. Simvastatin (5 and 10 mg/kg) attenuated morphine dependence as indicated by a less severe antagonist-precipitated withdrawal syndrome. Administration of naloxone was associated with the increased expression of TNF-α, GFAP, Iba1 and iNOS in the brain samples of morphine dependent mice, while the nine days treatment with both 5 and 10 mg/kg simvastatin reduced such changes.
Conclusion: The obtained results showed that the protective effects of simvastatin against both tolerance to nociceptive effects of morphine as well as withdrawal-induced behavioral profile are meaningful. Inhibition of glia activity as well as antioxidant effects of pharmaceutical simvastatin further proves its neuroprotective property.

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1. Introduction

Despite the indispensability of opioids such as morphine, in the treatment of moderate and severe pain conditions, the repeated administration of these drugs is along with some limitations. The analgesic performance of opioids greatly diminishes after chronic use. Consequently, expected actions of these drugs decrease. This phenomenon is known as tolerance which can be overcome by increasing the dose. In addition, following abrupt discontinuation or administration of antagonist, signs and symptoms of withdrawal appear which are as a result of dependence [1–3].

Contribution of activated glial cells (microglia and astroglia) to the development of tolerance and withdrawal syndrome after discontinuation of chronic morphine exposure has been demonstrated [4,5]. Activation of microglia leads to the central sensitization and reduction in the analgesic effects of morphine

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via production of many substances such as free radicals, nitric oxide, proinflammatory cytokines, prostaglandins, neurotrophic factors, and excitatory amino acids [5].

Simvastatin is a synthetic statin with lipophilic nature and is identified as a member of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, the rate-limiting enzyme at reducing low-density lipoprotein (LDL) cholesterol biosynthesis. This drug is commonly used for the hypercholesterolemia treatment and prevention of coronary heart disease [6]. Up to now, many cholesterol-independent pleiotropic effects of this drug, including protection against alzheimer's disease [7], Parkinson's disease [8], stroke [9], multiple sclerosis [10], depression, improving mood [11,12], seizure [13], peripheral neuropathy [14] and intracerebral hemorrhage [15] have been documented by experimental and clinical evidences.

In regards to the neuroprotective effects of simvastatin reported in previous studies, in this work we focused on the protective effects of simvastatin against tolerance to analgesic activity, moreover, naloxone-precciptead withdrawal of repeated administration of morphine.

In addition, we measured changes in the gene expression of glial fibrillary acidic protein (GFAP), an astroglia activation marker, ionized calcium-binding protein (Iba1), a microglia activation marker, induced nitric oxide synthase (iNOS), and a pro-inflammatory mediator, tumor necrosis alpha (TNF-α), after induction of withdrawal with naloxone in morphinized mice.

2. Materials and methods

2.1. Animals

A total number of about 96 animals were used for conducting tolerance (n = 48) and withdrawal (n = 48) regimes of study. The used male *Swiss* albino mice of weighing 25–35 g were maintained on a 12-h light/dark cycle with free access to food and water under controlled temperature (23 °C ± 2). Each animal was used only once. All animal care and experimental procedures were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals [16]. This study was approved by Institutional Ethics Committee, School of Medicine, Sabzevar University of Medical sciences, Iran (No: Medsab. Rec.93.94). An expert person was responsible for all handling process of animals, and all experiments were performed in the same environment at a time between 8:00 AM- 4:00 PM

2.2. Material

Morphine hydrochloride was purchased from Darupakhsh (Iran) and dissolved in saline. Naloxone (5 mg/kg) was supplied by Darupakhsh (Iran), dissolved in saline and administered by intraperitoneal (i.p.) route [18]. Simvastatin (Sobhan, Iran) emulsion was prepared in Tween 80% and was intraperitoneally injected to animals at the doses of 2.5, 5 and 10 mg/kg, twice daily according to the previous reports [9,14].

2.3. Morphine tolerance

In the control group, animals were injected with saline and Tween 80 (i.p.). Morphine was administered subcutaneously (s.c.) at the dose of 20 mg/kg, twice daily, for 9 days (negative control) [17]. In morphine + simvastatin groups, simvastatin was intraperitoneally (i.p.) injected to animals, at the doses of 2.5, 5 and 10 mg/kg, twice daily, about 30 min before morphine, according to the previous reports [9,14]. A group of animals were only treated with 10 mg/kg simvastatin for 9 days.

2.4. Morphine withdrawal

For conducting this protocol, another group of negative control, naïve animals treated with vehicle only, groups treated with simvastatin alone and morphine + simvastatin animals (N = 6-8), as mentioned in morphine treatment section, were used. Two hours after administration of morphine on day nine, animals received a single dose of naloxone (5 mg/kg, i.p.). Just after naloxone injection, each animal was placed in a Plexiglas box (base area: 20 × 20 cm, height: 35 cm in).Withdrawal signs, including jumping, rearing, paw tremor and teeth chattering during a period of 30 min, were recorded by count of each measure upon the presentation.

2.5. RNA extraction and quantitative real-time-polymerase chain reaction q (RT-PCR)

At the end of behavioral study, following the observation of withdrawal manifestations, animals were decapitated and brain tissues were removed from mice treated nine days with vehicle only, morphine as well as morphine plus simvastatin followed by naloxone challenge, on the last day of treatment. The tissues were immediately frozen in liquid nitrogen and stored at −80 ◦C until use for extraction of total RNA. Total RNA was extracted from the homogenized sample (approximately, 1 mg tissue) using TriPure Isolation Reagent (Roche Diagnostics, Deutschland GmbH, Germany).To avoid DNA contamination, extracted RNAs were treated with RNase-free DNase I (Thermo Fischer scientific, USA) followed by heat inactivation in the presence of EDTA. Total RNA (2 μg) was reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit (Takara Bio Inc, Japan) according to manufacturer's instructions. Real- time PCR was carried out using SYBR green PCR master mix (YTA, Iran) on CFX96 Touch™ Real-Time PCR Detection System − Bio-Rad (Philadelphia, USA) with the primers presented in Table 1. The following thermal conditions were applied: 10 min at 94 °C and 39 cycles of 15 s at 94 °C, 20 s at 57 °C and 30 s at 72 °C. The data were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression, using comparative threshold cycle method [19]. Each reaction was performed in triplicates.

2.6. Statistical analysis of results

The mean ± SEM was used to express the variability of results. Mixed model ANOVA, one-way ANOVA and *t*-test were used to analyze the data as appropriated which was followed by appropriate simple effects analyses and post-test (Bonferroni for mixed model ANOVA, Tukey for one-way ANOVA). The data were processed with the applications of SPSS version 19 software (Chicago: SPSS Inc.) The statistical significance level was set to $p < 0.05$.

Table 1
Primers specific for mouse inducible nitric oxide synthase (iNOS), glial fibrillary acidic protein (GFAP), ionized calcium-binding adapter 1(Iba1), tumor necrosis alpha (TNF-α) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), forward and reverse.

	Forward primer sequence	Reverse primer sequence
iNOS	AGCCTAGTCAACTGCAAGAG	TCTTGTATTGTTGGGCTGAGA
GFAP	AGAAAACCGCATCACCATTCC	CAGGGCTCCATTTTCAATCTG
TNF-α	CTACTGAACTTCGGGGTGAT	CTTGGTGGTTTGTGAGTGTG
Iba1	CAGACTGCCAGCCTAAGACA	AGGAATTGCTTGTTGATCCC
GAPDH	GCTAGGACTGGATAAGCAGGG	GCCAAATCCGTTCACACCG

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