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# Contributions of spinal D-amino acid oxidase to chronic morphine-induced hyperalgesia

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ABSTRACT

Spinal D-amino acid oxidase (DAAO) is an FAD-dependent peroxisomal flavoenzyme which mediates the conversion of neutral and polar D-amino acids (including D-serine) to the corresponding α-keto acids, and simultaneously produces hydrogen peroxide and ammonia. This study has aimed to explore the potential contributions of spinal DAAO and its mediated hydrogen peroxide/D-serine metabolism to the development of morphine-induced hyperalgesia. Bi-daily subcutaneous injections of morphine to mice over 7 days induced thermal hyperalgesia as measured by both the hot-plate and tail-immersion tests, and spinal astroglial activation with increased spinal gene expression of DAAO, glial fibrillary acidic protein (GFAP) and pro-inflammatory cytokines (interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)). Subcutaneous injections of the potent DAAO inhibitor CBIO (5-chloro-benzo[*d*]isoxazol-3-ol) prevented and reversed the chronic morphine-induced hyperalgesia. CBIO also inhibited both astrocyte activation and the expression of pro-inflammatory cytokines. Intrathecal injection of the hydrogen peroxide scavenger PBN (phenyl-*N*-*tert*-butylnitrone) and of catalase completely reversed established morphine hyperalgesia, whereas subcutaneous injections of exogenous D-serine failed to alter chronic morphine-induced hyperalgesia. These results provided evidence that spinal DAAO and its subsequent production of hydrogen peroxide rather than the D-serine metabolism contributed to the development of morphine-induced hyperalgesia.



## 1. Introduction

Opioids, in particular morphine, are gold-standard analgesics that are widely used to enable the remission of intra-operative, postoperative or chronic pain. However, the use of these drugs is plagued by the dangers of developing tolerance and hyperalgesia. Tolerance is characterized by a progressive lack of response to opioids which can be overcome by increasing the dosage, whereas hyperalgesia is a sensitization process by which opioids paradoxically cause hypersensitivity to pain, especially if the dosing is intermittent or naloxone-interrupted [1,2]. It is generally believed that a strong association exists between opioid-induced tolerance and hyperalgesia, and that opioid-induced tolerance is associated with chronic pain [2,3]. Opioid-induced nociceptive tolerance, opioid hyperalgesia and some forms of neuropathic pain are thought to share certain common mechanical components. These various conditions all depend on the ability of NMDA (*N*-methyl-D-aspartate) receptor activation to mediate excitatory activity, and the ability of NMDA receptor antagonists to attenuate or reverse these phenomena [3–5]. Morphine analgesic tolerance, hyperalgesia and physical dependence have also been shown to be associated with the activation of spinal astrocytes [6–8]. However, morphine-induced hyperalgesia was recently demonstrated to be dissociated from tolerance, as morphine hyperalgesia, but not tolerance, requires both the μ-opioid receptor-dependent expression of P2X4 receptors in the microglia and the μ-opioid receptor-independent gating on the release of brain-derived neurotrophic factor by P2X4 receptors [9]. In addition, the N-subtype calcium channel blocker ziconotide was shown to inhibit formalin-induced pain, spinal nerve ligation-induced painful neuropathy and post-operative pain, but it did not significantly attenuate morphine antinociceptive tolerance [10–12].

The spinal dorsal horn is a principle site of action for morphine analgesia, and this structure has been implicated in morphine hyperalgesia and tolerance. D-Amino acid oxidase (DAAO, EC1.4.3.3), an FAD-dependent peroxisomal flavoenzyme, is generally considered to be an astroglial enzyme in the spinal dorsal horn [13,14], although DAAO also shows immunoreactivity in neurons

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of the brain [15]. The upregulation of spinal DAAO has been demonstrated to be correlated with spinal nerve ligation-induced painful neuropathy [16], bone cancer inoculation-induced mechanical allodynia [17] and chronic morphine-induced antinociceptive tolerance [18]. In addition, the mutation and knockdown of the spinal DAAO gene and the inhibition of DAAO enzymatic activity were shown to reduce formalin-induced tonic pain [19–22], neuropathic pain [16,23], bone cancer pain [17], and sleep deprivation-induced mechanical pain hypersensitivity [24]. Furthermore, gene ablation and enzymatic inhibition of the spinal DAAO prevented and reversed chronic morphine-induced tolerance to antinociception [18,25]. However, it is not known whether inhibition of the spinal DAAO enzymatic activity reduces morphine-induced hyperalgesia.

With a strict stereospecificity and a broad but differential substrate specificity, DAAO catalyzes the oxidative deamination of both neutral and polar D-amino acids such as D-serine, D-alanine and D-proline. The products of this process are ammonia in addition to the relevant α-keto acids and hydrogen peroxide, the latter of which is less active and more stable reactive oxidative species (ROS) [13,26,27]. Inhibition of the DAAO enzymatic activity causes a reduction in the spinal hydrogen peroxide level [18,22] and an increase in the D-serine level in the brain and plasma [23,28,29]. It would be interesting to study the role that the DAAO-mediated hydrogen peroxide/D-serine metabolism plays in morphine-induced hyperalgesia. D-Serine has been identified as an endogenous full agonist at the strychnine-insensitive glycine binding B site of the NMDA receptor and a modulator of glutamate-mediated receptor activation [30,31], which is known to mediate morphine-induced tolerance and hyperalgesia. On the other hand, our recent studies have demonstrated that a blockade of hydrogen peroxide production was responsible for the antinociceptive effects of the DAAO inhibitors and gene silencers that targeted DAAO in cases of formalin-induced tonic pain [22] and morphine antinociceptive tolerance [18].

In this study, we hypothesized that spinal DAAO contributes to the generation and maintenance of chronic morphine-induced hyperalgesia via the synthesis of hydrogen peroxide. We performed the following protocols: (1) testing whether the potent DAAO inhibitor CBIO (5-chloro-benzo[*d*]isoxazol-3-ol) [32] reduced chronic morphine- and acute treatment of remifentanil-induced hyperalgesia as measured by the hot-plate and tail-immersion tests; (2) testing whether CBIO inhibited chronic morphine-induced spinal astroglial activation and increased expression of spinal pro-inflammatory cytokines (interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)); (3) testing whether the nonselective hydrogen peroxide scavenger phenyl-*tert*-*N*-butylnitrone (PBN) [22,33] and the hydrogen peroxide decomposition enzyme catalase [22,34] reversed chronic morphine-induced hyperalgesia; and (4) testing whether D-serine and MK-801, the latter of which is a potent, selective and non-competitive antagonist of the NMDA receptor [35], reduced chronic morphine-induced hyperalgesia.

## 2. Materials and methods

### 2.1. Drugs and chemicals

Morphine hydrochloride, remifentanil and CBIO (5-chloro-benzo[*d*]isoxazol-3-ol) were obtained from Sinopharm (Shanghai, China), Humanwell Healthcare Co. (Shanghai, China) and Maybridge (Cornwall, UK), respectively. PBN (phenyl-*N*-*tert*-butylnitrone), D-serine, MK-801 and catalase (*Micrococcus lysodeikticus*) were purchased from Sigma–Aldrich (Shanghai, China). All of these testing drugs were freshly dissolved in normal saline.

**Table 1**
The sequences of the forward and reverse primers used for gene amplification.

| Gene | Primers |
|---|---|
| GAPDH (glyceraldehyde-3-phosphate dehydrogenase) | Forward: TCCATGACAACTTTGGCATTG<br>Reverse: CAGTCTTCTGGGTGGCAGTGA |
| GFAP (glial fibrillary acidic protein) | Forward: GAATCGCTGGAGGAGGAGAT<br>Reverse: GCCACTGCCTCGTATTGAGT |
| DAAO (D-amino acid oxidase) | Forward: GGTTCCAAGACAGTTACGCTCG<br>Reverse: AGGGTGGGCTCCAGTTTACA |
| IL-1β (interleukin-1β) | Forward: TGGTGTGTGACGTTCCCATT<br>Reverse: CAGCACGAGGCTTTTTTGTTG |
| IL-6 (interleukin-6) | Forward: TCCATCCAGTTGCCTTCTTG<br>Reverse: GGTCTGTTGGGAGTGGTATC |
| TNF-α (tumor necrosis factor-α) | Forward: CCAGACCCTCACACTCAGATCA<br>Reverse: CACTTGGTGGTTTGCTACGAC |

### 2.2. Animals

Adult female Swiss mice weighing 18–25 g were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). Following delivery, the mice were housed in groups of five or six in plastic cages with clean bedding in a controlled environment with a temperature of 22 ± 2 °C and humidity of 60%. Light was also controlled with a 12-h light/dark cycle (with lights on at 7 a.m.) for 3–7 days, allowing for acclimatization prior to the experiments. The animals were given free access to food and water ad libitum. The same numbers of animals were randomly assigned to each experimental study group. All procedures were approved by the Laboratory Animal Use Committee of Shanghai Jiao Tong University School of Pharmacy.

### 2.3. Real-time quantitative PCR analysis

All mice were killed by decapitation and their lumbar enlargements were collected and mechanically homogenized in an electric micro-homogenizer at 10,000 rpm for 20 s on ice in TRIzol (Invitrogen, Grand Island, NY, USA). A fraction (1 μg) of the total RNA sample was reversely transcribed using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Real-time quantitative PCR was carried out with Mastercycler ep realplex (Eppendorf AG, Hamburg, Germany) using SYBR Green I dye (Tiangen Biotech, Beijing, China). PCR conditions were optimized to achieve a linear relationship between the initial RNA concentration and the PCR product. Triplicates of each real-time PCR reaction were performed in accordance with the following protocol: 10 min pre-denaturation for one cycle at 95 °C; 10 s denaturation at 95 °C; 60 s annealing and extension at 60 °C. The forward and reverse primers for DAAO, glial fibrillary acidic protein (GFAP), IL-1β, IL-6, TNF-α and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were synthesized by Shanghai DNA BioTechnologies (Shanghai, China), and are listed in Table 1. The amplification of the housekeeping gene GAPDH was measured for each sample as an internal control for sample loading and normalization. A melting curve was used to verify the primer specificity. The $2^{-\triangle\triangle Ct}$ calculations were done to quantify the relative amounts of gene expression for both the target and housekeeping genes as follows: $\triangle\triangle Ct = (Ct_{Target} - Ct_{Gapdh})_{morphine\text{-}treatment\ samples} - (Ct_{Target} - Ct_{Gapdh})_{control\ samples}$, with Ct used as the cycle threshold [36]. The percentage of the genes of interest relative to the housekeeping gene was calculated as % mRNA expression = $2^{-\triangle\triangle Ct} \times 100\%$.

### 2.4. Hot-plate and tail-immersion tests [18,25]

To test the effects of various drugs on the mice, hot-plate and tail-immersion tests were subsequently performed on the same animals. These tests started at around 9 a.m. every morning, 15 h