Dexmedetomidine inhibits visceral pain via suppression of PKCγ and JAK2/STAT3 signaling pathways

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Abstract: Objective: Dexmedetomidine (DEX) served as an anesthetic for a long time. This study was directed toward probing into its antalgic effects on acute inflammatory visceral pain (AIVP) elicited by trinitrobenzene sulfonic acid (TNBS) and to assess if DEX's antinociceptive effects were mediated by PKCγ or JAK2-STAT3 signaling pathways. Methods: TNBS induction was performed in rats to establish AIVP model. Abdominal withdrawal reflex (AWR), mechanical withdrawal threshold (MWT), and thermal withdrawal latency (TWL) were measured to examine the pain behavior of rat model. Results: Western blot (WB), real-time PCR and cellular fractionation assays were also conducted. In mDEX and hDEX groups, the AWR score was significantly reduced (P < 0.01), while TWL and MWT values were dramatically increased (P < 0.05) compared with those in AIVP group. Moderate to high DEX dosages inhibited the release of pro-inflammatory cytokines, like IL-1β, IL-6, TNF-α, and PGE2, and stimulated the release of IL-2. WB and real-time PCR revealed that mDEX and hDEX administration inhibited PKCγ and JAK2/STAT3 at both the protein and mRNA levels. Moreover, PKCγ and JAK2/STAT3 were dephosphorylated by mDEX and hDEX treatment. We found that PKCγ was translocated to the membrane, while JAK2 and STAT3 were mainly located in the nucleus after DEX treatment (DEXT). Conclusion: Taken together, we conclude that DEX's analgesic effect in AIVP is modulated by PKCγ inhibition and JAK2/STAT3 signaling pathways.

Keywords: Dexmedetomidine, acute inflammatory visceral pain trinitrobenzene sulfonic acid, JAK2, STAT3

Introduction

Visceral pain (VP) is a particularly serious problem in clinic. Inflammatory VP features incessant occurrence of referred pain, and incorrect location [1]. Almost 10-40% of common population experiences regular VP. Although it was previously thought that VP differs significantly in terms of neurophysiological mechanisms and transmission pathways [2]. Merely a small number of analgesics specially for VP are applicable to common pain control, and wherein opioids assume a crucial part in controlling serious pain and unable to be replaced by other analgesic drugs. Nevertheless, the dosage-relevant side effects are likely to occur, such as constipation, urine retention and nausea [3].

Morris et al. [4] found that colitis could be triggered by co-administration intra-rectally of ethanol and trinitrobenzenesulfonic acid (TNBS) at the dose of 0.1 g/kg. Ethanol effectively destroy intestinal barrier, and consequently TNBS could interact with colon tissue proteins [5]. Histopathological observation displayed that the principal characteristic of TNBS-triggered colitis involves the development of transmural inflammation which was particularly analogous to the histopathological lesions that developed in Crohn's disease. Therefore, TNBS-triggered colon inflammation is usually applied to establish a VP model [6].

Some α2-adrenoceptor agonists, such as detomidine and xylazine, are generally used as anesthetics to calm down animals for a long time [7]. DEX, a highly selective α2-adrenoceptor agonist, serves as a sedative in clinic. DEX connects with corresponding receptors through an 8-fold stronger affinity over clonidine that has been approved for treating refractory pain. It has been reported that DEX, relative to clonidine, has stronger analgesic effects and it exerts antinociceptive effect following intraspi-
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nal application [8]. Additionally, DEX causes fewer side effects relative to opioids. DEX administration could attenuate fentanyl demand in course of laparoscopic operation [9] and abdominal hysterectomy [10]. Currently, no studies have investigated if DEX mitigates acute and chronic inflammatory VP or explored the underlying mechanisms.

Being a molecule activated by nociception, protein kinase C (PKC) plays an important role in the modulation of pain development and transmission. It has been reported that PKC possessed a regulatory effect on pain regulation [11]. However, it is unclear whether PKC is involved in visceral hypersensitivity induced by trinitrobenzene sulfonic acid (TNBS). Genetic alignment has demonstrated that the PKC family consists of at least eleven isoenzymes, which can be divided into four subclasses: conventional, novel, and atypical PKC subtypes, and PKC-related kinases [12]. Each subtype is different in terms of tissue expression, suggesting their importance in a multitude of cellular functions. Therefore, the assessment of the PKC involvement in visceral pain is required.

During central sensitization, secreted pro-inflammatory cytokines activate the JAK/STAT pathway [13]. Among the JAK family, JAK2 is constitutively expressed in the central nervous system and can specifically activate the downstream sensor STAT3, which is involved in the visceral hypersensitivity in the spinal cord [14]. The cytokine-activated JAK2/STAT3 pathway consequently responds to the nerve injury and hypersensitivity [14], while intrathecal injection of JAK2/STAT3 suppressors ameliorates this hypersensitivity.

In the current study, an AIVP rat model of TNBS-triggered inflammatory VP was applied to determine DEX antalgic efficacy and evaluate its influence on PKCγ activation and JAK2-STAT33 pathways in visceral hyperalgesia.

Material and method

Sixty adult male SD rats (mean weight: 0.4 kg) were confined in wire-bottom cages and fasted for 1 h prior to electrophysiology and behavior experiments. All animal experiments were carried out according to the instructions of animal care and use committee.

TNBS administration

TNBS was used for eliciting AIVP in rats’ colon as described before [4]. Sixty SD rats were randomly and evenly assigned to six groups: control (no treatment), sham (0.25 ml v), AIVP model group, low dose DEX (lDEX; 300 μg/kg) in 5% DMSO, moderate (mDEX; 3 mg/kg) and high dose (hDEX; 10 mg/kg) DEX groups. In brief, the rats were subdurally injected with saline or DEX, and 15 min later, they were intraperitoneally injected with nembutal (50 mg/kg) for anesthesia. Finally, TNBS (diluted with ethanol at the ratio of 1:1; 0.5 mL) was slowly injected to descending colon. The inflammation occurrence was demonstrated by biochemistry and histopathology through determining myeloperoxidase activity and staining colon tissue with hematoxylin & eosin stain, respectively [15].

The nociceptive score was graded to four types: 0-25, the treated paw is indistinguishable from the contralateral one; 26-50, the rat puts little weight on the treated paw; 51-75, the rat avoids surface contact with the injected paw; 76-100, the rat shakes, flaps, or bites the injected paw.

AWR, MWT, and TWL measurements

The abdominal withdrawal reflex (AWR) was determined as depicted before [16] using colorectal distention (CRD) stimuli (80, 60, 40, and 20 mmHg). Each stimulus lasted for 20 s and then another followed after 5-min interval. The value was the average of three measurements.

The mechanical withdrawal threshold (MWT) was determined via a von Frey filament to stimulate the posterior paw center perpendicularly for no more than 4 s [17]. A positive response referred to raising or biting the treated paw. The maximum force was 15 g, when the corresponding MWT reached the maximum. Each stimulus was performed five times and alternated with a 0.5-min interval.

The mechanical withdrawal latency (TWL) was determined via a von Frey filament to stimulate the posterior paw center perpendicularly for no more than 4 s [17]. A positive response referred to raising or biting the treated paw. The maximum force was 15 g, when the corresponding MWT reached the maximum. Each stimulus was performed five times and alternated with a 0.5-min interval.

Using a thermal stimulator, the thermal withdrawal latency (TWL) was determined. It referred to the interval between thermal radiation initiation and withdrawal response. The stimulation with a regulated intensity was adopted, which was decreased after 20 s to avoid causing thermal injury. Each measurement was conducted five times and alternated with a 3-min
interval. TWL referred to the average of three median values.

**Electromyographic measurements of viscero-motor responses (VRs)**

The VRs in AIVP rats was determined by electromyography (EMG) [18]. Briefly, rats inhaled Et\textsubscript{2}O for anesthesia and fixated in a Bollman cage. They were connected by an Ag electrode (d=0.8 mm) with a Biological Function device.

**ELISA**

Serum and peritoneal fluid were collected from peripheral and peritoneal blood, accordingly. Interleukin 2 (IL-2) levels in serum and pro-inflammatory cytokine levels were measured using ELISA kits according to the guidance supplied by manufacturer. The cytokine levels were determined through their corresponding standardized curves.

**WB**

RIPA buffer was used for lysing frozen spinal dorsal horn tissues. Protein concentrations were measured using BCA Protein Quantitation Kit (Genscript). Proteins were isolated with 10% SDS-PAGE and subsequently blotted electrophoretically to polyvinylidene difluoride (Immobilon) membranes, followed by blockade with 5% milk for 60 min at approximately 25°C and culture with primary antibodies overnight at 4°C. Antibodies raised in rabbit against pro-inflammatory cytokines or in rat against pPKC\textgamma\ ((1:500, 9375, Cell Signaling Technology), JAK2 (1:2500, ab39636, Abcam), pJAK2 (1:1000, ab195055, Abcam), STAT3 (1:2500, ab5073, Abcam), and pSTAT3 (1:500, ab30647, Abcam) were used. These membranes were incubated with horseradish peroxidase-bound secondary antibodies (Amersham) for 60 min at about 25°C, followed by chemiluminescent detection.

**RNA isolation and real-time PCR**

RNA was isolated with Trizol. Messenger RNA (mRNA) levels were measured via PCR system with GAPDH as the internal control. The primer sequence of each gene is listed below: PKC\textgamma\ F: 5’-AGG TGC TGA GAG CGA AGC TCC GC-3’, R: 5’-TCC GCC CCT GTC CTT CCT ATC TC-3’; JAK2 F: 5’T-TTT GAA GAC AGG GAC CCT ACA CAG-3’, R: 5’-TCA TAG CGG CAC ATC TCC ACA-3’; STAT3 F: 5’-CAC CCA TAG TGA GCC CTT GGA-3’, R: 5’T-GTG CAG TGA CCA GGA CAG A-3’; GAPDH F: 5’-CAA GGT CAT CCA TGA CAA CTT TG-3’, R: 5’-GTC CAC CAC CCT GTT GCT GTA G-3’. Quantitative PCRs were performed in reaction solvent (20 μL) with SYBR Green PCR Mastermix added. Transcription levels were determined against the average of controls and standardized to the endogenous reference via 2\textsuperscript{-ΔΔCT} method.

**Statistical analysis**

Data were analyzed by SPSS software (v18.0, IBM® SPSS Statistics). Data were expressed as mean ± SD. t test or ANOVA was adopted to investigate the data. P < 0.05 meant statistically significant difference.

**Results**

**Influence of DEXT on AWR, MWT, TWL, and EMG responses**

Following AIVP initiation, the AWR values in treated rats were dramatically increased compared to those in control group (P < 0.01; **Figure 1A**). In contrast, TWL and MWT scores were visibly reduced (P < 0.05; **Figure 1B, 1C**). The abdomen EMG of CRD, together with AWR response was simultaneously documented. A higher EMG amplitude implied an enhanced response to VP. **Figure 1D** displayed that rats exposed to the AIVP model exhibited higher EMG amplitudes against untreated rats (P < 0.05).

The AWR scores in mDEX and hDEX groups were lower relative to those in AIVP model and lDEX groups (P < 0.05; **Figure 2A**). Further, MWT and TWL scores in mDEX and hDEX groups increased following DEXA compared to those in AIVP model and lDEX groups (P < 0.05; **Figure 2B and 2C**). The findings displayed that DEXA augmented pain threshold, TWL and MWT scores in AIVP model. The amplitudes of the EMG signals in mDEX and hDEX rats were also dramatically reduced compared with those in AIVP model and lDEX rats.

**Inflammatory responses in AIVP rats pre-treated with DEX**

To identify the influence of DEXA on pro-inflammatory cytokine production (hereinafter refe-
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Figure 3A-E displayed that the expression levels of pro-inflammatory cytokines were notably elevated in AIVP model, while there was a pronounced decrease in IL-2 expression. However, mDEX and hDEX pre-treatment led to a visible decrease in the expression of pro-inflammatory cytokines, while IL-2 levels rose. In summary, mDEX and hDEX pre-treatment significantly decreased PICP except for elevated levels of IL-2 (P < 0.05), compared to those in AIVP model. The findings confirmed that TNBS-triggered acute inflammation (AI) was alleviated through prior mDEX and hDEX administration.

Influence of DEX on activating PKCγ pathway

Next, we took advantage of real-time PCR and WB to evaluate if PKCγ participated in the induction of AIVP by TNBS and identify the modulating effects of DEX pre-treatment on PKCγ signaling in our AIVP model. In a first step, PKCγ expression in tissue lysates was detected. PKCγ expression was significantly increased after TNBS treatment, which was counteracted by high-dose DEXA (P < 0.01, Figure 4A and 4B). To explore whether activated PKCγ was down-regulated by DEXA, the phosphorylated form of PKCγ was additionally evaluated. Figure 4A and 4C displayed that TNBS treatment dramatically augmented the extent of PKCγ phosphorylation, suggesting that TNBS-triggered AI was mediated via PKCγ signaling. Besides, we

Figure 1. TNBS induces AIVP in rats. A. Difference in AWR values between control and AIVP model groups after TNBS treatment. B. Difference in MWTs between control and AIVP model groups after TNBS treatment. C. Difference in TWL values between control and AIVP model groups after TNBS treatment. D. Abdominal EMG responses to TNBS treatment in AIVP animal model (n=10). The assay has been performed three times, and the data were expressed as average ± SD.
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found that DEXT notably reduced the ratio of pPKCγ level to total PKCγ level \( (P < 0.01) \). In addition, WB was performed to examine changes in PKCγ membrane translocation in the spinal cord after different treatments. TNBS injection induced a significant increase in pPKCγ level. Its membrane-bound translocation and cytoplasmic location in AIVP model was dramatically increased. DEX administration reduced notably both fractions, but especially the membrane-bound form (Figure 4D, 4E), demonstrating that DEX inhibited AIVP progression through activating PKCγ signaling.

**DEX suppression of JAK2/STAT3 pathway activation**

It has been indicated that chronic pain can activate the JAK2/STAT3 pathway in rats’ spinal dorsal horn [19]. To evaluate DEX’s regulatory effects on JAK2/STAT3 signaling in vivo, we conducted WB and real-time PCR to determine the activation of STAT3e and expression levels of JAK2 and STAT3. Figure 5A-C displayed that mRNA and protein levels of JAK2 and STAT3 in AIVP model group were pronounced enhanced. Nevertheless, DEXA led to a decrease in JAK2 and STAT3. Regarding activated JAK2 and STAT3 level, trends observed were consistent with their expression results. DEXT dramatically decreased the phosphorylated forms of JAK2 and STAT3 (Figure 5D, 5E; \( P < 0.01 \)). Furthermore, to explore the activation level of JAK2/STAT3 signaling pathways, the nuclear and cytoplasmic distributions of STAT3 and pSTAT3 were examined by WB after cellular fractionation. Treatment by TNBS resulted in a dramatic augmentation of the nuclear fraction of both
pSTAT3 and STAT3, while administration of high-dose DEX reversed this augmentation (Figure 5F, 5G).

**Discussion**

Commonly, AIVP occurs frequently in patients with inflammatory diseases. AIVP levels are difficult to estimate because of knowledge gaps in reference to pathogeny, location and acute dilation. Nowadays, behavior tests were conducted to assess AIVP in term of analgesic efficacy, AWR, etc. [20], which can be considered as inadequate diagnostic approaches. Here, we show that in rats TNBS stimulation of the bowel results in AIVP. Further, DEXA reduces behavioral VRs to pain and suppresses signaling through PKCγ, JAK2, and STAT3 pathways. The antinociceptive efficacy of DEX in some VP models has been confirmed [21]. For instance,
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DEX has been reported to ameliorate TNBS-triggered colitis through eliciting immunoregulation effect, and showed anti-inflammatory effect through augmenting IL-4 and IL-10 levels via Th2 pathway [22].

IL-6 is one of the crucial pro-inflammatory cytokines activating the JAK/STAT pathway. IL-6 is suggested to be involved in neuropathic pain [23]. Anti-IL-6 antibodies could inhibit hypersensitivity caused by spinal nerve lesions [23]. These studies suggest that IL-6 production can activate the JAK2/STAT3 signaling and promote the development of visceral hypersensitivity. In the present study, an increased IL-6 production was observed after TNBS treatment, indirectly indicating the occurrence of visceral hypersensitivity and activation of the JAK2/STAT3 signaling pathway.

Dexmedetomidine (DEX) has been widely demonstrated to regulate the mRNA transcription and several signal transductions. During ischemia and reperfusion (I/R) injury, DEX suppressed JAK2 phosphorylation and STAT1 and STAT3, along with deregulation of the expressions of cleaved caspase 3, ICAM-1 and MCP-1 proteins [24]. In septic rat model, DEX significantly reduced pulmonary inflammation and inhibited CLP-triggered augmentation of TNF-α and IL-6 through inhibiting TLR4/MyD88 expression and activating NF-κB [25]. In our study, we observed an increased accumulation of phosphorylated JAK2 and STAT3 proteins in spinal dorsal horns of rats in the AIVP groups. The majority of pSTAT3 proteins were distributed to the nuclear fraction of nerve cells, demonstrating that AIVP induced by TNBS also activated the JAK2/STAT3 signaling pathway in the spinal dorsal horn. The JAK/STAT3 signaling pathway can be triggered by chronic pain, and JAK/STAT3 is possibly a promising therapeutic target for hypersensitivity. There are several reports about the connection of this pathway to acute pain. Cheppudira et al. revealed a regulatory role of JAK-STAT in acute bladder hyperreflexia and referred pain induced by bladder inflammation [26]. The suppression of the JAK/STAT3 pathway with specific inhibitors ameliorated mechanical allodynia [27]. The induced hypersensitivity was accompanied by increased mRNA levels of STAT3 and pSTAT3 [28]. These studies displayed that chronic pain evokes hypersensitivity via activation of phosphorylated JAK/STAT3 proteins but not their unphos-

Figure 4. Influence of DEXA on PKCy pathway in AIVP rats. The spinal cord was exposed after excising vertebral plate; and L6-S1 were rapidly dissected and frozen with liquid N\(_2\). Protein was separated on gel eluted with a 4-12% gradient. A. Western blot, showing the protein level of PKCy and phosphorylated PKCy. B. Real-time PCR determined the mRNA expression of PKCy. C. Calculated band density of phosphorylated PKCy and PKCy in each group was normalized to that of actin band, and then relative band density of phosphorylated PKCy and that of PKCy band are shown. D, E. Cellular fractionation was performed to separate membrane and cytoplasm fractions, and PKCy was determined in each part by WB. These assays have been performed three times, and the data obtained were presented as average ± SD.
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Figure 5. Influence of TNBS treatment and DEXA on JAK2/STAT3 pathway in AIVP model. The spinal cord was exposed after excising vertebral plate, and L6-S1 were rapidly dissected and frozen with liquid N₂. Protein was separated on gel eluted with a 4-12% gradient. A. Protein expression and phosphorylation of JAK2 and STAT3 were measured via WB. B, C. Real-time PCR to determine mRNA expression of JAK2 and STAT3 from samples of each group. D, E. Calculated band density of phosphorylated JAK2 and STAT3 bands, as well as JAK2 and STAT3 expressing bands, were normalized to that of actin band in each group, and then relative band density of phosphorylated JAK2 and STAT3 are shown. F, G. Cellular fractionation was used to separate the nuclear and cytoplasmic fraction. WB analysis of pSTAT3 and STAT3 for each fraction. These assays have been performed three times, and the data obtained were presented as average ± SD.

In short, the data show that DEXA suppresses TNBS-triggered VP and possesses a notable inhibition effect on AIVP-associated PICP in an AIVP rat model. Injection of DEX decreases PKCγ, JAK2, and STAT3 activation. The study suggests that DEX's antinociceptive efficacy is possibly regulated by inhibiting the inflammatory response relevant to AIVP and that DEX is probably a potential candidate for VP therapy.

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Disclosure of conflict of interest

None.

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