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RESEARCH PAPER

D-Amino acid oxidase-mediated increase in spinal hydrogen peroxide is mainly responsible for formalin-induced tonic pain

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BACKGROUND AND PURPOSE

Spinal reactive oxygen species (ROS) are critically involved in chronic pain. D-Amino acid oxidase (DAAO) oxidizes D-amino acids such as D-serine to form the byproduct hydrogen peroxide without producing other ROS. DAAO inhibitors are specifically analgesic in tonic pain, neuropathic pain and cancer pain. This study examined the role of spinal hydrogen peroxide in pain and the mechanism of the analgesic effects of DAAO inhibitors.

EXPERIMENTAL APPROACH

Formalin-induced pain behaviours and spinal hydrogen peroxide levels were measured in rodents.

KEY RESULTS

Formalin injected into the paw increased spinal hydrogen peroxide synchronously with enhanced tonic pain; both were effectively prevented by i.t. fluorocitrate, a selective astrocyte metabolic inhibitor. Given systemically, the potent DAAO inhibitor CBIO (5-chloro-benzo[*d*]isoxazol-3-ol) blocked spinal DAAO enzymatic activity and specifically prevented formalin-induced tonic pain in a dose-dependent manner. Although CBIO maximally inhibited tonic pain by 62%, it completely prevented the increase in spinal hydrogen peroxide. I.t. catalase, an enzyme specific for decomposition of hydrogen peroxide, completely depleted spinal hydrogen peroxide and prevented formalin-induced tonic pain by 65%. Given systemically, the ROS scavenger PBN (phenyl-*N*-*tert*-butylnitrone) also inhibited formalin-induced tonic pain and increase in spinal hydrogen peroxide. Formalin-induced tonic pain was potentiated by i.t. exogenous hydrogen peroxide. CBIO did not increase spinal D-serine level, and i.t. D-serine did not alter either formalin-induced tonic pain or CBIO's analgesic effect.

CONCLUSIONS AND IMPLICATIONS

Spinal hydrogen peroxide is specifically and largely responsible for formalin-induced pain, and DAAO inhibitors produce analgesia by blocking spinal hydrogen peroxide production rather than interacting with spinal D-serine.

Abbreviations

CBIO, 5-chloro-benzo[*d*]isoxazol-3-ol; DAAO, D-amino acid oxidase; E_{max}, maximum effect; PBN, phenyl-*N*-*tert*-butylnitrone; ROS, reactive oxygen species; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1

Introduction

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, including hydroxyl radicals, superoxides, peroxynitrites and lipid peroxyl radicals. ROS, formed as a natural byproduct of the normal metabolism of oxygen, are highly reactive due to the presence of unpaired valence shell electrons and play important roles in cell signalling

(Angermuller *et al.*, 2009). In pathological conditions, however, increased intracellular ROS levels cause cell damage, ranging from cytoplasmic swelling to death. ROS are implicated in many degenerative neurological conditions including Alzheimer's disease, Parkinson's disease, amyotropic lateral sclerosis, as well as brain dysfunction due to injury or aging (Knight, 1997). ROS scavengers and antioxidants have been extensively used to demonstrate that spinal free radicals are critically involved in central sensitization-mediated pain including formalin-induced tonic pain (Viggiano *et al.*, 2005; Hacimuftuoglu *et al.*, 2006; Chung *et al.*, 2008), capsaicin-induced secondary mechanical hyperalgesia (Lee *et al.*, 2007) and neuropathic pain (Tal, 1996; Khalil *et al.*, 1999; Kim *et al.*, 2004; Kim *et al.*, 2006). The currently used 'spin-trap' scavengers, such as phenyl-*N*-*tert*-butylnitrone (PBN) (Goldstein and Lestage, 2000), 5, 5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxy (TEMPOL) and *N*-acetylcystein are non-specific in that they scavenge nearly all types of ROS indiscriminately (Muscoli *et al.*, 2003). Thus, finding specific scavengers for each free radical could prove to be particularly beneficial, especially for hydrogen peroxide, which is less active but much more stable and may present as a final form of ROS (Angermuller *et al.*, 2009).

High levels of D-amino acid oxidase (DAAO) expression and enzyme activity are found in the mammalian kidney, liver, brain, spinal cord and to a lesser extent in leucocytes, the small intestine, epididymis and in the preputial and adrenal glands, although expression patterns vary between species (Kappor and Kapoor, 1997; Pollegioni *et al.*, 2007). In the spinal cord, DAAO is found almost exclusively in astrocytes (Kappor and Kapoor, 1997; Angermuller *et al.*, 2009), which have been extensively demonstrated to be an important target for pain (Watkins *et al.*, 1997; Lan *et al.*, 2007). The biology of DAAO has been extensively studied in the fields of chiral inversion (Wang *et al.*, 2012) and in the development of schizophrenia (Williams, 2009; Smith *et al.*, 2010; Verrall *et al.*, 2010). A series of compounds, including CBIO (5-chloro-benzo[*d*]isoxazol-3-ol), 'Compound 8' (4*H*-thieno[3,2-b]pyrrole-5-carboxylic acid), AS057278 (5-methylpyrazole-3-carboxylic acid) and 'Compound 2' (3-hydroxyquinolin-2-(1*H*)-one) (Williams, 2009; Smith *et al.*, 2010; Verrall *et al.*, 2010; Gong *et al.*, 2011), has emerged recently as novel inhibitors of DAAO with relatively high potencies and specificity for pharmacotherapy in the treatment of positive, negative and cognitive symptoms of schizophrenia in animal studies.

The role of spinal DAAO in pain transmission and transduction have also been studied previously (Fang *et al.*, 2005; Zhao *et al.*, 2008; 2010; Huang *et al.*, 2010; Chen *et al.*, 2011; Gong *et al.*, 2011). DAAO inhibitors such as CBIO, 'Compound 8', AS057278 and sodium benzoate are effective in reducing formalin-induced tonic pain, spinal nerve ligation-induced mechanical allodynia and bone cancer pain. However, they do not reduce acute pain such as formalin-induced acute nociception, thermal-induced acute pain in the tail flick and hot-plate tests, and carrageenan-induced acute inflammatory thermal hyperalgesia (Zhao *et al.*, 2008; Huang *et al.*, 2010; Gong *et al.*, 2011). The DAAO inhibitor SEP-227900 has been investigated clinically for the treatment of chronic neuropathic pain (http://www.sumitomo-chem.co.jp; Williams, 2009). However, even though DAAO may be involved in central sensitization, the underlying mechanism of the analgesic effect of DAAO inhibitors is not completely understood.

DAAO is a peroxisomal flavoprotein that catalyses the oxidative deamination of D-amino acids to α-keto acids and the byproduct hydrogen peroxide without producing other ROS (Pollegioni *et al.*, 2007; Angermuller *et al.*, 2009; Wang *et al.*, 2012). The spinal DAAO gene and enzymatic activity have been shown to be upgraded after spinal nerve ligation (Zhao *et al.*, 2010), leading to activation of spinal DAAO in central sensitization pain states through the production of hydrogen peroxide, a less active but more stable ROS that is responsible for pain. Peripheral hydrogen peroxide has been shown to cause pain and hyperalgesia (Sawada *et al.*, 2008; Keeble *et al.*, 2009). Therefore, it is probable that DAAO inhibitors produce analgesia by reducing the spinal level of hydrogen peroxide.

Recent evidence suggests that the regions in the CNS where NMDA subtype of glutamate receptors are densely distributed contain a high level of D-serine, which acts as an endogenous agonist on the glycine binding B site of the NMDA receptors and modulates glutamate-mediated receptor activation (Schell *et al.*, 1995). Activation of NMDA receptors is involved in central sensitization and mediates chronic pain (Cook *et al.*, 1987; Ying *et al.*, 2006). DAAO is involved in D-serine metabolism as elevated D-serine concentrations in plasma and brain are observed in DAAO mutant mice (Hashimoto *et al.*, 1993; Almond *et al.*, 2006) and after inhibition of DAAO activity (Adage *et al.*, 2008; Duplantier *et al.*, 2009). It was also reported that DAAO mutant mice demonstrate hyperalgesia (Wake *et al.*, 2001), and when DAAO was administered i.t., it acted as an analgesic (Ying *et al.*, 2006). Hence, it is also possible that DAAO inhibitors produce analgesia by interacting with spinal D-serine.

Hence, in the present study we examined the specific role of spinal hydrogen peroxide in tonic pain and the mechanism of the analgesic effect of the potent DAAO inhibitor CBIO (Gong *et al.*, 2011), in the formalin test. The series of experiments included the following protocols: (i) exploring the correlation between formalin-induced pain behaviours in the paw and the spinal level of hydrogen peroxide and its origin from astrocytes; (ii) testing the potentiating effect of i.t. administered exogenous hydrogen peroxide on formalin-induced tonic pain; (iii) examining the effects of CBIO, in comparison with PBN and catalase, a specific enzyme for hydrogen peroxide decomposition, on formalin-induced pain, spinal hydrogen peroxide levels and exogenous hydrogen peroxide-potentiated pain; and (iv) investigating the interactions between spinal D-serine and CBIO in formalin-induced pain. Our data demonstrated that DAAO-produced spinal hydrogen peroxide is largely (60%) and specifically responsible for formalin-induced tonic pain, and DAAO inhibitors produce analgesia by blocking spinal hydrogen peroxide production rather than interacting with spinal D-serine. Preliminary results were presented at the 14th Chinese Neuropharmacology Conference in abstract form (Lu *et al.*, 2010).

Methods

Experimental animals

Adult male Wistar rats (200–240 g body weight) and Swiss mice (22–26 g) were purchased from Shanghai Experimental Animal Institute for Biological Sciences (Shanghai, China). Animals were housed in groups of five to six for mice or two to three for rats in plastic cages with soft bedding and free access to food and water under a 12-12 h reversed light–dark cycle (07 h –19 h) at a constant temperature of 22 ± 2°C. All animals were acclimatized to these conditions for 3–5 days before any experimental procedures. All animal care and experimental procedures were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University School of Pharmacy and were in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

Drugs and reagents

Hydrogen peroxide and formalin were purchased from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). Formalin solutions were diluted from original formaldehyde solution to 1% or 5% v/v (formaldehyde/saline); 30% hydrogen peroxide solution was diluted in pH 7.4 PBS (sodium chloride 137 mM, potassium chloride 2.7 mM and phosphate buffer 10 mM). PBN, D-serine and catalase (*Micrococcus lysodeikticus*) were purchased from Sigma-Aldrich Chemical Company (Shanghai, China), while CBIO was obtained from May bridge PLC (Cornwall, UK). All of the drugs and reagents were freshly dissolved in sterile normal saline solution (0.9% NaCl solution) purchased from Shanghai Treeful Pharmaceutical Co. (Shanghai, China).

Mouse and rat formalin test

The rat formalin test followed the procedure of Gong *et al.* (2011). Animals were acclimatized individually to the observation cage for 30 min before the injection of 50 μL of 5% formalin in 0.9% saline s.c. on the dorsal side of the left hind paw. The animals were then immediately placed in a transparent polycarbonate box. Nociceptive behaviour in rats was manually quantified by counting the number of paw flinches induced by the formalin injection in 1 min epochs, and measurements were taken at 10 min intervals beginning immediately after formalin injection and ending 90 min. The mouse formalin test was performed by injection of 10 μL 5% or 1% formalin into the paw, as previously described with slight modifications (Hunskaar *et al.*, 1985). Briefly, the duration of nociceptive behaviours (licking/biting) was manually quantified in 5 min epochs continuously for 60 min, or the pooled durations at 0–5 min and 20–40 min were considered as the acute nociception and tonic pain, respectively.

Behavioural assessments of mechanical allodynia and heat hyperalgesia

For assessment of mechanical allodynia, the hind limb withdrawal threshold evoked by stimulation of the hind paw with a 2290 CE electrical von Frey hair (IITC Life Science Inc., Woodland Hills, CA) was determined while the mouse stood on a metal grid. The monofilaments, which produced forces ranging from 0.1 to 2.5 g, were applied to the foot pad with increasing force until the mouse suddenly withdrew its hind limb. The lowest force producing a withdrawal response was considered the threshold; this was based on three repeated measurements and the mean of these threshold values for each hind paw at each time point was used.

Heat hyperalgesia was assessed by placing the hind paw of the mouse above a radiant heat source (set to a low intensity of 45°C) and measuring the paw-withdrawal latency after this noxious heat stimulus, using a 390 G Plantar Test (IITC Life Science Instruments) as described by Hargreaves *et al.* (1988) and Gong *et al.* (2011). The cut-off latency was 30 s to avoid tissue damage. The paw-withdrawal latencies were evaluated at different time points, not less than 15 min, before and after control and test substance administration. Each test was calculated as a mean of three repeated measurements.

I.t. injection in mice

Mouse i.t. injections followed previously described procedures (Mestre *et al.*, 1994). Briefly, a 10 μL microsyringe with tubing for delivering testing drugs was inserted into the skin and through the L5–L6 intervertebral space directly into the subarachnoid space. A flick of the mouse's tail provided a reliable indicator that the needle had penetrated the spinal arachnoid mater; control and test agents in 5 μL (enough to spread consistently to caudal thoracic vertebrae, which contained the lumbar enlargement of the spinal cord) were subsequently injected into the subarachnoid space. The injection success rate, confirmed by 2% lidocaine, was consistently found to be over 95% in our laboratory.

Intraventricular catheterization and injection in rats and mice

The methods for intraventricular catheterization and injection in rats and mice were as described by Testylier *et al.* (1998). Briefly, animals were anaesthetized with pentobarbital sodium (50 mg·kg^{-1}) by i.p. injection and placed on a stereotaxic apparatus (Stoelting Company, Wood Dale, IL). For rats, after the skull had been exposed, stainless steel screws were inserted to stabilize the needle, which was implanted into the frontal cortex (AP:0 mm, H:2 mm) and into the somatosensory cortex (AP:3 mm, H:1.5 mm relative to the bregma). A cannula for intraventricular injection was inserted at AP: 0 mm (left side) and H: 4 mm relative to the bregma. For mice, a small hole was drilled into the skull, and a 0.45 mm needle was advanced into the right lateral ventricle (AP: 0.8 mm from the bregma; L:1.6 mm from the midline; depth:4 mm below the skull surface). After closure of the wound, the animals were returned to their home cages and allowed to recover for 2–3 days without using analgesics before entering the terminal experiments. Our preliminary study used 5 μL Indian ink dye to ensure the accuracy at the completion of the surgical operation. Control and test agents were slowly delivered in 1 min through the injection needle (diameter: 0.3 mm) connected to a 10 μL microsyringe via a polyethylene tube, which was held in the same place for 5 min.

Measurement of DAAO activity

This assay was performed as described previously (D'Aniello *et al.*, 1993; Gong *et al.*, 2011). Rats were killed by decapitation, and the lumbar enlargements of the spinal cord as well as kidneys were quickly removed, placed on ice and weighed. The tissues were homogenized in 0.1 M Tris–HCl buffer (pH 8.2) and centrifuged (1,700× *g*) for 10 min at 4°C. The protein content was measured by the method of Coomassie brilliant blue staining. D-alanine with 0.1 M Tris–HCl buffer was added to the supernatant to a final concentration of 1.5 mM and incubated at 37°C for 30 min. The K_m value of D-alanine in this assay was 5.8 mM; 25% trichloroacetic acid was later added to the assay mixture followed by centrifugation (21 000× *g*, 5 min). The supernatant was mixed with 2,4-dinitrophenylhydrazine (1 mM in 1 M HCl) and incubated at 37°C for 10 min. Finally, sodium hydroxide (1.5 M) was added followed by incubation at 37°C for 10 min. The absorbance was read at 450 nm on a Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of ketonic acid was calculated with reference to the standard curve of ketonic acid in the tissue homogenates. The enzyme activity was expressed as pyruvate production $nmol^{-1}$ of the lumbar enlargements of the spinal cord or kidneys.

Hydrogen peroxide assay

Mice were killed by decapitation, and their lumbosacral spinal cords were quickly dissected out. The tissues were immediately cooled in liquid nitrogen, and the precipitate was removed by centrifugation (15 000× *g*) for 10 min at 4°C. The concentration of hydrogen peroxide in the spinal cord was measured by a commercial assay kit, which used the ferrous ion oxidation–xylenol orange method (Dai *et al.*, 2010). Briefly, the supernatant was mixed with the reaction solution containing 250 μM 6 H_2O·ammonium irons (II) sulphate hexahydrate, 25 mM H_2SO_4, 100 mM sorbitol and 125 μM xylenol orange and incubated at room temperature for 30 min. The absorbance was read at 595 nm on a Varioskan Flash spectral scanning multimode reader. The concentrations of hydrogen peroxide were derived from standard curves of hydrogen peroxide.

Measurement of spinal D-serine level

Mice were killed by decapitation, and the spinal cord was quickly removed, placed on ice and weighed. The spinal cord was cut into pieces and ground, on ice, using a tissue mechanical grinder, the supernatant was then transferred into a vial and methanol was added. The solution was subjected to repeated freeze–thaw cycles, for three times, and then transferred into a 3 kDa Millipore® Ultra filtration device. The filtrates were collected and freeze-dried for measurement of D-serine by capillary electrophoresis with laser-induced fluorescence detection, as previously described (Zhao *et al.*, 2005). Briefly, the sample solution was transferred to a 0.3 mL micro-centrifuge vial, followed by the addition of 0.1 M borate buffer solution (pH 10) and 2 mM NBD-F solution in sequence. The mixed solution was heated at 60°C for 5 min before the addition of the derivative solution (40 mM HP-β-CD), which was then injected for capillary electrophoresis separation without further purification.

Calculations and statistical analysis

For dose–response curve analysis, the parameters, that is minimum effect (E_{min}), maximum effect (E_{max}), half-effective dose (ED_{50}) and Hill coefficient (n), were calculated from individual dose–response curves using a computer program (GraphPad Prism, version 5.01, GraphPad Software Inc., San Diego, CA). To determine the parameters of dose–response curves, values of response (Y) were fitted by nonlinear least-squares curves to the relation $Y = a + bx$, where $x = [D]^n/(ED_{50}{}^n + [D]^n)$, to give the value of ED_{50} and b (E_{max}) yielding a minimum residual sum of squares of deviations from the theoretical curve (Wang and Pang, 1993).

Data are presented as means ± SEM, and statistical significance was evaluated by one-way or two-way ANOVA followed by *post hoc* Student–Newman–Keuls test. A P-value of 0.05 or less was used to indicate statistical significance.

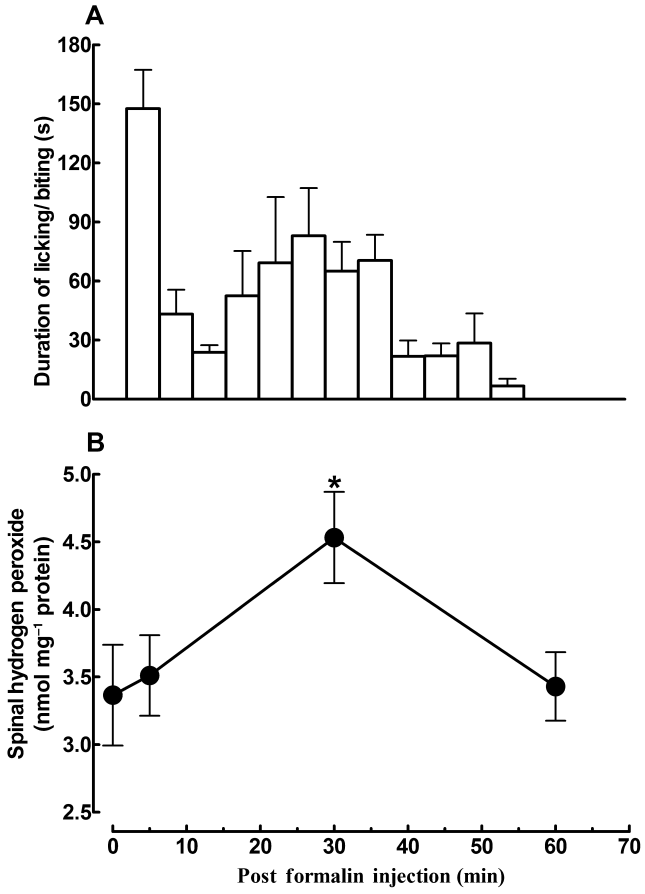

Figure 1

Time courses of pain behaviours (A) and spinal hydrogen peroxide levels (B) in Swiss mice, produced by injection of 10 μL of 5% formalininto the paw. (A) The duration of spontaneous pain behaviours (paw licking and biting) was manually quantified in 5 min epochs continuously for a period of 60 min. (B) Spinal hydrogen peroxide level was determined using the ferrous ion oxidation–xylenol orange method. Data are presented as means ± SEM ($n = 6$ in each group). *Denotes statistical significance ($P < 0.05$ by one-way ANOVA) compared with saline control group.

Results

Spinal astrocyte-derived hydrogen peroxide is specifically responsible for formalin-induced tonic pain

The effects of paw-injection of formalin on pain behaviours and spinal level of hydrogen peroxide were tested. In one group of mice (n = 6), the duration of spontaneous pain behaviours (paw licking and biting) were manually quantified in 5 min epochs continuously for a period of 60 min after s.c. injection of 10 μL of 5% formalin. Formalin produced a characteristic bi-phasic licking/biting response, consisting of an initial rapidly decaying acute phase (within 5 min after formalin injection) followed by a slowly rising and long-lived tonic phase that declined 60 min after injection (Figure 1A). To make the procedure simple, counts at 0–5 min and 20–40 min after formalin were pooled, respectively, and represented the neurogenic acute nociception and central sensitization tonic pain for the following studies.

For spinal hydrogen peroxide levels, four groups of mice (n = 6 in each group) received s.c. injection of 10 μL normal saline or 5% formalin, respectively, and spinal hydrogen peroxide concentrations were measured 30 min after saline injection and 5, 30 and 60 min after formalin injection (to match the time course of pain behaviours). The baseline value of spinal hydrogen peroxide was 3.4 ± 0.3 nmol·mg^{-1} protein. Formalin increased spinal hydrogen peroxide with the same time course as pain behaviours, that is the spinal hydrogen peroxide level after formalin injection remained the same at 5 min but was significantly increased at 30 min by 33.2% (P < 0.05 by one-way ANOVA) then decreased back to the initial level at 60 min after formalin (Figure 1B).

As DAAO is almost exclusively found in astrocytes in the CNS, including the spinal cord (Kappor and Kapoor, 1997), we evaluated whether formalin-produced spinal hydrogen peroxide was affected by i.t. fluorocitrate. Fluorocitrate is a metabolic poison that is selectively taken up by astrocytes to inhibit the tricarboxylic acid enzyme aconitase and has been widely used as a selective inhibitor of astrocyte activity

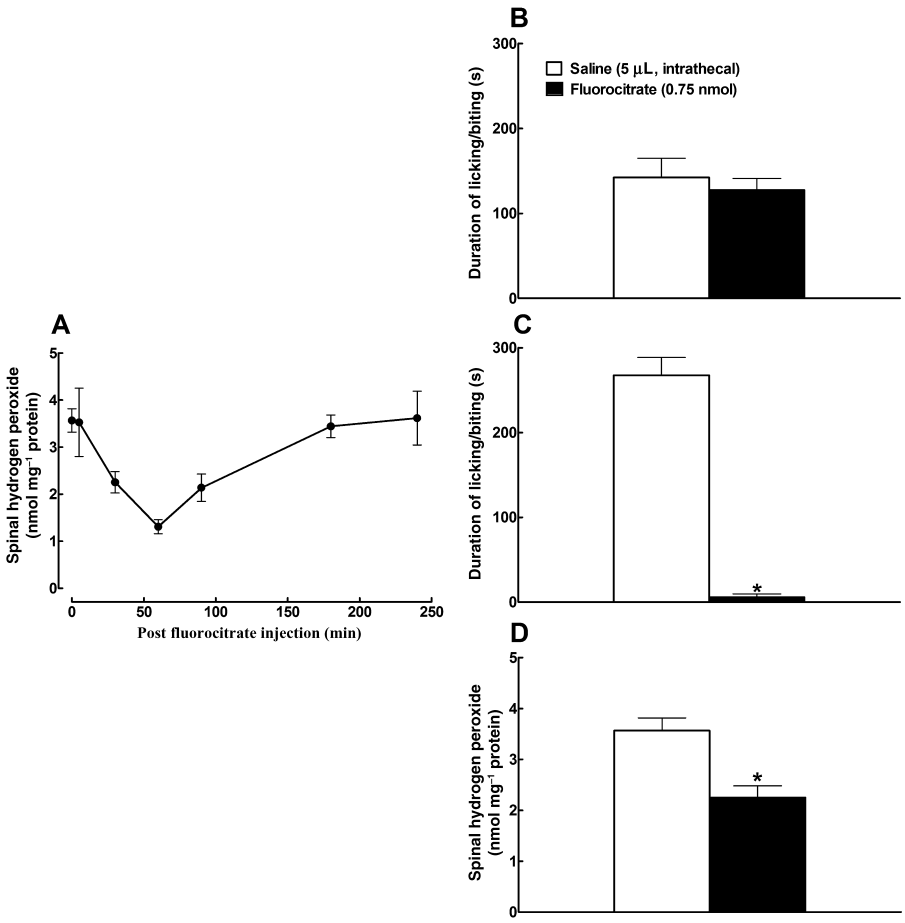

Figure 2

Effects of i.t. injection of fluorocitrate (0.75 nmol) on basal spinal hydrogen peroxide level (A) and formalin-induced acute nociception (B), tonic pain (C) and spinal hydrogen peroxide level (D) in Swiss mice. Hydrogen peroxide level was measured by the ferrous ion oxidation–xylenol orange method. The accumulative biting duration from 0–5 and 20–40 min after formalin injection represents acute nociception and tonic pain, respectively. Data are presented as means ± SEM (n = 6 in each group) * Denotes statistical significance (P < 0.05 by one-way ANOVA) compared with each saline control.

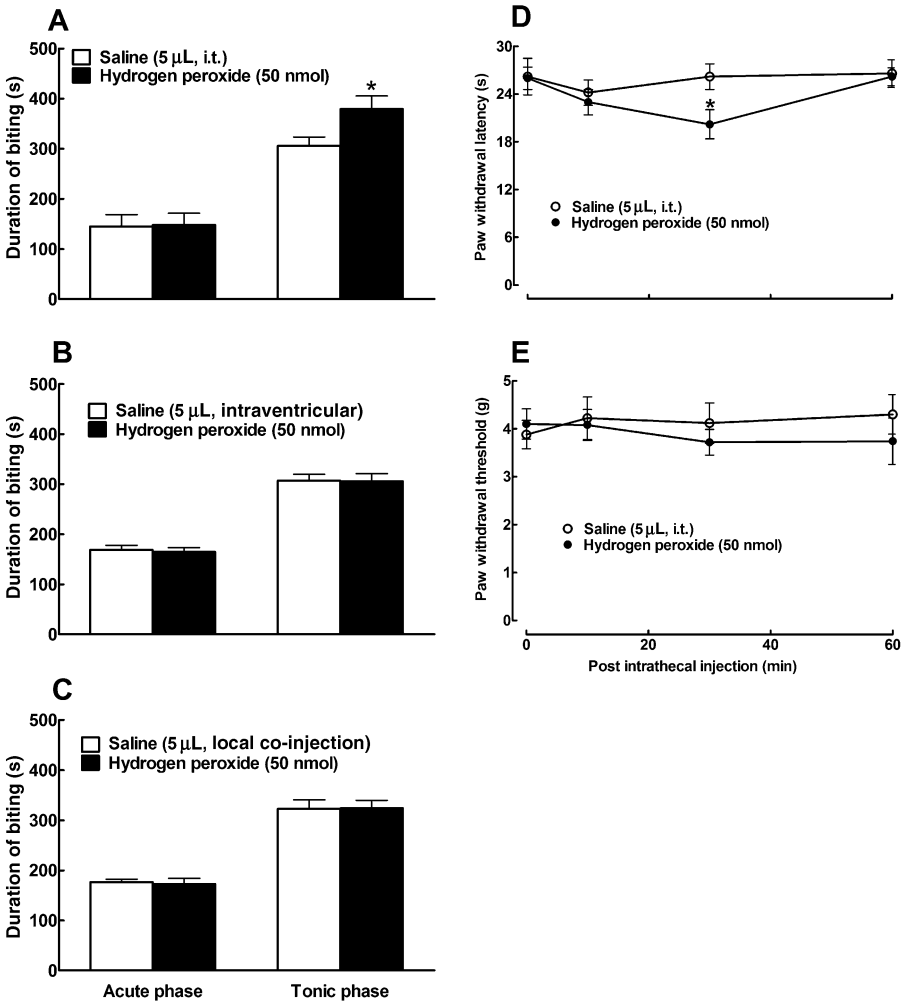

Figure 3

Effects of i.t. (A), intraventricular (B) and paw (C) co-injections of 50 nmol hydrogen peroxide on formalin-induced acute nociception and tonic pain in Swiss mice. Mice received i.t., intraventricular or paw co-injection of 5 μL saline or 50 nmol hydrogen peroxide 10 min (or the same time for paw co-injection of formalin) before the formalin challenge. The accumulative biting duration from 0–5 and 20–40 min after formalin injection represents acute nociception and tonic pain, respectively. Effects of i.t. injection of 50 nmol hydrogen peroxide on heat hyperalgesia (D) and mechanical allodynia (E) in Swiss mice. Data are presented as means ± SEM (n = 6 in each group). * Denotes statistical significance (P < 0.05 by one-way ANOVA) compared with each saline control.

(Swanson and Graham, 1994). First, seven groups of mice (n = 6 in each group) received an i.t. injection of 5 μL saline or 0.75 nmol fluorocitrate, and basal spinal hydrogen peroxide levels were determined 0, 0.5, 1, 1.5, 2, 3 and 4 h later. Fluorocitrate produced a reversible blockade of spinal hydrogen peroxide production with full recovery at 4 h and peak effect at 1 h post injection (Figure 2A); the latter was selected for the following study. No apparent motor side effects of fluorocitrate were observed during the study period.

The effects of fluorocitrate on formalin-induced acute nociception, tonic pain and increased spinal level of hydrogen peroxide were determined separately. Six groups of mice (n = 6 in each group) received i.t. injection of 5 μL saline or 0.75 nmol fluorocitrate followed by formalin injected at different time points (0.5 vs. 1 h) to match observed acute nociception and tonic pain 1 h post fluorocitrate injection. Compared with the saline control, fluorocitrate was not effective in reducing formalin-induced acute nociception (Figure 2B) but completely blocked tonic pain (P < 0.05 by one-way ANOVA; Figure 2C) and significantly reduced spinal hydrogen peroxide level (P < 0.05; Figure 2D).

The effects of exogenous hydrogen peroxide administered i.t., intraventricularly or locally on formalin-induced pain were tested. Six groups of mice (n = 6 in each group) each received i.t., intraventricular or paw (local) co-injection of 5 μL normal saline or 50 nmol hydrogen peroxide 10 min (or the same time for the paw co-injection of formalin) before the formalin challenge. Hydrogen peroxide given i.t. potentiated formalin-induced tonic pain by 24.0% (P < 0.05 by one-way ANOVA) but was not effective in causing acute nociception (Figure 3A). In contrast, neither intraventricular injection nor local co-injection of hydrogen peroxide altered either formalin-induced acute nociception or tonic pain (Figures 3B and C). The lack of response of hydrogen peroxide in the paw

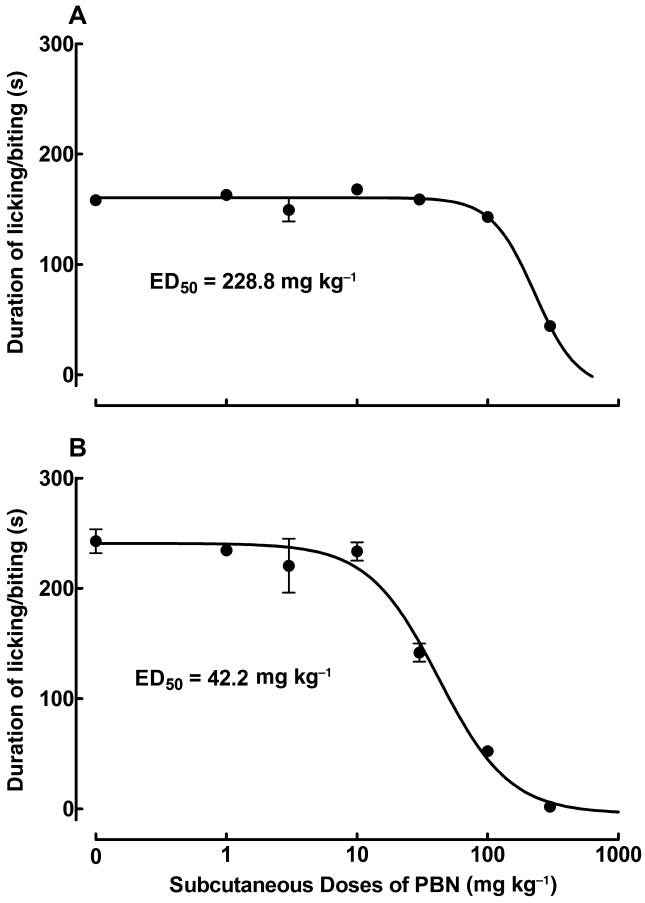

Figure 4

Dose–responses for effects of s.c. injection of phenyl PBN on formalin-induced acute nociception (A) and tonic pain (B) in Swiss mice. Mice received s.c. injection of saline (10 mL·kg^{-1}) or a variety of doses of PBN 30 min before formalin. The accumulative biting duration from 0–5 and 20–40 min after formalin injection represent acute nociception and tonic pain, respectively. Data are presented as means ± SEM (n = 6 in each group).

was in accordance with a recent publication showing that much higher doses (starting from approximately 2 μmol) were required to achieve pain responses (Keeble *et al.*, 2009). In addition, two groups of mice (n = 6 in each group) received i.t. injection of normal saline (5 μL) or hydrogen peroxide (50 nmol). The pain thresholds were tested before and 15, 30 and 60 min after injection by von-Frey hairs and radial heat. Hydrogen peroxide produced reversible heat hyperalgesia, with the peak effect occurring 0.5 h after injection (Figure 3D), but not mechanical allodynia (Figure 3E).

The effect of systemic injection of PBN on formalin-induced pain was tested. Seven groups of mice (n = 6 in each group) received s.c. injection of the vehicle (normal saline, 10 mL·kg^{-1}) or PBN (1, 3, 10, 30, 100 or 300 mg·kg^{-1}) 30 min before formalin. PBN produced a dose-dependent reversal of formalin-induced licking and biting both in the acute phase (Figure 4A) and tonic phase (Figure 4B), consistent with previous results (Hacimuftuoglu *et al.*, 2006). We further differentiated the effects of PBN on both phases by dose–response analysis, and demonstrated that PBN was roughly 4.4-fold less potent on acute nociception, ED_{50} 228.8 ± 2.2 mg·kg^{-1} (Figure 4C), than on tonic pain, ED_{50} 42.2 ± 6.5 mg·kg^{-1} (Figure 4D) (P < 0.05 one-way ANOVA). PBN 100 mg·kg^{-1} inhibited tonic pain by 78.47% but negligibly affected acute nociception and was therefore selected for the later study. No apparent motor side effects of PBN were observed during the study period.

CBIO produced analgesia by blockade of endogenous spinal hydrogen peroxide production rather than interactions with spinal D-serine

The analgesic effect of CBIO was tested. Seven groups of mice (n = 8 in each group) received s.c. injection of saline (10 mL·kg^{-1}) or CBIO (0. 1, 0. 3, 1, 3, 10, or 30 mg·kg^{-1}) 30 min before formalin challenge. Compared with the saline control, CBIO prevented formalin-induced tonic pain in a dose-dependent manner; 10 mg·kg^{-1} caused a maximal effect (Figure 5A) but did not prevent the acute nociception up to 30 mg·kg^{-1} (Figure 5B). Dose–response analysis of CBIO by best fit showed that maximum inhibition (E_{max}) of formalin-induced tonic pain was 61.5%, and the ED_{50} was 0.9 mg·kg^{-1} (Figure 5C).

We had previously shown that i.t. injection of 1 μg CBIO significantly (P < 0.05) inhibited formalin-induced tonic pain by 66.5% but not acute nociception in Wistar rats (Gong *et al.*, 2011). In order to further determine any other action site(s) of CBIO, four groups of Wistar rats (n = 6 in each group) received intraventricular or local (paw) injections of 10 μL saline or 1 μg CBIO 30 min before formalin injection. Paw injection of formalin in control rats also produced a characteristic bi-phasic flinching response consisting of an initial, rapidly decaying acute phase (within 10 min after formalin injection) followed by a slowly rising and long-lived (10–90 min) tonic phase. Compared with the saline control, 1 μg CBIO given either by the intraventricular route (Figure 6A) or co-injection with formalin (Figure 6B) was not effective in reducing tonic pain or acute nociception.

The effect of i.t. exogenous catalase, a specific enzyme for decomposition of hydrogen peroxide, on formalin-induced pain was further tested. Eight groups of mice (n = 6 in each group) received i.t. injection of the vehicle (saline, 5 μL) or catalase (0.75, 2.5, 7.5, 25, 75, 250 and 750 units) 30 min before formalin. Catalase given i.t. did not affect formalin-induced acute nociception (Figure 7A) but effectively prevented tonic pain in a dose-dependent manner (Figure 7B), with an ED_{50} of 12.1 U and E_{max} value of 63.7% (Figure 7C).

We tested whether CBIO and PBN, at effective analgesic doses, blocked spinal DAAO activity *ex vivo*. Three groups of mice (n = 5 in each group) received s.c. injection of saline (10 mg·kg^{-1}), CBIO (10 mg·kg^{-1}) or PBN (100 mg·kg^{-1}). One hour later, spinal cords were removed, and their DAAO enzymatic activities were assayed by measurement of pyruvate production from D-alanine (1.5 mM) as a substrate. CBIO completely inhibited spinal DAAO enzymatic activity (P < 0.05 by ANOVA; Figure 8A), whereas PBN did not alter spinal DAAO enzymatic activity (Figure 8B).

We examined the effects of CBIO, PBN and catalase, given at doses effective at reducing tonic pain but not acute

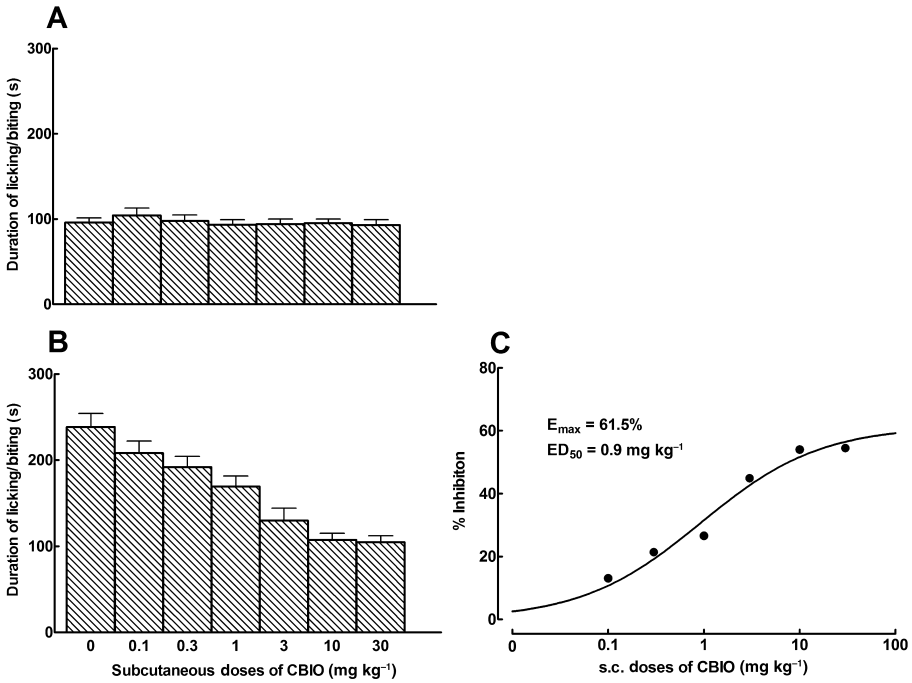

Figure 5

Dose–responses for effects of s.c. injection of CBIO on formalin-induced acute nociception (A) and tonic pain (B) in Swiss mice. Mice received s.c. injection of saline or various doses of CBIO 30 min before formalin challenge. The accumulative biting duration from 0–5 and 20–40 min after formalin injection represents acute nociception and tonic pain, respectively. (C) Dose–response analysis for effects of CBIO on formalin-induced tonic pain. Data are presented as means ± SEM (n = 8 in each group).

nociception, on the formalin-induced increase in spinal hydrogen peroxide levels. First, mice were divided into four groups, each containing 5 and 30 min subgroups (n = 6 in each group): that is (i) saline + saline groups; (ii) saline + formalin groups; (iii) CBIO + formalin groups; (iv) PBN + formalin groups. These mice received s.c. injection of normal saline (10 mL·kg^{-1}), CBIO (10 mg·kg^{-1}) or PBN (100 mg·kg^{-1}) 30 min before 10 μL saline or 5% formalin. Spinal hydrogen peroxide levels were measured at 5 and 30 min post-formalin, respectively.

At the 5 min point (acute phase), the baseline level of hydrogen peroxide was 3.0 ± 0.1 nmol·mg^{-1} protein in the saline control group; formalin did not significantly alter the spinal hydrogen peroxide level. CBIO and PBN had no effect on spinal hydrogen peroxide levels compared with the formalin group (Figure 9A). In a separate study, the marginal effect of PBN was confirmed when a higher dose of 300 mg·kg^{-1} inhibited spinal hydrogen peroxide by 15.5% (n = 6, P < 0.05 by one-way ANOVA). At the 30 min point (tonic phase), the baseline level of hydrogen peroxide was 2.4 ± 0.2 nmol·mg^{-1} in the saline control group; formalin markedly increased the spinal hydrogen peroxide level by 65.4% (P < 0.05 by two-way ANOVA followed by *post hoc* Student–Newman–Keuls test). Both CBIO (10 mg·kg^{-1}) and PBN (100 mg·kg^{-1}) caused a complete reversal of the increase in spinal hydrogen peroxide to the normal level (P < 0.05) (Figure 9B).

Second, the effect of an i.t. injection of catalase on the spinal hydrogen peroxide level was tested. Three groups of mice (n = 5 in each group) received an i.t. injection of saline (5 μL) or catalase (750 U) 30 min before a s.c. injection of 50 μL of saline or 5% formalin. Thirty minutes later, the mice were killed, and the spinal hydrogen peroxide levels were measured. Compared with the basic level in the saline control group, s.c. formalin significantly increased spinal hydrogen peroxide level by 53.1% (P < 0.05 by two-way ANOVA followed by *post hoc* Student–Newman–Keuls test); i.t. catalase completely prevented the increase in spinal hydrogen peroxide induced by formalin (P < 0.05) (Figure 9C).

We then investigated whether systemic treatment with CBIO (10 mg·kg^{-1}) or PBN (100 mg·kg^{-1}) was able to prevent the potentiating effect of i.t. exogenous hydrogen peroxide on formalin-induced pain. Mice were divided into six groups (n = 6–12 in each group): (i) saline + saline group; (ii) CBIO + saline group; (iii) PBN + saline group; (iv) saline + hydrogen peroxide group; (v) CBIO + hydrogen peroxide group; (vi) PBN + hydrogen peroxide group. These mice received s.c. injections of normal saline (10 mL·kg^{-1}), CBIO (10 mg·kg^{-1}) or PBN (100 mg·kg^{-1}) and 30 min later received an i.t. injection of 5 μL saline or 50 nmol hydrogen peroxide 15 min before formalin.

When given systemically, neither CBIO, PBN nor i.t. exogenous hydrogen peroxide had any effects on formalin-induced acute nociception. There were also no interactions between either CBIO, PBN or exogenous hydrogen peroxide on the acute nociceptive response (Figure 10A). For tonic pain, CBIO and PBN markedly reduced formalin-induced pain by 63.9% and 98.8%, respectively (P < 0.05 by two-way

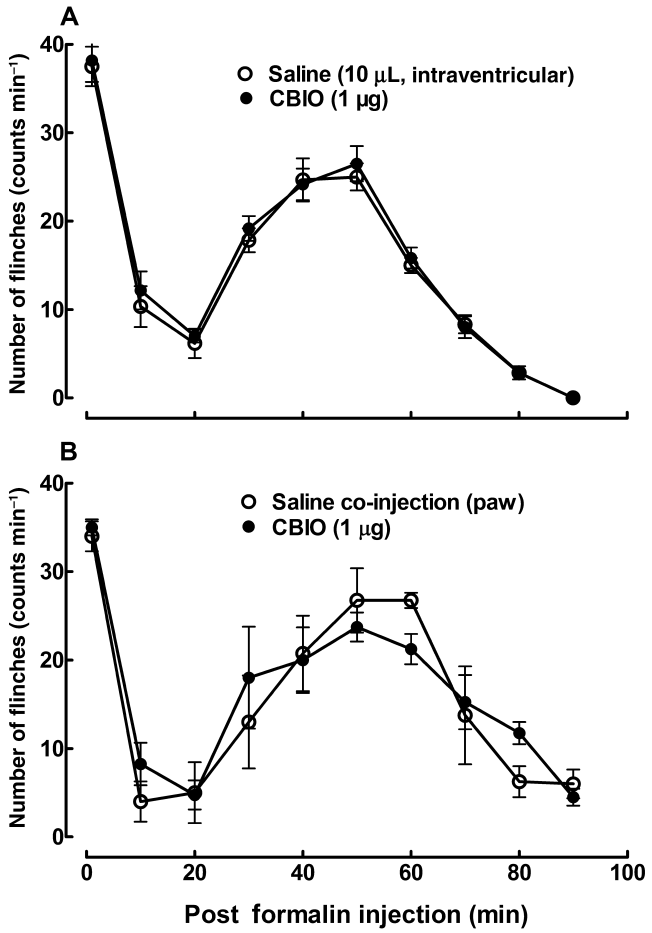

Figure 6

Effects of intraventricular (A) injection and paw co-injection (B) of CBIO on formalin-induced pain behaviours in Wistar rats. Rats received intraventricular and paw co-injection of saline or 1 μg CBIO 30 min (or the same time for co-injection of formalin) before formalin challenge. Data are presented as means ± SEM (n = 6 in each group).

ANOVA followed by *post hoc* Student–Newman–Keuls test), and exogenous hydrogen peroxide significantly potentiated the formalin-induced pain by 32.6% ($P < 0.05$). CBIO did not prevent the hydrogen peroxide-mediated potentiation of formalin-induced tonic pain, but PBN completely prevented this effect of hydrogen peroxide ($P < 0.05$) (Figure 10B).

We finally tested the possibility that CBIO produces analgesia by interacting with spinal D-serine in addition to blockade of spinal hydrogen peroxide production. We first examined whether CBIO given systemically increased spinal D-serine level using capillary electrophoresis with laser-induced fluorescence detection. The D-serine level in the spinal cord homogenates from control mice was below the limit of detection; s.c. injection of CBIO up to 30 mg·kg^{-1} did not raise the spinal D-serine level to the detection limit. Furthermore, two groups of mice received, respectively, s.c. co-injection of saline (10 mL·kg^{-1}) + D-serine (30 mg·kg^{-1}) and CBIO (30 mg·kg^{-1}) + D-serine (30 mg·kg^{-1}). The spinal D-serine level was measured at 60 min after injection. The spinal level of D-serine post D-serine injection in the saline control group was 121.0 ± 52.2 nmol·g^{-1} tissue. CBIO significantly increased the spinal D-serine level by 56.6% ($P < 0.05$ by one-way ANOVA) (Figure 11A).

We further tested the effects of D-serine, given i.t. alone or in combination with an s.c. injection of CBIO, on formalin-induced tonic pain. Mice were divided into four groups (n = 4–6 in each group): (i) saline + saline group; (ii) saline + D-serine group; (iii) CBIO + saline group; and (iv) CBIO + D-serine group. These mice received an s.c. injection of saline (10 mL·kg^{-1}) or CBIO (10 mg·kg^{-1}) and 30 min later received an i.t. injection of 5 μL saline or 100 μg D-serine 15 min before 5% formalin. The D-serine dose was selected according to the literature (Muth-Selbach *et al.*, 2009). I.t. injection of CBIO prevented formalin-induced tonic pain by 62%, compared with the saline control group ($P < 0.05$ by two-way ANOVA followed by *post hoc* Student–Newman–Keuls test). However, exogenous D-serine did not alter either formalin-induced tonic pain or the CBIO-induced analgesia (Figure 11B).

The ineffectiveness of D-serine on pain in the formalin test was consistent with previous findings, but it may be due to a possible saturated endogenous D-serine level stimulated by the high concentration (5%) of formalin (Ahmadi *et al.*, 2003). Therefore, 1% formalin was also applied. Two groups of mice (n = 6 in each group) received an i.t. injection of 5 μL saline or 100 μg D-serine 15 min before 1% formalin challenge. This lower concentration (1%) of formalin induced less (50%) licking and biting than the higher concentration (5%) but the i.t. injection of D-serine was still ineffective in affecting formalin-induced tonic pain (Figure 11B).

Discussion

Spinal hydrogen peroxide is specifically and largely responsible for formalin–induced tonic pain

First described by Dubuisson and Dennis (1977), the formalin model is widely used for evaluating the effects of analgesic compounds in laboratory animals and reflects both the acute and tonic responses to a noxious chemical stimulus. Injection of formalin provokes a bi-phasic response in which the phasic response is separated from the more enduring tonic response by a quiescent inter-phase (Coderre *et al.*, 1993; Jett *et al.*, 1997). The first phase of activity corresponding to direct activation of primary afferent fibres by formalin appears immediately following injection and lasts for approximately 5 min (Puig and Sorkin, 1996). Existing evidence suggests that the tonic component of the response reflects the combined effects of afferent input and central sensitization in the dorsal horn (Cook *et al.*, 1987; Dickenson and Sullivan, 1987; Coderre *et al.*, 1990), which is shared in chronic pain syndromes such as chronic neuropathic pain (Jett *et al.*, 1997), cancer pain (Mantyh *et al.*, 2002) and morphine tolerance (Mao *et al.*, 1995).

By application of a variety of non-specific free radical scavengers and antioxidants (Muscoli *et al.*, 2003), ROS have been extensively demonstrated to be primarily involved in the development and maintenance of central sensitization in

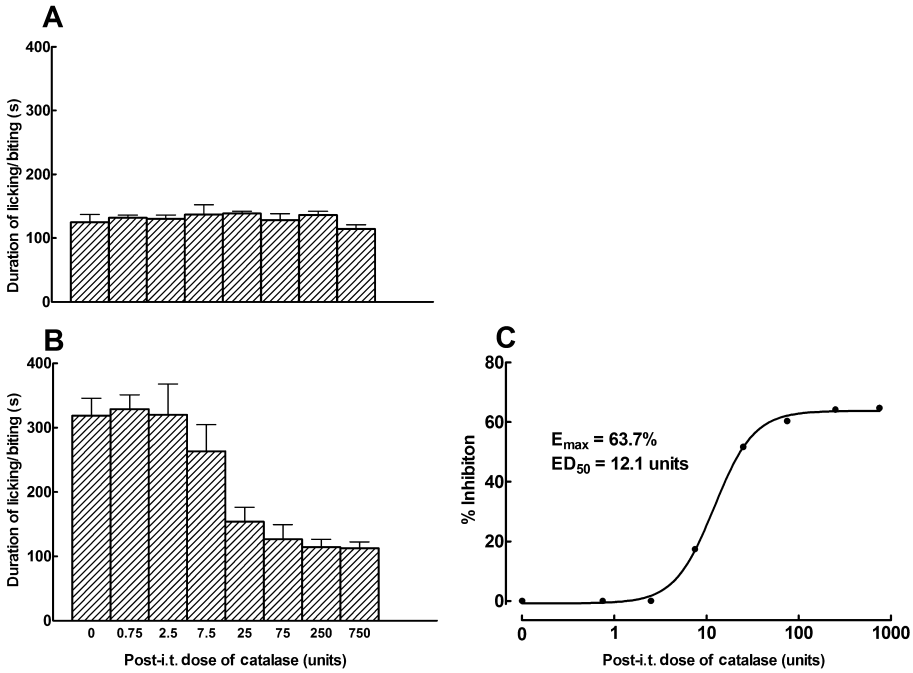

Figure 7

Dose–responses for effects of i.t. administration of catalase on formalin-induced acute nociception (A) and tonic pain (B) in Swiss mice. Mice received i.t. administration of saline (5 μL) or doses of catalase 30 min before formalin. The accumulative biting duration from 0–5 and 20–40 min after formalin injection represented acute nociception and tonic pain, respectively. Dose–response analysis for effects of catalase on formalin-induced tonic pain (C). Data are presented as means ± SEM (n = 6 in each group).

chronic pain conditions (Tal, 1996; Khalil *et al.*, 1999; Kim *et al.*, 2004; 2006; Viggiano *et al.*, 2005; Hacimuftuoglu *et al.*, 2006; Lee *et al.*, 2007; Chung *et al.*, 2008). It was reported that given systemically PBN blocks mechanical allodynia in a dose-dependent fashion in neuropathic rats. Neither development of tolerance nor loss of potency occurred with repeated injections of PBN. PBN injection was equally effective if given before or after the neuropathic lesion. The site of action of PBN was spinal rather than peripheral (Kim *et al.*, 2004). Moreover, 5,5-dimethyl-1-pyrroline-*N*-oxide, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxy and N-acetylcysteine, acting primarily at spinal levels, blocked formalin-induced tonic pain in rats and mice (Viggiano *et al.*, 2005; Hacimuftuoglu *et al.*, 2006; Chung *et al.*, 2008), although the acute nociception was also shown to be blocked by these free radical scavengers (Hacimuftuoglu *et al.*, 2006). Indeed, our study confirmed that PBN specifically and almost completely blocks the formalin-induced pain response and the increased spinal hydrogen level in the tonic phase, whereas it has negligible effects on both the nociceptive response and spinal hydrogen level during the acute phase. ROS consist of hydroxyl radicals, superoxides, peroxynitrites and lipid peroxyl radicals, among which hydrogen peroxide is less active but much more stable (Angermuller *et al.*, 2009).

Recent studies have found that peripheral hydrogen peroxide causes pain or hyperalgesia (Sawada *et al.*, 2008) via effects on primary afferent nociceptive neurons that expressed transient receptor potential vanilloid 1 (TRPV1) (Keeble *et al.*, 2009), and particularly transient receptor potential ankyrin 1 (TRPA1) (Sawada *et al.*, 2008); the latter was demonstrated to mediate formalin-induced pain (McNamara *et al.*, 2007). Our study focused on the role of spinal hydrogen peroxide, and our results, for the first time, demonstrate that increased spinal hydrogen peroxide is specifically and largely responsible for formalin-induced central sensitization-mediated tonic pain (but not acute nociception). (i) Paw injection of formalin increased spinal hydrogen levels at 20–40 min but not at 0–5 min after injection, with exactly the same time course as pain behaviours. These results are consistent with a previous publication where whisker pad injection of formalin induced a significant increase in the extracellular level of hydrogen peroxide into the subnucleus caudalis of the spinal trigeminal nucleus in rats, with a peak between 15 and 30 min after injection (Viggiano *et al.*, 2005). (ii) Spinal, but not intraventricular or paw (local) co-injection of exogenous hydrogen peroxide, specifically potentiates formalin-induced tonic pain, which was completely prevented by PBN but not CBIO. I.t. injection of exogenous hydrogen peroxide also produced heat hyperalgesia but not mechanical allodynia. (iii) More importantly, the specific and potent DAAO inhibitor CBIO in addition to PBN completely prevented formalin-induced the increase in spinal hydrogen peroxide and inhibited tonic pain (but not acute nociception) by approximately 60%. (iv) Lastly, our study showed that i.t. administration of catalase, an enzyme that functions to protect the cell from the toxic effects of hydrogen peroxide by specifically catalyzing the decomposition of hydrogen peroxide to water and oxygen (Chelikani *et al.*, 2004), completely depleted spinal hydrogen peroxide

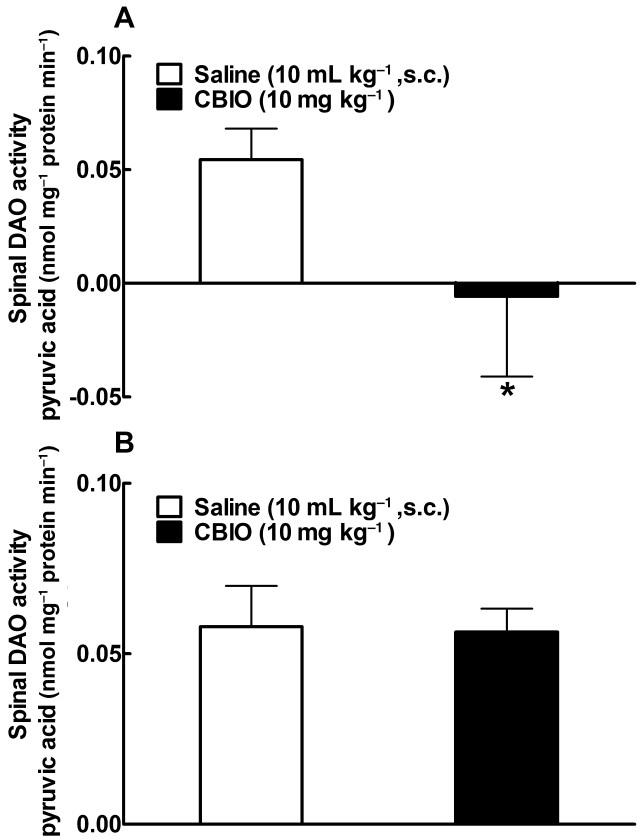

Figure 8

Ex vivo effects of s.c. injections of CBIO (10 mg·kg^{-1}, A) and PBN (100 mg·kg^{-1}, B) on spinal DAAO enzymatic activity (measured with 1.5 mM D-alanine as substrate) in Swiss mice. Data are presented as means ± SEM (n = 5 in each group). *Denotes statistical significance (P < 0.05 by one-way ANOVA) compared with each saline control.

and specifically prevented formalin-induced tonic pain by the same inhibition of 64%.

It is noteworthy to point out that CBIO, catalase and PBN prevented the formalin-induced increase in the spinal levels of hydrogen peroxide. In addition, PBN almost completely blocked the formalin-induced tonic pain, whereas CBIO and catalase only inhibited this effect of formalin by approximately 60%. This latter result is consistent with those obtained with other DAAO inhibitors as well as mutation and knock-down of the DAAO gene (Zhao *et al.*, 2008; 2010; Chen *et al.*, 2011; Gong *et al.*, 2011). The differential effects of CBIO, catalase and PBN on the spinal hydrogen peroxide level and pain responses indicate that spinal hydrogen peroxide plays a major role (approximately 60%) in formalin-induced pain compared to the other ROS. This notion is validated by the findings that DAAO is only involved in the production of hydrogen peroxide and does not produce the other ROS due to deamination of D-amino acids (Pollegioni *et al.*, 2007; Angermuller *et al.*, 2009; W *et al.*, unpublished observations), and CBIO is ineffective at scavenging exogenous hydrogen peroxide (the present study). Another alternative explanation for how spinal hydrogen peroxide mediates pain rests on the notion that interactions of each ROS is essential for the role of ROS in pain, and hydrogen peroxide is an important component for the interactions. Our study further reveals that formalin-induced spinal hydrogen is probably derived from astrocytes within the spinal cord in a DAAO-dependent manner. DAAO is found almost exclusively in the central region of the peroxisomal matrix compartment of peroxisomes in astrocytes (Angermuller *et al.*, 2009) in the spinal cords of mice, rats and humans (Kappor and Kapoor, 1997). Therefore, we speculate that formalin stimulates the release of D-amino acids, which are oxidized by DAAO located in astrocytes to produce hydrogen peroxide in the spinal cord. It was shown that i.t. pretreatment with fluorocitrate, a metabolic poison that selectively inhibits astrocyte activity, specifically prevents formalin-induced increase in spinal hydrogen peroxide and tonic pain but not acute nociception, consistent with previous behaviour results in which fluorocitrate given i.t. and through the cerebellomedullary cistern reduced the responses to formalin injections in the paw (Watkins *et al.*, 1997) and the orofacial area (Lan *et al.*, 2007), respectively.

It is well accepted that activation of NMDA receptors mediates central sensitization (Woolf and Thompson, 1991; Basbaum *et al.*, 2009). The signal transduction between DAAO-derived hydrogen peroxide and activation of NMDA receptors within the spinal cord is not yet completely known. Hyperalgesia and increased phosphorylated NMDA receptor subunit 1 expression has been observed in both spinal nerve ligated neuropathic rats and capsaicin-treated rats. Systemic injection of PBN and the antioxidant vitamin E dramatically reduced this hyperalgesia and blocked the enhancement of spinal phosphorylated NMDA receptor subunit 1 in both pain models. These data suggest that ROS are involved in NMDA receptor activation and thus contribute to neuropathic and capsaicin-induced pain (Kim *et al.*, 2006; Gao *et al.*, 2007).

DAAO inhibitors produce analgesia via blockade of endogenous spinal hydrogen peroxide production rather than interactions with D-serine

CBIO produced a dose-dependent analgesia in tonic pain in the formalin test with maximum inhibition of approximately 60% in mice, consistent with our recent findings in rats (Gong *et al.*, 2011). CBIO and the DAAO inhibitor sodium benzoate were also effective at blocking neuropathic pain and that associated with bone cancer (Huang *et al.*, 2010; Zhao *et al.*, 2010). The site of the analgesic effect of CBIO was spinal rather than supraspinal or peripheral as only i.t. administration was effective. Blockade of *in vitro* spinal DAAO activity by DAAO inhibitors, including CBIO, was positively correlated to their blockade of formalin-induced analgesia *in vivo* (Gong *et al.*, 2011). In this study, we further proved that CBIO but not PBN at entirely effective analgesic doses inhibited spinal DAAO activity completely *ex vivo*, indicating that CBIO produces analgesia via blockade of spinal DAAO. This is consistent with our previous finding that mutation and knock down of the DAAO gene reduced formalin-induced tonic pain (Zhao *et al.*, 2008; Chen *et al.*, 2011).

CBIO s.c. at an effective analgesic dose (10 mg·kg^{-1}) blocked the formalin-induced increase in spinal hydrogen

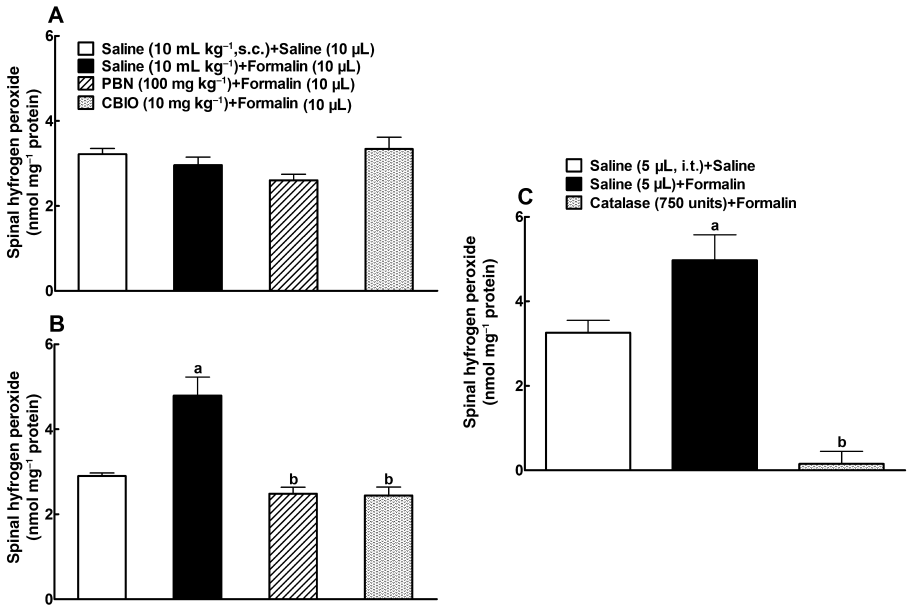

Figure 9

Effects of s.c. injection of CBIO (10 mg·kg^{-1}) and PBN (100 mg·kg^{-1}) on formalin-increased spinal hydrogen peroxide levels at 5 min (A) and 30 min (B) after formalin in Swiss mice. Mice received s.c. injection of saline, CBIO or PBN 30 min before saline or formalin. (C) The effect of i.t. injection of catalase (750 U) on formalin-induced increase in spinal hydrogen peroxide level 30 min after formalin in Swiss mice. Spinal hydrogen peroxide level was measured by the ferrous ion oxidation–xylenol orange method. Data are presented as means ± SEM (n = 5–6 for each group).[a] Denotes statistical significance (P < 0.05 two-way ANOVA followed by *post hoc* Student–Newman–Keuls test) compared with the saline control; [b] denotes statistical significance (P < 0.05) compared with the formalin control.

peroxide as well as tonic pain in mice but was not effective in blocking the potentiation of formalin-induced tonic pain induced by exogenous hydrogen peroxide. These results indicate that CBIO produces analgesia by blocking the production of endogenous hydrogen via oxidative deamination of neutral and polar D-amino acids, rather than by scavenging hydrogen peroxide or blocking its subsequently effector pathway. This is further supported by the finding that the ROS scavenger PBN inhibited the potentiation of formalin-induced tonic pain induced by exogenous hydrogen peroxide.

The effect of D-serine and activation of NMDA receptors have been extensively addressed (Schell *et al.*, 1995). Reduction of D-serine by DAAO via oxidative deamination was thus considered to be an antinociceptive factor (Wake *et al.*, 2001; Ying *et al.*, 2006). It was reported that mutation of DAAO potentiated formalin-induced central sensitization and pain. NMDA receptor mediated excitatory postsynaptic currents recorded from spinal cord dorsal horn neurons are also significantly potentiated in mutant mice (Wake *et al.*, 2001). Also i.t. exogenous DAAO has been shown to block formalin-induced pain (Ying *et al.*, 2006). However, our data clearly do not support the above hypothesis as: (i) we have shown that formalin-induced tonic pain is significantly reduced in DAAO mutant mice compared with wild-type mice (Zhao *et al.*, 2008), and administration i.t. of the exogenous enzyme of DAAO did not inhibit formalin-induced tonic pain (Lu *et al.*, 2010), in sharp contrast to the results of Wake *et al.* (2001) and Ying *et al.* (2006). (ii) Knock down of the spinal DAAO gene by i.t. injections of siRNA/DAAO in the polyethyleneimine complexation or adenoviral vector effectively blocked formalin-induced tonic pain (Chen *et al.*, 2011). (iii) We and other laboratories have consistently demonstrated that a series of DAAO inhibitors, including CBIO, 'Compound 8', AS057278 and sodium benzoate, produced analgesia rather than algesia in neuropathic pain (Fang *et al.*, 2005; Zhao *et al.*, 2010), pain associated with bone cancer (Huang *et al.*, 2010) and formalin-induced tonic pain (Zhao *et al.*, 2008; Gong *et al.*, 2011; the present study) in animals. In addition, the DAAO inhibitor SEP-227900 has been under early clinical investigation for the treatment of chronic neuropathic pain (http://www.sumitomo-chem.co.jp; Williams, 2009). (iv) Although systemic co-injection of CBIO with D-serine increased spinal D-serine level, consistent with previous findings (Ferraris *et al.*, 2008; Smith *et al.*, 2009), CBIO, when given alone, did not increase endogenous D-serine level in the brain (Ferraris *et al.*, 2008; Hashimoto *et al.*, 2009) and probably does not in the spinal cord either, although this was not clearly proved in this study due to detection limits. (v) Even if DAAO inhibitors could increase endogenous spinal D-serine content, the raised level would probably not be sufficient to activate spinal NMDA receptors or produce algesia, as we demonstrated that i.t. injection of exogenous D-serine did not alter the tonic pain induced by low or high concentrations of formalin or CBIO's analgesia.

The brain DAAO-mediated decreased levels of D-serine lowers the functional activity of NMDA receptors, which is believed to be one of the underlying mechanisms in the development of schizophrenia (Williams, 2009; Smith *et al.*, 2010; Verrall *et al.*, 2010). This hypothesis has prompted the development of DAAO inhibitors for the treatment of

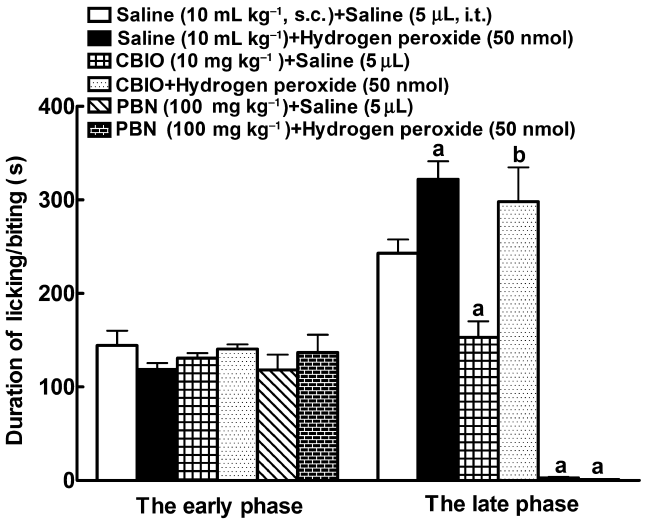

Figure 10

Effect of s.c. injections of CBIO (10 mg·kg^{-1}) and PBN (100 mg·kg^{-1}) on spinal exogenous hydrogen peroxide-induced potentiation of formalin-induced acute nociception (A) and tonic pain (B) in Swiss mice. Mice received s.c. injections of saline, CBIO or PBN and 30 min later received i.t. injection of 5 μL saline or 50 nmol hydrogen peroxide 15 min before formalin. The accumulative biting duration from 0–5 and 20–40 min after formalin injection represents acute nociception and tonic pain, respectively. Data are presented as means ± SEM ($n = 6$ in each group).[a] Denotes statistical significance ($P < 0.05$ two-way ANOVA followed by *post hoc* Student–Newman–Keuls test) compared with the saline control; [b] denotes statistical significance ($P < 0.05$) compared with the CBIO group.

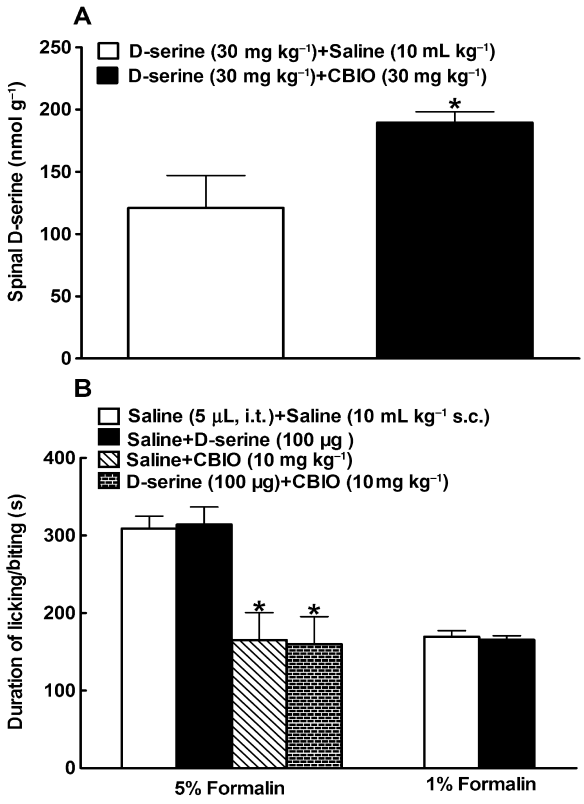

Figure 11

Effects of s.c. injection of CBIO on spinal D-serine level (A) and D-serine injected i.t. alone or in combination with s.c. injection of CBIO on formalin-induced tonic pain (B) in Swiss mice. (A) Mice received a s.c. co-injection of saline and D-serine or CBIO and D-serine; 60 min later, the spinal level of D-serine was measured by capillary electrophoresis with laser induced fluorescence detection. (B) Mice received s.c. injection of saline, D-serine, CBIO and the combination of D-serine and CBIO and 30 min later received i.t. injection of saline or D-serine 15 min before 1% or 5% formalin. The accumulative biting duration 20–40 min post formalin represents tonic pain. Data are presented as means ± SEM ($n = 4$ and $n = 6$ in each group in A and B, respectively). *Denotes statistical significance ($P < 0.05$ by one-way or two-way ANOVA followed by *post hoc* Student–Newman–Keuls test) compared with each saline control.

schizophrenia (Williams, 2009; Smith *et al.*, 2010) in animal studies and clinical investigations (http://clinicaltrials.gov/ct2/show/NCT00960219?term=DAAO+inhibitors&rank=1). However, the studies published using inhibitors have yielded fairly modest and inconsistent behavioural effects, in contrast to the fairly robust effects of exogenous D-serine, as well as conflicting results of increased brain endogenous D-serine levels. This may be due to the potency and pharmacokinetic properties of these inhibitors (Williams, 2009; Smith *et al.*, 2010). Alternatively, with regard to the pain modulation demonstrated in this study, the differential efficacies of D-serine and DAAO inhibitors may be due to the fact that DAAO inhibitors produce an anti-schizophrenic effect by inhibiting endogenous hydrogen peroxide production rather than increasing D-serine. Indeed, the role of brain ROS in the development of schizophrenia has been addressed recently (Prabakaran *et al.*, 2004).

In summary, the stimulation of spinal hydrogen peroxide is specifically and mainly (approximately 60%) responsible for formalin-induced tonic pain with a mechanism of central sensitization, similar to that of neuropathic pain and pain associated with cancer, while the other ROS play a minor role. Formalin-stimulated spinal hydrogen peroxide is probably derived from astrocytes after DAAO-mediated deamination of neutral and polar D-amino acids. DAAO inhibitors such as CBIO produce analgesia by reduction of spinal endogenous hydrogen peroxide rather than interacting with spinal D-serine. DAAO inhibitors such as CBIO might be used as probe drugs for studying the role of endogenous hydrogen peroxide in degenerative neurological diseases where DAAO is present.

Acknowledgements

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Conflict of interest

The authors report no conflicts of interest.

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