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Short communication

Intraplantar injection of tetrahydrobiopterin induces nociception in mice

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HIGHLIGHTS

- Tetrahydrobiopterin induces pro-nociceptive effects following peripheral injection.
- Local pre-treatment with morphine attenuate tetrahydrobiopterin evoked nociception.
- Tetrahydrobiopterin did not act through prostaglandin release.
- Nitric oxide inhibition does not mediate tetrahydrobiopterin evoked nociception.
- TRPV1 activation is not involved in tetrahydrobiopterin evoked nociception.

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ABSTRACT

Tetrahydrobiopterin (BH4) is implicated in the development and maintenance of chronic pain. After injury/inflammation, the biosynthesis of BH4 is markedly increased in sensory neurons, and the pharmacological and genetic inhibition of BH4 shows analgesic effects in pre-clinical animal pain models. Intrathecal injections of BH4 have been shown to induce and enhance pain-like behaviours in rats, suggesting that under chronic pain conditions BH4 may act by facilitating central sensitisation. So far it is unknown whether BH4 acts on peripheral sites of the somatosensory system or whether BH4 *per se* provokes nociceptive pain behaviours. The purpose of this study was therefore to investigate the acute nociceptive effects of intraplantar injection of BH4. BH4 was found to induce dose-dependent licking/biting of the paw lasting 5 min, which was not observed following an injection of biopterin (inactive BH4 metabolite). Paw swelling, measured as paw thickness and weight, was not observed after BH4 injection. To explore possible mechanisms of action of BH4, the effect of local pre-treatment with indomethacin, N_ω-nitro-L-arginine methyl ester, N_ω-nitro-L-arginine, capsazepine and ruthenium red was tested. Morphine served as a positive control. Intraplantar pre-injection of morphine dose-dependently inhibited BH4-induced nociception, while none of the other compounds showed any statistical significant antinociception. These results suggest that BH4 exhibits nociceptive properties at peripheral sites of the somatosensory system, proposing an as yet unexplored involvement of BH4 in peripheral nociceptive processes. However, this appears not to be mediated through nitric oxide and prostaglandin release or by activation of the transient receptor potential vanilloid 1.

1. Introduction

Tetrahydrobiopterin (BH4) is an essential co-factor for the activity of the enzymes tyrosine hydroxylase and tryptophan hydroxylase as well as for the three isoforms of nitric oxide synthase (NOS). BH4 is therefore crucial for the biosynthesis of catecholamines, serotonin and nitric oxide (NO) [1,2]. Given the pleiotropic biochemical properties of BH4, it is implicated in a number of pathological conditions, such as cardiovascular diseases, neurological diseases and psychiatric disorders [1,3]. The BH4

Abbreviations: BH4, tetrahydrobiopterin; BP, biopterin; i.pl., intraplantarily; L-NAME, N_ω-Nitro-L-arginine methyl ester hydrochloride; L-NNA, N_ω-Nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; PGE_2, prostaglandin E2; TRPV, transient receptor potential vanilloid.

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pathway has also been recognised as a potential, new target for the development of novel analgesics [2,4]. This was triggered by findings that demonstrated a link between BH4 biosynthesis and pain persistence in humans and rodents [5,6].

The biosynthesis of BH4 is highly controlled by the *de novo* synthetic pathway; the salvage pathway and the recycling pathway [1,2]. Biosynthesis of BH4 proceeds from GTP *via* three reactions catalysed by guanosine triphosphate cyclohydrolase 1,6-pyruvoyltetrahydrobiopterin synthase and sepiapterin reductase. Under normal conditions, sensory neuron activity of the synthetic pathway is low and the salvage and recycling pathways preserve BH4 homeostasis [2]. However, after peripheral injury or inflammation, guanosine triphosphate cyclohydrolase 1 activity markedly increases in sensory neurons accompanied by increased BH4 biosynthesis [5]. The inhibition of enzymes involved in the biosynthesis of BH4 markedly reduces the pathological increase of BH4, which was found to induce analgesia in pre-clinical rodent models of pain [5,7]. Recently, genetic inhibition of BH4 biosynthesis in the *hph-1* mouse model showed a decrease in pain-like behaviours, particularly after pain sensitisation or inflammation [6].

The exact site(s) of action of BH4 in painful conditions is still not completely elucidated. A previous study showed that intrathecal injection of BH4 induced heat hypersensitivity in naïve rats and increased pain-like hypersensitivity following peripheral injury, suggesting that a pathological increase in BH4 production may present pro-nociceptive properties at central sites of the somatosensory system [5]. Whether BH4 is involved in pain through peripheral nociceptive processes or provokes nociceptive pain behaviours at peripheral sites is still unknown. Therefore, to address this question this study aimed to investigate whether BH4 *per se* induces signs of nociception and paw swelling by intraplantar administration into the hind-paw of mice. To verify that the observed nociceptive behaviours were in fact due to BH4, the inactive metabolite biopterin (BP) was applied as a negative control.

The exact mechanism(s) involved in the pain-producing effect of BH4 is also not completely understood. This study therefore aimed to explore possible targets of BH4 in pain. It has been suggested that BH4 partly acts through an increased NO release [5]. As a further step in determining the mechanisms of action of BH4, this study evaluated the effect of intraplantar pre-injections of a cyclooxygenase inhibitor, NOS inhibitors and transient receptor potential vanilloid 1 (TRPV1) antagonists on BH4-induced nociception. Morphine was included as a positive control.

2. Materials and methods

2.1. Animals

Experiments were conducted using 8–10 week-old female Hsd-WIN:NMRI mice (Harlan, Germany). After arrival the mice were allowed at least 1 week of habituation and were housed as previously described [3]. Food and tap water were available *ad libitum* on a 12:12 h light–dark cycle (lights on at 6:00 a.m.). Experiments were conducted between 8:00 a.m. and 4:00 p.m. by an observer blinded to both the treatment groups and the doses given within each treatment. All testing procedures were approved by the Danish Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries (authorisation no. 2012-15-2934-00484) and conducted according to the guidelines of the National Institutes of Health (8th edition, revised 2011). All efforts were made to minimise animal suffering and the number of animals used.

2.2. Chemicals and drug injections

(6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride (BH4), 6-biopterin (BP), N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), N_ω-nitro-L-arginine (L-NNA), indomethacin, capsazepine, ruthenium red and capsaicin were purchased from Sigma–Aldrich (Denmark). Morphine hydrochloride was purchased from Nomeco (Denmark).

BH4 was dissolved in sterile 0.0667 M NaH_2PO_4 and 0.0667 M Na_2HPO_4 (1:4) with 0.1% (w/v) ascorbic acid and 0.44% (w/v) NaCl to avoid any injection of an acidic solution [8–10]. In this vehicle, the BH4 solution had a pH of 6.5–7.0. BP was dissolved in 0.308 M NaOH and the pH adjusted to 8.5 with HCl [11]. Further adjustment of pH was not possible, since BP precipitates below 8.5. Morphine, L-NAME, L-NNA and ruthenium red were prepared in sterile 0.9% (w/v) NaCl. Indomethacin was dissolved in 0.1 M $NaHCO_3$ with 0.302% (w/v) NaCl and capsazepine in 5% (v/v) DMSO, 5% (v/v) ethanol, 2% (w/v) Tween 80 and 88% (v/v) saline. These compounds were either given intraplantarily (i.pl.; 10 μL/paw) 15 min or intraperitoneally (20 mL/kg) 30 min prior to i.pl. injection of BH4 or capsaicin. The doses of the compounds were based on previous findings showing antinociception in mice [10,12–15].

2.3. Behavioural testing

BH4-induced spontaneous pain behaviours were measured as previously described for the formalin test [6]. Briefly, 20 μL of 10, 20, 30 or 60 μg/paw BH4 solution was injected i.pl. into the right hind-paw and the total time spent licking/biting the hind-paw was recorded for a period of 15 min to the nearest second at 5 min intervals. The vehicle and BP served as negative controls. Mechanical and heat pain-like hypersensitivity (Supplementary Fig. 1) was measured using von Frey and Hargreaves test, respectively, as previously described [6]. The capsaicin pain model served as a positive control for the antinociceptive effects of capsazepine and ruthenium red (Supplementary Fig. 3) [12]. The procedure was similar to that of BH4, though 10 μL of 0.2 μg/paw capsaicin solution was injected i.pl. and nociceptive behaviours recorded within 5 min.

2.4. Measurement of paw swelling

Paw swelling was measured as paw thickness (mm) and paw wet weight (mg) immediately after behavioural testing corresponding to 15 min after the BH4 injection (Supplementary Fig. 2). Paw thickness was measured at the dorsal–plantar axis at the metatarsal level using a digital caliper. For quantification of paw wet weight, mice were euthanised by cervical dislocation and hind-paws were cut from the tarsus and weighted on an analytical balance.

2.5. Data analysis

For drug treatment, raw data from the 5 min sampling are expressed as a % of the vehicle response according to the equation: % vehicle = (post-treatment value)/(vehicle value) × 100. Data were analysed by either one-way analysis of variance (ANOVA) followed by pair-wise comparisons using the Fisher's LSD test or two-tailed unpaired *t*-test (SigmaPlot 12.03, Systat Software Inc.). Data are presented as mean+ SEM or ±SEM. $p < 0.05$ is considered statistically significant.

3. Results and discussion

3.1. BH4-induced nociception in mice

The purpose of the current study was to investigate if BH4 *per se* provokes acute nociception when injected into the hind-paw of mice. Indeed, BH4 injection induced several signs of nociception including licking, biting, flinching, favouring and lifting of the paw. The most prominent nociceptive behaviour seemed to be licking/biting of the paw. To simplify the scoring of the nociceptive