

DENATONIUM BENZOATE ATTENUATES INFLAMMATION AND PAIN AND DECREASES PGE2 LEVELS IN RATS

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Abstract

Denatonium benzoate (DB) is an agonist of bitter taste receptors (TAS2Rs). TAS2Rs are detected in many non-sensory tissues, and their activation could lead to different effects. The aim of our study was to evaluate the anti-inflammatory, anti-hyperalgesic activity of DB and the changes in plasma levels of PGE2 after administration of DB. Male Wistar rats were divided into 5 groups (n = 6) and treated intraperitoneally (i.p.) with: saline, diclofenac sodium 25 mg/kg bw, DB 10 mg/kg bw and DB 15 mg/kg bw, respectively. The following methods were applied: i) carrageenan-induced paw oedema; ii) carrageenan-induced hyperalgesia and iii) enzyme-linked immunosorbent assay (ELISA). DB in doses of 10 and 15 mg/kg bw significantly inhibited paw oedema at the 2nd, 3rd and 4th hour after carrageenan injection. The anti-hyperalgesic activity was observed in rats treated with 10 and 15 mg/kg bw DB only at the 4th hour after carrageenan administration. The levels of PGE2 significantly decreased in groups treated with DB in both doses. Our results indicate that DB possesses anti-inflammatory and anti-hyperalgesic effects in the carrageenan-induced models of exudative inflammation and hyperalgesia. This activity may be related to decreased PGE2 levels.

Rezumat

Benzoatul de denatonium (DB) este un agonist al receptorilor pentru gustul amar (TAS2R). Aceștia sunt prezenți în multe țesuturi non-senzoriale, iar activarea lor poate genera efecte diferite. Scopul studiului nostru a fost evaluarea activității antiinflamatoare și anti-hiperalgeică a DB, precum și dinamica nivelurilor plasmatice ale PGE2 după administrarea DB. Șobolanii masculi Wistar au fost împărțiți în 5 grupuri (n = 6) și tratați intraperitoneal (i.p.) cu: soluție salină, diclofenac sodic 25 mg/kgc, DB 10 mg/kgc și, respectiv, DB 15 mg/kgc. S-au utilizat următoarele metode: i) edemul labei indus de caragenan, ii) hiperalgezie indusă de caragenan și iii) ELISA. DB în doze de 10 și 15 mg/kgc a inhibat semnificativ edemul labei după a 2-a, a 3-a și a 4-a oră de la injectarea caragenanului. Activitatea anti-hiperalgeică a fost observată la șobolanii tratați cu 10 și 15 mg/kgc DB numai după a 4-a oră de la administrarea caragenanului. Nivelurile de PGE2 au scăzut semnificativ în grupurile tratate cu DB în ambele doze. Rezultatele noastre indică faptul că DB posedă efecte antiinflamatorii și anti-hiperalgeice în modelele de inflamație exudativă și hiperalgezie induse de caragenan. Această activitate poate fi legată de scăderea nivelurilor de PGE2.

Keywords: denatonium benzoate, anti-inflammatory, anti-hyperalgesic, prostaglandin E2

Introduction

Inflammation is a protective response and an innate defence mechanism against potential harmful stimuli. The manifestations of inflammation include redness, heat, swelling and pain. Acute inflammation consists of two stages: (1) vascular and (2) cellular, which could be reproduced in experimental animals. The early stage could be induced by injection of histamine or serotonin. The most common agent used to evaluate

the second stage is carrageenan. However, carrageenan and kaolin could reproduce both phases of inflammation [17, 19].

The tissue damage unlocks the release of pro-inflammatory cytokines, chemokines, prostaglandins and many others from the injured cells and the infiltrating immune cells. These mediators exert their effects *via* binding to their respective receptors, some of which are also expressed on the peripheral terminals of nociceptors. This results in changes in the

activation thresholds of the nociceptors and increased sensitivity of the peripheral terminals of nociceptors to stimuli. This phenomenon is referred to as peripheral sensitization and contributes to inflammatory pain hypersensitivity or hyperalgesia. As a result, the response to mild painful stimuli is amplified, and pain could be evoked even by sensations such as pressure or warmth at the site of inflammation. In carrageenan-induced inflammation, hyperalgesia is initiated 1 hour after the application and lasts up to 6 hours [5, 10].

However, tissue injury triggers the release of mediators not only at the site of inflammation but also from the central terminals of nociceptors. Glutamate and substance P increase the production of PGE2 or pro-inflammatory cytokines in the spinal cord leading to additional excitation and disinhibition of dorsal horn neurons. This mechanism, termed central sensitization, also contributes to pain hypersensitivity and the generation of abnormal responses to sensory signals from the periphery. The physiological role of hypersensitivity is to protect injured tissues from further damage and reduce mechanical stress [4].

Many pain syndromes have an inflammatory origin. The biochemical mechanisms that determine the close link between pain and inflammation are based on the transformation of arachidonic acid to prostaglandin H2 (PGH2). That leads to the activation of prostaglandin synthases that convert PGH2 to prostaglandins and thromboxanes. PGE2, as a product of PGH2 conversion, possesses many controversial activities. It regulates the immune response by inducing pro- and anti-inflammatory reactions depending on the target cell and is one of the main players in pain [18, 22].

Based on the connection between pain and inflammation is logical to suggest that managing the inflammation influences the pain. Nonsteroidal anti-inflammatory drugs (NSAIDs) with an inhibitory effect on the enzyme cyclooxygenase (COX) and the following prostaglandin synthesis are used to reduce the inflammatory pain.

The bitter taste receptors (TAS2Rs) are G-protein receptors initially found on the tongue. Their location in other tissues has been revealed recently, which triggered intensive research in the field. TAS2Rs are detected on human airway smooth muscles, immune cells, the gastrointestinal tract and the human brain [8, 13]. Activation of TAS2Rs could lead to different effects depending on their localization.

Grassin-Delyle *et al.* reported that denatonium benzoate (DB), a bitter substance and agonist of TAS2Rs, reduces the cytokine release in human lung macrophages stimulated with lipopolysaccharide [8]. Our previous research showed its anti-inflammatory activity in histamine-induced rat paw oedema [25].

The aim of our study was to evaluate the anti-inflammatory effect of denatonium benzoate in the carrageenan-induced model of inflammation, the anti-

hyperalgesic activity of denatonium benzoate in the carrageenan-induced model of hyperalgesia, and the changes in plasma levels of PGE2.

Materials and Methods

Drugs and solutions

Denatonium benzoate and λ -carrageenan were purchased from Sigma. The solution for diclofenac sodium injection (Almiral[®]) was purchased from a pharmacy store. Carrageenan and denatonium benzoate were dissolved in saline.

Animals

Male Wistar rats with an average weight of 170 - 230 g were used. Animals were housed under standard laboratory conditions: temperature $22 \pm 1^\circ\text{C}$, humidity 45%, 12:12 hours light/dark cycle, food and water *ad libitum*. Approvals from the Bulgarian Food Safety Agency and the Ethics Committee of the Medical University – Plovdiv, Bulgaria were obtained before the experiments. The study was conducted according to the following guidelines: Universities Federation for Animal Welfare, the EU Directive 2010/63/EU for animal experiments and the relevant national and institutional rules and regulations. The groups used in the experiments are shown in Table I.

Table I

Groups used in the experiments and substances with which they are treated

Group	Substance
Control	Saline
GR1	Saline + λ -carrageenan
GR2	Diclofenac sodium 25 mg/kg bw + λ -carrageenan
GR3	DB 10 mg/kg bw + λ -carrageenan
GR4	DB 15 mg/kg bw + λ -carrageenan

Anti-inflammatory activity (Carrageenan-induced paw oedema)

Twenty-four male Wistar rats (weight 170 - 230 g) were divided into four groups ($n = 6$) and treated intraperitoneally as follows: 1st group (GR1) – treated with saline (0.1 mL/100 g bw), 2nd group (GR2) – treated with diclofenac sodium in a dose of 25 mg/kg bw, 3rd group (GR3) – treated with DB 10 mg/kg bw and 4th group (GR4) – treated with DB 15 mg/kg bw. The volume of each injection was 100 μL /100 g bw. One hour after the treatment, the animals received a subplantar injection of 100 μL of a 1% solution of λ -carrageenan in saline into the right hind paw (Table 1). Hind paw volume was measured immediately before carrageenan injection and at the 1st, 2nd, 3rd and 4th hour with a Plethysmometer (Ugo Basile, Gemonio, Italy).

The paw oedema was calculated according to the formula:

$$\text{Percentage of increase (\%)} = [(V_n - V_0)/V_0] \times 100,$$

V_n = the volume of the right hind paw registered after carrageenan injection at the n -th hour; V_0 = the

pain threshold in rats treated with diclofenac sodium when compared to the group GR1 at the third hour

of the experiment ($p < 0.05$), and this effect persisted to the fourth hour ($p < 0.01$) (Figure 2).

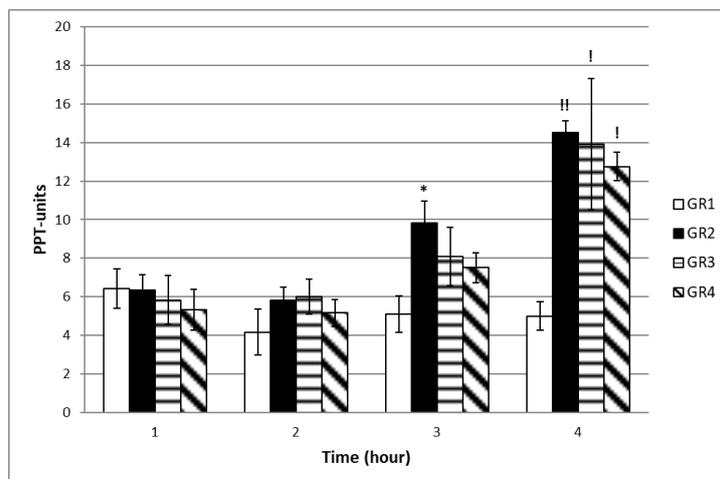


Figure 2.

Anti-hyperalgesic effect of denatonium benzoate in carrageenan-induced hyperalgesia and single-dose treatment.

* $p < 0.05$ compared to GR1 at 3rd hour; !! $p < 0.01$ compared to GR1 at 4th hour; ! $p < 0.05$ compared to GR1 at 4th hour

Plasma PGE2 levels after denatonium benzoate treatment

According to our results, the levels of PGE2 in the control group (saline-treated animals) were 350 ± 57 pg/mL. After induction of the pain by carrageenan injection, the levels of PGE2 in carrageenan treated animals (GR1) were 960 ± 130 pg/mL. Diclofenac sodium reduced the PGE2 levels in GR2 to 505 ± 120 pg/mL. After treatment with DB 10 mg/kg bw (GR3), the levels were 689 ± 180 pg/mL. The plasma levels of PGE2 in GR4 (rats treated with DB 15 mg/kg bw) were 618 ± 193 pg/mL. Significant differences

were observed between control group and all tested groups: GR1 ($p < 0.001$), GR2 ($p < 0.01$), GR3 ($p < 0.001$) and GR4 ($p < 0.001$). The levels of PGE2 significantly decreased in groups treated with diclofenac and DB in both doses compared to saline-treated animals GR1 ($p < 0.001$ for the three groups). A significant difference in PGE2 levels was observed between diclofenac (GR2) and DB 10 mg/kg bw treated group (GR3) ($p < 0.05$). However, there is no significant difference between diclofenac (GR2) and DB 15 mg/kg bw treated group GR4 (Figure 3).

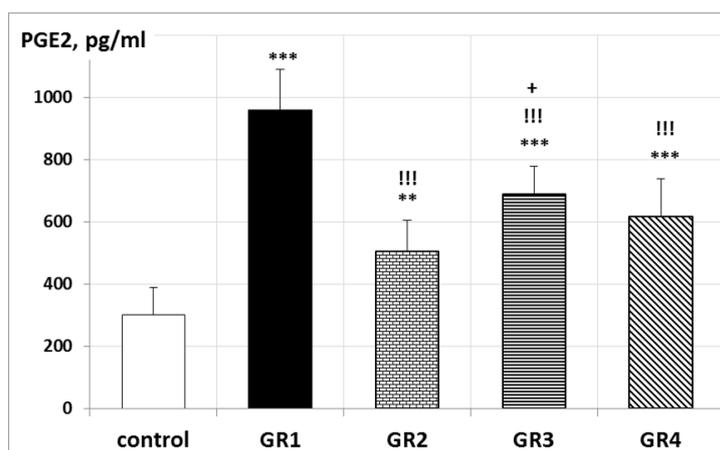


Figure 3.

Levels of PGE2 after denatonium benzoate treatment

*** $p < 0.001$ compared to control; ** $p < 0.01$ compared to control; !!! $p < 0.001$ compared to GR1; + $p < 0.05$ compared to GR2

Injection of carrageenan in the hind paw of rodents triggers the release of many pro-inflammatory mediators from cells engaged in the inflammatory response. The earliest derived mediators are histamine, serotonin and bradykinin. The process is followed by increased

PGs levels and migration of leukocytes to the inflamed tissues. The volume of the inflamed hind paw reaches its maximum approximately 3 h after the injection, followed by a slow decline. The initial phase (0 to 2.5 h of injection) is governed by the release of histamine

or serotonin. The second phase (measured 3h after carrageenan application) is related to the release of bradykinin, protease, prostaglandins [2, 5]. Our results show a well-defined anti-inflammatory effect of DB on the 2nd, 3rd and 4th hour after the carrageenan challenge, which clearly states its influence on the late phase of inflammation.

Therefore, we hypothesized that the inhibitory effect of DB on carrageenan-induced inflammation could be due to COX inhibition and the subsequent decrease in PGE2 synthesis. Our results show decreased levels of PGE2 in the peripheral blood of rats treated with DB, and the effect was dose dependent.

Carrageenan-induced animal model of acute inflammation is often used to evaluate the effect of NSAIDs and other substances, which inhibit COX activity on oedema formation and hyperalgesia. The enzyme plays a critical role in the synthesis of PGs [5]. The enzyme COX catalyses the biotransformation of arachidonic acid to prostanoids – lipid mediators that contribute to inflammatory pain. The most prominent influence on pain processing is credited to PGE2. Reduced production of prostanoids, mainly PGE2, by inhibiting COX-1 and/or COX-2 is considered the main mechanism of inflammatory pain suppression by NSAIDs. However, COX inhibitors could induce side effects on the gastrointestinal tract and kidneys, and the selective COX-2 inhibitors are associated with an increased cardiovascular risk [4, 9, 12].

Carrageenan injection in the paw triggers the release not only of pro-inflammatory mediators at the site of contact (the paw), but also in the spinal cord and other regions in the central nervous system (CNS). The increased production of PGE2 in the CNS increases inflammation and induces hyperalgesia. Selective COX-2 inhibitors reduce the levels of PGE2 in the cerebrospinal fluid and alleviate the hyperalgesia, while microinjection of PGE2 in the brain causes hyperalgesia. Peripheral oedema, neuron hyperexcitability and pain sensation are enhanced by increased spinal cord levels of PGE2 [9]. According to Ibuki *et al.*, thermal hyperalgesia was observed 1 hour after the carrageenan injection in the paw, and the condition persisted during the whole observation period of 6 h. The systemic application of COX-2-inhibitor completely abolished the increased sensitivity. However, the exact CNS regions on which PGE2 or other prostaglandins act to promote hyperalgesia remain unknown. The involvement of sites in the spinal cord, supraspinal CNS regions and medial preoptic area are proposed [10].

Pulkkinen *et al.* reported that treatment with denatonium does not evoke PGE2 release in the organ baths fluid, as assessed with enzyme immune analysis [20]. Our results showed decreased levels of PGE2 in the blood of rats treated with DB. A possible explanation of the difference between our *in vivo* results and the results obtained by Pulkkinen *et al.* *in vitro* lays in a

smaller amount of TAS2R receptors, expressed in the guinea pig aorta smooth muscle strips. The authors reported the expression of TAS2R4 and TAS2R10 receptors as targets of the activity of denatonium [20]. However, the TAS2R46 receptor has not been included in this study. The authors proposed that the smooth muscle relaxation induced by denatonium is related to TAS2R10 receptor activation due to the higher affinity of denatonium to this receptor. According to Meyerhof *et al.*, denatonium activates eight isoforms (4, 8, 10, 13, 39, 43, 46 and 47) of TAS2Rs in humans [16]. Based on this comparison, we propose that the effect of denatonium on the plasma levels of PGE2 is not related to the activation of TAS2R4 or TAS2R10 receptors. However, our results are performed in rats, where other isoforms of TAS2Rs (e.g. TAS2R46) are also expressed [15].

Moreover, TAS2R46 are also expressed on the surface of several types of cells and are involved in the inflammatory processes. The receptor was found in leucocytes, monocytes, neutrophils, etc. [3]. Based on this, we could propose that the anti-inflammatory activity of DB is a result of lowered levels of PGE2, and these effects may be related to the activation of TAS2R46 receptors.

The hypothesis of the involvement of TAS2R46 receptors in the anti-inflammatory and anti-hyperalgesic effects of denatonium is supported by the following results of the evaluation of the effects of several compounds of a natural origin, which also activate TAS2R46 receptors.

Brucine and strychnine are selective agonists of TAS2R10 and TAS2R46 receptors [16]. Denatonium also activates these receptors. Brucine has reduced the swelling in carrageenan-induced rat paw oedema (2nd hour) and the levels of PGE2 in the inflamed paw (4th hour) *vs.* controls. The authors proposed that the anti-inflammatory effect of brucine may be related to decreased synthesis of PG. However, the mechanism of this activity has not been revealed [24].

Another compound, artemorin, activates a broader wide of TAS2R (isoforms 4, 10, 14, 46 and 47) and has shown anti-inflammatory activity *in vitro* (cell culture of mouse macrophages - RAW 264.7). The mechanism of action also remains unknown [7, 16].

Anti-inflammatory and anti-hyperalgesic properties are reported also for parthenolide -a compound with natural origin with agonistic effect on the TAS2Rs (isoforms 4, 8, 10, 14, 44 and 46) [16, 23]. Moreover, Feltenstein *et al.* reported anti-inflammatory and anti-hyperalgesic effects of the compound in carrageenan-induced rat paw oedema [6].

Conclusions

The results of the present study indicate that denatonium benzoate decreases plasma levels of PGE2 and possesses an anti-inflammatory effect in the

carrageenan-induced model of exudative inflammation and an anti-hyperalgesic activity in the carrageenan-induced model of hyperalgesia.

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Conflict of interest

The authors declare no conflict of interest.

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