Original Research Article

Denatonium benzoate decreases the effect of histamine *in vitro* and in rats

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Abstract

Purpose: To evaluate the effect of denatonium benzoate (DB) in histamine-induced model of inflammation and the effect of the selective H1 receptor agonist (2-(2-Pyridyl) ethylamine) on rat gastric smooth muscle strips pretreated with DB.

Methods: The anti-inflammatory effect of DB was evaluated in vivo on histamine-induced rat paw edema. In vitro studies on spontaneous muscle contraction were performed on smooth muscle strips isolated from rat gastric corpus.

Results: The results showed a well-defined anti-inflammatory effect of DB (15 mg/kg) during the early stage of rat paw edema at the 15th ($p < 0.001$), 30th ($p < 0.01$) and 60th min ($p < 0.001$) compared to control. In vitro experiments indicated reduced spontaneous contractile activity of smooth muscle strips to H1 receptor agonist in the presence of DB (0.5 µM). The vascular effects of histamine are mediated by H1 receptors. Substances, which reduce the effect of histamine on the H1 receptors could influence the early stage of histamine-induced inflammation.

Conclusion: The results show that the anti-inflammatory activity of DB probably is related to its antagonistic activity on histamine H1 receptors. The results would contribute to the search for new anti-inflammatory drugs.

Keywords: Denatonium benzoate, Inflammation, Histamine, Muscle contraction

INTRODUCTION

Denatonium benzoate (DB) is a synthetic bitter compound used to activate bitter taste receptors (TAS2Rs) in different cell types. It is known as an agonist of eight subtypes of TAS2Rs - 4, 8, 10, 13, 30, 39, 43, and 46 [1,2]. The TAS2Rs are G-protein receptors initially found on the tongue.

Recently, their location in other tissues has been revealed, which triggered intensive research in the field. TAS2Rs are detected on human airway smooth muscles, immune cells, in the gastrointestinal tract, and the human brain [2,3], suggesting that their activation could lead to their participation in different physiological functions. Many gene variants of TAS1R-TAS2R are

related and correlate with the expression of genes associated with maintaining of the homeostasis. Its disturbance, caused by different stress inducers (diseases as diabetes, inflammatory or tumor processes), plays a role in premature human aging. A link has been found between the expression of some taste receptor genetic variants and longevity [4].

The number and subtypes of TAS2Rs vary widely across different species - humans have 25 TAS2Rs and rodents 35 TAS2Rs [5]. Furthermore, activation of TAS2Rs results in a different effect depending on the concentration of the bitter compound, the type of the tissue and the region of the tissue. For example, in smooth muscles, TAS2R agonists evoked concentration-dependent contractility changes in mouse gastric smooth muscles [6]. Regarding the skeletal muscles, our previous research showed that DB decreased the force of rat abdominal muscle contractions [7].

TAS2Rs are also expressed in human blood leukocytes [8,9] and some researchers have described the anti-inflammatory properties of the TAS2R agonists [8]. DB reduces lipopoly-saccharide-induced pro-inflammatory cytokine secretion by human lung macrophages [2].

The fact that the anti-inflammatory activity of DB and its molecular mechanisms are still not clear, indicates that there are not enough studies conducted on the effect of this agonist on the early phase of inflammation.

Histamine, a biogenic amine, is the main regulator of acute inflammation and hypersensitive allergic reaction. It is also involved in the regulation of important physiological processes such as cell proliferation and differentiation, hematopoiesis and tissue regeneration [10]. It is released from storage granules in mast cells as part of the allergic response to tissue damage and/or an antigen. The different effects of histamine are mediated by four types of histamine receptors (H1, H2, H3, and H4) that belong to the superfamily of G-protein coupled receptors [11].

As the main signal transducers, G proteins transmit the extrinsic stimuli inside the cells. Histamine H1 receptor is expressed in different cell types, like neurons, endothelial cells, muscle cells, hepatocytes, chondrocytes, monocytes, neutrophils, eosinophils, DCs, T and B lymphocytes etc. [12]. Its activation is linked not only with pro-inflammatory gene expression but also with smooth muscle relaxation.

Based on a brief review of the literature it was hypothesized that DB influences on histamine H1 receptors. Therefore, the aim of the study was to evaluate the effect of DB on histamine-induced rat paw inflammation *in vivo* and to examine the effect of the selective H1 receptor agonist (2-(2-Pyridyl) ethylamine) *in vitro* on rat gastric smooth muscle strips pretreated with DB.

EXPERIMENTAL

Drugs and solutions

Denatonium benzoate, histamine dihydrochlo-ride, acetylcholine and 2-(2-Pyridyl) ethylamine were purchased from Sigma. Solution for injection of diclofenac sodium (Almiral®) was purchased from a pharmacy store.

The composition of the preparation solution was as follows: Na^+ (143); K^+ (5.84) and Ca^{2+} (3.7 mmol/L), while the composition of modified Krebs' solution (KS) was: Na^+ - 143; K^+ - 5.84; Ca^{2+} - 2.5; Mg^{2+} - 1.19; Cl^- - 133; HCO_3^- - 16.7; $H_2PO_4^-$ - 1.2 and glucose - 11.5 mmol/L.

Animals

Male Wistar rats (175 – 230 g) were kept under standard laboratory conditions (temperature 22 ± 1 °C, humidity 45 %, 12-h dark/light cycle, food and water *ad libitum*).

Approvals from the Bulgarian Food Safety Agency (permit no. 252/22.11.2019) and Ethics Committee of the Medical University - Plovdiv, Bulgaria (protocol no. 1/13.02.2020) were obtained before the experiments. The study was conducted according to the International Council for Ethical Guidelines for Animal Breeding Labs for Researchers, ARRIVE, and the EU Directive 2010/63/EU for animal experiments [13,14].

In vivo evaluation of the effect of DB in histamine-induced inflammation

Twenty-four male Wistar rats (175 – 230 g) were divided into four groups (n = 6) and treated intraperitoneally as follows: 1-st group (controls) – treated with saline (0.1 ml/100 g bw), 2-nd group – treated with diclofenac in a dose of 25 mg/kg bw, 3-rd group – treated with DB 10 mg/kg bw (dissolved in saline), and 4-th group – 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 subplantar injection of 100 μL of a 0.1 % solution of histamine in saline into the right paw [15]. Before the injection of histamine and 5, 15, 30, 60, 90 and 120 min after the treatment the anti-

inflammatory effect was studied using Plethysmometer (UgoBasile, Gemonio, Italy) as described previously [16].

Change in paw -volume (P) was calculated as in Eq 1.

$$P\ (\%) = \{(V_n - V_0)/V_0\}100 \ldots\ldots (1)$$

where $P\ (\%)$ = increase in paw volume, V_n = the volume of the right hind paw registered after histamine injection at the n-th minute; and V_0 = the volume of the right hind paw registered for the same animal before histamine injection.

In vitro study on the effect of DB on rat smooth muscle contractility

Isolation of smooth muscle strips

Male Wistar rats weighing 175 – 230 g were euthanized and the smooth muscle preparations without mucosa were isolated. Preparations were obtained while cutting the muscle tissue into strips (20.0 ± 1.5 mm length, 3.0 ± 0.5 mm width). The samples were immediately rinsed in cooled (4 °C) preparation solution. They were randomly allocated and isometrically fixed in individual organ baths prefilled with 15 mL modified Krebs' solution (KS) with temperature 35.5 ± 0.25 °C.

The isolated strips were connected to an isometric force transducer (TRI 201, LSi LETICA; Pnlab S.L., Barcelona, Spain) and constantly oxygenated with 95 % O_2 and 5 % CO_2. Tension of 7 mN was applied to achieve isometrical recording. The muscle strips were allowed an equilibration period of 20 min before the recording of contractile activity [17].

Concentration-response curve for DB

The normal contractile activity was recorded after the equilibration period. Baseline tone (BT), frequency, mean amplitude (Amean) and area under the curve (AUC) were analyzed. The concentration-effect curve was obtained based on experiments on the following concentrations of DB: 0.03, 0.1, 0.3, 0.5, 1, 3, 7, 10, 30 and 60 µM. DB was added to the organ baths and the change in the spontaneous contractile activity was recorded for a 5-min period. The strips were washed out with KS before adding a higher concentration. The cut-off time for each experiment was 45 minutes after muscle isolation.

Effect of 2-(2-Pyridyl) ethylamine on spontaneous smooth muscle contraction of strips before and after treatment with DB

The normal contractile activity was recorded as described previously [18]. Briefly, BT, frequency, Amean and AUC were analyzed for a 5-min period (baseline period). The data was used for further comparative analysis.

2-(2-Pyridyl) ethylamine (1 µM) was added to the organ baths and changes in spontaneous activity were recorded for 5 min. The strips were washed out with KS and 0.5 µM DB was added to the organ baths. After 30 min, in the presence of DB, 1 µM 2-(2-Pyridyl) ethylamine was added to the organ baths. The aforementioned parameters (BT, frequency, Amean and AUC) were analyzed again for a 5-min period.

For each test, seven preparations from different animals were used. Each parameter was presented as a relative increment corresponding to the baseline period data.

Statistics

Statistical analysis was performed using SPSS 17.0. The normal distribution was evaluated with One-sample Kolmogorov-Smirnov test. In case of normal distribution, One-way ANOVA and Bonferroni post hoc test were employed for further analysis. Data, which did not show normal distribution, were analyzed using non-parametric Wilcoxon signed rank test.

The results of *in vivo* experiments are reported as mean ± SEM, and those of *in vitro* evaluations are presented as mean, 25 and 75 % percentiles. The number of tested preparations is given as *n*. Results were considered significant at $p < 0.05$.

RESULTS

Effect of DB on histamine-induced rat paw edema

The results showed a reduction in paw volume at the 15-th minute after histamine injection in the group treated with lower dose of DB (10 mg/kg) when compared to rats treated with diclofenac (31.5 ± 2.9 vs 53.5 ± 4.1%; p < 0.05), as shown in Figure 1.

The paw volume of rats treated with higher dose DB (15 mg/kg bw) was significantly lower than control rats (11.9 ± 6.3 vs 51.9 ± 6.1%; *p* < 0.001) and rats, treated with diclofenac (11.9 ± 6.3 vs 53.5 ± 4.1%; *p* < 0.001).
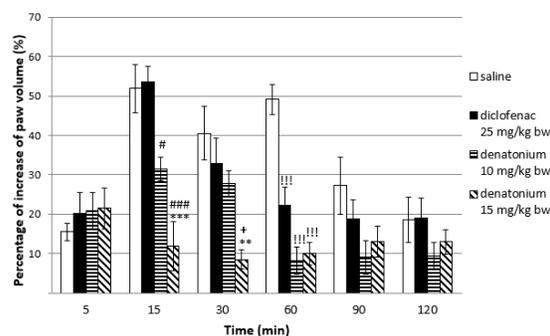
Figure 1: Anti-inflammatory effect of denatonium benzoate in histamine-induced paw edema after single administration. ***$p < 0.001$ compared to saline at 15 min; #$p < 0.05$ compared to diclofenac at 15 min; ###$p < 0.001$ compared to diclofenac at 15 min; **$p < 0.01$ compared to saline at 30 min; +$p < 0.05$ compared to diclofenac at 30 min; !!!$p < 0.001$ compared to saline at 60 min

The results showed a well-defined anti-inflammatory effect of DB in a dose of 15 mg/kg that persisted at the 30 minute after histamine application when a significant decrease was observed in comparison to the groups treated with saline (8.5 ± 2.3 vs 40.5 ± 6.8 %; $p < 0.01$) and diclofenac (8.5 ± 2.3 vs 32.9 ± 6.4 %; $p < 0.05$). One hour after the histamine injection, all tested substances showed eminent antiphlogistic effect. Comparing the paw volume change, a significant decrease was detected in the groups treated with diclofenac (22.2 ± 4.8 vs 49.2 ± 3.8 %; $p < 0.001$), DB 10 mg/kg (8.2 ± 3.5 vs 49.2 ± 3.8 %; $p < 0.001$), and DB 15 mg/kg (10.0 ± 2.9 vs 49.2 ± 3.8 %; $p < 0.001$) in comparison to controls.

Concentration-response curve for denatonium benzoate (DB)

Figure 2 represents the concentration-response curve for DB. The maximal muscle contraction induced by the addition of 10 µM acetylcholine to the organ baths was taken as 100 %.

Figure 3 shows the effect of 0.5 µM DB on the spontaneous contractility of smooth muscle strips isolated from rat gastric corpus.

In vitro effect of DB on spontaneous smooth muscle contraction of strips treated with H1 agonist

When added to organ baths containing solely KS, 2-(2-Pyridyl) ethylamine evoked a statistically significant increase in AUC, the amplitude, and BT. These parameters underwent changes when DB was present in the organ baths, as shown in Fig.4. The mean AUC was

reduced from 1.92 (1.79 – 1.96) to 1.42 (1.38 – 1.48) before and after addition of 0.5 µM DB ($p < 0.05$; $n = 7$). The Amean of the contraction was also diminished (1.82 (1.77 – 1,88) vs 1.39 (1.35 – 1.44); $p < 0.05$; $n = 7$), respectively. The BT also declined when 2-(2-Pyridyl) ethylamine was added to organ baths, containing KS and 0.5 µM DB (1.53 (1.42 – 1.64) in comparison to KS (1.97 (1.95 – 1.99); $p < 0.05$; $n = 7$; Wilcoxon signed rank test). No change on the phasic contraction frequency was observed.
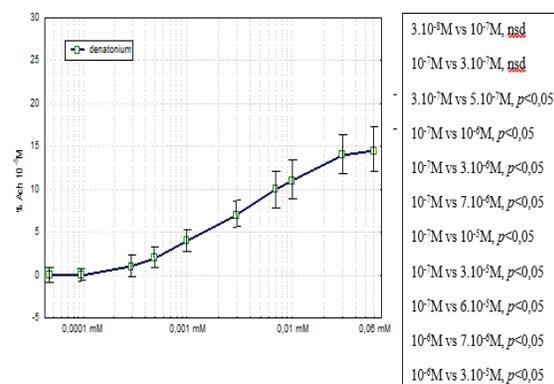

Figure 2: Left- Cumulative concentration-response curve of 3.10^{-8} M ÷ 6.10^{-5} M denatonium benzoate on the spontaneous contractility of smooth muscle strips isolated from rat gastric corpus. Results are presented as a percentage of the reaction of 10^{-5} M acetylcholine. Each point is calculated as mean ± SEM of seven preparations. Right- statistical analysis (Repeated Measures ANOVA and Bonferroni's Multiple Comparison Test); nsd- no statistically significant difference
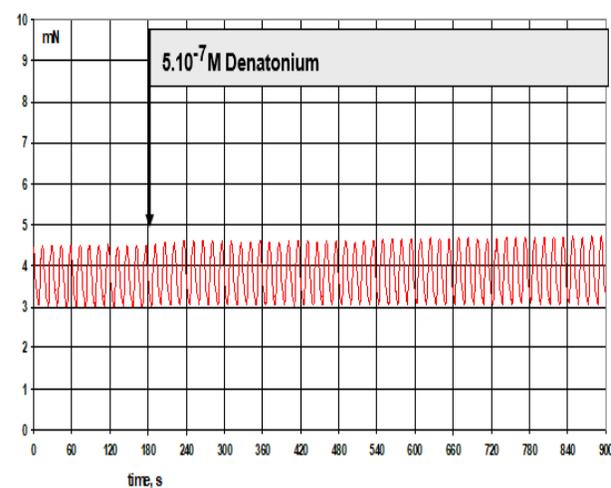

Figure 3: Change in the spontaneous contractility of smooth muscle strips isolated from rat gastric corpus under the influence of 5.10^{-7} M denatonium ($n = 7$). Arrow indicates the initial point of denatonium benzoate-treatment
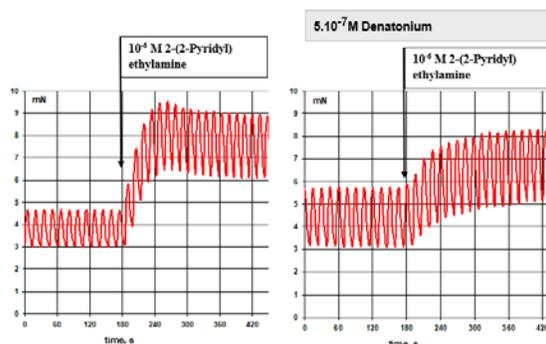
Figure 4: Change in the spontaneous contractility of smooth muscle strips isolated from rat gastric corpus under the influence of 10^{-6} M 2-(2-Pyridyl) ethylamine (*n*=7). 2-(2-Pyridyl) ethylamine (10^{-6} M) was added to the organ baths before (left) and after (right) 30 min of treatment of the strips with 5.10^{-7} M denatonium. Arrows indicate the initial point of 2-(2-Pyridyl) ethylamine treatment

DISCUSSION

The results obtained from the histamine model of inflammation confirm the anti-inflammatory activity of DB which is demonstrated in several studies [2,8,19]. The mechanism of this effect is not fully understood.

DB is an agonist of TAS2Rs and some of these receptors (TAS2R4, TAS2R10 and TAS2R46) are found on the surface of mast cells. Other results showed significant inhibition of histamine and PGD_2 release from IgE-receptor–activated primary human mast cells [19]. Orsmark *et al* detected the same subtypes in human leukocytes, including B and T-lymphocytes and suggested that DB could possess anti-inflammatory and bronchodilator activity [8]. The presence of TAS2Rs in human lung macrophages and reduced LPS-induced cytokine production after DB- treatment has been reported [2].

During the process of acute inflammation, three phases could be distinguished and they are regulated by different mediators. The processes in the early phase (0 to 15 min) are related to the release of histamine, serotonin, and bradykinin [20]. The study confirmed that histamine injection in the rat paw results in vasodilation and increased vascular permeability due to activation of the endothelial H1 receptors [21]. The results allow us to postulate that the effect of DB on the early stage of inflammation could be related to inhibition of synthesis, release or action of histamine. The vascular effects of histamine are mediated by endothelial H1 receptors and a possible explanation of the observed anti-inflammatory effect of DB would be that the substance reduces the effect of histamine on the H1 receptors. To test this hypothesis, *in vitro* experiments to reveal the influence of DB on the histamine-H1 receptors were performed.

In the *in vitro* studies, the concentration of 0.5 µM was the lowest concentration of DB in which a statistically significant contractile effect was observed.

Avau *et al* report that the effect of DB on gastric smooth muscle contractility is concentration and region-dependent. Increasing concentrations of DB increases the contractility and the effect reaches its peak at 100 µM. Higher concentrations of DB (1 mM) induce smooth muscle relaxation [6]. Concentrations, ranging from 1 µM to 100 µM, evoke concentration-dependent contraction of smooth muscle strips isolated from gastric fundus and antrum of rodents. The results could be explained by the activation of TAS2Rs, given the abundant presence of different TAS2Rs subtypes in the fundus and the antrum [6,22,23]. Based on these results, we performed our experiments on smooth muscle strips isolated from the gastric corpus. We presumed that in this zone of the rat stomach the expression of TAS2Rs is poor. To avoid the influence of the substance on the bitter taste receptors and a cumulative contractile effect of TAS2Rs and H1 receptor activation, we performed the experiments using the concentration of 0.5 µM DB. This is the lowest concentration of DB which induces the minimal statistically significant contractile effect. Thus, the strong contractile effect of TAS2Rs observed at higher concentrations of DB is avoided and this allows us to study the effect of DB on other receptor type.

Figure 3 demonstrates the efficiency of the selected model used in the study, allowing the calculation of DB concentration (0.5 µM), which induces acceptable contractile effect.

The observed effects after treatment with DB and H1 agonist are unlikely to be related to bitter taste receptors activation and the changes in the contractility are dominated by histamine receptors. Thus, after the addition of H1 agonist, we could study the effect of DB on histamine receptors.

The smooth muscle response to H1 receptor agonist was reduced in the presence of DB (Fig.4). The result allows us to postulate a possible effect of DB on the histamine receptors. This hypothesis could also explain the observed anti-inflammatory effect of DB during the early stage of histamine-induced rat paw edema. The

vascular effects of histamine are mediated by endothelial histamine-H1 receptors [21] and substances, which block these receptors, could influence the early phase of histamine-induced inflammation.

CONCLUSION

Intraperitoneal injection of denatonium benzoate (DB) attenuates the initial phase of histamine-induced paw inflammation in rats, and also decreases the response of smooth muscle strips to histamine-H1 receptor agonist. The reduced effect of histamine on histamine-H1 receptors likely contributes to the anti-inflammatory activity of DB in rat paw edema. The results constitute a strong basis for the search for new anti-inflammatory drugs.

DECLARATIONS

Acknowledgement

Conflict of interest

No conflict of interest is associated with this research.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Plamen Zagorchev conceived and designed the study, collected and analyzed the data, and supervised the study. Vesela Kokova participated in the study design, the data collection, the statistical analysis and the writing of the manuscript. Elisaveta Apostolova participated in the design of the study, collected and analyzed the data, performed the statistical analysis and wrote the manuscript. Milena Draganova-Filipova participated in the writing of the manuscript. All authors read the article and approved the final version of the manuscript.

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