Effect of probenecid on the pain-related behaviour and morphological markers in orofacial formalin test of the rat

Running title: Probenecid in orofacial formalin test

Annamária Fejes-Szabó M.Sc.[1], Zsuzsanna Bohár M.Sc.[1,2], **Gábor Nagy-Grócz M.Sc.[1]**, Enikő Vámos M.Sc., Ph.D.[1], Lilla Tar M.D.[1], Borbála Pődör M.Sc.[3], János Tajti M.D., Ph.D.[1], József Toldi M.Sc., Ph.D., D.Sc.[2,4], László Vécsei M.D., Ph.D., D.Sc.[1,2,#,]* and Árpád Párdutz M.D., Ph.D.[1,#]

[1] Department of Neurology, Faculty of Medicine, Albert Szent-Györgyi Clinical Centre, University of Szeged, Semmelweis u. 6., Szeged, H-6725, Hungary

[2] MTA-SZTE Neuroscience Research Group, Semmelweis u. 6., Szeged, H-6725, Hungary

[3] Department of Physiology & Biophysics, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, B3H 1X5, Canada

[4] Department of Physiology, Anatomy and Neuroscience, University of Szeged, Közép fasor 52., Szeged, H-6726, Hungary

[#] These authors contributed equally to this work.

* Corresponding author:

László Vécsei M.D., Ph.D., D.Sc.

Department of Neurology, Faculty of Medicine, Albert Szent-Györgyi Clinical Centre, University of Szeged, Hungary

Semmelweis u. 6, H-6725 Szeged, Hungary

Tel.: +36 62 545351

Fax: +36 62 545597

E-mail: vecsei.laszlo@med.u-szeged.hu

Abstract

Probenecid has been widely used in the treatment of gout, but evidence suggest that **it may also have** anti-nociceptive effect**s** in different inflammatory and pain conditions. **We** examined the potential modulatory effect**s** of probenecid on **behavioural and morphological markers** in the orofacial formalin test of the rat.

One hour after pre-treatment with vehicle or probenecid (1 mmol/kg body weight) intraperitoneally, 50μl 1.5% formalin solution or physiological saline was injected subcutaneously into the right **whisker pad** of rats. The rubbing activity directed to the injected whisker pad was then measured for a period of 45 minutes. Four hours after formalin injection, the caudal part of spinal trigeminal nucleus was removed and subjected to c-Fos **and neuronal nitric oxide synthase (nNOS)** immunohistochemistry **and to interleukin-1β and NAD(P)H:quinone oxidoreductase 1 (NQO1) Western blot.**

There was a significant decrease in formalin-induced biphasic behavioural response and c-Fos and nNOS immunoreactivity in the rats who were pre-treated with probenecid. However there were no alterations in expression of interleukin-1β or NQO1 after formalin administration.

Our results suggest that probenecid has an anti-nociceptive effect in **the** trigeminal inflammatory pain model. **This effect may be through influencing the release of prostaglandin E2 or desensitizing the transient receptor potential channel subtype A member 1 or the transient receptor potential channel subtype V member 2 or the effect may be through modulating kynurenic acid levels in the central nervous system.** Thus, probenecid might be a potential candidate for the treatment of trigeminal activation related pain conditions.

Key words: caudal part of spinal trigeminal nucleus; c-Fos; **interleukin-1β; NAD(P)H:quinone oxidoreductase 1; neuronal nitric oxide synthase;** orofacial formalin test; probenecid-

Introduction

Probenecid (PROB, *p*-(di-*n*-propylsulfamyl)benzoic acid) has primarily been used to enhance the renal retention of penicillin and to treat gout, due to its ability to increase the renal excretion of uric acid . Besides its effects on the kidney, **research data suggest that PROB may have anti-inflammatory and anti-nociceptive properties as well.** PROB blocks the multidrug resistance-associated protein 4 (MRP4), an organic anion transporter , which is crucial in the release of key inflammatory mediators such as prostaglandin E1 and prostaglandin E2 (PGE2) . In addition, PROB inhibits the pannexin-1 channel , which may be relevant in neuronal inflammatory processes . On the other hand, PROB **is an agonist of** the transient receptor potential channel subtype A member 1 (TRPA1) and the transient receptor potential channel subtype V member 2 (TRPV2) **, which play an important role in pain sensation . It is also well known, that an agonist-mediated desensitization of these TRPA1 and TRPV2 can be induced , leading to analgesia. These results strongly suggest that PROB might be effective in mitigating inflammation and nociception. In contrast, Bang and co-workers raise the possibility that PROB can elicit the pain induced by inflammation through its agonism on TRPV2 .**

Fundamental research and pharmacological studies investigating the trigeminal inflammation and nociception often use the orofacial formalin test, a reliable model for studying somatic pain causing the activation and sensitization of trigeminal system , which is crucial in the pathomechanism **of headaches such as** migraine - one of the **most common** neurological disorders . Formalin solution administered subcutaneously into the upper lip gives rise to a tissue injury, nociception and inflammation , activating the primary nociceptors, enhancing the immunoreactivity of c-Fos, a marker of neuronal activation in the caudal part of spinal trigeminal nucleus (TNC) and resulting **in an increase in neuronal**

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nitric oxide synthase (nNOS), which is a suggested marker of central sensitization . The induction of formalin causes a biphasic nociceptive behavioural response consisting of rubbing acitivity during an early and short-lasting (3–4 min) first phase of rubbing is followed by **a tonic** prolonged (20–22 min) **second** phase, the phases being separated by a 9–10 min quiescent period . The first phase is thought to be due to the direct chemical stimulation of the nociceptors by the formalin solution, while **the second phase is a manifestation of the** processes induced in the spinal cord during the first phase, together with peripheral inflammation . **The inflammatory mediators such as PGE2 and interleukin-1β (IL-1 β) and the oxidative stress may have important role in the formation of changes induced by subcutaneous (s.c.) formalin injection .**

Based on previous experimental results, it has been proposed that PROB, through its different receptorial actions, may have anti-nociceptive effect that can be seen in the orofacial formalin test. Thus the aim of the current study was to investigate whether PROB exerts **a modulatory** effect in this tonic trigeminal pain model through the examination of behavioural and morphological markers.

Materials and Methods

1. Animals:

The procedures used in this study followed the guidelines of the eighth edition of the Guide for the Care and Use of Laboratory Animals and the Use of Animals in Research of the International Association for the Study of Pain and the directive of the European Economic Community (86/609/ECC). They were approved by the Committee of Animal Research at the University of Szeged (I-74-12/2012) and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XXIV./352/2012.). **Sixty** adult male Sprague-Dawley rats weighing 200-250 g were used. The animals were raised and housed under standard laboratory conditions (in an air-conditioned, humidity-controlled and ventilated room), with drinking water and regular rat chow available *ad libitum* on a **12h-12h** dark-light cycle. Each rat was used only once.

2. Drug administration:

The rats were divided into two groups (n=**30** per group). The animals in the Placebo group received only intraperitoneal vehicle solution (physiological saline, 1.5 ml) as pre-treatment. In the PROB group, the rats were pretreated with an intraperitoneal injection of PROB (Sigma-Aldrich; 1 mmol/kg body weight, diluted to 1.5 ml, pH 7.4). Both groups of animals were divided further into two subgroups (n=1**5**): 1 h**our** after the PROB or vehicle pre-treatment, half of the animals received a s.c. injection of 50 µl 1.5% formalin solution (0.55% formaldehyde, Sigma-Aldrich) diluted in physiological saline, administered *via* a 26-gauge needle into the right **whisker pad** (Placebo-Form and PROB-Form), while the other half of

the rats were injected with s.c. 50 µl physiological saline without formalin (Placebo-Phys and PROB-Phys).

3. Behavioural test:

The testing procedures were performed during the light phase (between 8 a.m. and 2 p.m.) in a quiet room. The test box was a 30 x 30 x 30 cm glass terrarium with mirrored walls. For the off-line analysis of rubbing activity directed to the whisker pad, the behaviour of the individually tested rats was recorded with a video camera (Logitech HD Webcam C615) situated 1 m above the terrarium. **One hour after pre-treatment with vehicle (n=20) or PROB (n=20) and** after a 10-min habituation in the test box, the **whisker pads** of the **rats were** injected with s.c. formalin **(n=10 per subgroup)** or physiological saline **(n=10 per subgroup)** and the animal**s were** replaced immediately **back** in the chamber for 45 min. The rats did not receive any food or water during the observation period. The test box was cleaned and decontaminated after each animal. An observer blind to the experimental procedures analysed the recorded videos. The 45-min recording period was divided into 15 x 3-min blocks and the total time (number of seconds) spent on rubbing the injected area with the ipsilateral fore- or hindpaw was measured in each block and defined as the nociceptive score for that block. Earlier literature findings led us to use the grooming activity as control, measured in animals that received s.c. physiological saline .

*4. c-Fos **and nNOS** immunohistochemistry:*

Four **hours** after the formalin **or physiological saline** injection, the rats **(n=5-9 per subgroup)** were perfused transcardially with 100 ml phosphate-buffered saline (PBS), followed by 500 ml 4% paraformaldehyde (Merck) in phosphate buffer under deep chloral

hydrate (**0.4 g/kg body weight**, Sigma-Aldrich) anaesthesia. The medullary segment **containing** the TNC between +1 and -5 mm from the obex was removed, postfixed overnight for immunohistochemistry in the same fixative and cryoprotected (10% sucrose for 2 h, 20% sucrose until the blocks sank, and 30% sucrose overnight). Before sectioning, each **segment** was marked with a small incision on the ventral and left (contralateral) side of the tissue block, allowing side discrimination during the quantification process. 30-μm transverse cryostat sections were cut through the rostrocaudal axis from the beginning of the TNC **and were** serially collected in wells containing cold PBS. Each well contained **every tenth** section at 0.3-mm intervals along the rostrocaudal axis (15 levels/**sections**). The free-floating sections were rinsed in PBS and immersed in 0.3% H_2O_2 (Scharlau Chemie) in PBS for 30 min to suppress endogenous peroxidase activity. After several rinses in PBS containing 1% Triton X-100 (PBST; VWR International), sections were incubated at room temperature overnight in PBST containing rabbit anti-rat c-Fos polyclonal antibody (Santa Cruz Biotechnology, sc-52) at a dilution of 1:2000 **or at 4°C for two nights in PBST containing rabbit anti-rat nNOS polyclonal antibody (EuroProxima, B220-1) at a dilution of 1:5,000.** The immunohistochemical reaction was visualized by using Vectastain Elite avidin-biotin kits (Vector Laboratories, PK6101). Briefly, the sections were incubated at room temperature for 2 h**ours** in PBST containing goat anti-rabbit biotinylated secondary antibody. After several rinses in PBST, and incubation at room temperature for 2 h**ours** in PBST containing avidin and biotinylated horseradish peroxidase, the sections were stained with 3,3'-diaminobenzidine (Sigma-Aldrich) intensified with nickel ammonium sulphate (Scharlau Chemie). The specificity of the immune reactions was checked by omitting the primary antiserum.

The counting of immunoreactive (IR) cells **in the TNC** was performed by an observer blind to the experimental procedures **under the 10x objective of a Nikon Optiphot-2 light**

microscope in every tenth transverse section in each animal. Before the counting, the location of each section along the rostrocaudal axis and the location of the TNC on each medullary section were determined by means of The Rat Brain in Stereotaxic Coordinates Atlas . **The c-Fos neurones with obvious specific nuclear staining and the nNOS-IR neurones with cytoplasmic and dendritic staining and a nucleus were taken into consideration and** were counted in the TNC both ipsilaterally and contralaterally to the formalin **or physiological saline** injection.

5. IL-1β and NAD(P)H:quinone oxidoreductase 1 (NQO1) Western blot:

Four hours after the formalin or physiological saline injection, the rats (n=5 per subgroup) were deeply anaesthetized with chloral hydrate as described above, perfused transcardially with 100 ml ice cold PBS and the ipsi- and contralateral side of the TNC between 0 and -4 mm from the obex was removed. Until measurements, the samples were stored at −80°C. The TNC segments were sonicated in ice cold lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% igepal, 0.1% cholic acid, 2 μg/ml leupeptin, 2 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml pepstatin, 2 mM EDTA and 0.1% sodium dodecyl sulphate (SDS) (all chemicals were from Sigma-Aldrich). The lysates were cleared from cellular debris by centrifugation at 12,000 RPM for 10 min at 4°C and supernatants were aliquoted and stored at -20°C. Protein concentration was measured according to BCA protein assay method with BCA Protein Assay Kit (Novagen) using bovine serum albumin as a standard. Samples were cooled on ice during the whole procedure. Prior to loading into the gels, each sample was mixed with sample buffer, and denatured by boiling for 3 min. Equal amounts of protein samples (20 μg/lane) were separated by standard SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 12% Tris–Glycine gel and electrotransferred onto

Amersham Hybond-ECL nitrocellulose membrane (0.45 μm pore size, GE Healthcare). The Page Ruler Prestained Protein Ladder (Fermentas, 10-170 kDa) was used to determine approximate molecular weights. Following the transfer, membranes were blocked for one hour at room temperature in Tris-buffered saline containing Tween 20 (TBST, MP Biomedicals) and 5% non fat dry milk powder and then incubated in TBST containing 1% non fat dry milk and (i) rabbit anti-rat IL-1β (H-153) polyclonal antibody (Santa Cruz Biotechnology, sc-7884, dilution: 1:200, incubation: overnight at 4°C) or (ii) rabbit anti-rat NQO1 (H-90) polyclonal antibody (Santa Cruz Biotechnology, sc-25591, dilution: 1:200, incubation: overnight at 4°C) or (iii) mouse anti-rat β-actin monoclonal antibody (Calbiochem, CP01, dilution: 1:100,000, incubation: overnight at 4°C). Next day after several rinses in TBST, membranes were incubated in TBST containing 1% non fat dry milk and horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology; sc-2030 and sc-2031) for 2 hours at room temperature. Protein bands were visualized after incubation of membranes with the SuperSignal West Pico Chemiluminescent Substrate (Pierce) using Carestream Kodak BioMax Light film (Kodak).

For densitometric analyses, films were scanned and quantified using Java ImageJ 1.47v analysis software (National Institutes of Health). The β-actin was used as sample loading control and normalization protein as well. The results from each animal were further normalized to its own contralateral side.

6. Statistical analysis:

In the behavioural study, comparisons were made of the rubbing activity in each block of 3-min in each of the subgroups (Placebo-Form, Placebo-Phys, PROB-Form and PROB-Phys) by using two-way repeated measures ANOVA. Subgroups were used as the between-subject

factor and 15 blocks of 3-min as the within-subject factor for the analysis. When Mauchly's test of sphericity proved significant, the Greenhouse-Geisser correction was performed. Pairwise comparisons of subgroup means were based on the estimated marginal means with Sidak adjustment for multiple comparisons.

To compare statistical differences between subgroups in the first and second phases, the two phases were determined on the basis of significant results detected during two-way repeated measures ANOVA **described above**. The first phase was found to be the first of the 15 blocks, while the second phase was the sum of the fifth to the eleventh blocks. To determine significant differences between subgroups in the first or second phase, we used one-way ANOVA followed by the Tamhane *post hoc* test.

In terms of the immunoreactivity, the numbers of IR neurones in the various subgroups (Placebo-Form, Placebo-Phys, PROB-Form and PROB-Phys) were compared at each level of 0.3-mm (15 levels) along the rostrocaudal axis by using two-way repeated measures ANOVA. Since there was no significant difference in the number of c-Fos- **and nNOS**-IR neurones between the contralateral sides in the subgroups injected with **s.c.** formalin and the contra- and ipsilateral sides in the subgroups injected with **s.c.** physiological saline (data not shown), in the course of the statistical analysis the data obtained from the contralateral sides of the subgroups injected with **s.c.** formalin were used as controls. The contra- and ipsilateral sides of the formalin-injected subgroups were used as the between-subject factor, and the 15 levels along the rostrocaudal axis as the within-subject factor for the analysis. When Mauchly's test of sphericity proved significant, the Greenhouse-Geisser correction was performed. Pairwise comparisons of subgroup means were based on the estimated marginal means with Sidak adjustment for multiple comparisons.

Statistical analysis of the Western blot data was carried out using one-way ANOVA followed by the Sidak *post hoc* test. Since there was no significant difference in band

densities between the contralateral sides in the subgroups injected with s.c. formalin and the contra- and ipsilateral sides in the subgroups injected with s.c. physiological saline (data not shown), in the course of the statistical analysis the data obtained from the contralateral sides of the subgroups injected with formalin were used as controls.

All tests were two-sided, and probability levels $p<0.05$ were considered to be statistically significant. Group values are reported as means ± S.E.M.

Statistical analysis of measurements was carried out with IBM SPSS Statistics, version 20 (IBM Corporation) software.

Results

1. Nociceptive response

The **behavioural pattern observed in the rats** is in accordance with previous findings . After the formalin injection, the rats immediately withdrew their heads, often accompanied by vocalization. Following their return to the observation box, the rats started to rub their whisker pad continuously and intensely with the ipsilateral forepaw **accompanied** often **by** the contralateral forepaw, and occasionally scraped the perinasal area with the ipsilateral hindpaw after a period of ~20 s**ec**. This period, referred to as the first phase, lasted ~3-4 min, and was followed by a quiescent period of 9-10 min, separating the first phase from the second phase. The second phase was characterized by less intense, but continuous rubbing of the face, predominantly with the ipsilateral forepaw **consorted** often **by** the contralateral forepaw as well. This tonic phase lasted ~20-22 min. In the PROB-Form subgroup, the behavioural pattern in both phases was clearly less pronounced than that in the Placebo-Form subgroup, and such behaviour was not witnessed at all in the Placebo-Phys and PROB-Phys subgroups, where the animals displayed very little rubbing/grooming activity.

The comparison of the 3-min blocks revealed significant differences in rubbing activity between the Placebo-Form and Placebo-Phys subgroups in the first (***$p < 0.001$) and fifth to eleventh (**$p < 0.01$; ***$p < 0.001$) blocks (Fig. 1). The PROB pre-treatment significantly decreased the formalin-induced nociceptive behaviour in each block ($^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; Fig. 1). However, it should be noted that PROB did not attenuate the effect of formalin completely in the first block, where the time spent on rubbing was still significantly **higher** than that in the Placebo-Phys subgroup (*$p < 0.05$; Fig. 1). There was no significant difference between the Placebo-Phys and PROB-Phys animals in any block, nor was between the PROB-Form and PROB-Phys subgroups in any other block apart from the first (Fig. 1).

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The face rubbing activity in the Placebo-Form subgroup was significantly higher during both the first (***p < 0.001) and the second phase (***p < 0.001) than that in the Placebo-**Phys** subgroup (Fig. 2). PROB significantly decreased the nociceptive behaviour in both phases (**p < 0.01; ***p < 0.001; Fig. 2), however this effect was not complete in the first phase (*p < 0.05; Fig. 2).

2. c-Fos in the TNC

Microscopic examination of the immunostained transverse sections of the TNC revealed c-Fos immunoreactivity in the nuclei of the neurones. In the Placebo-**Form sub**group, unilateral s.c. formalin injection produced an increase in the number of c-Fos-IR neurones in **the dorsal, superficial area** of the transverse sections of the ipsilateral TNC as compared with the non-treated contralateral side (Fig. 3/A). This increase was significant at different levels along the rostrocaudal axis (between -0.3 and -3.3 mm), in accordance with the somatotopic representation (**p < 0.01; ***p < 0.001; Fig. 3/C).

In the PROB-**Form sub**group, the number of c-Fos-IR neurones at the different levels along the rostrocaudal axis also increased in the ipsilateral TNC. This effect was similar, but less pronounced than that in the Placebo-**Form sub**group (Fig. 3/B), i.e. PROB significantly decreased the formalin-related activation of the second order trigeminal neurones at several levels of the TNC (between -0.3 and -2.4 mm; [#]p < 0.05; [##]p < 0.01; [###]p < 0.001; Fig. 3/C). On the contralateral sides of the TNCs, there were no significant differences either between the subgroups or between the different levels along the rostrocaudal axis (Fig. 3).

3. nNOS in the TNC

On the immunostained transverse sections, the nNOS-IR neurones showed cytoplasmic and dendritic staining. The unilateral s.c. formalin injection gave rise to an increase in the number of nNOS-IR neurones in the dorsal, superficial area of the ipsilateral TNC compared to the non-treated contralateral side in the Placebo-Form subgroup (Fig. 4/A). Along the rostrocaudal axis, significant increase can be observed at different levels (between -2.1 and -2.7 and between -3.3 and -3.9 mm; $*p < 0.05$; $**p < 0.01$; Fig. 4/C). In the PROB-Form subgroup, there was no difference in the number of nNOS-IR neurones between ipsilateral and contralateral TNC (Fig. 4/B), i.e. PROB significantly decreased the number of the second order trigeminal neurones expressing nNOS at several levels of the TNC (between -2.1 and -2.7 and between -3.3 and -3.9 mm; [#]$p < 0.05$; [##]$p < 0.01$; Fig. 4/C) at the injected side. On the contralateral sides of the TNCs, there were no significant differences either between the subgroups or between the different levels along the rostrocaudal axis (Fig. 4).

4. IL-1β and NQO1 in the TNC

The Western blot analysis of the TNC segments did not show significant difference between the contra- and ipsilateral sides for any of the subgroups either in the case of IL-1β or in the case of NQO1 (data not shown), which means that IL-1β or NQO1 expression did not change four hours after the unilateral s.c. formalin injection and PROB pre-treatment did not have any modulatory effect on the expression of IL-1β or of NQO1.

Discussion

This is the first study showing that PROB produced a pronounced anti-nociceptive behavioural effect in the orofacial formalin test especially in the second phase. PROB exhibited no effect on the **baseline** activity of the control animals, which received a s.c. saline injection in the whisker pad. In addition to the modulation of the nociceptive behaviour, PROB also mitigated the formalin induced c-Fos and nNOS expression in the TNC – considered markers of activation and sensitization of these neurones .

This anti-nociceptive effect of PROB along with its inhibitory effect on the trigeminal activation and sensitization can be the result of various mechanisms:

PROB inhibits MRP4, an organic anion transporter , which releases key inflammatory mediators such as prostaglandin E1 and PGE2 from cells in a time- and ATP-dependent manner , and these mediators contribute to inflammation and hyperalgesia . PGE2 released from cultured rat trigeminal ganglion nerve cells can sensitize transient receptor potential channel subtype V member 1 (TRPV1) (a key factor in the development of sensitization during nociception). Moreover, PGE2 can induce calcitonin gene-related peptide (CGRP) release (a general event after the activation of first order trigeminal neurones) from cultured trigeminal ganglion cells . PGE2 can also act on the trigeminal system at the level of the central nervous system **by enhancing the capsaicin-induced CGRP release in slice preparations of the TNC** . The expression of cyclooxygenase-2 (an inducible enzyme of prostaglandin synthesis) has also been detected in the neurones and glial cells in the trigeminal ganglion and in the neurones of the TNC . In addition, prostaglandin receptors play a relevant role in trigeminal nociception and in the pathomechanism of trigeminal activation . They are present in the trigeminal ganglion , co-expressed with TRPV1, with a functional interaction between them in the trigeminal system , and are involved in mediating

CGRP release from cultured rat trigeminal neurones . Quantitative autoradiography has revealed that TNC has a moderate density of PGE2 binding sites as well, and the mRNA of all prostaglandin receptors are also present there . Accordingly, a possible mechanism of action of PROB is the inhibition of the release of PGE2, a key player in trigeminal nociception and an important molecule in migraine genesis through the blockade of MRP4.

PROB also inhibits the pannexin-1 channel , which may be relevant in inflammation and hence in pain processing. The pannexin-1 channels are mainly expressed in the spinal cord ; moreover, they are present in the postsynaptic neurones and are crucial in caspase-1 activation, which leads to the production and release of interleukin-1beta (IL-1β) in neurones, astrocytes and macrophages . The release of IL-1β from trigeminal ganglion satellite cells may contribute to the mechanisms underlying trigeminal inflammatory hyperalgesia . Up-regulation of astrocytic IL-1β in the TNC can lead to central sensitization *via* its receptors present on trigeminal neurones in the same area . The interleukin-1 receptor antagonists **were effective in modulation of changes caused by noxious stimulation** . Consequently, these results clearly indicate that IL-1β plays a role in trigeminal activation. **However the results of our Western blot measurement showed that there were not changes in the expression of IL-1β at the level of TNC four hours after s.c. formalin administration and therefore it is not possible that PROB exerts such effect on this level, but one can not exclude the possibility that PROB affects IL-1β in the periphery, at the inflammation site or in the trigeminal ganglion.**

It is important to note that some data suggest that PROB is able to activate **some of the transient receptor potential channels, including TRPV2 and** TRPA1 **which** play an important role in pain perception **and are present in the trigeminal system** . **However, the agonist-mediated desensitization of these receptors is also known , which can cause an anti-nociceptive effect . PROB can desensitize the TRPA1 and may act similarly on**

TRPV2 as well, which might account of its anti-nociceptive properties in the present experiments.

It is a well-known fact that PROB can increase the concentration of kynurenic acid - an endogenous tryptophan metabolite - in the central nervous system . This phenomenon can be caused by inhibition of organic anion transporters by PROB , which are involved in the transport of kynurenic acid taking place from brain through the blood-brain barrier or by increasing of concentration of tryptophan in the central nervous system . Experimental data suggest that kynurenic acid can affect nociception and elevation in its concentration in the brain can inhibit the trigeminal activation , probably due to an antagonistic effect on ionotropic glutamate and α7-nicotinic acetylcholine receptors or due an agonistic action on G-protein-coupled receptor-35 , which receptors play important role in pain processing and trigeminal activation .

Several lines of evidence confirm the role of oxidative stress in the induction of tissue damage e.g. neuronal activation caused by inflammation in orofacial formalin test . However, in the present study, we could not support this hypothesis, since the NQO1 expression, a marker of oxidative stress , remained unchanged four hours after s.c. formalin injection.

Conclusions

The results of our study provide evidence that pre-treatment with PROB can have anti-nociceptive effects and can modulate the changes induced by trigeminal inflammation in both behavioural and morphological markers in the rat orofacial formalin test. PROB **may** exert this effect through inhibition of the inflammatory processes **which could be through** blocking the MRP4, which induces the release of key inflammatory mediators such as PGE2 **or through desensitization of TRPA1 and TRPV2 or by enhancing concentration of kynurenic acid in the brain**. These properties and the **relatively good side effect profile can** make PROB a possible pharmacological candidate in the treatment of trigeminal activation related pain.

Acknowledgements

List of abbreviations

CGRP: calcitonin gene-related peptide

IL-1β: interleukin-1beta

IR: immunoreactive

MRP4:   multidrug resistance-associated protein 4

nNOS: neuronal nitric oxide synthase

NQO1:   NAD(P)H:quinone oxidoreductase 1

PBS: phosphate-buffered saline

PBST: phosphate-buffered saline containing 1% Triton X-100

PGE2:   prostaglandin E2

PROB: probenecid

s.c.:   subcutaneous

TNC: caudal part of spinal trigeminal nucleus

TRPV1:   transient receptor potential channel subtype V member 1

TRPV2:   transient receptor potential channel subtype V member 2

TRPA1:   transient receptor potential channel subtype A member 1

Figure legends

Fig. 1. Diagram showing the time (number of seconds) spent rubbing the injected area with the ipsilateral fore- or hindpaw in each block of 3 min in all 4 subgroups. In the Placebo-Form subgroup (black diamonds), the two phases of formalin action are clearly distinguishable. The rubbing activity in the first (***$p < 0.001$) and in the fifth to eleventh (**$p < 0.01$; ***$p < 0.001$) blocks was significantly higher in the Placebo-Form subgroup (black diamonds) than in the Placebo-Phys subgroup (dark-grey squares). The PROB pre-treatment significantly reduced the formalin-induced nociceptive behaviour in each of the above-mentioned blocks ($^{##}p < 0.01$; $^{###}p < 0.001$) in the PROB-Form subgroup (black triangles) relative to the Placebo-Form subgroup. There was no significant difference between the findings on the Placebo-Phys and PROB-Phys animals in any block.

Fig. 2. Diagrams showing the rubbing activity in the first (A) and the second (B) phase in all 4 subgroups. In the Placebo group, the subcutaneous formalin injection (Placebo-Form) induced a significant increase in rubbing activity in both the first and the second phase (***$p < 0.001$) as compared with that in the saline-treated animals (Placebo-Phys). In both phases, pre-treatment with PROB had a significant effect on mitigating the formalin-induced increase in the time spent in rubbing (**$p < 0.01$; ***$p < 0.001$) as compared with the Placebo-Form subgroup.

Fig. 3. (A) c-Fos-immunoreactive (IR) neurones (black arrow) in the superficial laminae of a transverse section of **medulla containing** the caudal part of the spinal trigeminal nucleus (TNC) in the Placebo-**Form sub**group. Subcutaneous formalin injection resulted in a higher number of c-Fos-IR neurones on the ipsilateral side than on the contralateral side. Scale bar: 500 μm. (B) c-Fos-IR neurones on the formalin-treated ipsilateral sides of sections in the TNC in the two **sub**groups. The formalin-induced increase in IR cells was more prominent **on the ipsilateral side** in the Placebo-**Form sub**group (B/a) than in the probenecid (PROB) (B/b)-pretreated animals. Scale bar: 200 μm. (C) Diagram showing the mean number of c-Fos-IR cells in the superficial laminae of the TNC at different levels along the rostrocaudal axis (mean ± S.E.M, n=**9** per **sub**group). In the Placebo-**Form sub**group, formalin produced a significantly higher number of c-Fos-IR neurones on the formalin-treated side of the TNC as compared with the contralateral side at different levels along the rostrocaudal axis (between -0.3 and -3.3 mm), in accordance with the somatotopic representation (**$p < 0.01$; ***$p < 0.001$). PROB pre-treatment significantly decreased the effect of formalin between -0.3 and -2.4 mm ([#]$p < 0.05$; [##]$p < 0.01$; [###]$p < 0.001$).

Fig. 4. (A) Immunostained transverse sections of medulla containing the caudal part of the spinal trigeminal nucleus (TNC) from the Placebo-Form and probenecid (PROB)-Form subgroup. Black arrow shows the neuronal nitric-oxide synthase (nNOS)-immunoreactive (IR) neurones in the superficial area of the TNC. On the ipsilateral side (A/b), more nNOS-IR neurones can be observed than on the contralateral side (A/a) in the Placebo-Form subgroup. After formalin injection, there is a lower number of nNOS-IR neurones on the ipsilateral side in the probenecid (PROB)-Form subgroup (A/d) than in the Placebo-Form subgroup

(A/b). In these two subgroups, difference between the contralateral sides cannot be observed. Scale bar: 200 μm. (B) Diagram showing the mean number of nNOS-IR cells in the superficial area of the TNC at different levels along the rostrocaudal axis (mean ± S.E.M, n=5 per subgroup). Formalin produced a significant increase in number of nNOS-IR neurones on the formalin-treated side of the TNC in the Placebo-Form subgroup as compared with the contralateral side at different levels along the rostrocaudal axis (between -2.1 and -2.7 and between -3.3 and -3.9 mm; $*p < 0.05$; $**p < 0.01$). The effect of formalin was significantly decreased by pre-treatment with PROB between -2.1 and -2.7 and between -3.3 and -3.9 mm ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$). There was no significant difference between the contralateral sides.

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