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To cite this version:

Accepted Manuscript

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PII: S1094-5539(08)00108-9
DOI: 10.1016/j.pupt.2008.11.001
Reference: YPUPT 876

To appear in: *Pulmonary Pharmacology & Therapeutics*

Received Date: 6 August 2008
Revised Date: 29 October 2008
Accepted Date: 4 November 2008


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TYPE OF CONTRIBUTION: Regular paper

DATE OF PREPARATION: 2nd August 2008

NUMBER OF TEXT PAGES: 12

NUMBER OF TABLES: 0

NUMBER FIGURES: 3

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ETHANOL POTENTIATES THE TRPV1-MEDIATED COUGH IN THE GUINEA PIG

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Abbreviated title: Ethanol exacerbates cough in guinea pig

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Abstract:
Ethanol is a chemical irritant able to induce a large variety of effects in the airways. It has been reported that ethanol sensitises the transient receptor potential vanilloid 1 (TRPV1) to various stimuli and inhalation of ethanol enhances the cough mediated by TRPV1 activation (capsaicin) in patients suffering of airway sensory hyperreactivity. Here, we set out to investigate whether ethanol sensitizes the cough induced by TRPV1 activation in a guinea pig model and the possible mechanism of such exacerbating effect. Aerosolized resiniferatoxin (RTX, 0.5 µM) and hypertonic saline (7%) produced a cough response dependent and independent of TRPV1 activation, respectively. Ethanol (3%, 10 min) inhalation, that per se did not cause any tussive response, significantly increased the number of coughs evoked by RTX inhalation without affecting hypertonic saline (7%) induced cough. Potentiation by ethanol of the tussive response to RTX was prevented by the PKC inhibitor, GF109203X (GFX).
In conclusion, ethanol selectively exaggerates, via a PKC-dependent pathway, the cough response evoked by TRPV1 stimulation. The present results may contribute to explain respiratory distresses sometimes associated to alcohol consumption, including cough and asthma.

Keywords: cough, guinea pig, vanilloid receptor-1 (TRPV1), resiniferatoxin, protein kinase C (PKC).
**Introduction**

Ethyl alcohol, (ethanol) is known to produce a variety of neuronal actions, including an inhibitory effect, possibly by facilitating the opening of gamma aminobutyric acid-A (GABA-A) receptor-chloride channels [1]. An opposite excitatory action of ethanol has recently been described in primary sensory neurons [2] expressing the transient receptor potential vanilloid-1 (TRPV1) [3]. TRPV1 is a cation channel rather selectively expressed in a subset of nociceptive primary sensory neurons with C and A-δ fibers, which are activated by noxious temperature, low extracellular pH [3,4], and a variety of lipids, including anandamide, N-arachidonoyl dopamine, and certain eicosanoids [5-7]. The excitatory action of ethanol on TRPV1 is most likely due to its ability to lower the threshold temperature for the channel activation [2]. Ethanol has been shown also to dramatically potentiate TRPV1 activation by protons and anandamide [2].

Different intracellular pathways have been reported to contribute to TRPV1 sensitization. Protein kinase A (PKA) has been shown to sensitize TRPV1 [8] and other mechanisms independent of PK [9] seem to contribute to TRPV1 exaggerated responses. Type C protein kinase (PKC) contributes to the exaggeration of TRPV1-mediated responses by activation of G protein coupled receptors, including the proteinase activated receptor-2 (PAR2) [10], the B2 bradykinin receptor [11] or by agonists of tyrosine kinase receptors [12,13].

In damaged or irritated skin or mucosal surface, exposure to ethanol is often associated to a burning pain sensation, that has proposed to be due to the sensitizing effect of ethanol on TRPV1 [2]. A typical response mediated by TRPV1 activation in experimental animals and man is cough [14], and alcoholic drinks are capable of triggering a wide range of irritative and defensive responses in alcoholic drink-sensitive individuals, including rhinitis, itching, facial swelling, headache, cough and asthma [15,16-18]. It has been reported that ethanol-containing corticosteroid medicines cause bronchoconstriction in susceptible asthmatic patients supposedly because of the alcohol included in the formulation [19]. Moreover, inhalation of ethanol 5 and 25% enhanced the cough reaction to capsaicin [20] in patients with airway sensory hyperreactivity.

Thus, the purpose of the present study was to investigate whether ethanol could exaggerate the cough response produced by TRPV1 stimulation in a guinea pig model and whether PKC could contribute to the modulation of the evoked tussive response.
Experimental procedures

Animals. Male Dunkin-Hartley guinea pigs (250-350 g, Pampaloni, Italy) were acclimatized in cages, (24 ± 0.5 °C) for 1 week after delivery, and were allowed free access to water and standard rodent diet (Morini, Italy). All experiments complied with the national guidelines and were approved by the regional ethics committee.

Cough study

Experimental set-up. After the period of acclimatisation to laboratory conditions, animals were individually placed in a transparent perspex box (20 x 10 x 10 cm, Vetrotecnica, Italy) ventilated with a constant airflow of 400 ml/min. Pro-tussive stimuli were nebulised via a mini-ultrasonic nebuliser (Model 2511; PulmoSonic, DeVilbiss). The particle size produced had an aerodynamic mass median diameter of 0.9 µm and the output of the nebuliser was 0.4 ml per min. Identification of a cough response was achieved by three different approaches: 1) by observing/counting the typical cough posture of the guinea pig (by a trained and blind observer) during the challenge; 2) by the presence of the investigator who confirmed the cough sounds during the challenge (transmitted from the microphone in the cage to the recorder and to the outside speakers); 3) by the subsequent analysis of the sound waves recorded into a personal computer. The cough sounds were recorded, digitally stored and counted by a blind observer.

Study Protocols. All experiments were carried out at the same time of day starting at 9.00 a.m. To elicit cough, guinea pigs were exposed for 10 min to aerosols of RTX (0.5 µM) and hypertonic saline (7% sodium chloride, 1.2 M). To evaluate the potential modulatory role of ethanol against the TRPV1-mediated cough, aerosolised ethanol (1-3%) was administered to guinea pigs for 10 min prior to the cough challenge. In experiments aimed at evaluating the role of PKC on cough exacerbation, the PKC blocker, GF109203X (GFX, 1 µM) was aerosolised for 10 min prior to the aerosol with ethanol 3%.

The effect of aerosolised ethanol was also investigated against hypertonic saline (7% sodium chloride), a stimulus that induces cough in a TRPV1-indipendent manner. Hypertonic saline was administered for 10 min after the administrations of 3% ethanol. To prevent the possible contribution of bronchoconstriction in the tussive response to the diverse stimuli, all guinea pigs were intraperitoneally administered with the β-adrenoceptor agonist, terbutaline (0.5 mg/kg), five minutes prior to the beginning of the experiment.
Drugs. Agents were obtained from the following companies: resiniferatoxin, (Tocris, UK); GF109203X (GFX), (Alexis Corporation, Switzerland). The stock concentration of RTX (1 mM) and GFX (1 mM) were prepared in 100% DMSO. All the other drugs were dissolved in isotonic solution.

Data analysis. Values were presented as mean ± SE. Data are compared using Student’s t-test or standard one way analysis of variance (ANOVA) following by post hoc Bonferroni’s test. A p value < 0.05 was considered significant. A minimum of 6 guinea pigs were used to test the effect of vehicle or of each single dose of the drugs.

Results
Aerosolised ethanol (1-3%, per 10 min) did not induce a significant increase in the number of coughs (1.2 ± 1.0, n = 8) if compared to that produced by isotonic saline (0.9% sodium chloride, 1.0 ± 0.2, 10 min, n = 10, data not shown).

In contrast, aerosolised RTX (0.5 µM), administered for 10 min, produced a number of coughs (10.9 ± 0.8, n = 13, fig. 1) significantly higher than those induced by aerosolised isotonic saline (0.9% sodium chloride, 1.1 ± 1.3, n = 12). Pre-treatment with aerosolised ethanol (1% and 2%) did not modify the number of coughs induced by RTX (fig. 1). In contrast, aerosolised 3% ethanol (per 10 min) consistently and significantly enhanced RTX-induced cough (45 ± 8% of increase, n = 12, fig. 1).

Furthermore, an aerosol pre-treatment with GFX practically abolished the cough exacerbation produced by ethanol 3% (fig. 2) suggesting a crucial role of PKC in the observed cough exacerbation.

In the experimental set aimed to evaluate the selectivity of the exacerbating effect of ethanol, 3% ethanol was aerosolised per 10 min prior to the cough challenge with hypertonic saline (7%, sodium chloride) and no modification of the tussive response were observed between ethanol-treated animals (6.3 ± 1.5, n = 7) and animals that received aerosolised hypertonic saline (7.3 ± 0.3, n = 6) (fig. 3).

Finally, in an independent experimental set, after 10 min of aerosolisation with 3% ethanol, guinea pig blood samples were collected and alcoholemia was quantified. No measurable plasmatic levels of ethanol were found in any of the blood samples (data not shown).
Discussion

The present results show that ethanol potentiates TRPV1-mediated cough by a PKC-dependent mechanism in a guinea pig model. Specifically, ethanol at doses that per se did not produce any measurable cough response, exaggerated RTX-evoked cough. The ability of ethanol to affect TRPV1 seems to derive from its property to sensitize the channel to other stimuli. For instance, in electrophysiological experiments ethanol reduced the threshold temperature for TRPV1 activation by 8°C [2]. Thus, ethanol, reducing the threshold temperature for TRPV1 activation from 43°C [3] to 35°C [2] allowed the physiological temperature to activate the channel. TRPV1 activation thresholds to anandamide and protons are also lowered by ethanol pre-treatment [2]. Interestingly, ethanol activity on the cough reflex appeared to be selective, since it did not affect the tussive response evoked by hypertonic saline, a tussigenic agent [21,22] which is thought to induce cough through a mechanism totally independent from the TRPV1 activation [22,23]. The fact that no detectable ethanol plasma levels were attained after the exposure to the higher tested aerosolised concentration (3%), indicates that ethanol enhanced TRPV1-dependent cough through a peripherally restricted action.

TRPV1 channel activity is remarkably up-regulated by different inflammatory mediators which promote channel phosphorylation by either activation of PKC or cAMP-dependent PKA mechanism. Phosphorylation of TRPV1 increases the probability of the ion channel to open in response to heat, protons and endogenous lipids [11,24]. Thus, TRPV1 phosphorylation seems to play an important role in the regulation of channel activity that finally results in channel sensitization. PKC is known to phosphorylate several cellular components, including enzymes and membrane-bound receptors and ion channels that are key regulators in the process of nociceptor excitation and sensitization (for review see: [9]). Interestingly, PKC has been shown to arbitrate the effects of ethanol on receptors [25] and membrane-bound transporters [26], but the mechanism underlying this modulation has not been elucidated. Ethanol causes also translocation of PKC in a similar manner to that induced by phorbol esters [27] and similarly to ethanol, phorbol esters act on PKC activating TRPV1 channels and decreasing its threshold of activation to heat [28,29]. Because ethanol has been associated to PKC translocation [30] and PKC appears to play an integral role in TRPV1 sensitisation, we hypothesize that PKC could mediate ethanol-induced sensitization of TRPV1 in the airways in vivo.

In this respect, we have previously shown that the PKC activator, 12-O-tetradecanoylphorbol-13-acetate (TPA), that per se does not cause cough, significantly potentiated citric acid- and RTX-induced cough in guinea pig, an effect that was completely reversed by GFX [31]. The
aforementioned results reinforce the hypothesis that PKC promotes activation of TRPV1 indirectly via phosphorylation of some residues of the protein [32]. Involvement of PKC in ethanol-mediated potentiation of TRPV1-dependent cough was indicated by the observation that PKC inhibition by GFX, abated the exaggerated response induced by pre-exposure to ethanol. The selectivity of this effect is supported by the fact that ethanol did not show any modulatory effect against hypertonic saline-induced cough. Furthermore, as previously shown, treatment with aerosolised TPA did not exacerbate the cough induced by hypertonic saline[31]. We shown here that aerosolized ethanol enhances the cough response to TRPV1 agonists via a PKC-dependent mechanism without interfering with a cough reflex induced by a TRPV1-independent mechanism. A recent finding showed that a positive correlation between the tussive response to capsaicin and the number of TRPV1-positive nerves in patients with chronic cough exists [33]. Moreover patients with airways sensory hyperresponsiveness exhibited an exacerbated cough reflex to capsaicin after inhalation of ethanol at concentrations significantly higher (5 and 25%) than those used in the present work (3%) [20]. Here we described that the mechanism of action of such exacerbating effect involves the activation of PKC. This observations may suggest that an increased network of TRPV1 sensory fibres may predispose susceptible individuals to cough exacerbation by a large variety of conditions, including accidental, iatrogenic or alimentary exposure to ethanol.

Grants: This work was in part supported by ARCA, Padua and MUIR, Rome.

CONFLICT OF INTERESTS: Authors have no, real or perceived competing of interest that relate to this manuscript.
References


Legends to Figures:

Fig. 1: Dose dependent effect of aerosolised ethanol (EtOH, 1-3%) on resiniferatoxin (RTX, 0.5 µM)-induced cough in the guinea pig. Aerosolised ethanol was administered for 10 min prior to the cough challenge with RTX. *, $P < 0.05$, ANOVA and Bonferroni’s test vs. vehicle (VEH). Each column is presented as mean (S.E.M.) of at least 6 experiments.

Fig. 2: Effect of aerosolized GF109203X (GFX, 1 µM) on ethanol (EtOH, 3%)-induced resiniferatoxin (RTX, 0.5 µM) dependent cough exaggeration in the guinea pig. #, $P < 0.05$, ANOVA and Bonferroni’s test vs. vehicle (VEH1); *, $P < 0.05$, ANOVA and Bonferroni’s test vs. vehicle (VEH2). Each column is presented as mean (S.E.M.) of at least 6 experiments.

Fig. 3: Effect of aerosolized ethanol (EtOH, 3%) on cough induced by inhalation of hypertonic saline (HS, 7% sodium chloride) in the guinea pig. Each column is presented as mean (S.E.M.) of at least 6 experiments. Student’s t-Test test was used and no statistically significance was observed between the two groups.
**Graph 1**

The graph illustrates the number of coughs (10 min) in response to different concentrations of EtOH (EtOH (1%), EtOH (2%), EtOH (3%)). Treatment with RTX (0.5 µM) showed a significant reduction in coughs compared to the vehicle (VEH) control. Statistical significance is indicated by an asterisk (*).
Hypertonic Saline (7%)

VEH

EIOH (3%)

n of coughs (10 min)