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Ethanol and Opioids Do Not Act Synergistically To Depress Excitation in Carotid Body Type I Cells

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Objective The combination of opioids and ethanol can synergistically depress breathing and the acute ventilatory response to hypoxia. Multiple studies have shown that the underlying mechanisms for this may involve calcium channel inhibition in central neurons. But we have previously identified opioid receptors in the carotid bodies and shown that their activation inhibits calcium influx into the chemosensitive cells. Given that the carotid bodies contribute to the drive to breathe and underpin the acute hypoxic ventilatory response, we hypothesized that ethanol and opioids may act synergistically in these peripheral sensory organs to further inhibit calcium influx and therefore inhibit ventilation.

Methods Carotid bodies were removed from 56 Sprague–Dawley rats (10–21 days old) and then enzymatically dissociated to allow calcium imaging of isolated chemosensitive type I cells. Cells were stimulated with high K^+ in the presence and absence of the μ-opioid agonist [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) (10 μM), a maximal sublethal concentration of ethanol (3 g L^{-1}, 65.1 mM) or a combination of both.

Results DAMGO alone significantly inhibited Ca^{2+} influx but this effect was not potentiated by the high concentration of ethanol.

Conclusion These results indicate for the first time that while opioids may suppress breathing via an action at the level of the carotid bodies, ethanol is unlikely to potentiate inhibition via this pathway. Thus, the synergistic effects of ethanol and opioids on ventilatory parameters are likely mediated by central rather than peripheral actions. *NeuroReport* 32: 1307–1310 Copyright © 2021 The Author(s). Published by Wolters Kluwer Health, Inc.

Introduction

The drive to breathe is generated in the brainstem and modulated by chemoreceptive inputs located peripherally (e.g. carotid bodies) and centrally [e.g. nucleus tractus solitarius (NTS)] [1]. The carotid bodies respond to hypoxic and hypercapnic blood gas conditions leading to increased breathing, thereby restoring blood gas homeostasis [2]. It is known that opioid receptors are found in high concentration within these central and peripheral chemoreceptive regions [3,4] and activation of these opioid receptors depresses breathing [5,6]. The underlying mechanism by which opioids cause this depression seems to be via activation of signaling through G_i-coupled G-proteins that lead to cellular inhibition [7]. Interestingly, it has previously been shown that the chemosensitive type I cells within the carotid bodies contain a large number of functional μ-opioid receptors and their activation leads to a significant attenuation of cellular activity via inhibition of calcium influx [4]. It is of note that an opioid receptor antagonist, methylnaltrexone, which cannot cross the blood-brain barrier, significantly

reversed breathing depression induced via morphine in response to hypoxia [8]. Moreover, naloxone, a μ-opioid receptor antagonist, enhanced carotid bodies responses to hypoxia, an effect opposite to that seen when opioids were administered [9]. Taken together, these data suggested that the peripherally located carotid bodiess could play a key role in how opioids depress breathing.

Extreme risk of mortality occurs when opioids are used in conjunction with other depressants. For instance, concurrent consumption of alcohol and opioids synergistically depressed breathing and significantly increased mortality [10]. In one trial in humans, consuming oxycodone while receiving a simultaneous intravenous infusion of ethanol resulted in reduced baseline breathing, attenuated breathing during hypoxic challenges, and increased instances of apnea compared to the effects of either drug alone [10]. Indeed, ethanol use has long been known to cause respiratory depression [11,12].

In extremely high concentrations, ethanol inhibits calcium currents in neurons, leading to decreases in excitability [13,14]. Importantly, in the NTS, a brain region known to modulate breathing patterns, ethanol caused a significant decrease in cellular responses through a mechanism mediated by an inhibition of calcium influx [15]. It is evident that opioids and ethanol have potent

effects centrally leading to the depression of breathing. However, the effect of ethanol in conjunction with opioids on the peripheral chemoreceptors within the carotid bodies remains to be investigated.

Given that opioids and ethanol can inhibit cellular excitation via inhibition of calcium influx [4,7,13] we tested the hypothesis that the synergistic depressive effect on breathing of using opioids and ethanol concurrently might be mediated, in part, by depression of carotid body type I cell calcium responses to stimuli. In this study, we examine a sub-lethal concentration of ethanol [16] together with a high concentration of the peptide μ-opioid agonist [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) 10 μM (DAMGO Kd at μ-opioid receptors 3.5 nM; [17]). We are therefore probing the maximal effects of combining opioids and ethanol on carotid body type I cells.

Methods
Ethical approval
All studies described herein were performed in accordance with protocols approved by Wright State University's Institutional Laboratory Animal Care and Use Committee. This includes adhering to the standards put in place by the National Institute of Health in their published guide for the care and use of laboratory animals (NIH publication No. 80-23).

Type I cell isolation
On each experimental day, two neonatal, Sprague–Dawley rats ($n = 56$; 10–21 days old; Harlan) were anesthetized with isoflurane (4–5% in oxygen); a toe pinch tested withdrawal reflexes to ensure the proper plane of anesthesia was achieved. Carotid bodies were removed and placed in ice-cold Dulbecco's phosphate-buffered saline (DPBS) without Ca^{2+} or Mg^{2+}. Rats were then euthanized humanely via decapitation while still under anesthesia. Noncarotid body tissue was removed from the organs before they were transferred into an enzyme cocktail solution (0.4 mg ml^{-1} collagenase type I, 220 μ mg^{-1} (Worthington Biochemical Corp.), 0.2 mg ml^{-1} trypsin type I, 8550 BAEE μ mg^{-1} (Sigma) in DPBS with low $CaCl_2$ (86 μM) and $MgCl_2$ (350 μM)) and incubated for 20 min at 37 °C. Carotid bodies were then lightly pulled apart and incubated again for 7 min. The tissue was further separated via trituration before centrifugation at $110 \times g$ for 5 min. Isolated cells were resuspended in tissue culture media [Ham's F12 (Sigma) with 10% heat inactivated fetal bovine serum (Biowest) added], centrifuged again, resuspended in tissue culture media, and then plated onto 12 mm diameter poly-d-lysine coated glass coverslips. Cells were incubated for 2 h at 37 °C in a humidified, 5% CO_2/air incubator to allow adherence to the coverslips. Cells were used within 8 h after being dissected. These methods yield isolated carotid body type I cells that remain oxygen-sensitive [18].

Calcium imaging
Intracellular calcium levels in carotid body type I cells were visualized using a calcium-sensitive, fluorescent dye, FURA-2AM as previously described by [4]. Briefly, coverslips with cells adhered were placed in 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES) buffered extracellular solution (in mM: 140 NaCl, 4.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 11 glucose, 10 HEPES, adjusted to pH 7.4 with NaOH at 37 °C) containing FURA-2AM (5 μM; Invitrogen) for 30 min and allowed to load in the dark at room temperature. Cells were next washed for 15 min at room temperature in the dark with extracellular solution free of dye before being mounted in a chamber (0.4 mL, Warner Instruments, RC-25F) and gravity-perfused with heated extracellular HEPES solution (8 mL min^{-1}, 37 °C, pH 7.4). Cells were visualized with an inverted microscope (Nikon TE2000U) with a 40× oil immersion objective lens (CFI Super Fluor). Cells loaded with FURA-2 were excited every 5 s with a 50 msec exposure to 340 nm and 380 nm light generated by passing white light, generated from a 175 W xenon arc lamp (Lambda-LS; Sutter), through a filter wheel (Lambda 10-3; Sutter) containing 340 nm and 380 nm light filters (Chroma). Emission was recorded at 510 nm with a charge coupled device camera (Photometrics; Coolsnap HQ2). To prevent the photodamage of cells, neutral density filters (0.7 optical density; Chroma) were placed in the light path before cellular exposure. Image acquisition was controlled by Metafluor imaging software (Molecular Devices; v7.1.2). Baseline recordings were made while cells were perfused with a standard HEPES buffered salt solution before exposure to a high K$^+$ extracellular solution stimulus caused a rapid rise of intracellular calcium; only cells responding to the stimulus with a rapid and reversible rise in calcium were recorded. After calcium levels returned to baseline, cells were exposed to either ethanol (3 g L^{-1}, 65.1 mM), a μ-opioid receptor agonist: DAMGO (10 μM; Sigma-Aldrich E7384) or both ethanol and DAMGO, for 3 min before being challenged again with the high K$^+$ stimulus while continually in the presence of the drug. Cells were then allowed to return to baseline while still in the presence of the drug. Extracellular solution was HEPES buffered salt solution. The high K$^+$ stimulus was (in mM): 64.5 NaCl, 80 KCl, 2.5 $CaCl_2$, 1, $MgCl_2$, 11 glucose, 10 HEPES adjusted to pH 7.4 with KOH at 37 °C [4,18]. DAMGO-Enkephalin acetate salt was dissolved in double-distilled water before being aliquoted, frozen (–20 °C), and then thawed before use.

Data analysis
Within imaging software, regions of interest (ROI) were drawn over entire single cells and the calcium signal recorded as the averaged amount within the ROI. Cellular responses were analyzed by subtracting the peak calcium response during drug exposure from the peak calcium control response (e.g. the delta between the first and

second calcium peaks). Individual cellular responses (i.e. the delta between the two responses) were grouped based on treatment and analyzed using a Student's *t*-test comparing group delta averages; significance set at $P < 0.05$.

Results

Isolated carotid body chemosensitive cells were depolarized with a high potassium challenge and the change in Fura-2 fluorescence was recorded. Cells were then exposed to ethanol, DAMGO or ethanol and DAMGO before receiving the same depolarizing high potassium stimulus. Thus, any inhibitory effects of ethanol, DAMGO or the ethanol DAMGO combination on the excitatory Fura-2 response to potassium could be calculated. Data are presented as the percent change of fluorescence inhibited, plus/minus the SEM. Cells exposed to ethanol alone were not significantly inhibited ($n = 16$; $2.68 \pm 0.80\%$; Fig. 1a,b). However, similar to previous findings, DAMGO alone did significantly inhibit the Fura-2 response ($n = 14$; $6.07 \pm 0.97\%$; $P < 0.05$, Fig. 1b,d) and importantly the ethanol (ETOH) + DAMGO combination also showed significant inhibition of the Fura-2 signal ($n = 16$; $6.20 \pm 1.00\%$; $P < 0.05$; Fig. 1c,d). DAMGO and the DAMGO + ethanol groups were both noted to have significant inhibition of Fura-2 responses compared to the ETOH alone ($P < 0.05$ for both analyses, Fig. 1d). There was no significant difference

in the percent of inhibition between the DAMGO and ETOH + DAMGO treated groups.

Discussion

It is well established that the combined use of ethanol and opioids synergistically depresses breathing and the acute hypoxic ventilatory response [10,19]. Many studies have demonstrated ethanol and opioids inhibit respiration via actions on centrally-located breathing modulators [6,20]. However, the involvement of peripheral breathing modulators (e.g. the carotid bodies) that may mediate, in part, this synergistic depression of breathing has not yet been fully elucidated. The carotid bodies express functional μ-opioid receptors and their activation attenuate carotid body type I cell activity [4]. However, the effect of a highly intoxicating concentration of ethanol on carotid body type I cell activity had not yet been characterized before this study.

The simple but novel experiments described in this brief article demonstrate that ethanol at a highly intoxicating concentration does not inhibit Ca^{2+} entry in carotid body type I cells nor does it potentiate the inhibitory effects of DAMGO, a peptide μ-opioid receptor agonist.

These calcium-imaging experiments suggested that ethanol does not inhibit voltage-gated calcium influx in carotid

Fig. 1.
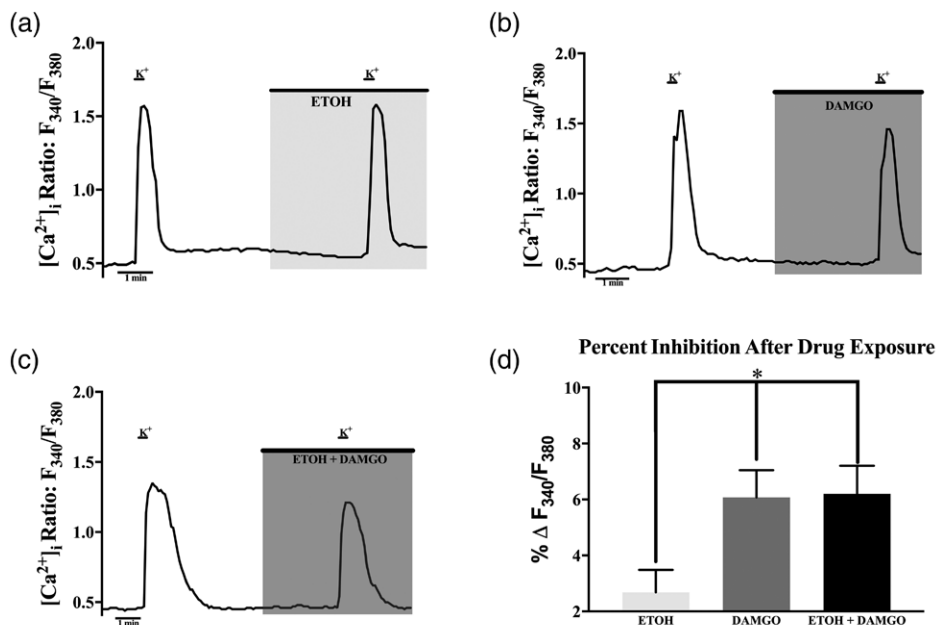
Effects of ethanol (ETOH; 3 g L^{-1}, 65.1 mM), DAMGO (10 μM), or both combined, on carotid body type I cell Fura-2 fluorescence ratios (F_{340}/F_{380}). (a) Responses to a high K^+ HEPES solution are shown both before and after 3 min of exposure to ethanol in an example recording. (b) Responses to a high K^+ solution are shown both before and after 3 min of exposure to DAMGO in an example recording. (c) Responses to a high K^+ solution are shown both before and after 3 min of exposure to ethanol and DAMGO. (d) Bar chart showing the average percent inhibitions for the second high K^+ response compared to the first. DAMGO and the ETOH + DAMGO treated group responses were significantly more depressed than those of ETOH treated cells alone ($P < 0.05$ for both analyses). There was no significant difference between the DAMGO and the ETOH + DAMGO treated groups responses. DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; ETOH, ethanol; HEPES, 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid.

body type I cells. Previous literature has demonstrated that extremely intoxicating concentrations of ethanol inhibit both n-methyl d-aspartate (NMDA) and L-type calcium channel-mediated calcium influx in centrally-located neurons [13,21]. For example, at extremely high concentrations of ethanol compared to those used in this study (~10 g L^{-1}), Walter and Messing [13] observed a 50% inhibition of calcium influx in neuronal cells and Wang *et al*., [22] showed that ethanol does not decrease pituitary L-type calcium channel conductance but decreases the probability of the channel being in the open state. It is important to note however that a plasma concentration of 10 g L^{-1} is a lethal concentration with death usually occurring at a concentration of around 3 g L^{-1} [16]. Furthermore, other mechanisms of calcium entry may also be inhibited by ethanol, [21] showed that ethanol inhibited a NMDA-receptor mediated calcium influx in neurons. Despite these previously documented effects, we did not observe an ethanol-elicited inhibition of intracellular calcium levels as we hypothesized. As both NMDA-receptors and L-type calcium channels are expressed in carotid body type I cells these results were somewhat surprising [23,24]. However, cell-specific differences in channel isoforms, expression levels and G-protein receptor coupling may explain why ethanol inhibits neurons centrally but not peripherally, in the carotid bodies. Importantly, these studies from the 1990s also used extreme concentrations of ethanol that would be considered exceptionally lethal [15], whereas we opted to use more a more appropriate, highly intoxicating, concentration.

These results suggest that while opioids may suppress breathing via an action at the level of the carotid body, ethanol is unlikely to potentiate inhibition via this pathway. Thus, the synergistic effects of ethanol and opioids on ventilatory parameters are likely mediated by central rather than peripheral actions.

Conflicts of interest
There are no conflicts of interest.

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