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Research Report

Chronic phenytoin treatment reduces rat carotid body chemosensory responses to acute hypoxia

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ABSTRACT

Ventilation is peripherally controlled by afferent activity arising from the peripheral chemoreceptors. In the rat, chemosensory activity is conveyed to the central nervous system through axons of neurons located in the nodose-petrosal-jugular-complex. These neurons have distinct electrophysiological properties, including a persistent Na^+ current. Acute blockade of this current with phenytoin and other anti-epileptic drugs reduces normoxic chemosensory activity and responses to acute hypoxia. However, because anti-epileptic therapy is prolonged and there is no information on the effects of chronic phenytoin treatment on peripheral chemosensory activity, we studied the effects of long-lasting phenytoin treatment (~25 days) on afferent chemosensory activity, on a wide range of oxygen inspiratory fractions. Osmotic pumps containing dissolved phenytoin (166 mg/mL) or vehicle (daily flow: 60 μL) were implanted subcutaneously in male adult Sprague Dawley rats. At the end of the treatment, the animals were anesthetized and carotid sinus nerve activity was recorded in vivo. Afferent chemosensory activity in normoxia was not significantly different between control (71.2 ± 2.2 Hz) and phenytoin treated (95.4 ± 2.1 Hz) rats. In contrast, carotid body chemosensory responses to acute hypoxic challenges were markedly reduced in phenytoin treated rats, specifically in the lowest part of the hypoxic range (control 133.5 ± 18.0 Hz vs phenytoin treated 50.2 ± 29.4, at 5% F_IO_2). Chronic phenytoin treatment severely impaired the chemosensory responses to acute hypoxia, suggesting that long-term phenytoin treatment in patients may result in a reduced peripheral respiratory drive together with a reduction in the respiratory responses to hypoxic challenges.

1. Introduction

Phenytoin (5,5-diphenylimidazolidinedione; 5,5-diphenylhydantoin) is an anti-epileptic drug widely used for the treatment of epilepsy, which is generally treated with pharmacological agents intended to reduce neuronal excitability. Phenytoin is a Na^+ channel blocker that has no effect on fast activation and inactivation kinetics, responsible for the transient Na^+ current (I_{NaT}), but blocks the persistent Na^+ current (I_{NaP}), stabilizing the Na^+ channel in a non-conducting state (Catterall, 1999; Kuo and Bean, 1994) by accelerating its slow inactivation kinetics (Quandt, 1988; Colombo et al., 2013). The ability of phenytoin to block the I_{NaP} contributes to its anticonvulsant effect by decreasing membrane excitability and preventing the spread of the aberrant electrical activity from epileptogenic regions. Despite the known effects of phenytoin on epilepsy pathophysiology, less is known about its side effects on respiratory control. Donnelly and colleagues showed that acute treatment with phenytoin severely impaired normoxic ventilation and the hypoxic ventilatory response in rats (Faustino and Donnelly, 2006, 2006a). Furthermore, the same authors suggested that the carotid body chemoafferent pathway is a primary site of action of phenytoin. The carotid body (CB) is the main arterial chemoreceptor that peripherally drives ventilation in mammals. Afferent activity is generated by the synaptic drive of sensory nerve terminals of petrosal ganglion neurons by transmitters released by the CB receptor (glomus, Type-I) cells (Gonzalez et al., 1994; Iturriaga and Alcayaga, 2004; Nurse, 2014; Prabhakar, 2000). The generated afferent activity is conveyed to the central nervous system through sensory fibers of the

Abbreviations: I_{NaT}, transient Na^+ current; I_{NaP}, persistent Na^+ current; CB, carotid body; F_IO_2, oxygen inspiratory fraction; f_x, chemosensory discharge frequency; Δf_x, changes in chemosensory discharge frequency

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glossopharyngeal nerve, projecting to the nucleus of the solitary tract. Although the transmitters released by glomus cells are considered essential in the generation of afferent chemosensory activity (Iturriaga and Alcayaga, 2004; Nurse, 2014; Piskuric and Nurse, 2013), the electrical characteristics of petrosal ganglion neurons may also influence the afferent activity (Donnelly et al., 1998). These neurons present fast action potentials with almost no overshoot, a short duration after hyperpolarization and they discharge tonically when depolarized by long-lasting intracellular current pulses (Donnelly, 1999). In the rat, chemosensory afferent activity is decreased by reduced extracellular Na^+ and TTX (Donnelly et al., 1998), and petrosal ganglion neurons present TTX-sensitive sodium currents, both transient and I_{NaP} types (Faustino and Donnelly, 2006a). Interestingly, acute inhibition of I_{NaP} by phenytoin reduces both basal discharge in normoxia and the maximal discharge induced by acute reduction of the oxygen inspiratory fraction (F_IO_2; from 21% to 12%) in identified chemosensory neurons in vitro (Faustino and Donnelly, 2006, 2006a). Thus, acute phenytoin treatment markedly alters CB-chemoafferent hypoxic responses. In contrast to what is known about the effects of acute phenytoin treatment on CB-mediated chemoreflex responses, no information is available about the effects of chronic phenytoin administration on CB chemosensory function. Indeed, understanding the outcome of chronic phenytoin treatment on CB function is of potential clinical value since most patients with epilepsy receive chronic prescription of the drug. Thus, the main purpose of this study was to determine in rats the long-lasting effects of phenytoin treatment on CB chemosensory activity at several oxygen levels.

2. Results

Fig. 1 depicts representative electroneurograms obtained from one sham operated rat and one phenytoin treated rat. Under normoxic conditions (F_IO_2=21%) CB chemosensory afferent discharges, over the background noise threshold, were not significantly different between sham and phenytoin treated rats (Fig. 1A, B). Increasing F_IO_2 to 100% for 20 s produced a marked reduction in the afferent activity (Fig. 1A, B), that recovered to

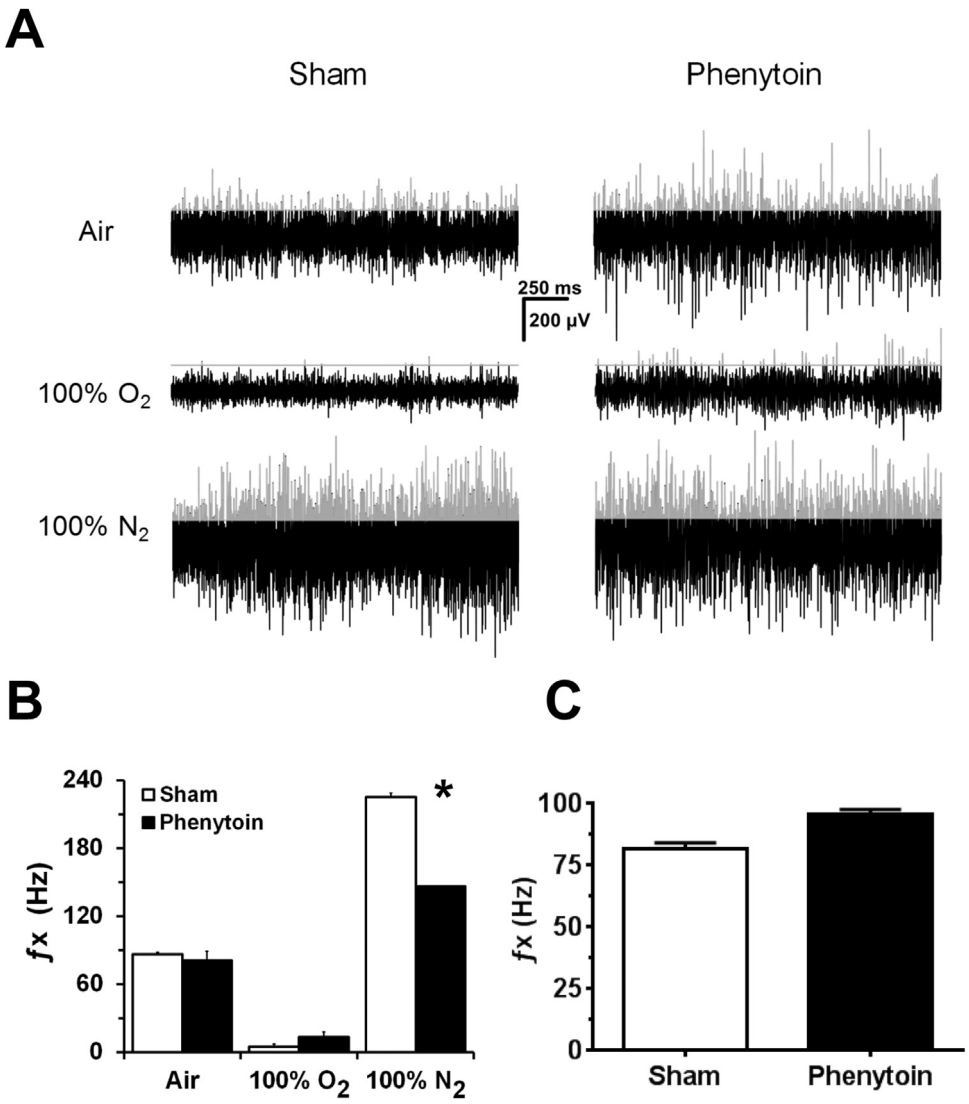

Fig. 1. Chemosensory frequency discharge (f_x) is modified by oxygen inspiratory fraction (F_IO_2) and phenytoin treatment. A) Chemosensory discharges recorded from the carotid nerve of one sham and one phenytoin treated rat are reduced in hyperoxia (100% O_2) and increased in hypoxia (100% N_2). Gray lines indicate the threshold for spike recognition and counted spikes. B) Mean chemosensory frequency discharges (f_x) computed in two consecutive one second intervals, from the upper recordings, during normoxia (Air; F_IO_2=21%), hyperoxia (F_IO_2=100% O_2) and hypoxia (F_IO_2=0%) in the sham (empty bars) and the phenytoin treated (filled bars) rat. * $P < 0.05$, Student´s *t*-test. C) Mean chemosensory frequency discharges in normoxia, computed during 5 normoxic intervals (30 s) on each animal prior to a change in F_IO_2, are not significantly different ($P > 0.05$, two way repeated measures ANOVA) between sham (n =9; empty bars) and phenytoin treated (n =7; filled bars) rats.

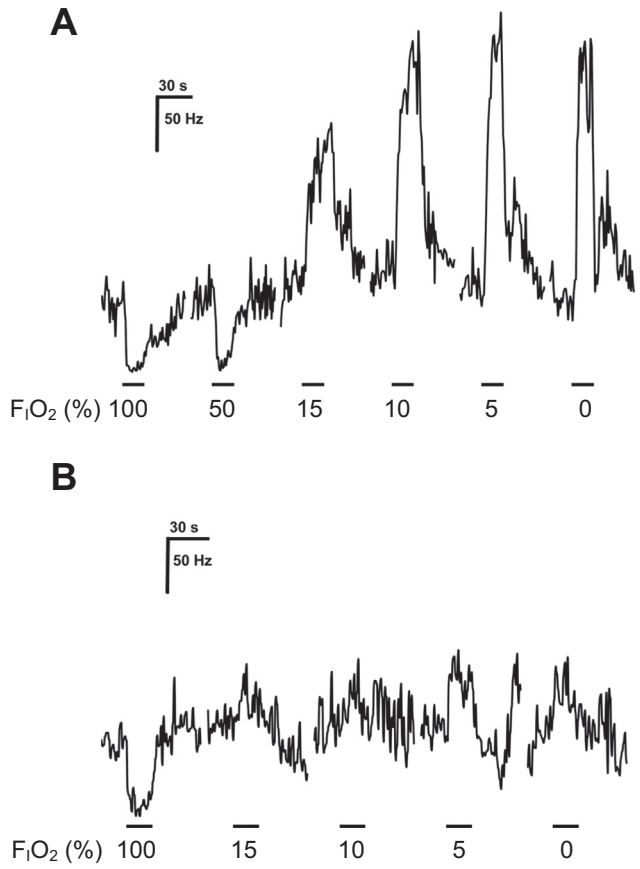

Fig. 2. Chemosensory discharge frequency (f_x) is modified by acute changes in oxygen inspiratory fraction (F_IO_2) in a single rat. Discharge frequency recordings in a sham rat (A) and a phenytoin treated (B) rat, showing that changes in F_IO_2 transiently modified f_x, reducing it if F_IO_2 was above 21%, while F_IO_2s below that level increased f_x. Reductions of f_x induced by hyperoxia were similar in both animals, but hypoxic responses were largely reduced in the phenytoin treated animal.

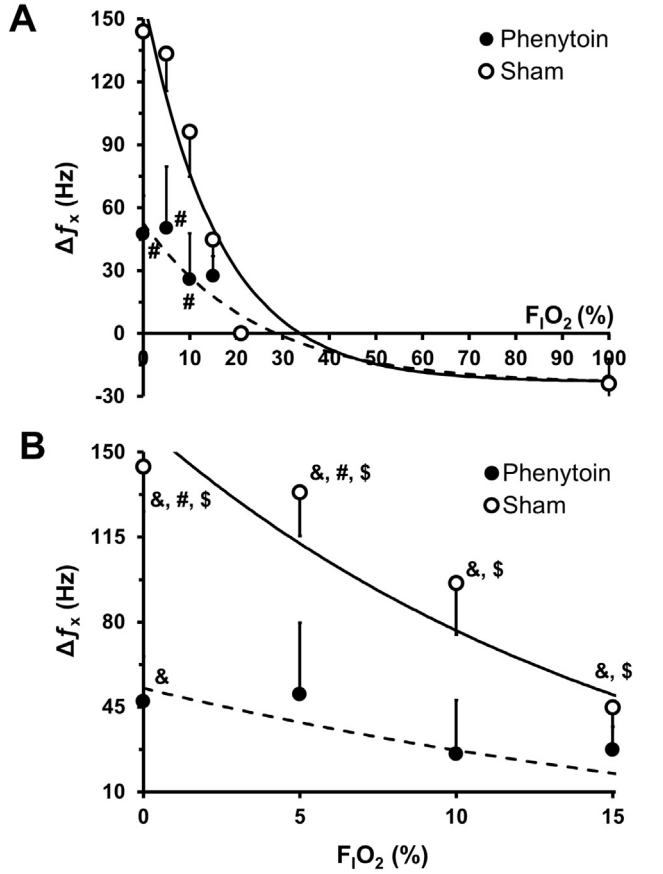

Fig. 3. Mean relationship between oxygen inspiratory fraction (F_IO_2) and changes in chemosensory discharge frequency (Δf_x) in sham (n = 11; empty circles, continuous line) and phenytoin treated (n = 8; filled circles, segmented line) rats. A) The overall mean relationships were significantly different ($P < 0.05$, two way repeated measures ANOVA) between phenytoin treated and sham operated animals, being significantly different (#, $P < 0.05$; Bonferroni's multiple comparisons test after two way repeated measures ANOVA) the mean responses between treatments when F_IO_2 was equal or below 10%. B) Expanded rendition of the relationships, showing significant differences ($P < 0.05$, Holm-Sidak's multiple comparisons test after two way repeated measures ANOVA) between responses within each treatment: and, different form response to $F_IO_2 = 100\%$; #, different form response to $F_IO_2 = 15\%$; $, different form response to $F_IO_2 = 21\%$.

baseline levels within 60 s after returning to normoxia in both conditions (Fig. 2). Reducing F_IO_2 to 0% for 20 s produced a rapid increase in CB chemosensory discharge frequency (f_x). The maximal value was reached within 10 s (Figs. 1A, 2) and returned to baseline levels within 30 s of return to normoxia in both experimental conditions. The mean CB discharge frequencies recorded in normoxia and hyperoxia from these two animals were similar in both conditions (Fig. 1B; $P > 0.05$, Student *t*-test), but frequency responses to hypoxia were significantly reduced ($P < 0.05$; Student *t*-test) in amplitude by phenytoin treatment (Fig. 1B). Indeed, the mean normoxic discharge frequency (Fig. 1C), measured in five 30 s intervals prior to changes in F_IO_2, was not significantly different ($P > 0.05$, two way repeated measures ANOVA) between sham operated (77.2 ± 2.2 Hz; n = 9) and phenytoin treated (95.4 ± 2.1 Hz; n = 7) rats. In all animals, changing F_IO_2 for 20 s in the 100–0% range produced changes in fx that were related to the magnitude and direction of the change. Increases in F_IO_2 over the normoxic (21%) value produced a marked reduction of the discharge, while decreases of F_IO_2 below normoxic values produced increases in f_x (Figs. 2, 3). The responses to hyperoxia were similar in magnitude and duration in sham and phenytoin treated rats, but the magnitude of the responses to hypoxia were largely reduced by phenytoin treatment (Figs. 2, 3). Nonetheless, the relationship between the changes in f_x (Δf_x) and the F_IO_2 was similar in phenytoin treated rats and sham rats (Fig. 3).

The relationship between mean Δf_xs and F_IO_2 shows that the responses induced by hypoxia were reduced in phenytoin treated rats ($P < 0{,}05$, two way repeated measures ANOVA), with mean Δf_xs for F_IO_2s equal or below 10% (Fig. 3A) being significantly lower ($P < 0.05$; Bonferroni's multiple comparisons test) in phenytoin treated rats. In the lower part of the F_IO_2 range, responses induced in sham operated rats, increased significantly over normoxic values ($P < 0.05$, Holm-Sidak's multiple comparisons test after two way repeated measures ANOVA) for all the hypoxic challenges, and increases in f_x were maximal when F_IO_2 was 5% or less (Fig. 3B). The mean Δf_xs induced by changes in F_IO_2 in phenytoin treated rats were not significantly different from basal normoxic f_xs ($P > 0.05$; Holm-Sidak's multiple comparisons test after two way repeated measures ANOVA). The only significant difference ($P < 0.05$; Holm-Sidak's multiple comparisons test after two way repeated measures ANOVA) was between the extreme values of the F_IO_2 range (100% and 0%). In addition, we assessed the effects of phenytoin treatment on CB hypoxic sensitivity and oxygen threshold (Fig. 4). Compared to sham operated animals, phenytoin treated rats display a significant ($P < 0.05$, Students *t*-test) 2.2-fold reduction in CB hypoxic sensitivity, from 278.0 ± 23.0 Hz in sham rats to 128.0 ± 8.4 Hz in phenytoin treated ones (Fig. 4B). Accordingly, the oxygen threshold (% of F_IO_2) required to elicit a hypoxic response in phenytoin treated rats was markedly shifted towards hyperoxic values (Fig. 4C; $F_IO_2 = 37.4\%$) when compared to the values obtained in sham rats (Fig. 4C; $F_IO_2 = 14.8\%$).

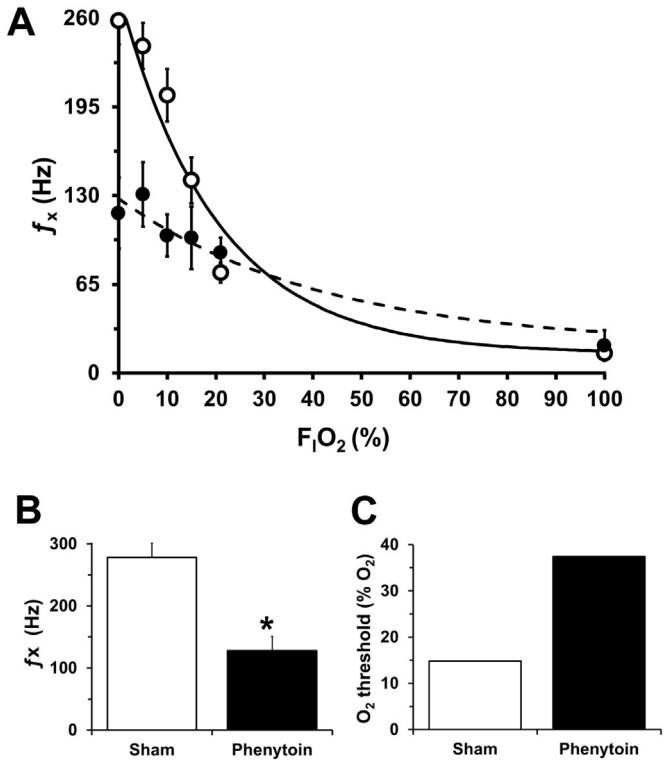

Fig. 4. Effects of chronic phenytoin treatment on CB chemosensory function hypoxic sensitivity and oxygen threshold. A) Summary data showing CB chemosensory exponential-fitted curves in response to several F_IO_2 in sham rats (continuous line) and phenytoin treated rats (segmented line). B) CB hypoxic sensitivity in sham and phenytoin treated rats. Note that phenytoin reduced CB hypoxic sensitivity by 2.5-fold. C) The oxygen threshold required to elicit a CB chemosensory response is shifted towards hyperoxic values in phenytoin treated rats.

3. Discussion

Our results show that CB chemosensory responses to acute hypoxia are significantly reduced by chronic phenytoin treatment in the rat. These findings extend previous observations of the effect of acute phenytoin treatment on CB chemosensory responses in rats (Faustino and Donnelly, 2006, 2006a). Previous work shows that acute phenytoin has no effects on the conduction velocity, amplitude, and duration of the intracellularly recorded action potentials, suggesting that reductions in the afferent activity originate at the level of action potential generation (Faustino and Donnelly, 2006, 2006a). On the other hand, a reduction of the amplitude of the fast conducting component of the sciatic nerve compound action potential (CAP) has been reported, after acute (Uemura et al., 2014) and chronic (Zafeiridou et al., 2016) phenytoin treatment, suggesting a reduction in cell excitability. Similar reduction in CAP amplitude has been reported by acute phenytoin treatment on the mostly unmyelinated cervical sympathetic trunk (Elliot, 1990). Although we did not measure the amplitude of the CAP in our preparation, the amplitude of individual spikes over background noise in normoxic conditions were not different in amplitude or duration between sham and phenytoin treated rats. Moreover, mean discharge was not significantly different between sham and phenytoin treated rats in the normoxic condition. Thus, significant changes in the discharge appear to be unrelated to chronic phenytoin treatment.

On the other hand, petrosal ganglion neurons are insensitive to hypoxia (Alcayaga et al., 1999, 2000; Nurse and Zhang, 2001), which suggests that the effect of chronic phenytoin treatment on the responses to acute hypoxia cannot be directly mediated by the effects of oxygen on neuronal fibers or terminals. It is noteworthy that phenytoin effects are more important in hypoxic than in hyperoxic responses. However, if I_{NaP} is a key component in the generation and the increase of the discharge frequency of chemosensory afferents (Donnelly et al., 1998; Faustino and Donnelly, 2006) in the whole range (0–100% O_2), its effects could be low or even undetectable at high F_IO_2s and became increasingly relevant as F_IO_2 decreases.

Our whole nerve recordings also contain activity from baroreceptor fibers whose terminals are located in the carotid sinus. Baroreceptor discharges are reduced with decreases in arterial pressure and conversely increased when arterial pressure is elevated (Brown, 1980; Landgren, 1952). Because hypoxic stimulation produces hypotension (Magnusson and Cummings, 2015; Mendoza et al., 2014) the observed increases in CB chemosensory responses to acute hypoxia could not depend on barosensory activity. Moreover, if baroreceptor activity was indeed present in our recordings it would presumably be reduced during hypoxia, and thus the observed changes in chemosensory activity would underestimate the real magnitude of the changes.

It is widely accepted that afferent chemosensory activity results from synaptic activation mediated by neurotransmitters released by CB chemoreceptor (type 1, glomus) cells (Gonzalez et al., 1994; Iturriaga and Alcayaga, 2004; Nurse, 2014; Prabhakar, 2000). It has been reported that acute phenytoin treatment has no effect on the CB catecholamine secretion, suggesting that phenytoin would not modify the synaptic communication between CB chemoreceptor cells and the nerve terminals (Faustino and Donnelly, 2006, 2006a). However, although catecholamine and dopamine release have been used as markers of CB function (González et al., 1994; Ureña et al., 1994) the measured release does not appear to be closely related to the afferent activity (Donnelly, 1996; Iturriaga et al., 1996; 2000). It is noteworthy that the direct effect of dopamine on petrosal ganglion neurons appears to be species-specific, ranging from modulator to a neuronal activator (Iturriaga et al., 2009). On the other hand, synaptic communication between CB chemoreceptor cells and petrosal ganglion chemosensory neurons can be modified by long-lasting adaptation processes, such as chronic normobaric hypoxia (Alcayaga et al., 2012; Icekson et al., 2013). Moreover, phenytoin inhibits K^+-induced glutamate and GABA release from central synaptosomes in rats (Kammerer et al., 2011, Kammerer et al., 2011a). Similarly, phenytoin reduces presynaptic Ca^{2+} increases, induced by caged glutamate release in cultured cortical neurons (Chou et al., 2014), and by 4-AP in hippocampal nerve endings (Sitges et al., 2016). The above data indicate that in addition to its action on I_{NaP}, phenytoin may also modify the synaptic communication acting directly on the exocytotic pathways. Prolonged (4 weeks) phenytoin treatment in rats differentially modifies gene expression in the central nervous system, with the hippocampus being more affected than the frontal cortex (Mariotti et al., 2010). The changes in gene expression suggest increased glutamate degradation and N-methyl-D-aspartic receptor (NMDAR) expression, increase in anti-apoptotic and antioxidant pathways, and vesicle motility and fusion (Mariotti et al., 2010). Thus, phenytoin can differentially modify key components of synaptic communication and protect against oxidative stress. The preceding data indicate that a possible direct effect of phenytoin on synaptic transmitter release by glomus cells and/or sensory nerve terminals cannot be ruled out.

Phenytoin is used clinically in the chronic treatment of epilepsy and trigeminal neuralgia (in infants and adults), and in the acute treatment of eclampsia. The antiepileptic effects of phenytoin are mainly explained by its selective action on Na channels (Lingamaneni and Hemmings, 2003), specifically reducing I_{NaP} (Segal and Douglas, 1997) and thus reducing the probability of high frequency neuronal discharge (Brumberg et al., 2000). However, phenytoin may also reduce excitatory synaptic transmission

(Cunningham et al., 2000) and enhance inhibitory synaptic transmission to high frequency stimulation, without affecting low frequency transmission (Cunningham et al., 2000).

Phenytoin shown to produce a dose-dependent reduction of the anoxia induced gasping frequency in guinea pigs (Naiman and Williams, 1964), increasing significantly the survival time after the onset of anoxia in guinea pigs and cats. Bilateral chemodenervation had similar effects to those of phenytoin in control animals, although phenytoin further increased survival time in chemodenervated animals (Naiman and Williams, 1964), suggesting actions in both peripheral chemoreceptors and in the central nervous system.

Phenytoin has no direct effect on mitochondrial function (Santos et al., 2008) or oxidative stress (Santos et al., 2008a), even at concentrations beyond its therapeutic use (Rundfeldt and Löscher, 1993), but phenytoin metabolites could induce a reduction in mitochondrial function (Santos et al., 2008) and an increase in oxidative stress (Santos et al., 2008a). These changes in mitochondrial function and oxidative stress can also modify chemosensory activity and peripheral ventilatory control (Del Rio et al., 2010, 2011). Thus, phenytoin actions on synaptic transmission, mitochondrial and oxidative stress may also modify afferent chemosensory activity and impact on the relative importance of peripheral chemosensory drive.

4. Conclusions

Chronic phenytoin treatment has a profound effect on the afferent chemosensory discharges induced by 30 s hypoxic challenges. The reductions in responses to hypoxia suggest that chronic phenytoin treatment may reduce excitability of afferent neurons and/or modify the synaptic communication between carotid body receptor cell and neuronal terminals. Moreover, antiepileptic drugs with similar molecular targets than phenytoin may produce similar long lasting effects. Further studies are necessary to elucidate the exact mechanisms that underlie the long term effects of phenytoin treatment.

5. Experimental procedures

All research and animal care were performed according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Research Commission on Life Sciences, National Research Council (National Academy Press, Washington, DC 1996) and the guidelines of the Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT, Chile) and were approved by the Bioethics and Biosafety Commission from the Universidad de Chile.

5.1. Animals

For these studies, nineteen male Sprague Dawley rats (204.7 ± 5.5 g) were used. The animals were fed with standard chow diet ad libitum and kept on a 12-h light/dark schedule (08:00–20:00 h). Rats were treated for approximately 25 days with either phenytoin or vehicle administered via osmotic pump. Laboratory personnel performing experiments were blinded to experimental group. After 25 days of treatment rats were assessed for changes in CB afferent activity in response to several F_iO_2 levels (0–100%).

5.2. Drug treatment

Rats were randomly assigned to the following treatment groups: i) phenytoin group was administered 10 mg/day for 25 days of phenytoin dissolved in 40% DMSO in NaCl 0.9% via chronic implantation of a mini-osmotic pump (Alzet® 2ML4, DURECT Corporation, USA) or ii) vehicle (sham operated group, 40% DMSO in NaCl 0.9%) via osmotic pump. Briefly, the animals were anesthetized with 2% isoflurane in O_2 and an incision was performed between the rat scapulae. A subcutaneous pocket was opened to place the osmotic pump in the back of the animal. The wound was closed with Michel suture clips (7.5 mm long x 1.75 mm wide) and covered with a gel mixture of local anesthetic (lidocaine, 2%) and *anti*biotics (bacitracin, 5 mg/g; neomycin 500 U.I./g). Eleven animals (202.3 ± 7.7 g) were implanted with an osmotic pump filled with vehicle (sham; 40% DMSO in NaCl 0.9%) and eight rats (210.8 ± 7.9 g) were implanted with a pump containing phenytoin (166 mg/mL) dissolved in the same vehicle (pump delivered rate 60 μL/day). Since osmotic pumps were not primed, phenytoin treated rats received an additional single i.p. phenytoin dose of 75 mg/kg, in the same vehicle at the beginning of the experiment.

5.3. Carotid body chemosensory recordings

Three weeks after surgery, animals were anesthetized with sodium pentobarbitone (60 mg/kg, i.p.) and placed in supine position in a custom made thermoregulated dissection table. The neck was opened through the midline, the trachea cannulated with a plastic tube and the carotid bifurcation exposed. The carotid nerves were separated from surrounding tissue and severed at both sides of their apparent origin in the glossopharyngeal nerve. One nerve was placed in paired Pt/Ir electrodes, connected in turn to an AC preamplifier (P55, Natus Neurology, USA), and covered with warm mineral oil. The recorded signal (electroneurogram) was band-pass filtered (10 Hz–1 KHz), amplified, fed to a custom made spike amplitude discriminator and counter, and the conditioned signal digitally counted in 1 s intervals to assess the chemosensory discharge frequency (f_x, in Hz). Animals breathed spontaneously throughout the experiment and were submitted to acute changes in oxygen inspiratory fraction (F_IO_2: 0–100%) for 30 s. The signals were digitally acquired at 2 KHz (PowerLab 8SP, ADInstruments, USA) and stored as binary files.

5.4. Data analysis and statistics

Data was displayed and analyzed using LabChart® 7 Pro (ADInstruments, USA) and Excel® spreadsheet (Microsoft Corp., USA) under Windows® 7 Professional (Microsoft Corp., USA) operating system. Changes in f_x (Δfx) were evaluated as the maximal or minimal response during a single F_IO_2 modification minus 95% confidence interval of the mean basal frequency (bas f_x), computed in a 30 s interval prior to every evoked response.

Results are presented as the mean ± standard error (SE). Statistical analyses were performed using GraphPad Prism version 6.00 for Windows® (GraphPad Software, La Jolla, CA, USA). Mean value comparisons were performed with Student´s *t*-test or Mann Whitney test, according to structure of data. Results from experimental groups were analyzed using repeated measures two-way ANOVA with multiple comparisons post-hoc tests. A p value < 0.05 was considered as statistically significant. All comparisons of experimental data were performed with two-tailed tests.

Declarations

6.1. Competing interests

No competing interests are declared by the authors.

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Authors' contributions

JA designed the study. JA, MPO and RDR carried out the experiments. JA and RDR analyzed data and prepared the manuscript. All authors read and approved the final manuscript.

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