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Therefore the correct authorship of this paper at the time should have read as follows:

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Abstract: The 2-nitrate-1,3-dibuthoxypropan (NDBP) is a new organic nitrate synthesized from glycerin. The aim of this study was to compare the cardiorespiratory responses evoked by NDBP to the clinically used organic nitrate glyceryl trinitrate (GTN). We found that intravenous injection of NDBP (1 to 15 mg/kg) and GTN (0.1 to 5 mg/kg) produced hypotension. NDBP induced bradycardia at all doses, while GTN induced tachycardia at three lower doses but bradycardia at higher doses. Hydroxocobalamin (20 mg/kg; HDX), a NO scavenger, blunted hypotension induced by NDBP (15 mg/kg), and its bradycardic effect. In addition, HDX blunted both hypotension and bradycardia induced by a single dose of GTN (2.5 mg/kg). Both NDBP and GNT altered respiratory rate, inducing a biphasic effect with a bradypnea followed by a tachypnea; HDX attenuated these responses. Our data indicate that NDBP and GTN induce hypotension, bradycardia and bradypnea, which are mediated by nitric oxide release.

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Dear Editor,

We would like to submit for your consideration our manuscript “Cardiorespiratory effects induced by 2-nitrate-1,3-dibuthoxypropan are reduced by nitric oxide scavenger in rats”. This work describes cardiovascular and respiratory effects of new synthetic organic nitrate. None of the data were published or submitted for publications elsewhere.

Respectfully yours,

A/Prof Eugene Nalivaiko

**Cardiorespiratory effects induced by 2-nitrate-1,3-dibuthoxypropan
are reduced by nitric oxide scavenger in rats**

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Abstract

The 2-nitrate-1,3-dibuthoxypropan (NDBP) is a new organic nitrate synthesized from glycerin. The aim of this study was to compare the cardiorespiratory responses evoked by NDBP to the clinically used organic nitrate glyceryl trinitrate (GTN). We found that intravenous injection of NDBP (1 to 15 mg/kg) and GTN (0.1 to 5 mg/kg) produced hypotension. NDBP induced bradycardia at all doses, while GTN induced tachycardia at three lower doses but bradycardia at higher doses. Hydroxocobalamin (20 mg/kg; HDX), a NO scavenger, blunted hypotension induced by NDBP (15 mg/kg), and its bradycardic effect. In addition, HDX blunted both hypotension and bradycardia induced by a single dose of GTN (2.5 mg/kg). Both NDBP and GNT altered respiratory rate, inducing a biphasic effect with a bradypnea followed by a tachypnea; HDX attenuated these responses. Our data indicate that NDBP and GTN induce hypotension, bradycardia and bradypnea, which are mediated by nitric oxide release.

Key words: arterial pressure, heart rate, respiratory rate, nitric oxide, organic nitrate

1. Introduction

Nitric oxide (NO) is a simple gas that can be formed *in vivo* by the action of the NO synthase, an enzyme that converts L-arginine to L-citrulline. NO plays a role in multiple physiological and pathological effects, including vasodilation, angiogenesis, immunity, platelet aggregation and neurotransmission (Ignarro, 2000; Moncada et al., 1991; Bolotina et al., 1994). In central nervous system (CNS), especially in the brainstem, NO has been implicated in the control of cardiorespiratory function (Krowicki et al., 1997; Kamendi et al., 2006). To accomplish its physiological effects, the classic signaling pathway induced by NO involves activation of the soluble guanylyl cyclase (sGC) catalyzing the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which in turn activates the cGMP-dependent protein kinase (PKG) (Arnold et al., 1977; McDonald and Murad, 1996).

The simplicity and versatility of NO molecule allows the synthesis of a great variety of NO donors (Miller and Megson, 2007; Muscará and Wallace, 1999; Munhoz et al., 2012). NO donors are substances that mimic the role of endogenous NO. These substances have been used for several cardiovascular diseases, mainly hypertension and coronary artery disease (Harrison and Bates, 1993; Naseem, 2005; Paulo et al., 2012). Two of the most known NO donors are the sodium nitroprusside (SNP) and glyceryl trinitrate (GTN). Despite their beneficial effects in cardiovascular system, they have some restrictions for their clinical applications due to undesirable side effects (Laursen et al., 1996). For instance, GTN has its effect attenuated during continuous treatment; it also promotes tolerance to other nitrates, a phenomenon known as cross-tolerance (Kosmicki, 2009). SNP has limited use due to the development of tolerance; furthermore, prolonged administration of this NO donor has been restricted due to the high toxicity caused by the release of cyanide (Ignarro, 2002).

1 The limitations named above for commonly used NO donors could be potentially overcome
2 with the synthesis of new NO donors. The 2-nitrate-1,3-dibuthoxypropan (NDBP) is a
3 synthetic compound obtained in our research center from glycerin (Santos, 2009). Previous
4 study from our research group showed that NDBP induces hypotension predominantly due to
5 the reduction in cardiac output by direct vagal activation (França-Silva et al., 2012b). The
6 present study aimed to further investigate the cardiorespiratory responses evoked by NDBP
7 and to compare this effect with the well-known NO donor, glyceryl trinitrate (GTN).
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18 **2. Material and Methods**

19 *2.1 Animals*

20 Male Wistar rats (200–250 g) were housed in conditions of controlled temperature ($21\pm1^{\circ}\text{C}$)
21 at a 12 h light-dark cycle (lights on 8am) with free access to food (Labina®, Purina, São
22 Paulo, Brazil) and tap water. All procedures described in the present study were approved by
23 the Institutional Animal Care and Use Committee of the Federal University of Paraíba
24 (CEPA/LTF protocol nº 0209/10). Rats were anesthetized with ketamine and xylazine (75 and
25 10 mg/kg, i.p., respectively) and the lower abdominal aorta and inferior vena cava were
26 catheterized via left femoral artery and vein using polyethylene tubing catheters. The catheters
27 were filled with heparinized saline and tunneled under the skin to the interscapular region.
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45 *2.2. Experimental protocol*

46 One day after the surgical procedures, blood pressure and heart rate were evaluated in one
47 group of conscious rats before and after administration of NDBP (1, 5, 10, 15 and 20 mg/kg).
48 In another group of animals, effects of GTN (0.1, 0.25, 0.5, 1, 2.5 and 5 mg/kg) were
49 assessed. From these results, the submaximal doses of 15 mg/kg and 2.5 mg/kg for NDBP and
50 GTN, respectively, were chosen for the following experiment conducted in two other groups
51 of rats. Either NDBP or GTN were administered twice, with hydroxycobalamin (HDX, a NO
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scavenger, 20 mg/kg) given 15 min before the addition of the second dose of the nitrate. In this second experiment, in addition to arterial pressure and heart rate measurements, we also recorded respiratory rate (see below).

2.3. Data acquisition and analysis

Arterial pressure was measured by connecting the arterial catheter to a pre-calibrated pressure transducer (MLT0380/D, ADInstruments, Sydney, Australia). Respiratory rate was acquired using a custom-built whole-body plethysmograph (Kabir et al., 2010; Carnevali et al., 2013). This consisted of a sealed Perspex cylinder (i.d. 95 mm, length 260 mm, volume 2.5 l) with medical air constantly flushed through it at a flow rate of 2.5 l/min. The output flow was divided into two lines using a T-connector. One line was attached to a differential pressure amplifier (model 24PCO1SMT, Honeywell Sensing and Control, Golden Valley, MN, USA), while the other line was open to the room air. Arterial and venous catheters were passed through the plethysmograph wall via a sealed port. Respiratory and AP signals were digitized at 1 kHz and acquired using PowerLab4/35 (ADInstruments, Australia) and a computer running the LabChart 7.0 software (ADInstruments, Australia). Respiratory rate, heart rate, systolic, diastolic and mean arterial pressure were computed online with subsequent off-line verification.

2.4. Drugs

NDBP was synthesized in the Department of Chemistry at the Federal University of Paraiba as described earlier (França-Silva et al., 2012b). It was dissolved in a mixture of saline and chremophor and diluted to the desired concentrations with saline as previously described (França-Silva et al., 2013a). GTN was obtained from Cristal Pharm[®] (Brazil) and diluted to the desired concentrations with saline. Hydroxycobalamin was obtained from Merck[®] (USA).

2.5. Statistical Analysis

Results are expressed as mean $\pm$ S.E.M. Data were analyzed by the Student's t-test or by the two-way repeated measures ANOVA followed by a Dunnett post-hoc test for multiple comparisons whenever appropriate. All statistical analyses were performed using GraphPad Prism (v. 5.0, GraphPad Software, Inc.). Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Effects NDBP and GTN on blood pressure and heart rate

Administration of NDBP elicited hypotensive and bradycardic responses as illustrated in Fig 1A. These effects were dose-dependent as shown in Fig. 3A. Intravenous bolus injection of GTN provoked similar hypotensive effect (Fig. 2A), also in a dose-dependent manner (Fig. 3B). Interestingly, GTN at three lower doses induced tachycardia while at three higher doses it caused bradycardia whereas NDBP caused bradycardia at all doses (Fig. 3B). The mean arterial pressure and heart rate values returned to basal levels within the first minute after injection of all doses of the nitrates. GTN was found to be more potent than NDBP in inducing a decrease in mean arterial pressure and heart rate ($p > 0.05$).

3.2 NDBP and GTN induce a more potent reduction in diastolic than in systolic arterial pressure.

Because the notion of the mean arterial pressure loses its sense during profound bradycardia elicited by both our NO donors, we also analyzed their effects separately on systolic and on diastolic arterial pressure values (SAP and DAP). The maximal variations in these parameters induced by NDBP and GTN are presented in Fig. 4. After administration of NDBP (1 to 20 mg/kg), there was a reduction in the SAP and this reduction was significantly accentuated in DAP (Figure 4A). Likewise, bolus injection of GTN (0.1 to 5 mg/kg) produced a dose-

dependent decrease in both SAP and DAP, with more prominent effect on the latter one (Fig. 4B).

3.3. Hypotension and bradycardia induced by NDBP and GTN are blunted by NO scavenger

In order to evaluate the NO involvement in the responses induced by NDBP or GTN, the cardiovascular effects were assessed before and after a bolus injection of a NO scavenger, HDX (20 mg/kg). Administration of HDX blunted hypotension induced by NDBP (-11 ± 5 vs. -66 ± 6 mmHg, $P < 0.05$, $n = 7$) and converted its bradycardic effect into the tachycardic ($+65 \pm 13$ vs. -356 ± 24 bpm, $P < 0.05$, $n = 7$) as shown in Fig 1B (raw data) and Fig. 5A (mean group values). HDX also blunted both hypotension (-47 ± 5 vs. -82 ± 8 mmHg, $P < 0.05$, $n = 6$) and bradycardia (-191 ± 28 vs. -378 ± 30 bpm, $P < 0.05$, $n = 6$) evoked by a single dose of GTN as illustrated in Fig 2B (raw data) Fig 5B (mean group values). HDX alone had no effect on any of the recorded variables (data not shown).

3.4. NDBP and GTN induce biphasic changes in respiratory rate

To determine whether NO donors change the respiratory function, we monitored the respiratory rate in conscious rats using whole-body plethysmography. NDBP induced a short-lasting reduction in respiratory rate (-78 ± 9 cpm; $P < 0.05$, $n = 6$) followed by a prolonged tachypnea (231 ± 28 cpm; $P < 0.05$, $n = 6$) as shown in Fig 1A (raw data) and in Fig 6A&C (mean group values). Similar biphasic effects were found following GTN administration: short-latency bradypnea (-95 ± 12 cpm; $P < 0.05$, $n = 6$) followed by a tachypnea (159 ± 19 cpm; $P < 0.05$, $n = 6$) as shown in Fig. 1B (raw data) and in Fig 6B&D (mean group values).

3.5. Bradypnea and tachypnea induced by NDBP and GTN are reduced by NO scavenger

To determine whether modifications in the respiratory rate were mediated via NO release, respiratory responses to nitrates were assessed before and after administration of HDX. The drug attenuated both brady- and tachypneic effects of NDBP as illustrated in Fig. 1. HDX also reduced the bradypnea induced by GTN, but did not alter its tachypneic effects. Mean group values for these experiments are presented in Fig. 6.

4. Discussion

NO donors provoke hypotension (Chen et al., 2002; Munhoz et al., 2012) associated with tachycardia or bradycardia (Alpert, 1990; França-Silva et al., 2012b), and our results with NTG are in full accordance with these and other previous studies. Fall in arterial pressure following NDBP replicates and confirms our recent findings that it induces hypotension and bradycardia in conscious spontaneously hypertensive and normotensive rats (França-Silva et al., 2012b). Administration of a NO scavenger HDX attenuated both hypotension and bradycardia caused by NDBP, confirming the role of NO in the cardiovascular responses elicited by this new organic nitrate. Likewise, HDX substantially reduced cardiovascular responses to GTN, in agreement with previous studies (Tinker and Michenfelder, 1976; Schubert et al., 2004; Bonaventura et al., 2006).

Several mechanisms could be involved in the hypotensive effects of NO donors. We have previously reported that NDBP induces vasodilatation in mesenteric artery; similar to other NO donors (Araújo et al., 2013; Shukur et al., 2013), this effect was mediated by NO/cGMP/PKG pathway (França-Silva et al., 2012a). Centrally-mediated hypotensive effects of NO donors are also well documented: direct administration of NO or a NO donors into the paraventricular nucleus (PVN) decreases sympathetic nerve activity and lowers arterial blood pressure (Horn et al., 1994; Zhang and Patel, 1998). Conversely, inhibition of NO synthesis in

1 the PVN results in sympathoexcitation (Zhang and Patel, 1998; Coleman et al., 2010). While
2 both central and peripheral neural mechanisms could be involved to some extent in the
3 hypotension described here, we have recently reported that the principal mechanism whereby
4 NDBP reduces AP in rats is short-lasting but dramatic reduction in cardiac output due to
5 profound bradycardia: indeed, prevention of this bradycardia by vagal blockade or by
6 transection of both vagi virtually eliminated the hypotensive effect of NBDP (França-Silva et
7 al., 2012b). This experiment also unambiguously confirmed that NO-induced bradycardia was
8 of central origin, and that peripheral sympatholytic effects of NO (eg. Kolo et al., 2004) plays a
9 minor, if any, role in this effect.
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24 The principal qualitative difference between the two NO donors studied in this work was in
25 their chronotropic effects. Indeed, at the low doses, GTN induced tachycardia whereas effects
26 of NBDP were tachycardic at all doses. NO donors like GTN and SNP are common
27 pharmacological tools for studying HR-baroreflex in experimental animals (Botelho-Ono et
28 al., 2011; Queiroz et al., 2013; Fukuma et al., 2011), and mentioned above tachycardic
29 responses were most likely baroreflex-mediated. Bradycardia elicited by higher doses of both
30 donors is likely due to the fact that direct activation of cardiac vagal neurons overrode the
31 baroreflex-mediated tachycardia. The question however remains as to why NBDP did not
32 cause tachycardia at any dose, including doses that provoked hypotension comparable to that
33 caused by low doses of NTG that were associated with tachycardia. For now this question
34 remains open; the possibility that the difference was due to non-NO action of the donors is
35 excluded since NBDP-induced bradycardia was completely abolished by NO scavenger.
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56 Usually, organic nitrates cause a greater reduction in diastolic blood pressure (DBP) than the
57 systolic pressure (SBP) (Bauer et al., 1997; Korzycka and Górska, 2005), similar results were
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1 found in our study for both NDBP and GTN. Commonly this selective effect of NO donors
2 are explained by their greater activity on the veins and are further implicated in reducing
3 preload and venous return.
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10 To the best of our knowledge, our study is the first to report the effects of exogenous NO
11 donors on the respiratory function. These effects were mediated by NO as they were
12 substantially attenuated by the NO scavenger HDX. Since short-term bradypnea observed in
13 our study is reminiscent to that elicited during activation of chemoreflex by i.v. KCN in rats,
14 it is not unreasonable to suggest that chemoreflex pathway could be involved in the effects of
15 NO in our study. While data on NO-dependence of the chemoreflex are quite limited
16 compared to the baroreflex, several studies provide evidence that allows to exclude
17 involvement of chemoreflex in the respiratory effects of NO donors. Firstly, at the level of
18 peripheral chemoreceptors, NO reduces, not increases, the discharge of the afferent fibers
19 (Sun et al., 1999). Secondly, direct microinjections of NO synthase inhibitors into the nucleus
20 of tractus solitaries, a chemoreflex relay region, did not affect respiratory rate (Granjeiro and
21 Machado, 1999). Lastly, and most importantly, chemoreflex-induced bradypnea is associated
22 with generalized vasoconstriction (Heard et al., 1996) whereas in our experiments we
23 observed falls in the AP.
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46 Our results do not allow us to conclude whether NO directly affected neuronal activity of the
47 respiratory pattern generator in the brainstem or activated descending pathways that modulate
48 its activity. Documented influences from the paraventricular nucleus could be safely excluded
49 as microinjection of NO donor in this nucleus did not affect respiratory rate (Reddy et al.,
50 2007).
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1 The effect of NO donors on respiration was biphasic, with initial bradypnea followed by a
2 period of tachypnea. This delayed tachypnea is unlikely to be a compensatory chemoreflex-
3 mediated response as it developed as a very short latency. As in rodents various sensory
4 stimuli provoke vigorous non-specific respiratory arousal responses (Kabir et al., 2010;
5 Nalivaiko et al., 2012), it may be that tachypnea observed in our rats was also the sign of
6 general arousal triggered by NO-dependent activation of interoceptive input.
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17 In conclusion, NDBP induced hypotension, bradycardia and bradypnea, all of which were
18 mediated by nitric oxide release. The major difference between NDBP and GTN (a clinically
19 used organic nitrate) is that the former did not provoke baroreflex-dependent tachycardia.
20 While the reason for this difference is not clear, NDBP appears to possess a more favorable
21 clinical profile compared to GTN as avoiding cardiac sympathetic activation during angina
22 relief could be beneficial in many instances.
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34 technical assistance in experimental procedures.
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Figure legends:

Fig 1. Representative tracings illustrating cardiovascular and respiratory responses to NDBP before (A) and after (B) administration of NO scavenger hydroxycobalamine.

Fig 2. Representative tracings illustrating the cardiovascular and respiratory responses to NTG before (A) and after (B) administration of NO scavenger hydroxycobalamin.

Fig 3. Effects of increasing doses of NDBP (A) and NTG (B) on the mean arterial pressure (MAP, top panels) and heart rate (HR, bottom panels). Values are means $\pm$ S.E.M.

Fig 4. Effect of NDBP (A) and GTN (B) on systolic arterial pressure (SAP, $\square$) and diastolic arterial pressure (DAP, $\blacksquare$). Values are means $\pm$ S.E.M. * $p < 0.05$ vs SAP.

Fig 5. Hydroxycobalamine attenuates hypotensive and bradycardic effects of NDBP (15 mg/kg, A) and GTN (2.5 mg/kg, B). White bars – responses elicited by NO donors post-vehicle; black bars – post-HDX. Values are means $\pm$ S.E.M. * $p < 0.05$ vs basal (post-vehicle) values.

Fig 6. Mean group values showing effects of a single dose of NDBP (15 mg/kg, A) and GTN (2.5 mg/kg, B) on the respiratory rate, in control and after blockade with HDX. Open symbols - NO donors were administered post-vehicle; closed symbol - post-HDX. Panels B and D are quantified results from A and B, respectively. Two white bars represent the delta values for the initial bradypnea and subsequent tachypnea after vehicle; black bars – corresponding values after HDX administration. * $p < 0.05$ vs basal (post-vehicle) values.

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