Nordexfenfluramine causes more severe pulmonary vasoconstriction than dexfenfluramine

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Am J Physiol Lung Cell Mol Physiol 286: L531–L538, 2004. First published November 7, 2003; 10.1152/ajplung.00247.2003.—The anorectic agent dexfenfluramine (dex) causes the development of primary pulmonary hypertension in susceptible patients by an unknown mechanism. We compared the effects of dex with those of its major metabolite, nordexfenfluramine (nordex), in the isolated perfused rat lung and in isolated rings of resistance pulmonary arteries. Nordex caused a dose-dependent and more intense vasoconstriction, which can be inhibited by the nonspecific 5-hydroxytryptamine type 2 (5-HT2) blocker ketanserin. Similarly a rise in cytosolic calcium concentration ([Ca2+]i) in dispersed pulmonary artery smooth muscle cells (PASMCs) induced by nordex could be prevented by ketanserin. Unlike prior observations with dex, nordex did not inhibit K+ current or cause depolarization in PASMCs. Removal of Ca2+ from the tissue bath or addition of nifedipine (1 μM) reduced ring contraction to nordex by 60 ± 9 and 63 ± 4%, respectively. The addition of 2-aminooxydiphenyl borate (2-APB), a blocker of store-operated channels and the inositol 1,4,5-trisphosphate receptor, caused a dose-dependent decrease in the ring contraction elicited by nordex. The addition of 2-APB (10 μM) and nifedipine (1 μM) completely ablated the nordex contraction. Likewise the release of Ca2+ from the sarcoplasmic reticulum by cyclopiazonic acid markedly reduced the nordex contraction while leaving the KCl contraction unchanged. We conclude that nordex may be responsible for much of the vasoconstriction stimulated by dex, through the activation of 5-HT2 receptors and that the [Ca2+]i increase in rat PASMCs caused by dex/nordex is due to both influx of extracellular Ca2+ and release of Ca2+ from the sarcoplasmic reticulum.

anorectic agent; primary pulmonary hypertension; 5-HT2 receptor; potassium channels

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before the administration of 10 μM norDEX. In additional studies, we eliminated the calcium in the bath solution 1 min before the norDEX by switching solutions.

Isolated perfused lung model. Male Sprague-Dawley rats were anesthetized and intubated with PE-200 tubing (inner diameter 1.44 mm, outer diameter 1.90 mm), a thoracotomy was performed, and the animal was heparinized (100 units). The PA was cannulated with a double-lumen cannula so that pressure measurements and perfusion could be obtained simultaneously. The left atrium was cannulated for efferent flow in a recirculating manner at a rate of 0.04 ml/min•kg body wt

Angiotensin II (0.15 μg) was administered at 5-min intervals. The 5-HT2 receptor antagonist ketanserin (1 μM) or vehicle was then administered at physiological values (frequency 70 breaths/min, tidal volume 1.5 ml), with a positive end-respiratory pressure of 2.5 cm H2O. To determine lung reactivity, we subjected the lungs to two consecutive cycles, each consisting of 10 min of normoxia, a bolus injection of angiotensin II (0.15 μg) into the afferent line, and, after 8 min, a 6-min hypoxic challenge. Lungs were accepted for study only if they had a baseline pressor response >8 mmHg to hypoxia. After return to baseline, the lungs were given a nitric oxide synthase inhibitor (N-nitro-L-arginine methyl ester, 50 μM) and perfused with a further 20 min. At this point, increasing doses of the test drugs dex or norDEX (1, 10, and 100 μM) were administered at 5-min intervals. The 5-HT2 receptor antagonist ketanserin (1 μM) or vehicle was then administered. We have found that this concentration of ketanserin prevents the vasoconstriction caused by 100 μM serotonin (data not shown).

Cell isolation. Rat pulmonary artery smooth muscle cells (PASMCs) were dispersed fresh on each day of experimentation. Resistance PAs (~200 μm diameter, 4th or 5th division) were dissected and placed in “Ca2+-free” Hanks’ solution for 10 min at 4°C. The Hanks’ solution contained (in mM): 140 NaCl, 4.2 KCl, 1.2 KH2PO4, 0.5 MgCl2, 10 HEPES, and 0.1 EGTA (pH 7.4). Arteries were then transferred to a solution containing 1 mg/ml papain, 0.75 mg/ml bovine albumin, and 0.85 mg/ml dithiothreitol and digested at 4°C for 30 min and then at 37°C for 13 min. Arteries were washed thoroughly with Hanks’ solution without EGTA (“low-Ca2+”) for at least 10 min and then maintained on ice in Hanks’ supplemented with 1 mg/ml glucose. Several digestions were done each day to ensure cell viability. This digestion protocol consistently produced high yields of viable, relaxed SMC.

Electrophysiology. Triturated cells were divided into aliquots on the stage of an inverted microscope (Nikon Diaphot 200) for conventional whole cell patch-clamp studies. After a brief period to allow partial adherence to the bottom of the recording chamber, cells were perfused with a normoxic solution of composition (in mM): 115 NaCl, 25 NaHCO3, 4.5 KCl, 1 KH2PO4, 1.5 CaCl2, 0.5 MgCl2, 10 HEPES, and 10 glucose (pH 7.4 with NaOH. PO2~120 mmHg by bubbling with 21% O2/5% CO2/balance N2). Electrode resistances ranged from 3 to 5 MΩ after fire polishing and when filled with a solution of composition (in mM): 140 KCl, 1.0 MgCl2, 10 HEPES, and 1 EGTA (pH 7.2 with KOH).

The patch-clamp amplifiers were Axopatch 200A and B (Axon Instruments, Foster City, CA) in all voltage- and current-clamp experiments. Offset potentials were nulled directly before formation of a seal. Capacitance and series resistance were corrected (usually

Fig. 1. A: representative traces showing changes in tension of endothelium-intact pulmonary artery (PA) rings stimulated by norDEX and dexfenfluramine (dex) (1–100 μM). B: effects of different doses of norDEX (n = 16) and dex (n = 12) on PA tension changes. Values are expressed as percent change of the constriction caused by phenylephrine (PE%). Means ± SE, **P < 0.01, difference from dex.
80%). No leak subtraction was made. Leakage current was monitored using hyperpolarizing steps (−30 mV) from the holding potential, a procedure that did not activate ion channels but allowed measurement of passive membrane properties and leak during the experiments. Cells expressing holding current at −70 mV of >20 pA before or during the recordings were discarded. The extracellular perfusate perfusion rate was 2 ml/min. Cells were voltage clamped at a holding potential of −70 mV, and currents were evoked by +20 mV steps to more positive potentials using test pulses of 200-ms duration at a rate of 0.1 Hz. Currents were filtered at 1 kHz and sampled at 2 or 4 kHz. For resting membrane potential ($E_m$) experiments, cells were held in current clamp at their resting potential. Data were filtered at 1 kHz and sampled at 2 or 4 kHz. Series resistance and leak were checked at the beginning and end of each $E_m$ experiment to eliminate artificial changes in potential. Data were recorded and analyzed with pClamp 6.04 software (Axon Instruments).

**Measurement of intracellular $\text{Ca}^{2+}$.** Cytosolic calcium concentration ($[\text{Ca}^{2+}]_i$) was measured by dual-excitation imaging using fura 2 (9). Single PASMCs were prepared as for patch-clamp experiments, transferred to imaging dishes (Molecular Probes, Eugene, OR), and incubated in low- $\text{Ca}^{2+}$ Hanks' solution with the cell-permeable acetoxymethyl ester form of fura 2 (0.1 μM) and Pluronic F-127 (0.8 μM) for 15 min at room temperature (15). The plates were then washed with HEPES buffer (see solution composition in Electrophysiology) containing 1.5 mM $\text{Ca}^{2+}$ and incubated at room temperature for a further 20 min. The plates were then washed again and placed on the microscope stage. The drugs were added directly to the cells as a bolus by microinjection. All drugs were given in 10 μl volumes to remove potential volume-induced artifacts. Saline injections of 10 μl had no effect on $[\text{Ca}^{2+}]_i$. Changes in $[\text{Ca}^{2+}]_i$ were recorded in individual cells with a MetaFluor (Universal Imaging, West Chester, PA)-driven 340/380 filter imaging system and cooled charge-coupled device camera (Photometrics, Tucson, AZ). Background fluorescence was recorded from each dish of cells and subtracted before calculation of the 340- to 380-nm ratio. Measurements were made every 5 s, and $[\text{Ca}^{2+}]_i$ was calculated according to the method of Grynkiewicz et al. (9). Dissociation constants of 325 nM were calculated from in vitro calibration. We determined maximal and minimal ratio values at the end of each experiment by first treating the cells with 1 μM ionomycin (maximal ratio) and then chelating all free $\text{Ca}^{2+}$ with 10 mM EGTA (minimal ratio). Intracellular free $\text{Ca}^{2+}$ was calculated with the formula $[\text{Ca}^{2+}]_i$ (in nM) = $K_d \times (F_0/F_s) \times (R_{\text{max}} - R)/(R_{\text{max}} - R)$, where $K_d$ is the dissociation constant, $F_0$ is the maximal 380-nm signal intensity, $F_s$ is the minimal 380-nm signal intensity, and $R$ is the ratio of 340-nm fluorescence to 380-nm fluorescence. Any cells not responding to ionomycin were discarded, as were cells showing significant photo bleaching. Peak increases in $[\text{Ca}^{2+}]_i$ were measured during each intervention, and data are given as averaged peak values.

**Drugs.** Nordex, dex, and ketanserin were obtained from RBI (Natick, MA). All other drugs and salts were obtained from Sigma (St. Louis, MO) except fura 2-AM (Molecular Probes). All the drugs were dissolved in normal saline, except ketanserin and nifedipine, which were dissolved in ethanol, and 2-APB, cyclopiazonic acid (CPA), fura 2, Pluronic F-127, and ionomycin, which were dissolved in DMSO as a stock solution. Vehicle controls were done for all experiments.

**Statistics.** The contractions for the ring experiments are expressed as a percentage of the contraction developed by 1 μM PE. Values are expressed as the means ± SE. Intergroup differences were assessed by a factorial ANOVA with post hoc analysis with Fisher’s least significant difference test (StatView II, version 4.0, Abacus Concepts). A paired t-test was used for evaluation of differences within groups. $P < 0.05$ was considered statistically significant.

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**Fig. 2.** A: representative traces of tension stimulated by nordex (1–100 μM) in both endothelium intact (EC+) and denuded (EC−) PA rings. B: no difference in effects of nordex on tension in endothelium intact (n = 16) or denuded (n = 6) PA rings. Values are expressed as percent change of the PE contraction. Means ± SE.
RESULTS

Isolated PA rings. PE-induced contraction of endothelium-intact and -denuded PA rings was 428.9 ± 33.9 and 429.4 ± 81.2 mg, respectively. Nordex was a more potent vasoconstrictor than dex in intact PA rings. At concentrations of 3, 10, 30, and 100 μM, it caused a greater increase in tension, expressed as a percentage of PE contraction (13.4 ± 3.3%, 33.2 ± 3.9, 35.7 ± 4.1, 32.3 ± 4.3%) than dex (3.1 ± 0.7, 5.4 ± 1.3, 8.4 ± 2.2, 8.5 ± 2.9%) (Fig. 1). The effect of nordex on PA ring tension was not affected by the denudation of endothelium (Fig. 2).

The effect of nordex (10 μM) on PA ring tension (both intact and denuded) was almost completely inhibited (from 32.0 ± 3.8, 35.5 ± 5.3% to 0.1 ± 1.9, 1.4 ± 1.6% of PE contraction) by the nonspecific 5-HT2 receptor antagonist ketanserin (1 μM) (Fig. 3).

The contraction caused by nordex was dose dependently inhibited by 2-APB, a blocker of store-operated calcium channels (SOCs) and the inositol 1,4,5-trisphosphate (IP3) receptors. The blockade was 56.7 ± 9.3% following the administration of 10 μM 2-APB (Fig. 4). Omitting bath calcium or adding 1 μM nifedipine inhibited 59.8 ± 8.5 and 62.9 ± 3.9% of the nordex contraction, respectively. Similarly, depleting SR calcium with 30 μM CPA almost completely inhibited the nordex-induced contraction (Fig. 4). CPA at 30 μM has no effect on the contraction of 60 mM KCl (372 ± 52 vs. 362 ± 38 mg, n = 9). The combination of 1 μM nifedipine and 10 μM 2-APB completely inhibited the contraction induced by nordex (Fig. 4).

Isoleted perfused lung model. Both nordex and dex cause an increase in PA pressure in isolated perfused rat lungs at concentrations of 1–100 μM. At 10 and 100 μM, more significant contraction (Δ) was caused by nordex than by dex (18.6 ± 3.3, 36.5 ± 4.3 mmHg vs. 5.6 ± 1.2, 15.0 ± 3.0 mmHg, Fig. 5). Ketanserin (1 μM) significantly decreased the PA pressure changes caused by D-norfenfluramine at 100 μM (from 43.8 ± 3.4 to 29.9 ± 3.9 mmHg) (P < 0.01) but not by dex at 100 μM (from 30.1 ± 5.2 to 29.1 ± 5.0 mmHg) (Fig. 5).

Electrophysiology. Patch-clamp studies in isolated PASMCs found that K+ currents and E_m were not significantly affected by 10 μM nordex (n = 5) (Fig. 6A) or 100 μM nordex (data not shown). The inhibition of K+ current by dex (100 μM) was the same in the presence and absence of ketanserin (1 μM) (data not shown, n = 5 for both groups). The outward K+ current and resting E_m of PASMCs were not significantly affected by 10 μM nordex (n = 4) (Fig. 6B) or 30 μM 2-APB (data not shown).

Effects of nordex on [Ca2+] in PASMCs. Average resting [Ca2+] in isolated PASMCs was calculated to be 131 ± 12 nM (n = 22). Nordex at 10 μM caused a significant increase in [Ca2+] of 182.2 ± 54.6 nM. Pretreatment of PASMCs with 1 μM ketanserin for 2 min before exposure to 10 μM nordex completely abolished the nordex-induced increase in [Ca2+] (Fig. 7).

DISCUSSION

Nordex is a more potent pulmonary vasoconstrictor than dex. The greater ability of nordex to cause vasoconstriction in the isolated lung or contraction of PA rings can be prevented

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Fig. 3. A: representative traces of inhibitory effect of 5-hydroxytryptamine type 2 receptor antagonist ketanserin (1 μM) on nordex-induced constriction in endothelium-denuded PA rings. B: in both endothelium intact (n = 8) and denuded (n = 12) PA rings, the effect of nordex (10 μM) on PA ring tension was almost completely inhibited by ketanserin (1 μM). Values are expressed as percent change of the PE constriction. Means ± SE. ***P < 0.001 different from nordex control.
Fig. 4. A: representative traces from different rings of the inhibitory effects of zero calcium in the bath solution, 2-aminoethoxydiphenyl borate [2-APB, sarcoplasmic reticulum (SR) calcium release channel blocker], nifedipine (NIF, L-type calcium channel blocker), and cyclopiazonic acid (CPA, depleting SR calcium) on nordex-induced constriction (10 μM). B: percent inhibition effects of different doses of 2-APB (3, 10, 30 μM; n = 5, 7, 7), 30 μM CPA (n = 9), 0 calcium (n = 5), NIF (n = 5), and the combination of 2-APB and NIF (n = 6) on the initial nordex-induced constriction (10 μM). **P < 0.01, ***P < 0.001 different from control.

Fig. 5. Nordex increases pulmonary arterial pressure (Ppa) more than dex in isolated perfused rat lungs. Note that ANG II and hypoxia have similar responses in both sets of lungs. Values between interventions indicate baseline measurements. Nordex (10 μM and 100 μM) caused more Ppa increase than the same dose of dex (***P < 0.01). Ketanserin (1 μM) can significantly decrease Ppa changes caused by nordex (###P < 0.01) but not dex. Values are means ± SE. L-NAME, N-nitro-L-arginine methyl ester.
by pretreatment with ketanserin, suggesting that it is mediated by 5-HT2 receptors. The observation is strengthened by the finding that ketanserin entirely prevents the rise in cytosolic calcium produced by nordex. The enhanced contraction that nordex stimulates in PA rings is not diminished by removal of endothelium, indicating that it is the result of a direct effect on the smooth muscle, rather than the result of the release of endothelium-derived substances such as endothelin or thromboxane A2. The concentrations of dex and nordex that were demonstrated to cause acute vasoconstriction in the isolated lung and PA ring (10 μM) are close to those in the blood of patients taking dex over a long time (0.1–1 μM).

The effect of zero calcium outside the SMC indicates that ~60% of the increase in cytosolic calcium induced by nordex enters from outside the cell. The effect of 2-APB would suggest that the remainder of the increase in calcium probably comes from the SR. This two-component model for the increase in cytosolic calcium is similar to that described in the response to 5-HT (27). It is possible that 2-APB may have effects in addition to blocking SOCs and the IP3 receptor (3), but the electrophysiology experiments described here make it unlikely that it has an effect on potassium current or Em (Fig. 6). The specificity of 2-APB is reinforced by the observation that it did not reduce the pressor response to KCl (60 mM) in isolated perfused rat lungs (data not shown, n = 5) or the contraction to 60 mM KCl in the PA rings (data not shown, n = 6). The observation that nifedipine inhibits much of the increase in cytosolic calcium caused by nordex might be
thought to implicate L-type calcium channels. However, although dex inhibits potassium current and causes membrane depolarization in PASMCS (16, 26), nordex surprisingly had no effect on potassium current or on \( E_m \). This apparent impasse may be explained by the report that the same concentration of nifedipine (1 \( \mu \)M) can also block the SOCs that replete the SR (6). Other papers support the concept that dihydropyridine agents, such as nifedipine, can inhibit SOCs as well as voltage-gated L-type calcium channels (4, 11, 28). Consequently these data would suggest that the vasoconstriction caused by nordex is the result of calcium release from the SR and calcium entry/repletion through the SOCs and not through voltage-gated L-type calcium channels. This interpretation is strengthened by the observation that prior treatment of PA rings with CPA to release SR stores of calcium will markedly inhibit nordex-induced contraction while leaving the contraction to KCl unchanged.

There is considerable evidence that dex and nordex can act as agonists at the 5-HT\(_{2B}\) receptor (7, 13, 22). Although dex binds weakly, nordex shows a high affinity for the human 5-HT\(_{2B}\) receptor (7, 22). The subsequent signaling cascade involves G proteins, phospholipase C, IP\(_3\), and diacylglycerol (25). Dex given chronically to hypoxic mice, with sufficient time for metabolism to nordex, increases right ventricular pressure and pulmonary vascular muscularization (13). These effects of both hypoxia and dex are markedly inhibited in mice lacking the 5-HT\(_{2B}\) gene. Thus it appears that a large part of the hemodynamics and vascular remodeling seen in response to dex/nordex is mediated by 5-HT\(_{2B}\) receptor. When dex is given acutely (5 min before hypoxia), there is no increase in measured levels of plasma nordex and only a small increase (10.220.32.246 on April 13, 2017 http://ajplung.physiology.org/ Downloaded from

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