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Original Contribution

Therapeutic hypercapnia prevents inhaled nitric oxide-induced right-ventricular systolic dysfunction in juvenile rats

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ABSTRACT

Chronic pulmonary hypertension in the neonate and infant frequently presents with right-ventricular (RV) failure. Current clinical management may include protracted treatment with inhaled nitric oxide (iNO), with the goal of reducing RV afterload. We have previously reported that prolonged exposure to iNO causes RV systolic dysfunction in the chronic hypoxia-exposed juvenile rat, which was prevented by a peroxynitrite decomposition catalyst. Given that inhalation of CO_2 (therapeutic hypercapnia) may limit oxidative stress and upregulated cytokine expression in the lung and other organs, we hypothesized that therapeutic hypercapnia would attenuate cytokine-mediated nitric oxide synthase (NOS) upregulation, thus limiting peroxynitrite generation. Sprague–Dawley rat pups were exposed to chronic hypoxia (13% O_2) from postnatal day 1 to 21, while receiving iNO (20 ppm) from day 14 to 21, with or without therapeutic hypercapnia (10% CO_2). Therapeutic hypercapnia completely normalized RV systolic function, RV hypertrophy, and remodeling of pulmonary resistance arteries in animals exposed to iNO. Inhaled nitric oxide-mediated increases in RV peroxynitrite, apoptosis, and contents of tumor necrosis factor (TNF)-α, interleukin (IL)-1α, and NOS-2 were all attenuated by therapeutic hypercapnia. Inhibition of NOS-2 activity with 1400 W (1 mg/kg/day) prevented iNO-mediated upregulation of peroxynitrite and led to improved RV systolic function. Blockade of IL-1 receptor signaling with anakinra (500 mg/kg/day) decreased NOS-2 content and had similar effects compared to NOS-2 inhibition on iNO-mediated effects, whereas blockade of TNF-α signaling with etanercept (0.4 mg/kg on alternate days) had no effects on these parameters. We conclude that therapeutic hypercapnia prevents the adverse effects of sustained exposure to iNO on RV systolic function by limiting IL-1-mediated NOS-2 upregulation and consequent nitration. Therapeutic hypercapnia also acts synergistically with iNO in normalizing RV hypertrophy, vascular remodeling, and raised pulmonary vascular resistance secondary to chronic hypoxia.

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Pulmonary hypertension (PHT) is characterized by increased pulmonary artery resistance and pressure, secondary to sustained pulmonary vasoconstriction and proliferation of pulmonary arterial wall smooth muscle [1]. Chronic PHT in the neonate and infant is generally associated with developmental disorders of the lung (such as chronic lung disease of prematurity) and, unlike PHT in older children and adults [2], frequently manifests with a malignant phenotype, culminating in a rapid onset of right-ventricular (RV)

Abbreviations: cGMP, cyclic guanosine monophosphate; DAPI, 4′,6-diamidino-2-phenylindole; DHE, dihydroethidium; E_a, arterial elastance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNO, inhaled nitric oxide; IL, interleukin; LV, left ventricular; NGS, normal goat serum; NOS-1, neuronal nitric oxide synthase; NOS-2, inducible nitric oxide synthase; NOx, nitrate/nitrite; PAAT, pulmonary arterial acceleration time; PHT, pulmonary hypertension; PV, pressure–volume; PVR, pulmonary vascular resistance; RNS, reactive nitrogen species; ROS, reactive oxygen species; RR, respiration rate; RV, right ventricular; RVESP, right-ventricular end-systolic pressure; RVET, right-ventricular ejection time; RVSV, right-ventricular stroke volume; TNF, tumor necrosis factor; TUNEL, terminal deoxyuridine triphosphate nick-end labeling; V_t, tidal volume; VTI, velocity time integral; 1400 W, *N*-[[3-(aminomethyl)phenyl]methyl]ethanimidamide, dihydrochloride.

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failure and premature death [3]. Such a phenotype may be anticipated by absent or poor acute responsiveness to pulmonary vasodilators, such as inhaled nitric oxide (iNO) [4].

The pathophysiology of chronic PHT is complex and multifactorial, but dysfunction of the vascular nitric oxide (NO)–cyclic guanosine monophosphate (cGMP) axis, which modulates relaxation and inhibits proliferation of smooth muscle [5], has been identified as a key component [6]. The goal of iNO, a widely used pulmonary vasodilator in neonates and infants [7], is to overcome endothelial dysfunction, characterized by decreased production of endogenous NO. In patients with only a partial improvement in hypoxemia, or where significant RV dysfunction is present, iNO may be utilized for prolonged periods with the goal of limiting RV afterload and of preventing or reversing vascular remodeling [8]. The long-term effects of this approach are currently unknown [9]; however, extrapulmonary effects of iNO, both beneficial [10] and potentially harmful [11,12], are now well described.

Concerns with the use of iNO are primarily related to the propensity of NO to react with superoxide anion to form peroxynitrite anion ($ONOO^-$), a potent oxidizing and nitrating agent [13–15]. Peroxynitrite is a potent pulmonary vasoconstrictor and contributes to chronic hypoxia or hyperoxia-induced pulmonary vascular remodeling in the newborn rat [16–18]. We have recently reported that sustained rescue treatment with iNO (20 ppm for 7 days from postnatal day 14 to 21) in juvenile rats with established chronic hypoxic PHT had no effect upon pulmonary vascular remodeling. Furthermore, exposure to iNO led to the novel and unexpected finding of greatly increased nitration in the right, but not left, cardiac ventricle [19]. Neutrophil numbers and peroxidase activity were not increased [19], arguing against a significant role for peroxidase-catalyzed nitration [20,21]. A critical role for increased $ONOO^-$ in RV systolic dysfunction secondary to iNO was suggested by ameliorating effects of treatment with a $ONOO^-$ decomposition catalyst, FeTPPS [19]. The upstream mechanisms leading to increased nitration were unclear; however, screening of a number of putative mediators revealed RV mRNA upregulation of both nitric oxide synthases (NOS) 1 and 2, as well as the cytokines tumor necrosis factor (TNF)-α and interleukins (IL)-1α and 1β [19].

Therapeutic hypercapnia, the exogenous inhalation of CO_2 to induce hypercapnic acidosis, has been shown by our group and others to have beneficial effects on the pulmonary circulation [22–26] and on cardiac function [27–29]. Putative contributors to injury reported to be attenuated by hypercapnic acidosis, predominantly studied in the lung, have included inflammatory cell influx [30,31], expression of proinflammatory cytokines [30,32], and oxidative and nitrative stress [23,30,32]. We have recently reported that rescue treatment with therapeutic hypercapnia (10% CO_2 for 7 days from postnatal day 14 to 21) in juvenile rats with established chronic hypoxic PHT partially reversed PHT and remodeling of pulmonary resistance arteries [25]. These effects were associated with evidence of improved vascular NO–GMP signaling [25]. Our goal in the present study was therefore to determine whether concurrent therapeutic hypercapnia would prevent RV systolic dysfunction secondary to iNO. We hypothesized that therapeutic hypercapnia would limit cytokine production in the right ventricle, thus attenuating NOS upregulation and consequent generation of reactive nitrogen species (RNS). A secondary goal was to determine whether hypercapnia and iNO would have synergistic effects in reversing PHT and vascular remodeling secondary to chronic hypoxia.

Experimental procedures

Materials

Nitric oxide (400 ppm, balance N_2) was from Praxair (Mississauga, ON, Canada). Etanercept (Enbrel) and anakinra (Kineret) were purchased from Amgen (Thousand Oaks, CA, USA) and 1400 W, a NOS-2-specific inhibitor [33], was purchased from Tocris Biosciences (Bristol, UK). Acids, alcohols, organic solvents, Permount, Superfrost/Plus microscope slides, and paraformaldehyde were from Fisher Scientific (Whitby, ON, Canada). Rabbit polyclonal antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cat. No. sc-25778) and IL-1β (Cat. No. sc-7884) and mouse monoclonal antibodies against NOS-2 (Cat. No. sc-7221) and IL-1α (Cat. No. sc-9983) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A mouse polyclonal antibody against TNF-α was from Hycult Biotech (Uden, The Netherlands; Cat. No. HP8001). A rabbit monoclonal antibody against cleaved caspase-3 (Cat. No. 9664) and a rabbit polyclonal antibody against total caspase-3 (Cat. No. 9662) were from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal anti-nitrotyrosine (Cat. No. 06-284) was from EMD Millipore (Billerica, MA, USA) and anti-NOS-1 (Cat. No. 610310) was from BD Biosciences (Mississauga, ON, Canada). Anti-NAD(P)H oxidase 4 (NOX4; Cat. No. NB 110–58851) was from Novus Biologicals (Littleton, CO, USA). Avidin–biotin–peroxidase complex immunohistochemistry kits, 6-diamino-2′-phenylindole (DAPI) aqueous mounting medium, 3,3′-diaminobenzidine staining kits, and hematoxylin counterstain were from Vector Laboratories (Burlingame, CA, USA). Normal goat serum (NGS) was from Wisent (St-Bruno, QC, Canada). Weigert's resorcin–fuchsin stain was from Rowley Biochemical (Danvers, MA, USA). Precast Tris–glycine 4–20% gels and polyvinylidene difluoride membranes were from Thermo Scientific (Kalamazoo, MI, USA). Dihydroethidium (DHE) was from Molecular Probes (Eugene, OR, USA). Peroxynitrite solution was from Cayman Chemical (Ann Arbor, MI, USA). Unless otherwise specified, all other chemicals and reagents were from Bioshop Canada (Burlington, ON, Canada).

In vivo interventions

Animal interventions were approved by the Animal Care Committees of the Hospital for Sick Children Research Institute and the Keenan Research Centre at St. Michael's Hospital, in accordance with standards defined by the Canadian Council on Animal Care. Litters of Sprague–Dawley rat pups were exposed to air (21% O_2) or normobaric hypoxia (13% O_2) from birth until postnatal day (PND) 21. Equal litter sizes ($n=10$–12) and sex distribution were maintained throughout the exposure period. From PND 14 to 21, pups were continuously exposed to iNO (20 ppm), as previously described [19], with or without ($<0.5\%$ CO_2) concurrent exposure to 10% CO_2. The concentration of CO_2 to which the animals were exposed was shown in previous dose–response studies to be maximally effective in attenuating PVR and pulmonary vascular remodeling in chronic hypoxia-exposed neonatal [23] and juvenile [25] rats. In separate experiments, pups were exposed to hypoxia and iNO while also receiving injections of etanercept (0.4 mg/kg; injected volume 5 μl/g body wt of 0.08 mg/ml in 0.9% (w/v) saline, intraperitoneally (ip), alternate days), anakinra (500 mg/kg; injected volume 3 μl/g body wt of 150 mg/ml aqueous solution containing 0.18 mg/ml disodium EDTA, 8.2 mg/ml NaCl, 2.4 mg/ml sodium citrate, and 1 mg/ml Tween 80, pH 6.5, ip, daily), or 1400 W (1 mg/kg; injected volume 5 μl/g body wt of 0.2 mg/ml in 0.9% saline, ip, daily) beginning on PND 14. Vehicle-treated controls were included for all experiments. Doses of etanercept (a soluble TNF-2 receptor protein that inhibits TNF-α signaling by binding endogenous ligand) and anakinra (a recombinant nonglycosylated human IL-1 receptor antagonist analogue that acts as a competitive antagonist) have previously been shown by our group to be effective at preventing inflammatory lung injury in the neonatal rat [26,34]. The appropriate dose of 1400 W was determined from previously published dose–response studies in adult rats [33]. Atmospheric concentrations

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