DESCRIPTION
THERAPEUTIC TREATMENT FOR LUNG CONDITIONS

RELATED APPLICATION INFORMATION

This patent application is based on and claims priority to U.S. Provisional Patent Application Serial No. 61/025,157, filed January 31, 2008, the entire contents of which are herein incorporated by reference.

TECHNICAL FIELD

The presently disclosed subject matter relates to the treatment of lung conditions, such as bronchopulmonary dysplasia (BPD) and chronic hypoxia-induced pulmonary hypertension, such as in infants.

BACKGROUND

Bronchopulmonary dysplasia (BPD) typically occurs in infants, particularly preterm infants, and is characterized as an acute injury to the lungs by either oxygen and/or mechanical ventilation, resulting in interference with or inhibition of lung alveolar and vascular development (Jobe *et al.* (2001) *Am J Respir Crit Care Med* 163:1723-1729). In animal models, inhaled NO improves both gas exchange and lung structural development, but the use of this therapy in infants at risk for BPD is controversial (Ballard *et al.* (2006) *N Engl J Med* 355:343-353).

Infants with chronic lung disease and cyanotic congenital heart disease frequently suffer from hypoxia. Because of its effects on both existing and developing pulmonary arteries, chronic hypoxia causes progressive changes in both the function and structure of the pulmonary circulation. Shimoda L, et al., *Physiol Res* (2000) 49:549-560; Subhedar, N. V., *Acta Paediatr suppl* (2004) 444:29-32. Ultimately, chronic hypoxia results in severe pulmonary hypertension culminating in right-sided heart failure and death.

Accordingly, approaches for the treatment of lung conditions, such as BPD and chronic hypoxia-induced pulmonary hypertension, and further such as in infants, representative a long-felt and continuing need in the art.

SUMMARY

The presently disclosed subject matter provides methods and compositions for treating lung conditions, such as bronchopulmonary dysplasia (BPD) and chronic hypoxia-induced pulmonary hypertension, in a subject.

In some embodiments, an effective amount of a nitric oxide precursor is administered to a subject suffering from BPD and/or associated complications and/or at risk for suffering BPD and/or complications associated with BPD. In some embodiments, the nitric oxide precursor comprises at least one of citrulline, a precursor that generates citrulline *in vivo*, a pharmaceutically acceptable salt thereof, and combinations thereof. In some embodiments, the nitric oxide precursor, such as citrulline, is administered orally. In some embodiments, the nitric oxide precursor, such as citrulline, is administered intravenously.

In some embodiments, an effective amount of a nitric oxide precursor is administered to a subject suffering from chronic hypoxia-induced pulmonary hypertension and/or associated complications and/or at risk for suffering chronic hypoxia-induced pulmonary hypertension and/or complications associated with chronic hypoxia-induced pulmonary hypertension. In some embodiments, the nitric oxide precursor comprises at least one of citrulline, a precursor that generates citrulline *in vivo*, a pharmaceutically acceptable salt thereof, and combinations thereof. In some embodiments, the nitric oxide precursor, such as citrulline, is administered orally. In some embodiments, the nitric oxide precursor, such as citrulline, is administered intravenously.

It is therefore an object of the presently disclosed subject matter to provide for treatment for a lung condition in a subject.

An object of the presently disclosed subject matter having been stated hereinabove, other objects will become evident as the description proceeds, when taken in connection with the accompanying drawings and examples as best described hereinbelow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of the urea cycle.

Figure 2 is a flow diagram of study procedures followed in the Examples.

Figure 3 is a bar graph showing mean pulmonary arterial pressure measurements in control (n=6), chronically hypoxic (n=11), and L-citrulline treated chronically hypoxic (n-6) piglets. All values are mean±SEM. *different from control; +different from chronically hypoxic; $p<0.05$, ANOVA with post-hoc comparison test.

Figure 4 is a bar graph showing calculated pulmonary vascular resistance in control (n=6), chronically hypoxic (n=11), and L-citrulline treated chronically hypoxic (n=6) piglets. All values are mean±SEM. *different from control; +different from chronically hypoxic; $p<0.05$, ANOVA with post-hoc comparison test.

Figure 5 is a bar graph showing exhaled Nitric Oxide in control (n=6), chronically hypoxic (n=11), and L-citrulline treated chronically hypoxic (n=5) piglets. All values are mean±SEM. *different from control; +different from chronically hypoxic; $p<0.05$, ANOVA with post-hoc comparison test.

Figure 6 is a bar graph showing nitrite/nitrate accumulation in lung perfusate in control (n=17), chronically hypoxic (n=9), and L-citrulline treated chronically hypoxic (n=5) piglets. All values are mean±SEM. *different from control; +different from chronically hypoxic; $p<0.05$, ANOVA with post-hoc comparison test.

Figure 7A is an image of an immunoblot for eNOS protein reprobed for actin for lung tissue from controls (n=3), chronic hypoxic (n=3), and L-citrulline treated chronic hypoxic (n=3) piglets.

Figure 7B is a bar graph showing densitometry of eNOS normalized to actin for lung tissue from controls (n=3), chronic hypoxic (n=3), and L-citrulline treated chronic hypoxic (n=3) piglets.

DETAILED DESCRIPTION

Preterm births continue to be the major challenge in obstetrics and neonatology, accounting for most of the perinatal mortality and long-term neurologic morbidity among newborns. BPD is one of many complications that can be associated with preterm birth. BPD can be associated with prolonged hospitalization of a preterm infant, multiple rehospitalizations during the first few years of life, and developmental delays. Fortunately, BPD is now infrequent in

infants of more than 1,200 g birth weight or with gestations exceeding 30 weeks (Jobe *et al.* (2001) *Am J Respir Crit Care Med* 163:1723-1729). The incidence of BPD defined as an oxygen need at 36 weeks postmenstrual age is about 30% for infants with birth weights < 1,000 g (Jobe *et al.* (2001) *Am J Respir Crit Care Med* 163:1723-1729). Some of these infants have severe lung disease, requiring ventilation and/or supplemental oxygen for months or even years.

Multiple factors contribute to BPD, and probably act additively or synergistically to promote injury. The traditional view has been that BPD is caused primarily by oxidant- and ventilation-mediated injury (Jobe *et al.* (2001) *Am J Respir Crit Care Med* 163:1723-1729). Mechanical ventilation and oxygen can interfere with alveolar and vascular development in preterm infants and has been attributed to the development of BPD (Jobe *et al.* (2001) *Am J Respir Crit Care Med* 163:1723-1729). Reduced numbers of alveoli can result in a large decrease in surface area, which has been associated with a decrease in dysmorphic pulmonary microvasculature. These anatomic changes are associated with persistent increases in white blood cells and cytokine levels in airway samples (Jobe *et al.* (2001) *Am J Respir Crit Care Med* 163:1723-1729).

Inflammation can also play a role in the development of BPD. Multiple proinflammatory and chemotactic factors are present in the air spaces of ventilated preterm infants, and these factors are found in higher concentrations in the air spaces of infants who subsequently develop BPD (Jobe *et al.* (2001) *Am J Respir Crit Care Med* 163:1723-1729). Other factors considered important to the development of BPD include: bombesin-like peptides, hyperoxia, hypoxia, poor nutrition, glucocorticoid treatment and the overexpression of the cytokines tumor necrosis factor-α, TGF-α, IL-6, or IL-11 (Jobe *et al.* (2001) *Am J Respir Crit Care Med* 163:1723-1729).

Diagnosing BPD generally comprises monitoring an infant's breathing over the initial weeks of life for signs of delayed lung development and a continued and/or increased dependence upon assisted breathing. Diagnostic tests that can be performed to assist in the diagnosis of BPD can include: blood oxygen tests, chest x-rays, and echocardiograms. BPD has traditionally been diagnosed when an infant requires supplemental oxygen at 36 weeks postmenstrual age. Newer definitions used in diagnosing and defining BPD

include specific criteria for 'mild,' 'moderate' and 'severe' BPD (Ryan, R. M. (2006) *J Perinatology* 26:207–209).

Treating BPD can include a mutli-faceted approach to treating the symptoms of the condition and providing an infant's lungs an opportunity to develop. Currently available treatments can comprise: surfactant administration to improve lung aeration, mechanical ventilators to compensate for respiratory failure, supplemental oxygen to insure adequate blood oxygen, bronchodilator medications to improve airflow in the lungs, corticosteroids to reduce swelling and inflammation of airways, fluid control to avoid pulmonary edema, treatments for *patent ductus arteriosus,* and proper nutrition.

Nitric oxide administration via inhalation has been demonstrated to improve lung development in infant animal models (Ballard *et al.* (2006) *N Engl J Med* 355:343-353). However, NO administration via inhalation is controversial for human subjects. Thus, in accordance with some embodiments of the presently disclosed subject matter, administering citrulline or other NO precursor to a subject suffering from BPD to thereby increase *in vivo* NO synthesis can provide an alternative to NO inhalation as a BPD treatment.

Because of its effects on both existing and developing pulmonary arteries, chronic hypoxia causes progressive changes in both the function and structure of the pulmonary circulation. Shimoda L, et al., *Physiol Res* (2000) ;49:549-560; Subhedar, N. V., *Acta Paediatr suppl* (2004);444:29-32. Ultimately, chronic hypoxia results in severe pulmonary hypertension culminating in right-sided heart failure and death. Currently the therapy for pulmonary hypertension in infants suffering from chronic cardiopulmonary disorders associated with persistent or episodic hypoxia is largely limited to improving the underlying cardiopulmonary disorder and attempts to achieve adequate oxygenation. Abman, S. H.; *Arch Dis Child Fetal Neonatal Ed* (2002) 87: F15-F18; Allen, J. and ATS subcommittee AoP, *Am J Respir Crit Care Med* (2003) 168: 356-396; Mupanemunda, R. H., *Early Human Development* (1997) 47: 247-262; Subhedar, N. V., *Acta Paediatr suppl* (2004) 444:29-32. Thus, in accordance with some embodiments of the presently disclosed subject matter, a novel therapeutic approach comprising administering citrulline to a subject suffering from chronic hypoxia-induced pulmonary hypertension is provided.

Cutrulline is a key intermediate in the urea cycle and in the production of nitric oxide (NO). In the urea cycle, citrulline is a precursor for the *de novo* synthesis of arginine. Arginine can be deaminated via arginase to produce urea, which can subsequently be excreted to rid the body of waste nitrogen, particularly ammonia. Alternatively, arginine can provide for the production of NO via nitric oxide synthase. As such, intact urea cycle function is important not only for excretion of ammonia but in maintaining adequate tissue levels of arginine, the precursor of NO.

Nitric oxide is synthesized by nitric oxide synthase using arginine as a substrate. The rate-limiting factor in the synthesis of NO is the availability of cellular arginine, and the preferred source of arginine for NO synthesis is *de novo* biosynthesized from citrulline. The *in vivo* synthetic pathway for arginine commences with ornithine. Ornithine is combined with carbamyl phosphate to produce citrulline, which in turn is combined with aspartate, in the presence of adenosine triphosphate, to produce argininosuccinate. In the final step, fumarate is split from argininosuccinate, to produce arginine. The degradative pathway for arginine is by the hydrolytic action of arginase, to produce ornithine and urea. These reactions form the urea cycle. *See also* Figure 1.

As an alternative to degradation for urea synthesis, arginine can provide the substrate necessary for NO synthesis via nitric oxide synthase. Additionally, exogenous citrulline can enter the urea cycle and provide for the *in vivo* synthesis of arginine, which can subsequently provide for NO synthesis. Accordingly, administering citrulline to subjects, including but not limited to subjects susceptible to or diagnosed with BPD or with chronic hypoxia-induced pulmonary hypertension can increase arginine synthesis and subsequently increase NO production to thereby prevent and/or treat BPD or chronic hypoxia-induced pulmonary hypertension. Citrulline precursors that generate citrulline *in vivo* can also be provided. As an alternative to citrulline, other NO precursors can be provided. For example, arginine, or a precursor that generates arginine *in vivo*, can be provided as an NO precursor.

I. THERAPEUTIC METHODS

The presently disclosed subject matter provides methods and compositions for increasing NO synthesis in a subject. In some embodiments, an effective amount of citrulline or other NO precursor is administered to a subject to increase NO synthesis. In some embodiments, the NO precursor is selected from the group including, but not limited to, citrulline, a precursor that generates citrulline *in vivo*, arginine, a precursor that generates arginine *in vivo,* or combinations thereof. In some embodiments, the citrulline or other NO precursor is administered orally. In some embodiments, the citrulline or other NO precursor is administered intravenously.

The presently disclosed subject matter also provides methods and compositions for treating BPD and/or associated complications in a subject. In some embodiments, an effective amount of citrulline or other NO precursor is administered to a subject suffering from BPD and/or associated complications and/or at risk for suffering complications associated with BPD. In some embodiments, the NO precursor is selected from the group including, but not limited to, citrulline, a precursor that generates citrulline *in vivo*, arginine, a precursor that generates arginine *in vivo,* or combinations thereof. In some embodiments, the citrulline or other NO precursor is administered orally. In some embodiments, the citrulline or other NO precursor is administered intravenously. In some embodiments, the subject to be treated is a subject suffering from an acute condition associated with BPD. Representative examples of such conditions are disclosed herein above.

The presently disclosed subject matter also provides methods and compositions for treating chronic hypoxia-induced pulmonary hypertension and/or associated complications in a subject. In some embodiments, an effective amount of citrulline or other NO precursor is administered to a subject suffering from chronic hypoxia-induced pulmonary hypertension and/or associated complications and/or at risk for suffering complications associated with chronic hypoxia-induced pulmonary hypertension. In some embodiments, the NO precursor is selected from the group including, but not limited to, citrulline, a precursor that generates citrulline *in vivo*, arginine, a precursor that generates arginine *in vivo,* or combinations thereof. In some embodiments, the

citrulline or other NO precursor is administered orally. In some embodiments, the citrulline or other NO precursor is administered intravenously. In some embodiments, the subject to be treated is a subject suffering from an acute condition associated with chronic hypoxia-induced pulmonary hypertension. Representative examples of such conditions are disclosed herein above.

In some embodiments, the nitric oxide precursor comprises at least one of citrulline, a precursor that generates citrulline *in vivo*, a pharmaceutically acceptable salt thereof, and combinations thereof. *See* Figure 1. In some embodiments, the nitric oxide precursor is selected from the group including, but not limited to, citrulline, arginine, or combinations thereof. In some embodiments, the nitric oxide precursor, such as citrulline, is administered orally. In some embodiments, the nitric oxide precursor, such as citrulline, is administered intravenously.

In some embodiments, the subject suffers from hypocitrullinemia. In some embodiments the hypocitrullinemia is characterized by plasma citrulline levels of ≤ 37 μmol/liter, in some embodiments, ≤ 25 μmol/liter, in some embodiments, ≤ 20 μmol/liter, in some embodiments, ≤ 10 μmol/liter, in some embodiments, ≤ 5 μmol/liter.

In some embodiments, the subject suffering from a condition as disclosed herein suffers from relative hypocitrullinemia. The term "relative hypocitrullinemia" refers to a state in which the subject suffering from a condition has reduced plasma citrulline as compared to a subject not suffering from a condition.

As used herein, the phrase "treating" refers to both intervention designed to ameliorate a condition in a subject (*e.g.*, after initiation of a disease process or after an injury), to ameliorate complications related to the condition in the subject, as well as to interventions that are designed to prevent the condition from occurring in the subject. Stated another way, the terms "treating" and grammatical variants thereof are intended to be interpreted broadly to encompass meanings that refer to reducing the severity of and/or to curing a condition, as well as meanings that refer to prophylaxis. In this latter respect, "treating" can refer to "preventing" to any degree, such as but not limited to in a subject at risk for suffering a condition, or otherwise enhancing the ability of the

subject to resist the process of the condition.

The subject treated in the presently disclosed subject matter in its many embodiments is desirably a human subject, although it is to be understood that the principles of the presently disclosed subject matter indicate that the presently disclosed subject matter is effective with respect to all vertebrate species, including warm-blooded vertebrates such as mammals and birds, which are intended to be included in the term "subject". In this context, a mammal is understood to include any mammalian species in which treatment is desirable, such as but not limited to agricultural and domestic mammalian species.

Thus, provided is the treatment of mammals such as humans, as well as those mammals of importance due to being endangered (such as Siberian tigers), of economical importance (animals raised on farms for consumption by humans) and/or social importance (animals kept as pets or in zoos) to humans, for instance, carnivores other than humans (such as cats and dogs), swine (pigs, hogs, and wild boars), ruminants (such as cattle, oxen, sheep, giraffes, deer, goats, bison, and camels), and horses. Also provided is the treatment of birds, including the treatment of those kinds of birds that are endangered, kept in zoos, as well as fowl, and more particularly domesticated fowl, i.e., poultry, such as turkeys, chickens, ducks, geese, guinea fowl, and the like, as they are also of economical importance to humans. Thus, provided is the treatment of livestock, including, but not limited to, domesticated swine (pigs and hogs), ruminants, horses, poultry, and the like.

II. PHARMACEUTICAL COMPOSITIONS

An effective dose of a composition of the presently disclosed subject matter is administered to a subject in need thereof. An “effective amount” is an amount of a composition sufficient to produce a measurable response (*e.g.,* a biologically or clinically relevant response in a subject being treated). Actual dosage levels of active ingredients in the compositions of the presently disclosed subject matter can be varied so as to administer an amount of the active compound(s) that is effective to achieve the desired therapeutic response for a particular subject. The selected dosage level will depend upon

the activity of the therapeutic composition, the route of administration, combination with other drugs or treatments, the severity of the condition being treated, and the condition and prior medical history of the subject being treated. By way of example and not limitation, doses of compositions can be started at levels lower than required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. The potency of a composition can vary, and therefore an "effective amount" can vary.

After review of the disclosure of the presently disclosed subject matter presented herein, one of ordinary skill in the art can tailor the dosages to an individual subject, taking into account the particular formulation, method of administration to be used with the composition, and particular disease treated. Further calculations of dose can consider subject height and weight, gender, severity and stage of symptoms, and the presence of additional deleterious physical conditions.

By way of additional examples, the amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the subject to be treated and the particular mode of administration. For example, a formulation intended for administration to humans can contain from 0.5 mg to 5 g of active agent compounded with an appropriate and convenient amount of carrier material which may vary from about 5 to about 95 percent of the total composition. For example, in a human adult, the doses per person per administration are generally between 1 mg and 500 mg up to several times per day. Thus, dosage unit forms will generally contain between from about 1 mg to about 500 mg of an active ingredient, typically 25 mg, 50 mg, 100 mg, 200 mg, 300 mg, 400 mg, 500 mg, 600 mg, 800 mg, or 1000 mg.

The nitric oxide precursor is administered in some embodiments in a dose ranging from about 0.01 mg to about 1,000 mg, in some embodiments in a dose ranging from about 0.5 mg to about 500 mg, and in some embodiments in a dose ranging from about 1.0 mg to about 250 mg. The nitric oxide precursor can also be administered in some embodiments in a dose ranging from about 100 mg to about 30,000 mg, and in some embodiments in a dose ranging from about 250 mg to about 1,000 mg. A representative dose is 3.8 g/m2/day of

arginine or citrulline (molar equivalents, MW L-citrulline 175.2, MW L-arginine 174.2).

Representative intravenous citrulline solutions can comprise a 100 mg/ml (10%) solution. Representative intravenous citrulline dosages can comprise 200 mg/kg, 400 mg/kg, 600 mg/kg, and 800 mg/kg. In some embodiments, for example but not limited to a 600 or 800 mg/kg dosage, the dose can be decreased by an amount ranging from 50 mg/kg and 100 mg/kg to mitigate observed undesired effects on systemic blood pressure. In some embodiments, doses can be administered one or more times during a given period of time, such as a day.

In some embodiments a pharmaceutical composition comprises an amount of citrulline effective to raise plasma citrulline level to treat a condition as disclosed herein in a subject. In some embodiments, the level is determined by comparing plasma citrulline levels in a subject to be treated to that observed in a subject not suffering from the condition. In some embodiments, the amount of citrulline is effective to raise plasma citrulline level in a subject to at least 5 μmol/liter, optionally at least 10 μmol/liter, optionally at least 20 μmol/liter, optionally at least 25 μmol/liter, and optionally about 37 μmol/liter.

In some embodiments, the presently disclosed subject matter provides pharmaceutical compositions comprising a nitric oxide precursor and a pharmaceutically acceptable carrier, such as a pharmaceutically acceptable carrier in humans. In some embodiments, the presently disclosed subject matter provides pharmaceutical compositions comprising citrulline or arginine in dosages as described above.

A composition of the presently disclosed subject matter is typically administered orally or parenterally in dosage unit formulations containing standard nontoxic pharmaceutically acceptable carriers, adjuvants, and vehicles as desired. The term "parenteral" as used herein includes intravenous, intra-muscular, intra-arterial injection, or infusion techniques.

Injectable preparations, for example sterile injectable aqueous or oleaginous suspensions, are formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile

injectable preparation can also be a sterile injectable solution or suspension in a nontoxic acceptable diluent or solvent, for example, as a solution in 1,3-butanediol.

Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables. Exemplary carriers include neutral saline solutions buffered with phosphate, lactate, Tris, and the like.

In a representative embodiment doses can be administered to a subject several times during a relevant treatment period, including but not limited to 1, 2, 3, 4, 5, 6 or more dosages.

It will be understood, however, that the specific dose level for any particular subject will depend upon a variety of factors including the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

EXAMPLES

The following Examples have been included to illustrate representavtive modes of the presently disclosed subject matter. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the following Examples are intended to be exemplary only in that numerous changes, modification, and alterations can be employed without departing from the spirit and scope of the presently disclosed subject matter.

EXAMPLES 1-4

The following Examples evaluate whether oral supplementation with L-citrulline during exposure of newborn piglets to 10 days of chronic hypoxia would prevent the development of pulmonary hypertension and the concomitant reduction in NO production.

METHODS EMPLOYED IN EXAMPLES 1-4

ANIMAL CARE

A total of 17 hypoxic and 17 control piglets were studied. *See* Figure 2. Control animals were studied on the day of arrival from the farm at 12 days of age. The hypoxic pigs (2 days old) were placed in a normobaric hypoxic chamber for 10 to 11 days. Normobaric hypoxia was provided using compressed air and nitrogen to create inspired oxygen of 8-11 % (PO_2 60-72 Torr) and CO_2 was maintained at 3-6 Torr by absorption with soda lime. The animals were monitored with daily weights and physical exam twice daily. They were fed ad lib with sow milk replacer from a feeding device in the cage.

L-CITRULLINE SUPPLEMENTATION

Six of the seventeen hypoxic piglets were supplemented with oral L-citrulline starting on the first day of the hypoxic exposure. *See* Figure 2. L-citrulline supplementation was provided at a dose of 0.13-gm/kilogram body weight twice a day using a syringe to deliver the dose orally. If it appeared to study personnel that the piglet had not ingested the majority of a dose, it was repeated. L-citrulline was mixed using a preparation (Sigma Pharmaceuticals, St. Louis, Missouri, United States of America, 98% purity) at a concentration of 0.13 grams per milliliter of distilled water and when completely dissolved passing this solution through a 0.20 Micron filter.

IN VIVO HEMODYNAMICS

In vivo hemodynamics were measured in 6 of the control piglets and all of the hypoxic piglets. *See* Figure 2. For these measurements, the animals were weighed and then preanesthetized with Ketamine (15 mg/kg) and Acepromazine (2 mg/kg) intramuscularly. A tracheostomy, venous and arterial catheters, and thermistor were then placed as previously described using intravenous pentobarbital for sedation. Fike, C. D. et al., *J Appl Physiol* (2000) 88:1797-1803. Pulmonary artery pressure, left ventricular end diastolic pressure, and cardiac output were measured. Cardiac output was measured by a thermodilution technique (model 9520 thermodilution cardiac output computer, Edwards Laboratory, Irvine, California, United States of America) using a thermistor in the aortic arch and the left ventricle catheter as an injection port. Cardiac output was measured at end expiration as the mean of three injections of

3 ml of normal saline (0°C). Exhaled NO was measured as described below. During the *in vivo* measurements, animals were ventilated with room air using a piston-type ventilator at a tidal volume of 15-20 cc/kg, end-expiratory pressure of 2 mmHg, and a respiratory rate of 15-20 breaths per minute.

EXHALED NITRIC OXIDE MEASUREMENT

For exhaled NO measurement in anesthetized animals, expiratory gas was sampled two to three times for 3 minute periods each and passed through a chemiluminescence analyzer (model 270B NOA; Sievers, Boulder, Colorado, United States of America) to measure NO concentration as previously described. Fike, C. D., et al., *American Journal of Physiology (Lung, Cellular and Molecular Physiology 18)* (1998) 274:L517-L526. Exhaled NO production (nmol/min) was calculated using minute ventilation and the measured exhaled NO concentration.

ISOLATED LUNG PERFUSIONS

The lungs were isolated and perfused *in situ* with a Krebs Ringer bicarbonate (KRB) solution containing 5% dextran, mol. wt. 70,000, at 37°C and ventilated with a normoxic gas mixture (21% O_2 and 5% CO_2) as previously described. Fike, C. D. et al., *J Appl Physiol* (2000) 88:1797-1803. The lungs were perfused for 30-60 min until a stable pulmonary arterial pressure was achieved. Perfusate samples (1 ml) were then removed from the left atrial cannula every 10 min for a 60-minute period. The perfusate samples were centrifuged, and the supernatant was stored at -80°C for future analysis of nitrite/nitrate (NOx^-) concentrations as described below. At the end of the perfusion, the volume of perfusate remaining in the circuit and reservoir was measured. In some cases, lung tissue was collected immediately following the perfusion, frozen with liquid nitrogen and then stored at -80 degrees for later measurement of eNOS content as described below.

NOX^- MEASUREMENT

A chemiluminescence analysis described previously was used to determine perfusate NOx^- concentration (nmol/ml) at each collection time. Fike, C. D. et al., *J Appl Physiol* (2000) 88:1797-1803; Turley, J. E. et al., *Am J Physiol Lung Cell Mol Physiol* (2003) 284:L489-L500 Perfusate (20 µl) was injected into the reaction chamber of a chemiluminescence NO analyzer (model

170B NOA, Sievers). The reaction chamber contained vanadium (III) chloride in 1 M HCl heated to 90°C to reduce nitrite and nitrate to NO gas. The NO gas was carried into the analyzer using a constant flow of N_2 gas via a gas bubble trap containing 1 M NaOH to remove HCl vapor. A standard curve was generated by adding known amounts of $NaNO_3$ to distilled water and assaying as described for the perfusion samples.

The perfusate NOx^- concentration (nmol/ml) was calculated for each collection time by multiplying the perfusate concentration of NOx^- at that sample collection time by the volume of the system (perfusion circuit + reservoir) at the sample collection time plus the amount of NOx^- removed with all previous samples. The rate of NOx^- production was determined from the slope of a linear regression line fit to the amount of NOx^- in the perfusate versus time for the first 60 minutes of the collection period.

PLASMA AMINO ACID MEASUREMENTS

On the day of hemodynamic measurements and/or lung perfusion study, for control and both L-citrulline treated and untreated chronic hypoxic animals, blood was drawn prior to starting the study and the plasma frozen at -80 degrees for later determination of amino acid levels. For the L-citrulline treated hypoxic animals, the time of obtaining the blood sample was approximately 12 hours after the last dose of L-citrulline so was a trough level. In some of the L-citrulline treated animals (n=3), after blood sampling for a trough level, a dose of L-citrulline was given via nasogastric tube. Following this dose, blood samples were drawn every 30 minutes for 90 minutes (the length of the *in vivo* studies). All samples were spun, the plasma collected and frozen at -80 degrees for amino acid analysis.

Concentrations of plasma citrulline and arginine were determined by amino-acid analysis on protein-free extracts. Amino acids were separated by cation-exchange chromatography using a Hitachi L8800 amino acid analyzer (Hitachi USA, San Jose, California, United States of America). Calibration of the analyzer was performed before testing of piglet samples.

WESTERN BLOT OF ENOS IN LUNG TISSUE

Using a standard immunoblot technique as previously described, we analyzed samples of whole lung homogenates from controls (n=3), untreated

hypoxic (n=3) and L-citrulline treated hypoxic (n=3) animals for eNOS. We used 10 micrograms of total protein, a dilution of primary eNOS antibody of 1:500 (BD transduction) and a dilution of secondary anti-mouse antibody conjugated to horseradish peroxidase of 1:5000. Fike, C. D., et al., *American Journal of Physiology (Lung, Cellular and Molecular Physiology 18)* (1998) 274:L517-L526.

CALCULATIONS AND STATISTICS

Pulmonary vascular resistance was calculated from the in vivo hemodynamic measurements: (Pulmonary arterial pressure – left ventricular end diastolic pressure) ÷ (Cardiac output/body weight).

Data are presented as means ± SE. The one-way ANOVA with Fisher's protected least significant difference (PLSD) post hoc comparison test was used to compare data between control, untreated hypoxic and L-citrulline treated hypoxic animals. A p-value less than 0.05 was considered significant. Meier, U., *Pharm Stat* (2006) 5:253-263.

EXAMPLE 1

IN VIVO HEMODYNAMIC MEASUREMENTS

Both L-citrulline treated and untreated chronic hypoxic animals had lower cardiac output and weights and higher LVEDP measurements on the day of study at 12-13 days of age than comparable age control piglets (Table 1). Measurements of aortic pressure and blood gas indices were similar (paO_2 was 74±5 Torr in control piglets, 74±8 Torr in untreated hypoxic piglets and 78±7 Torr in L-citrulline treated hypoxic piglets; paCO2 was 39±2 in control piglets, 41±4 in untreated hypoxic piglets and 30±1.0 in L-citrulline treated hypoxic piglets) among groups. Notably, as shown in Figure 3, L-citrulline treated hypoxic animals had significantly lower pulmonary artery pressures than untreated hypoxic animals (p-value of 0.01). Pulmonary artery pressures did not differ between normoxic controls and L-citrulline treated hypoxic animals (p=0.08).

In addition, as shown in Figure 4, calculated pulmonary vascular resistance in those hypoxic animals treated with L-citrulline (0.071±0.003) were significantly lower than those of untreated hypoxic animals (p-value of 0.001).

Furthermore, pulmonary vascular resistances were similar in L-citrulline treated hypoxic animals and normoxic controls (p-value of 0.07).

EXAMPLE 2

EXHALED NO OUTPUT AND PERFUSATE NOX^-

As shown in Figure 5, exhaled NO output in controls and L-citrulline treated hypoxic animals were higher than exhaled NO output in untreated hypoxic animals (p-values of 0.001 and 0.032 respectively). However, exhaled NO output did not differ between control and L-citrulline treated hypoxic animals (p=0.124).

As shown in Figure 6, lungs from both the control (p=0.02) and L-citrulline treated hypoxic (p=0.04) animals had significantly higher NOx^- accumulation rates than lungs from untreated hypoxic animals. Furthermore, there was no difference in the rate of NOx^- accumulation between lungs from L-citrulline treated hypoxic animals and normoxic controls.

EXAMPLE 3

PLASMA AMINO ACIDS

As shown in Table 2, although not reaching statistical significance (p=0.05), plasma L-citrulline levels in untreated chronic hypoxic piglets were less than trough L-citrulline levels in treated hypoxic piglets. Moreover, when drawn ninety minutes after a dose, levels of L-citrulline in treated hypoxic animals were almost twice that of the untreated chronic hypoxic animals (p=0.001). However, regardless of the time the sample was drawn, plasma arginine levels were not higher in L-citrulline treated chronic hypoxic animals when compared to untreated hypoxic animals.

EXAMPLE 4

WESTERN BLOT FOR LUNG ENOS PROTEIN

As shown in Figures 7A and 7B, the amount of eNOS protein present in the lung tissue of control animals was significantly higher than that present in the lungs of untreated hypoxic animals. Furthermore, the amount of eNOS protein present in the lung tissue of L-citrulline treated hypoxic piglets was not

significantly different from that in the untreated hypoxic animals and was significantly lower than eNOS protein levels in control animals.

TABLE 1

DATA FOR CONTROL, CHRONICALLY HYPOXIC AND L-CITRULLINE TREATED CHRONICALLY HYPOXIC PIGLETS

N=number of animals, Values are means±SEM, *p<0.05 vs. controls, ANOVA with post-hoc comparison test.

Treatment Group	**Weight at 12 days of age (kg)**	**Aortic Pressure (cm H_2O)**	**LVEDP (cmH_20)**	**Cardiac Output (ml/min/kg)**	**Arterial p**
Controls N=6	3.94±0.3	91±0.8	5.2±0.6	414±43	7.38±0.05
Chronic Hypoxic N=11	2.76±0.15*	100±4	7.4±0.5*	244±16*	7.38±0.01
Citrulline Hypoxic N=6	2.6±0.09*	97±6	7.2±0.4*	270±41*	7.36±0.02

TABLE 2

PLASMA AMINO ACID LEVELS FOR CONTROL, CHRONICALLY HYPOXIC AND L-CITRULLINE TREATED CHRONICALLY HYPOXIC PIGLETS

Treatment Group	Citrulline	Arginine
Controls N=10	71±6	112±16
Chronic Hypoxic N=8	111±23	51±10*
L-citrulline treated Hypoxic: trough N=6	161±5*	39±10*
L-citrulline treated Hypoxic: 90 min. N=3	219±36*†	43±5*

N=number of animals, Values are means±SEM, *$p<0.05$ vs. controls, † $p<0.05$ vs. untreated chronic hypoxics, ANOVA with post-hoc comparison test, Citrulline Trough- plasma level approximately twelve hours after L-citrulline dose, Citrulline 90 min- plasma level 90 minutes after administration of L-citrulline dose.

DISCUSSION OF EXAMPLES

In Examples 1-4, it was found that L-citrulline supplementation ameliorates the development of pulmonary hypertension in newborn piglets exposed to 10 days of chronic hypoxia. Other findings in this study are that both exhaled NO production and pulmonary vascular NOx^- accumulation rates are greater in L-citrulline-treated hypoxic piglets than in untreated hypoxic piglets. Thus, these findings show that L-citrulline supplementation significantly increased pulmonary NO production. The amount of eNOS protein was not increased in the treated hypoxic animals.

While it is not desired to be bound by any particular theory of operation, the mechanism by which L-citrulline mediates an increase in NO production is believed to be by increasing the amount of L-arginine available as a substrate for eNOS. Plasma levels of arginine in the L-citrulline treated animals in the present Examples were not significantly increased when compared with untreated hypoxic animals. This discordance between intracellular arginine and NO production, termed an "arginine paradox", appears to be present in view of the increase in NO production in the face of unchanged plasma arginine levels seen with L-citrulline supplementation in the present Examples. L-citrulline is a urea cycle intermediate metabolized to arginine by a recycling pathway of two enzymes, argininosuccinate synthase (AS) and argininosuccinate lyase (AL). These two enzymes, AS and AL, have been found co-located with eNOS in pulmonary endothelial cells. Boger, R. H., *Curr Opin Clin Nutr and Met Care* (2008) 11:55-61. It is thought that together these enzymes produce a separate subcellular pool of arginine used exclusively for NO synthesis. Tissue and plasma arginine levels cannot accurately measure this subcellular pool.

L-citrulline could also have improved NO production and eNOS function by additional mechanisms. Again, while it is not desired to be bound by any particular theory of operation, another potential action of L-citrulline in the present Examples is the prevention of the uncoupling of eNOS by maintaining adequate levels of its substrate arginine.

Again, while it is not desired to be bound by any particular theory of operation, L-citrulline might also have affected the bioavailability of NO by

compensating for increased NO degradation. During exposure to chronic hypoxia, superoxide production might increase from enzymatic sources other than eNOS, such as NADPH oxidase. Liu, et al., *Am J Physiol Lung Cell Mol Physiol* (2006) 290:L2-L10. This excess superoxide production might have directly interacted with NO to reduce its local production. In this case, it is possible that providing L-citrulline allowed enough NO production to compensate for the superoxide mediated reduction.

In summary, the present Examples show that L-citrulline ameliorates chronic hypoxia-induced pulmonary hypertension in newborn piglets. Also provided is evidence that the effectiveness of citrulline is due to increased NO production. Thus, L-citrulline is a useful therapy in neonates at risk of developing pulmonary hypertension due to chronic or intermittent unresolved hypoxia.

REFERENCES

The references listed below as well as all references cited in the specification are incorporated herein by reference to the extent that they supplement, explain, provide a background for or teach methodology, techniques and/or compositions employed herein.

Jobe *et al.* (2001) *Am J Respir Crit Care Med* 163:1723-1729.

Ballard *et al.* (2006) *N Engl J Med* 355:343-353.

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U.S. Patent No. 6,743,823.

It will be understood that various details of the presently disclosed subject matter may be changed without departing from the scope of the presently disclosed subject matter. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

CLAIMS

What is claimed is:

1. A method of treating bronchopulmonary dysplasia, the method comprising administering an effective amount of nitric oxide precursor to a subject in need thereof.

2. The method of claim 1, wherein the nitric oxide precursor is selected from the group consisting of citrulline, a precursor that generates citrulline *in vivo,* arginine, a precursor that generates arginine *in vivo,* and combinations thereof.

3. The method of claim 1, wherein the administering comprises oral administration, intravenous administration, and combinations thereof.

4. The method of claim 1, wherein the subject is an infant.

5. The method of claim 4, wherein the infant is a preterm infant.

6. The method of claim 1, wherein the nitric oxide precursor is administered in a dose ranging from about 100 mg to about 30,000 mg.

7. The method of claim 6, wherein the nitric oxide precursor is administered in a dose ranging from about 250 mg to about 1,000 mg.

8. The method of claim 1, wherein the subject suffers from hypocitrullinemia characterized by plasma citrulline levels of ≤ 37 μmol/liter.

9. A method of treating chronic hypoxia-induced pulmonary hypertension, the method comprising administering an effective amount of nitric oxide precursor to a subject in need thereof.

10. The method of claim 9, wherein the nitric oxide precursor is selected from the group consisting of citrulline, a precursor that generates citrulline *in vivo,* arginine, a precursor that generates arginine *in vivo,* and combinations thereof.

11. The method of claim 9, wherein the administering comprises oral administration, intravenous administration, and combinations thereof.

12. The method of claim 9, wherein the subject is an infant.

13. The method of claim 12, wherein the infant is a preterm infant.

14. The method of claim 9, wherein the nitric oxide precursor is administered in a dose ranging from about 100 mg to about 30,000 mg.

15. The method of claim 14, wherein the nitric oxide precursor is administered in a dose ranging from about 250 mg to about 1,000 mg.

16. The method of claim 9, wherein the subject suffers from hypocitrullinemia characterized by plasma citrulline levels of ≤ 37 μmol/liter.

17. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an amount of citrulline effective to raise plasma citrulline level to treat bronchopulmonary dysplasia or hypoxia-induced pulmonary hypertension in a subject, wherein the level is determined by comparing plasma citrulline levels in a subject to be treated to that observed in a subject not suffering from bronchopulmonary dysplasia or hypoxia-induced pulmonary hypertension.

18. The pharmaceutical composition of claim 17, wherein the amount of citrulline effective to raise plasma citrulline level in a subject to at least 5 μmol/liter, optionally at least 10 μmol/liter, optionally at least 20 μmol/liter, optionally at least 25 μmol/liter, and optionally about 37 μmol/liter.

19. The pharmaceutical composition of claim 17, wherein the pharmaceutical composition is adapted for intravenous or oral administration.