α2b-Adrenoceptor Deficiency Leads to Postnatal Respiratory Failure in Mice*1

Received for publication, March 31, 2010, and in revised form, August 18, 2010. Published, JBC Papers in Press, August 20, 2010, DOI 10.1074/jbc.M110.129205

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α2-Adrenoceptors belong to the family of adrenergic receptors, which regulate the neuronal release of norepinephrine as part of a negative feedback loop. Among the α2-adrenoceptors, the α2B-subtype may also influence developmental signaling pathways involved in angiogenesis of the placenta. Thus, the aim of the present study was to determine whether α2B-adrenoceptors are also involved in other developmental processes beyond placental angiogenesis. Ablation of α2B-adrenoceptors led to lethality of mutant mice during the first hours after birth. Despite normal breathing and drinking behavior, mutant mice developed cyanosis, which could be traced back to a defect in lung morphology with significantly reduced alveolar volume and thickened interalveolar septi. In α2B-deficient lungs and in isolated alveolar type II cells, expression of sonic hedgehog (SHH) was significantly increased, resulting in mesenchymal proliferation. In vitro α2B-adrenoceptor stimulation supressed expression of sonic hedgehog and the cell cycle genes cyclin D1 and Ki67. In vivo inhibition of enhanced SHH signaling by the smoothened antagonist cyclopamine partially rescued perinatal lethality, lung morphology, and altered gene expression in mutant mice. Thus, α2B-adrenoceptors in lung epithelia play an important role in suppressing sonic hedgehog-mediated proliferation of mesenchymal cells and thus prevent respiratory failure.

EXPERIMENTAL PROCEDURES

Generation of α2B-Adrenoceptor-deficient Mice—The targeted deletion of the α2B-adrenoceptor gene (Adra2b−/−) has been described previously (7). For this study, Adra2b−/− mice, which were backcrossed onto a C57BL/6 background (Harlan Winkelmann, Borchen, Germany) for 12 generations, were used (9). All animal procedures were approved by the responsible animal care committee of the University of Freiburg, Germany. Genotyping was performed with primers as described previously (9). Cyclopamine (18 mg/kg of body weight, Sigma-Aldrich, Taufkirchen, Germany) was applied subcutaneously to pregnant mice.

Histology and Immunohistochemistry—Lungs and hearts were fixed in 4% paraformaldehyde and embedded in paraffin or araldite. Cardiac sections were stained with hematoxylin and eosin. Semithin araldite sections of the lung were stained with methylene blue (11, 12). For immunodetection of sonic hedgehog, cryostat sections were incubated with primary antiserum (R&D Systems, Wiesbaden, Germany) followed by an Alexa Fluor 488 secondary antibody (Molecular Probes, Invitrogen, Darmstadt, Germany). Nuclei were visualized by brief incubation with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Blood slides were stained with Giemsa and May-Grunwald.

Isolation and Cultivation of Primary Cells—Alveolar type II cells were isolated as described with modifications (13). Cell
α₂B-Adrenoceptors Affect Lung Development

**FIGURE 1.** Perinatal survival and characterization of α₂B-adrenoceptor-deficient mice. a, genotype distribution of the targeted α₂B allele (Adra2b⁻⁻) was determined during the embryonic and perinatal period in mice derived from heterozygous intercrosses. Numbers above columns indicate the total number of mice investigated at the respective time points. b, and c, spontaneous breathing rate (b) and blood glucose (c) did not differ between wild-type and mutant mice immediately after birth (n = 8–14 per genotype for breathing rate, n = 18–21 for blood glucose). d, erythrocyte morphology (inset: Pappenheim staining; bars, 10 μm) and mRNA expression of hemoglobin chains in the liver on postnatal day P0 (n = 5–6 per genotype).

suspensions from bronchoalveolar lavage of neonatal mice were passed through 100-, 40- (BD Biosciences, Heidelberg, Germany), and 20-μm (Millipore, Schwalbach, Germany) filters and incubated with biotinylated anti-CD31, anti-CD45, and anti-CD16/32 antibodies (BD Biosciences). Then streptavidin-coated magnetic particles (Dynabeads, Invitrogen) were added. Magnetic particles were captured using a magnetic tube separator (Promega, Mannheim, Germany), and the remaining cells were plated on tissue culture slides. After 4–6 h of cultivation, cells were used for gene expression analysis or were cultivated for an additional 16 h and slips. After 4–6 h of cultivation, cells were used for gene expression analysis or were cultivated for an additional 16 h and stained with methylene blue for cell identification.

Gene Expression—Total RNA was prepared from lung samples and primary alveolar type II cells with the RNeasy kit (Qiagen, Hilden, Germany). For quantitative real-time PCR analyses, 35 μl of the amplification mixture (Qiagen, Quantitect SYBR Green kit) was used containing 20 ng of reverse-transcribed RNA and 300 nmol/liter primers (supplemental Table 1, Eurofins MWG Operon, Ebersberg, Germany). Reactions were incubated in triplicate on an MX3000P detector (Agilent Technologies, Waldbronn, Germany). The cycling conditions were: 15 s of polymerase activation at 95 °C and 40 cycles at 18–21 for blood glucose). d, erythrocyte morphology (inset: Pappenheim staining; bars, 10 μm) and mRNA expression of hemoglobin chains in the liver on postnatal day P0 did not differ between genotypes (n = 5–6 per genotype).

**FIGURE 2.** Cardiac function and structure in α₂B-adrenoceptor-deficient mice on postnatal day 0. a–g, electrocardiograms were recorded from α₂B-deficient and wild-type mice during isoflurane anesthesia on postnatal day 0. Adra2b⁻⁻/⁻ mice showed regular rhythm (c and d) and normal averaged ECGs (b and d). RR interval (e), QRS interval (f), and QTc interval (g, n = 8–13 per genotype), h and i, frontal sections through hearts on day P0 revealed normal cardiac structure (hematoxylin and eosin staining; bars, 500 μm) and closure of the ductus arteriosus (A, aorta; DA, ductus arteriosus (arrow); LV, left ventricle; RV, right ventricle). j, ductus arteriosus closure in Adra2b⁻⁻/⁻ and Adra2b⁻⁻/⁻ mice on day P0 (n = 17–18 per genotype, p = 0.34, Fisher’s test).

**RESULTS**

**Perinatal Lethality of α₂B-Adrenoceptor-deficient Mice**—Heterozygous intercrosses of α₂B-adrenoceptor-deficient mice resulted in a percentage of Adra2b⁻⁻/⁻ mice at weaning age that was significantly lower than the predicted Mendelian ratio (Fig. 1a, 3 Adra2b⁻⁻/⁻ out of 98 offspring at postnatal day P25). Between embryonic days E10.5 and E18.5, however, the genotype distribution did not deviate significantly from the pre-
dicted Mendelian ratio. Most $\alpha_{2B}$-deficient mice died during the day of birth (Fig. 1a). Newborn $\alpha_{2B}$-deficient mice showed normal breathing rate (Fig. 1b) and drinking behavior, as evidenced by the presence of visible milk in their stomach. At this time, blood glucose levels did not differ between genotypes (Fig. 1c). No alterations in hematopoiesis were observed, as evidenced by the presence of visible milk in their stomach. At this time, blood glucose levels did not differ early after birth (Fig. 1d). Hepatic mRNA expression of hemoglobin chains did not differ early after birth (Fig. 1d). However, during the next hours, $\alpha_{2B}^{-/-}$ mice were retarded in their weight gain and became cyanotic.

**Cardiac Phenotyping of $\alpha_{2B}$-deficient Mice**—Cardiac structure and function did not differ between wild-type and $\alpha_{2B}$-mutant mice on postnatal day 0 (Fig. 2). ECG recordings revealed normal cardiac rhythm with no signs for conduction deficits in $\alpha_{2B}$-deficient mice after birth (Fig. 2, a–g). Upon microscopical investigation of $\alpha_{2B}^{-/-}$ hearts, all valves and chambers were properly developed, and postnatal closure of the ductus arteriosus Botalli was normal (Fig. 2, h–j, $p = 0.34$). However, the right ventricle was significantly dilated in $\alpha_{2B}^{-/-}$ as compared with wild-type hearts (internal diameter in $\alpha_{2B}^{+/+}$, 564.8 $\pm$ 58.3 $\mu$m, $n = 5$, versus $\alpha_{2B}^{-/-}$, 937.4 $\pm$ 87.4 $\mu$m, $n = 4$, $p < 0.01$). Organ weights of heart, kidney, and liver were similar between genotypes, but $\alpha_{2B}$-deficient mice had significantly lower body and lung weights than wild-type mice (Table 1).

**Pulmonary Phenotype of $\alpha_{2B}$-deficient Mice**—Reduced lung weight, cyanosis, and dilatation of the right ventricle led us to investigate pulmonary development and morphology (Fig. 3). Between embryonic days E14.5 and E18.5, lungs from $\alpha_{2B}^{-/-}$ mice developed normally, and cellular versus alveolar/bronchial spaces were similarly distributed between genotypes (Fig. 3, a–f). However, early after birth, lungs of $\alpha_{2B}^{-/-}$ mice were less inflated and showed reduced alveolar spaces and thickened inter-alveolar septi (Fig. 3, j–l). Capillary density did not differ between genotypes. $\alpha_{2B}^{-/-}$ lungs were characterized by a significantly higher degree of mitosis and increased expression of the cell cycle markers cyclin D1 ($Ccn$) and Ki67 ($Mki67$) (Fig. 4, a–c). Quantitative real-time PCR revealed that $\alpha_{2B}$-adrenoceptors were expressed at 3.3- and 3.7-fold higher levels in the lung as compared with $\alpha_{2A}$- or $\alpha_{2C}$-adrenoceptors, respectively (Fig. 4d). To determine the cellular localization of $\alpha_{2B}$-adrenoceptors, bronchial epithelium, which stained positively for expression of SHH and interstitial lung tissue, were microdissected and subjected to quantitative real-time PCR analysis (Fig. 4e). Bronchial specimens showed 5-fold higher $\alpha_{2B}$-mRNA expression than interstitial cells, suggesting that

![FIGURE 3. Histology and morphometric analysis of lungs in $\alpha_{2B}$-adrenoceptor-deficient mice between embryonic day E14.5 and postnatal day P0. a–l, lungs from $\alpha_{2B}^{-/-}$ and wild-type mice were obtained from timed matings at the indicated days of development. Morphometric analysis of methylene blue-stained lung sections revealed normal distribution of alveolar/bronchial spaces versus cellular areas during embryonic development (c, f, and i) but increased interalveolar septi and cell areas in $\alpha_{2B}^{-/-}$ lungs on postnatal day P0 (A, alveoli; bars, 20 $\mu$m, E14.5 and 10 $\mu$m, E17.5–P0; $n = 3$–7 per genotype and time point, **, $p < 0.01$ versus $\alpha_{2B}^{+/+}$).](attachment://figure3.png)
α₂b-Adrenoceptors Affect Lung Development

Gene Expression in α₂b-Deficient Lungs on Postnatal Day 0—To search for the mechanism of the defect in perinatal lung development in mutant mice, genes that have previously been shown to play an essential role for lung development were determined in their expression on day P0 (supplemental Table 2). Out of 40 genes tested, 6 were increased in their expression in Adra2b⁻/⁻ lungs, including Ccnd1, Flt1, Mki67, Ptch1, Shh, and Vegfa (supplemental Table 2). Of these genes, sonic hedgehog (Shh) was most strongly up-regulated in Adra2b⁻/⁻ versus wild-type lung tissue (supplemental Table 2). Also, the SHH receptor patched (Ptch1) was significantly increased in its expression (supplemental Table 2). However, smoothened (Smo), which interacts with patched, did not differ in its expression between genotypes (supplemental Table 2).

Gene Expression in α₂b-Deficient Alveolar Type II (ATII) Cells on Postnatal Day 0—To test whether Shh is regulated in epithelial cells, ATII cells were isolated on postnatal day 0. To validate the cell isolation procedure, the expression of Ddr2, a fibroblast marker (16), Pecam, an endothelial marker (17), and Sph, an ATII marker (18), was assessed. Relative to Sph, expression of Pecam and Ddr2 was significantly reduced in primary cultured ATII cells as compared with intact lungs, confirming enrichment of ATII cells (Fig. 5a). Light microscopic analysis confirmed the purity of the cultivated ATII cells (18, 19) and did not indicate morphological differences between genotypes (Fig. 5b). The expression of Shh mRNA was 3-fold higher in Adra2b⁻/⁻ versus wild-type ATII cells (Fig. 5c).

DISCUSSION

The main finding of the present study is that α₂b-adrenoceptors are essential for perinatal survival and lung development in mice. α₂b-Adrenoceptors may modulate the expression of Shh between epithelial and mesenchymal cells by suppressing sonic hedgehog expression and signaling (Fig. 7). Ablation of α₂b-adrenoceptor expression led to early postnatal respiratory failure.

α₂b-Adrenoceptors contribute to presynaptic inhibition of neurotransmitter release (22) and elicit hypertension and vasoconstriction as postsynaptic receptors (7, 23, 24). In addition, the α₂b-subtype is unique among the adrenoceptors because it plays an essential role during embryonic development (9, 10). Although previous studies have demonstrated the involvement of α₂b-receptors in extraembryonic development (9, 10), i.e. placenta and yolk sac, the present study shows that α₂b-recep-

![FIGURE 4. Rate of mitosis, cell cycle gene expression, and α₂-adrenoceptor subtype distribution in the lung. a, Mito- 
sis rate. Adra2b⁻/⁻ lungs contained significantly more mitotic cells (inset, arrowhead) as compared with wild- 
type lungs on postnatal day 0 (bars, 10 μm). b and c, mRNA expression of cell cycle regulators cyclin D1 (Ccnd1, 
unpaired t test. d, α₂-adrenoceptor mRNA expression in bronchial epithelial cells and interstitial cells after microdissection from cryostat sections of wild-type lungs on day P0 (n = 4–6 per genotype).

d–i, Immunostaining of SHH in bronchial epithelia of the wild-type lung on day P0. e, lower panel, DAPI 
fluorescence to detect nuclei (bars, 20 μm). f, α₂-adrenoceptor mRNA expression in bronchial epithelial cells 
and interstitial cells after microdissection from cryostat sections of wild-type lungs on day P0 (n = 4 per group). 

α₂b-receptors are coexpressed with SHH in epithelial cells of the lung (Fig. 4f).

α₂b-Adrenoceptor Stimulation Suppresses Shh Expression—We hypothesized that α₂b-receptors might modulate expression of Shh to control perinatal lung development. Lung slices from wild-type and α₂b-deficient E17.5 embryos were maintained in DMEM in vitro and were stimulated with the α₂b-agonist medetomidine (Fig. 5, d–i). Medetomidine suppressed Shh mRNA expression in wild-type but not in α₂b-deficient lungs (Fig. 5d). Furthermore, SHH protein levels were lower in lung specimens that were cultivated in the presence of medetomidine (Fig. 5e). Lung section histology was not affected by the in vitro culture (Fig. 5f). Expression of cyclin D1 (Ccnd1) and the sonic hedgehog target genes Gli1 and Gli2 but not Gli3 was significantly reduced by the α₂-adrenoceptor agonist medetomidine (Fig. 5, g and i).
**FIGURE 5.** Expression of Shh and downstream signaling molecules in primary alveolar type II cells and in the lung in vitro. 

**a-c,** ATII epithelial cells from newborn Adra2b+/+ (a–c) or Adra2b−/− (b and c) mice were isolated, and marker gene expression (a) and morphology were determined. Low Pecam and Ddr2 to Spb mRNA ratios in primary ATII cells as compared with intact lungs indicate low levels of contaminating fibroblasts (Ddr2) or endothelial cells (Pecam) (a, n = 3–4 per group, *, p < 0.05, ***, p < 0.001 versus Adra2b+/+ lung). Primary cultivated ATII cells of Adra2b−/− neonatal mice did not show morphological differences but express significantly more Shh as compared with wild-type neonatal ATII cells (b and c, n = 5 per genotype, **, p < 0.01 versus Adra2b+/+; b, bars, 20 μm).

**d–i,** lung sections derived from E17.5 wild-type or Adra2b−/− deficient embryos were cultivated in vitro and stimulated with 25 nmol/liter medetomidine for 24 h. d and e, medetomidine suppressed sonic hedgehog mRNA (d) and protein levels (e) in wild-type lungs but not in Adra2b−/− tissue (d, f), lung histology did not differ between genotypes and treatment groups after in vitro incubation (bars, 10 μm; B, bronchi). g and i, expression of cyclin D1 (Ccnd1, g) and the SHH downstream target genes, Gli1 and Gli2 (i), was reduced by medetomidine in wild-type lungs in vitro. Gli3 expression was not significantly reduced (d–i, n = 6 per group, *, p < 0.05; **, p < 0.01 versus untreated Gli3, p = 0.052).
Adrenoceptors Affect Lung Development

FIGURE 6. Effect of cyclopamine treatment on perinatal lethality, lung structure, and gene expression. 

a, pregnant females from heterozygous crosses were treated with cyclopamine starting on day E17.5 (18 mg/kg of body weight, injected subcutaneously), and survival of Adra2b−/− offspring was compared with vehicle-treated Adra2b+/− mice. Numbers above columns indicate living Adra2b+/− mice (*, p = 0.017, log rank test). b–d, lung histology of cyclopamine-treated wild-type and mutant mice on day P1 did not differ between genotypes (bars, 10 μm; A, alveoli). e–g, cyclopamine treatment resulted in a significant suppression of Shh expression and the cell cycle markers Ccnd1 and Mki67 in Adra2b−/− lungs as compared with wild-type lungs on day P1 (n = 4–6 per group, *, p < 0.05 versus Adra2b+/−).

Several factors confirm the finding that perinatal lethality in Adra2b−/− mice is caused by lung failure. Several essential postnatal functions including breathing, drinking behavior, and cardiac rhythm appeared normal in α2b-deficient mice immediately after birth. Several hours after birth, mutant mice became cyanotic, which is consistent with the observed thickening of interalveolar septi and reduced air spaces in the lung. Thus, the distance for gas exchange between alveoles and capillaries may be increased in mutant mice, reducing gas exchange and ultimately causing respiratory failure. In accordance with this mechanism, hypoxia may elicit pulmonary vasoconstriction, which leads to increased vascular resistance and dilatation of the right ventricle. A similar chain of events has been observed in mice lacking pituitary adenylate cyclase-activating polypeptide type I receptors (25). In contrast to the embryonic period, where α2b is essential for vascular development in the placenta, pulmonary vascular development was not significantly altered by α2b-ablation as capillary density did not differ between wild-type and Adra2b−/− lungs. Other organ systems that have been suggested to be regulated by α2b-receptors also developed normally in Adra2b−/− mice. α2b-Receptors are abundantly expressed in hematopoietic cells in the fetal liver (26). However, erythrocyte counts and morphology as well as hemoglobin synthesis in the liver were normal as compared with wild-type mice.

Our data suggest that dysregulation of sonic hedgehog signaling in the lung is causally related to the developmental defect in the lung. SHH has previously been shown to play an important role in the determination of cell fate and embryonic patterning. General ablation of SHH expression caused severe lung malformations, hypoplasia, and deficient branching morphogenesis (27, 28). Transgenic re-expression of SHH in respiratory epithelial cells substantially rescued the peripheral lung morphogenesis defect observed in SHH-deficient mice (29). Overexpression of SHH under control of the surfactant protein C promoter led to a similar phenotype as observed in α2b-deficient mice, i.e. early postnatal lethality, thickened septi in the lung, and proliferation of mesenchymal cells (30). Similar to the α2b-deficient mice, SHH transgenic pups died soon after birth due to respiratory failure (30).

SHH may play a causal role in the phenotype elicited by ablation of α2b-expression (Fig. 7). In vitro, stimulation of α2b-receptors suppressed expression of SHH and cell cycle markers cyclin D1 and Ki67. These results indicate that SHH is under tonic inhibition by α2b-adrenoceptors in vivo. Furthermore, inhibition of enhanced SHH signaling by the smoothened antagonist cyclopamine partially rescued the perinatal lethality and the pulmonary phenotype. Cyclopamine has been demonstrated to specifically block hedgehog signaling by direct binding to the G protein-coupled receptor smoothened (20, 21). Further studies are required to identify the precise intracellular pathway linking α2b-receptors with SHH repression. In addition, it will be important to define the role of α2b-receptors in the development of pulmonary diseases, including lung fibrosis.
and inflammation. In conclusion, \( \alpha_{2B} \)-adrenoceptors suppress sonic hedgehog to control this important epithelial-mesenchymal signaling pathway, which is essential for lung development.

Acknowledgments—We thank Nadine Beetz for discussion and assistance with gene expression and ECG analysis and K. Julia Ellis for manuscript editing.

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