Endocannabinoid anandamide mediates hypoxic pulmonary vasoconstriction

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Endocannabinoids are important regulators of organ homeostasis. Although their role in systemic vasculature has been extensively studied, their impact on pulmonary vessels remains less clear. Herein, we show that the endocannabinoid anandamide (AEA) is a key mediator of hypoxic pulmonary vasoconstriction (HPV) via fatty acid amide hydrolase (FAAH)-dependent metabolites. This is underscored by the prominent vasoconstrictive effect of AEA on pulmonary arteries and strongly reduced HPV in FAAH−/− mice and wild-type mice upon pharmacological treatment with FAAH inhibitor URB597. In addition, mass spectrometry measurements revealed a clear increase of AEA and the FAAH-dependent metabolite arachidon酸 in hypoxic lungs of wild-type mice. We have identified pulmonary vascular smooth muscle cells as the source responsible for hypoxia-induced AEA generation. Moreover, either FAAH−/− mice or wild-type mice treated with FAAH inhibitor URB597 are protected against hypoxia-induced pulmonary hypertension and the concomitant vascular remodeling in the lung. Thus, the AEA/FAAH pathway is an important mediator of HPV and is involved in the generation of pulmonary hypertension.

Significance

Hypoxic pulmonary vasoconstriction (HPV) is an important physiological reflex, which is only found in the lung and adapts perfusion to ventilation. HPV is potentially involved in hypoxia-induced pulmonary hypertension (PH) occurring in respiratory disorders. In this study we show that the endocannabinoid anandamide (AEA) via its fatty acid amide hydrolase (FAAH)-dependent metabolites is involved in HPV and PH. We have identified pulmonary arterial smooth muscle cells as the source of hypoxia-induced AEA synthesis. Our results illustrate that the onset of PH is prevented in FAAH−/− mice or by treating wild-type mice with a FAAH antagonist for 3 wk of hypoxia. Thus, we demonstrate a previously undescribed signaling pathway underlying HPV and an alternative strategy for the treatment of common pulmonary diseases.


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A higher incidence of pulmonary hypertension is reported for females, and we have focused on female mice in the present study. To exclude prominent sex-dependent differences in the pulmonary vascular response to endocannabinoids, we have also tested the effect of AEA (10 μM) in male mice using the IPL system. A prominent vasoconstrictive response upon AEA application was observed; the magnitude was ~30% lower in male (n = 8) compared with female mice (n = 4). Thus, AEA is a specific and potent vasoconstrictor of pulmonary vessels in male and female mice.

**AEE-Induced Pulmonary Vasoconstriction Is Mediated by FAAH-Dependent Metabolites.** We next investigated the signaling pathway underlying AEA-induced vasoconstriction in pulmonary arteries. First, we determined expression of the most important receptor molecules and the key enzyme involved in metabolization of AEA in murine lungs and found only minimal CB1 but prominent CB2 receptor and FAAH expression (Fig. S2B). In IPL measurements, the vasoconstrictive effect of AEA was independent of CB1 and CB2 receptors because it was preserved in Cnr1−/− and Cnr2−/− mice (Fig. 1D). This was also corroborated by experiments with the CB1/CB2 receptor agonist HU-210, which did not alter pulmonary arterial tone (Fig. 2A). Although inhibition of CYP450 enzymes by 17-octadecynoic acid (ODYA) (1 μM) had no effect (Fig. S2F and Fig. 2D), inhibition of COX by indomethacin (Indo) (10 μM) and 5-LOX by nordihydroguaiaretic acid (NDGA) (10 μM) induced a prominent reduction of the AEA-dependent pulmonary vasoconstriction (Fig. 2D). The leukotriene receptor antagonist montelukast (1 μM), an approved antiasthma drug, also attenuated pulmonary arterial vasoconstriction by AEA (Fig. 2D and Fig. S2G); montelukast did not change 5-HT–induced vasoconstriction, underscoring the specificity for AEA-mediated vasoconstriction, we found that the nonhydrolyzable AEA analog Meth-AEA (10 μM) did not alter pulmonary vascular tone (Fig. 2B). We also tested the effect of the pharmacological blockade of FAAH with URB597 in Cnr1/Cnr2−/− mice, but this approach did not restore the AEA-induced vasoconstriction (Fig. 2B). These experiments underscore that the decreased vasoconstriction by AEA after inhibition of FAAH is due to the reduction or lack of FAAH-dependent metabolites and is not caused by AEA accumulation resulting in enhanced signaling via CB receptors. Because FAAH is known to metabolize AEA to arachidonic acid (AA), we also examined this downstream metabolite and found that AA (10 μM) induced a strong transient vasoconstriction in pulmonary arteries in the IPL (Fig. S2E). A contribution of further downstream metabolites like eicosaoids was likely, because enzymes producing eicosanoids, such as cyclooxygenase1 (COX1) and 2 (COX2) as well as 5-lipoxygenase (5-LOX), are highly expressed in lung tissue (Fig. 2C). To examine their involvement in AEA-dependent pulmonary vasoconstriction, we applied different pharmacological agents. Although inhibition of CYP450 enzymes by 17-octadecenoic acid (ODYA) (1 μM) had no effect (Fig. S2F and Fig. 2D), inhibition of COX by indomethacin (Indo) (10 μM) and 5-LOX by nordihydroguaiaretic acid (NDGA) (10 μM) induced a prominent reduction of the AEA-dependent pulmonary vasoconstriction (Fig. 2D). The leukotriene receptor antagonist montelukast (1 μM), an approved antiasthma drug, also attenuated pulmonary arterial vasoconstriction by AEA (Fig. 2D and Fig. S2G); montelukast did not change 5-HT–induced vasoconstriction, underscoring the specificity of AEE arterioconstriction.

![Image](https://example.com/image.png)
of this compound (Fig. S2D). These data suggest that AEA is a strong pulmonary vasconstrictor, and this effect is mediated by AEA hydrolysis to AA and COX- and LOX-dependent metabolites, especially leukotrienes. The proposed signaling pathways and the pharmacological inhibitors are summarized as a scheme in Fig. S3.

**FAAH Is a Mediator of Hypoxic Pulmonary Vasoconstriction.** Hypoxia-induced vasoconstriction (HPV) is unique for the pulmonary vasculature, and earlier studies have proposed that AA or eicosanoids may be involved (15, 16). We therefore explored whether the AEA/FAAH pathway and its metabolites could also play a role in acute HPV. For this purpose we ventilated mice with hypoxic gas in the IPL and measured the mean pulmonary arterial pressure increase during two subsequent hypoxic challenges (0% O₂ ventilation) after a 5-HT–induced contraction under normoxia. The hypoxia-induced vasoconstriction reached almost 60% of the 5-HT (10 μM) effect in wild-type animals, whereas it was strongly reduced to about 20% in FAAH⁻/⁻ mice (Fig. 3 A–C); there was no difference in baseline tone between hypoxic gas in the IPL and measured the mean pulmonary arterial pressure increase during two subsequent hypoxic challenges (0% O₂ ventilation) after a 5-HT–induced contraction under normoxia. The hypoxia-induced vasoconstriction reached almost 60% of the 5-HT (10 μM) effect in wild-type animals, whereas it was strongly reduced to about 20% in FAAH⁻/⁻ mice (Fig. 3 A–C); there was no difference in baseline tone between hypoxic gas in the IPL and normoxia. Positive control: brain. (B) LC-MRM measurements of AEA and AA levels in murine lungs yielded an increase after 2 and 6 h of hypoxic challenge. Thus, metabolization of AEA by the FAAH/LOX pathway in the lung is critically involved in HPV.

**Hypoxia Causes Elevated AEA and AA Levels in the Lung.** To directly assess AEA and AA levels in whole lungs under hypoxia, we used LC-MRM measurements. These could not be performed on lung tissue after IPL because cardiovascular arrest leads to unspecific increase of AEA and AA in the tissue. Therefore, mice were kept in hypoxic chambers (10% O₂) and killed at different time points, and the lung tissue was analyzed. We found that AEA

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**Fig. 3.** The AEA/FAAH axis is an important mediator of hypoxic pulmonary vasoconstriction (HPV). (A and B) Original recordings of PAP in the IPL demonstrate strongly reduced HPV (0% O₂) in FAAH⁻/⁻ (B) compared with WT mice (A); as control, 5-HT (10 μM) was used. (C) Statistical analysis of PAPs normalized to 5-HT reveals that genetic abrogation (FAAH⁻/⁻ mice) and pharmacological inhibition (URB, 10 μM) of FAAH and leukotriene receptor blockade by montelukast (1 μM) strongly diminish HPV. **P < 0.01, one-way ANOVA with Dunnett’s test.

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**Fig. 4.** Hypoxia increases AEA and AA levels in the lung. (A) LC-MRM measurements of AEA and AA levels in murine lungs yielded an increase after 2 and 6 h of hypoxic ventilation (10% O₂). The values are normalized to levels at normoxic ventilation (21% O₂). *P < 0.05, one-way ANOVA with Dunnett’s test. (B) PCR analysis reveals prominent expression of N-acyl-phosphatidyl-ethanolamine phospholipase D (NAPE-PLD) and weak expression of MAGL in murine lung tissue; as positive control, brain tissue was used. (C) Western blot analysis demonstrates strong protein expression of NAPE-PLD and FAAH in murine lung tissue but very low levels in the heart. Positive control: brain. (D–I) Immunohistochemistry of murine lung sections shows colocalization of NAPE-PLD (red) (D and F), FAAH (red) (G and H), and vascular smooth muscle cells (α-smooth muscle actin; green) (E, F, H, and I); nuclei are stained with hoechst (blue) (F and I). (Scale bar, 10 μm.)
and AA levels were significantly elevated after 2 and 6 h of hypoxic ventilation (Fig. 4I) but not earlier as would be expected from the IPL data. This is most likely due to the slower onset of strong hypoxia in spontaneously breathing mice kept in 10% O2. A similar time course for the development of HPV has been also reported for humans (17). The specificity of the elevation of AEA was further demonstrated by 2-AG measurements that displayed no change upon hypoxia exposure (n ≥ 5, *P > 0.05 for 0 h vs. 1, 2, 6, and 24 h).

Thus, hypoxia leads to increased levels of AEA and its metabolite AA in the lung.

**AEA Is Generated and Metabolized in Pulmonary Arterial Smooth Muscle Cells.** We next wanted to identify the cell type responsible for AEA generation in the lung and therefore investigated the expression of enzymes involved in AEA synthesis. We reasoned that elevated levels of AEA likely correlate with its increased synthesis by N-acetyl-phosphatidyl-ethanolamine phospholipase D (NAPE-PLD) (18), and we therefore focused on the gene expression analysis of this enzyme. Similar to FAAH, we found a strong signal for NAPE-PLD at the mRNA level in the whole lung, which was comparable to murine brain tissue (Fig. S2B and Fig. 4B). In contrast, MAGL, an enzyme that mainly metabolizes 2-AG, was only weakly expressed in the lung (Fig. 4B). Pulmonary mRNA expression of NAPE-PLD and FAAH was also confirmed at the protein level by Western blot analysis (Fig. 4C). Both enzymes showed only low expression levels in the heart (Fig. 4C). Similarly, this was also observed by immunostainings of cardiac vessels (Fig. 5A–L), which displayed only very weak expression levels of NAPE-PLD and FAAH, indicating a special role of these enzymes in pulmonary vasoregulation. To identify the cell types expressing NAPE-PLD and FAAH, we performed immunostainings in lung sections and found that pulmonary arterial smooth muscle cells of intrapulmonary arteries expressed both enzymes (Fig. 4D–I); this finding is in full agreement with the proposed key role of smooth muscle cells for the induction of HPV (10). In clear contrast, in the CD31+ endothelial cell layer of muscularized intrapulmonary arteries we could not detect NAPE-PLD and FAAH (Fig. 5M–R). Because smooth muscle cells of pulmonary arterioles appeared to be the main site of AEA generation and metabolization, we analyzed the expression of NAPE-PLD and FAAH in a human pulmonary arterial smooth muscle cell line (hPASMCs) and found again prominent protein expression of NAPE-PLD and FAAH (Fig. 5A and B). We used the same cell type to assess AEA production and metabolization.

First, we tested the conversion of exogenously applied AEA into AA after 1 h in hPASMCs. LC-MRM yielded clearly elevated AA levels compared with solvent control (Fig. 5C). Next, we examined the effect of hypoxia on AEA and AA levels in hPASMCs by LC-MRM. The analysis was performed after 5 h of hypoxia (0.1% O2) or normoxia (controls) because this time point had yielded maximal levels of AEA and AA under hypoxia in whole lungs (see also Fig. 4I). Our data in hPASMCs showed a significant elevation of AEA and AA under hypoxia (Fig. 5D), which was accompanied by an increase of NAPE-PLD protein expression by 34.7 ± 6.7%, n = 4, P < 0.05 (Fig. 5E). In contrast to the smooth muscle cells, bovine pulmonary endothelial cells showed no increase of AEA and AA levels after 5 h of hypoxia (n = 5, P > 0.05); similarly, AEA levels in human microvascular endothelial cells of the lung also did not increase significantly upon hypoxia compared with controls (n = 5, P > 0.05). Thus, hypoxia increases AEA and AA levels in pulmonary arterial smooth muscle cells.

**FAAH Is Involved in the Development of Pulmonary Arterial Hypertension.** Our experiments clearly demonstrate that the AEA/FAAH axis is strongly involved in the regulation of acute hypoxic vasoconstriction, and we therefore wondered whether this signaling pathway also plays a role in the generation of hypoxia-induced pulmonary hypertension. To examine this, wild-type and FAAH+/− mice were kept for 3 wk under normoxic (21% O2) or hypoxic (10% O2) conditions, and then functional and morphological analyses were performed. Hypoxia resulted, as expected, in an increase of vascular wall thickness compared with the relative vessel diameter in wild-type mice (Fig. 6A and D). However, such changes were not observed in FAAH+/− mice exposed to hypoxia (Fig. 6B and D). Similarly, the Fulton index was clearly elevated in hypoxic wild-type animals indicating right heart hypertrophy, whereas it was unaltered in FAAH−/− mice (Fig. 6E). These findings were also supported by catheter-based right ventricular systolic pressure (RVSP) measurements yielding strongly increased...
pressure in wild-type mice after 3 wk of hypoxia (Fig. 6A and Fig. 6F), whereas in FAAH−/− mice, no obvious changes could be detected (Fig. 6B and Fig. 6F).

LC-MRM measurements of AEA and AA levels under chronic hypoxic conditions revealed that 2 d after the onset of hypoxia, AEA and AA levels significantly decrease. In the following days (5 d and 7 d) a steady increase of AEA and AA levels was found under chronic hypoxic conditions reaching significance for AEA at day 7 (Fig. 6C).

To further corroborate the important role of the AEA/FAAH pathway in the generation of pulmonary hypertension and to determine if FAAH is a potential therapeutic target, we treated wild-type mice during 3 wk of hypoxia with URB597 (+URB) or only solvent as control (−URB) by daily i.p. injections (5 mg/kg). This treatment prevented hypoxia-induced remodeling and pulmonary hypertension, namely, the elevation of vascular wall thickness (Fig. 6C and D), Fulton index (Fig. 6E), and RVSP (Fig. 6D and Fig. 6F), whereas treatment of mice for only 3 d did not have protective effects (Fig. 6E). These findings suggest that degradation of AEA to vasoactive metabolites by FAAH is involved in the development of hypoxia-induced pulmonary hypertension.

Discussion

Endocannabinoids are emerging as unique mediators of organ homeostasis, and this concept also applies to the cardiovascular system. In fact, experimental evidence indicates their involvement in the regulation of systemic blood pressure (19) and cardiac output (2) and in atherosclerosis (20). Herein, we demonstrate that AEA mediates hypoxic pulmonary vasoconstriction and is also involved in pulmonary hypertension via its degradation to FAAH-dependent metabolites. Effects of endocannabinoids on vascular tone have been mainly attributed to direct endocannabinoid signaling via surface receptors (1, 2, 21) so far, whereas degradation pathways of endocannabinoids have been thought to play a minor role (4, 22). FAAH is the principal AEA-degrading enzyme, thereby limiting the effects of AEA at cannabinoid receptors. We found FAAH to be strongly expressed in the lung, whereas only low expression levels were detected in organs or vessels involved in systemic circulation (i.e., heart and tail artery), suggesting that this differential expression could mechanistically explain the importance of the AEA degradation pathway for pulmonary tone regulation. FAAH is known to metabolize AEA to AA and ethanolamine, and AA is the precursor of eicosanoids, a family of lipid mediators that are

Fig. 6. The AEA/FAAH axis is involved in the generation of hypoxia-induced pulmonary hypertension. (A) H&E stainings of intrapulmonary arteries of WT mice demonstrate increased vascular wall thickness after 3 wk of HX (10% O2) (Right) compared with NX (21% O2) (Left). (Scale bar, 20 μm.) (B) H&E stainings of intrapulmonary arteries of FAAH−/− mice display no change in vascular wall thickness after 3 wk of HX (10% O2) (Right) compared with NX (21% O2) (Left). (Scale bar, 20 μm.) (C) H&E stainings of intrapulmonary arteries of WT mice after 3 wk of HX (10% O2) with daily injections of URB (5 mg/kg) (Left) demonstrate reduced vascular wall thickness compared with solvent (Right). (Scale bar, 20 μm.) (D–F) Statistical analysis of vascular wall thickness (D), Fulton index (E), and RVSP (F) demonstrates increased values after 3 wk of HX compared with NX in WT mice; these changes were absent in FAAH−/− mice and could be abrogated by URB injection in WT mice. *P < 0.05, **P < 0.01, and ***P < 0.001, Student’s t test.
generated by COX-, LOX-, or CYP450-dependent pathways. Our pharmacological data reveal that AEA-induced pulmonary vasoconstriction is mediated by COX and LOX enzymes. These findings are in accordance with earlier studies, where AA and AA-dependent eicosanoids were shown to modulate pulmonary tone and contribute to HPV (15, 23, 24) and pulmonary hypertension (16, 25). A recent study suggests that pulmonary endothelial cytochrome P450-dependent hydroperoxide A2 generates AA and CYP450-dependent metabolites under hypoxia, resulting in vasoconstriction (26). Our data further extend this concept illustrating that hypoxia can also increase levels of an important precursor of vasoconstrictive eicosanoids and arachidonic acid in PASMCs. The complex pattern of AEA/AA levels over time obtained with our LC-MRM measurements also indicates that the balance of production and degradation to eicosanoids is involved in hypoxic pulmonary hypertension. We also provide evidence that the hypoxia-induced elevation of AEA and AA is restricted to PASMCs and does not occur in pulmonary endothelial cells. This is in line with the prevailing notion that sensor, transducer, and effector mechanisms of HPV reside in the PASMC (10). The endocannabinoid-mediated HPV is restricted to FAAH-dependent pathways because no hypoxia-induced increase of 2-AG, an endocannabinoid mainly metabolized by MAGL, could be found; MAGL was only weakly expressed in the lung, and 2-AG evoked no increase of pulmonary vascular tone. We focused on the role of AEA in HPV because it is the main and best characterized substrate of FAAH, but there may also be other fatty acid amides involved. AEA biosynthesis can be exerted by different enzymatic pathways, the most important being hydrolysis of phospholipid-derived NAPE by NAPE-PLD (18). The elevated NAPE-PLD protein expression in PASMCs under hypoxia can explain enhanced AEA levels, even though a contribution of other recently identified enzymes capable of AEA generation such as α/β-hydrolase 4 (Abh4) and glycophosphodiesterase 1 (GDE1) (27) or protein tyrosine phosphatase, nonreceptor type 22 (PTPn22) (28), cannot be excluded. Moreover, there are recent indications that reactive oxygen species (ROS) are involved in the modulation of pulmonary vascular tone by hypoxia (29). Because AEA has been reported to lead to ROS formation (30), a link between these two pathways also appears possible. Thus, we have identified the AEA/FAAH axis as a previously undescribed signaling pathway playing an important role in HPV and pulmonary hypertension. This could also provide alternative treatment options for clinically highly relevant pulmonary disorders, in particular, in the light of a recently developed FAAH inhibitor with a pharmacological activity restricted to peripheral organs (31).

Materials and Methods
Details for all the methods are found in SI Materials and Methods. Description of cell culture protocols, reverse transcription-PCR, Western blots, immunohistochemistry, and LC-MRM are given in SI Materials and Methods. Also see SI Materials and Methods for IPL and in vivo experiments.

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