Endocannabinoid anandamide mediates hypoxic pulmonary vasoconstriction

Daniela Wenzel[a,1], Michaela Matthey[a,1], Laura Bindila[b], Raissa Lerner[b], Beat Lutz[b], Andreas Zimmer[c], and Bernd K. Fleischmann[a,2]

Institutes of [a]Physiology I and [c]Molecular Psychiatry, Life and Brain Center, University of Bonn, 53127 Bonn, Germany; and [b]Institute of Physiological Chemistry, University Medical Center of the Johannes Gutenberg University Mainz, 55128 Mainz, Germany

Edited by Gregg L. Semenza, The Johns Hopkins University School of Medicine, Baltimore, MD, and approved September 26, 2013 (received for review May 7, 2013)

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 arachidonic acid 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.

pulmonary vascular tone | cannabinoid

Endocannabinoids have been shown to induce vasorelaxation in systemic vessels which is primarily mediated by the specific cannabinoid 1 and 2 (CB1/CB2) and also other G protein-coupled receptors (e.g., non-CB1/CB2 receptors) (1, 2). Based on these results, especially CB1 receptors have been proposed as promising therapeutic targets for the treatment of arterial hypertension (2). Endocannabinoids are also known to potentially act via their intracellular enzymatic metabolization by the fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL) to (vaso)active intermediates (3, 4), but these pathways are considered less important for the regulation of vascular tone in systemic vessels.

Pulmonary arteries are unique because of their prominent vasoconstriction in response to hypoxia. Hypoxic vasoconstriction is responsible for adapting perfusion to ventilation in the lungs and therefore also plays an important role in pathophysiological situations characterized by a high ventilation/perfusion mismatch such as acute lung injury or liver cirrhosis (5, 6). In addition, this mechanism potentially contributes to the onset of pulmonary hypertension in response to hypoxia occurring in high altitude or in various respiratory diseases such as chronic obstructive pulmonary disease or fibrosis (7–9). Pulmonary arterial smooth muscle cells are suggested to play a major role in hypoxic vasoconstriction (10), but the precise mechanisms and the underlying signals are still not well understood. Earlier experimental evidence suggested that the endocannabinoid anandamide (AEA) can either enhance (11) or reduce (12) pulmonary arterial tone, and this prompted us to reexplore the role of endocannabinoids in basic physiological and pathophysiological responses of pulmonary arteries using experimental in vitro, ex vivo, and in vivo approaches.

Results

Anandamide Increases Pulmonary Arterial Tone. The effect of AEA on pulmonary arterial tone was first assessed in large and small pulmonary arteries in mice. AEA had no effect on large pulmonary arteries in isometric force measurements using a myograph (Fig. S1*A*). In contrast, AEA induced a prominent increase of pulmonary arterial tone in the isolated perfused lung (IPL) system (Fig. 1 *A* and *D*). This model provides a reliable readout for pulmonary vascular tone, which is mostly determined by the resistance of small arteries. The effect of AEA was found to be dose-dependent, starting at a nominal AEA concentration of 100 nM (Fig. 1*B* and Fig. S1*B*). Quantitative analysis using liquid chromatography-multiple reaction monitoring (LC-MRM) measurements revealed that only 23.6 ± 4.8% ($n = 6$) of the exogenously applied dose of lipophilic AEA reached the lungs via the tubes of the perfusion system of the IPL; these data indicate that AEA evoked pulmonary vasoconstriction at concentrations that have been measured in the human blood (13, 14). The effect of AEA was specific because no response was observed upon perfusion with the solvent ethanol (Fig. 1 *C* and *D*) or the endocannabinoid 2-arachidonylglycerol (2-AG, 10 μM; Fig. S2*A*). We compared the vasoconstrictive effect of AEA with that of serotonin (5-HT), one of the strongest vasoconstrictors of pulmonary arteries, and found that the AEA-induced increase of vascular tone at equivalent concentrations was ∼50% higher (Fig. 1*D*). The strong effect of AEA on pulmonary vascular tone could imply its potential involvement in pathophysiological processes. Because in humans

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.

Author contributions: D.W. and B.K.F. designed research; D.W., M.M., L.B., and R.L. performed research; D.W., M.M., L.B., R.L., B.L., and B.K.F. analyzed data; and D.W., A.Z., and B.K.F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]D.W. and M.M. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: bernd.fleischmann@uni-bonn.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1308130110/-/DCSupplemental.

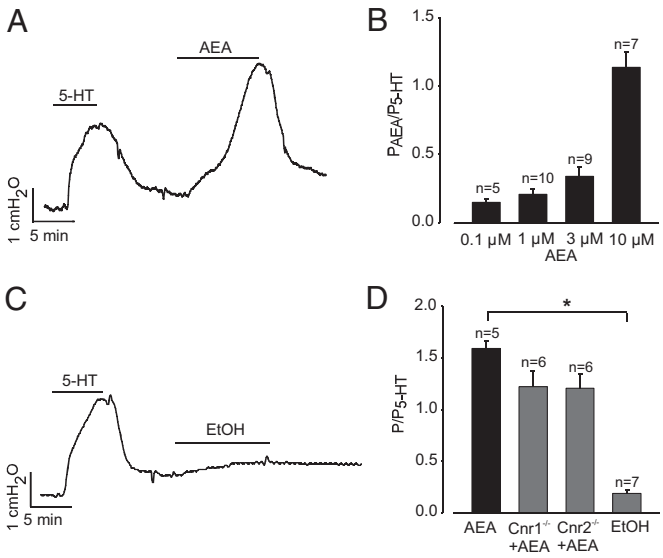

Fig. 1. The endocannabinoid AEA increases pulmonary arterial tone in the IPL model of mice. (*A*) Original recording illustrates an elevation of pulmonary arterial pressure (PAP) in the IPL upon application of the potent pulmonary vasoconstrictor serotonin (5-HT, 10 μM) or AEA (10 μM). (*B*) The AEA-induced PAP increase is dose-dependent; the contractile response to AEA is normalized to PAP values in presence of 5-HT (10 μM). (*C*) Original recording of PAP in the IPL reveals almost no effect upon application of the solvent ethanol (EtOH). Control: 5-HT (10 μM). (*D*) Statistical analysis of PAPs normalized to the 5-HT response indicates that AEA-induced vasoconstriction is preserved in *Cnr1*$^{-/-}$ and *Cnr2*$^{-/-}$ mice, whereas the solvent EtOH has no effect. *$P < 0.05$, one-way ANOVA with Dunnett's test.

a higher incidence of pulmonary hypertension is reported for females, 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.

AEA-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. S2*B*). In IPL measurements the vasonconstrictive effect of AEA was independent of CB1 and CB2 receptors because it was preserved in *Cnr1*$^{-/-}$ and *Cnr2*$^{-/-}$ mice (Fig. 1*D*). This was also corroborated by experiments with the CB1/CB2 receptor agonist HU-210, which did not alter pulmonary arterial tone (Fig. S2*C*). These data suggested that AEA metabolites may be involved in AEA signaling in pulmonary vessels. To examine their contribution, we took advantage of *FAAH*$^{-/-}$ mice and tested the effect of AEA on pulmonary arterial tone in the IPL. AEA had only a very small effect on pulmonary vascular tone in *FAAH*$^{-/-}$ mice (Fig. 2 *A* and *B*). These results were confirmed using the FAAH inhibitor URB597 (1 μM), which strongly reduced the vasconstrictive effect of AEA on pulmonary vessels (Fig. 2*B*) in wild-type mice. URB597 had no unspecific effects on pulmonary vasoreactivity because it did not alter the 5-HT–induced increase of vascular tone (Fig. S2*D*). As further proof of the FAAH dependence of AEA-mediated vasoconstriction, we found that the nonhydrolyzable AEA analog Meth-AEA (10 μM) did not alter pulmonary vascular tone (Fig. 2*B*). 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. 2*B*). 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. S2*E*). A contribution of further downstream metabolites like eicosanoids 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. 2*C*). To examine their involvement in AEA-dependent pulmonary vasoconstriction, we applied different pharmacological agents. Although inhibition of CYP450 enzymes by 17-octadecynoic acid (ODYA) (1 μM) had no effect (Fig. S2*F* and Fig. 2*D*), 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. 2*D*). The leukotriene receptor antagonist montelukast (1 μM), an approved antiasthma drug, also attenuated pulmonary arterial vasoconstriction by AEA (Fig. 2*D* and Fig. S2*G*); montelukast did not change 5-HT–induced vasoconstriction, underscoring the specificity

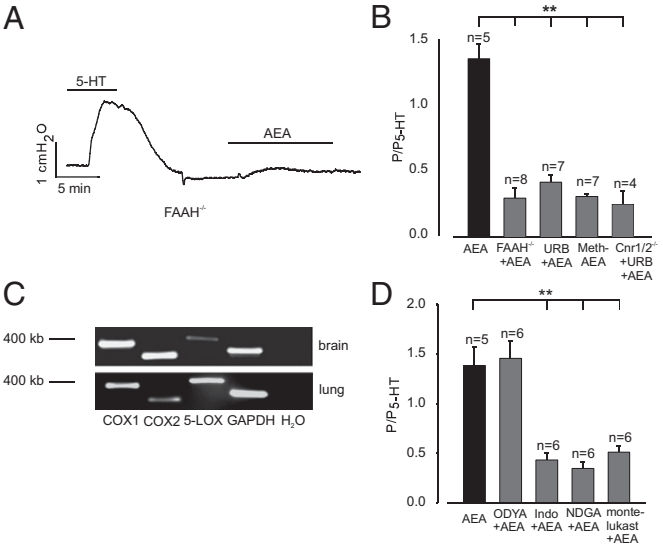

Fig. 2. AEA elevates PAP via the FAAH signaling pathway. (*A*) Original recording of PAP in the IPL illustrates only a minimal vasoconstrictive response by AEA (10 μM) in *FAAH*$^{-/-}$ mice. Control: 5-HT (10 μM). (*B*) Statistical analysis of PAPs normalized to the 5-HT response shows that vasoconstriction by AEA (10 μM) is mediated by FAAH-dependent AEA metabolites because it is strongly reduced in *FAAH*$^{-/-}$ mice and upon pharmacological inhibition of FAAH by URB597 (URB, 1 μM) in wild-type and Cnr1/2$^{-/-}$ mice. In addition, the nonhydrolyzable AEA analog Meth-anandamide (Meth-AEA, 10 μM) has no effect. **$P < 0.01$, one-way ANOVA with Dunnett's test. (*C*) PCR analysis reveals expression of cyclooxygenase1 (COX1), cyclooxygenase2 (COX2), and 5-lipoxygenase (5-LOX) in murine lung; as positive control, mouse brain was used. (*D*) Statistical analysis of PAPs normalized to the 5-HT response indicates that vasoconstriction by AEA (10 μM) is mediated by COX- and 5-LOX–dependent metabolites because it is diminished by the respective inhibitors Indo (10 μM) and NDGA (10 μM) or blockade of leukotriene receptors with montelukast (1 μM). The CYP450 inhibitor ODYA (1 μM) has no effect. **$P <$ 0.01, one-way ANOVA with Dunnett's test.

PHYSIOLOGY

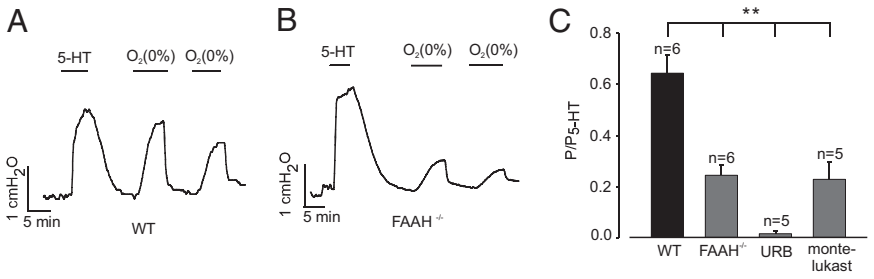

Fig. 3. The AEA/FAAH axis is an important mediator of hypoxic pulmonary vasoconstriction (HPV). (*A* and *B*) Original recordings of PAP in the IPL demonstrate strongly reduced HPV (0% O_2) in *FAAH*$^{-/-}$ (*B*) compared with WT mice (*A*); as control, 5-HT (10 μM) was used. (*C*) Statistical analysis 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.

of this compound (Fig. S2*D*). These data suggest that AEA is a strong pulmonary vasoconstrictor, 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% O_2 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 *FAAH*$^{-/-}$ (0.86 ± 0.5 cm H_2O, $n = 8$) and wild-type (0.45 ± 0.8 cm H_2O, $n = 5$, $P > 0.05$) animals. We also examined HPV in wild-type mice in the presence of the pharmacological FAAH inhibitor URB597 (1 μM and 10 μM). When URB597 was applied in increasing concentrations during the two hypoxic episodes (1 μM and 10 μM), it led to a strong reduction of the first and second hypoxic vasoconstrictive responses, respectively (Fig. S4*A*); when 10 μM of URB597 was used throughout the experiment, the hypoxia-induced vasoconstriction in lungs of wild-type mice was almost abolished (Fig. S4*B* and Fig. 3*C*). These results could not be explained by an accumulation of AEA and its degradation via other pathways besides FAAH (e.g., prostamide pathway), because the leukotriene receptor blocker montelukast (1 μM) also prevented HPV when applied before (Fig. 3*C*) or during (Fig. S4*C*) the 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% O_2) and killed at different time points, and the lung tissue was analyzed. We found that AEA

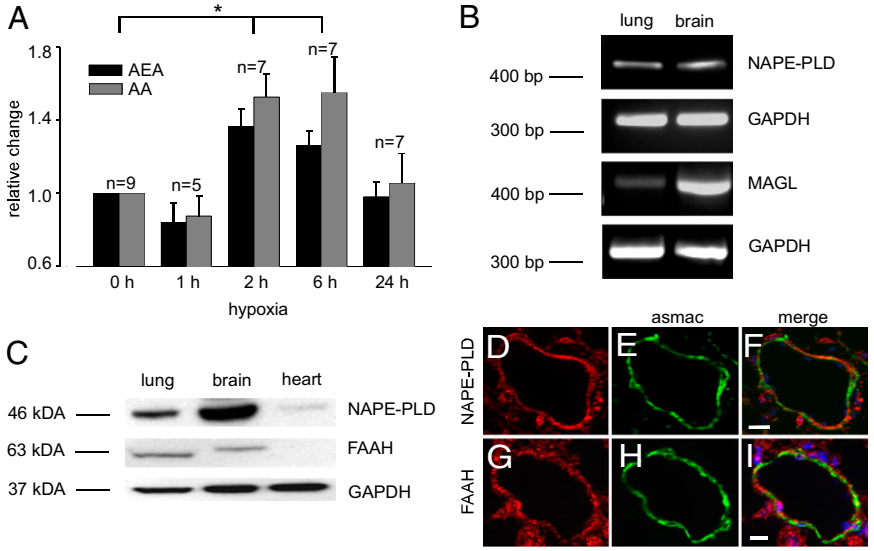

Fig. 4. Hypoxia increases AEA and AA levels in the lung. (*A*) LC-MRM measurements of AEA and AA levels in murine lungs yielded an increase after 2 and 6 h of hypoxic ventilation (10% O_2). The values are normalized to levels at normoxic ventilation (21% O_2). $^{*}P < 0.05$, one-way ANOVA with Dunnett's 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 *I*), 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. 4*A*) 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% O_2. 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 ($n \geq 5$, $^*P > 0.05$ 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*-acyl-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. S2*B* and Fig. 4*B*). In contrast, MAGL, an enzyme that mainly metabolizes 2-AG, was only weakly expressed in the lung (Fig. 4*B*). Pulmonary expression of NAPE-PLD and FAAH was also confirmed at the protein level by Western blot analysis (Fig. 4*C*). Both enzymes showed only low expression levels in the heart (Fig. 4*C*). Similarly, this was also observed by immunostainings of cardiac vessels (Fig. S5 *A–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. 4 *D–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. S5 *M–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. 5 *A* 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. 5*C*). 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% O_2) or normoxia (controls) because this time point had yielded maximal levels of AEA and AA upon induction of hypoxia in whole lungs (see also Fig. 4*A*). Our data in hPASMCs showed a significant elevation of AEA and AA under hypoxia (Fig. 5*D*), which was accompanied by an increase of NAPE-PLD protein expression by 34.7 ± 6.7%, $n = 4$, $P < 0.05$ (Fig. 5*E*). 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% O_2) or hypoxic (10% O_2) 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. 6 *A* and *D*). However, such changes were not observed in $FAAH^{-/-}$ mice exposed to hypoxia (Fig. 6 *B* 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. 6*E*). These findings were also supported by catheter-based right ventricular systolic pressure (RVSP) measurements yielding strongly increased

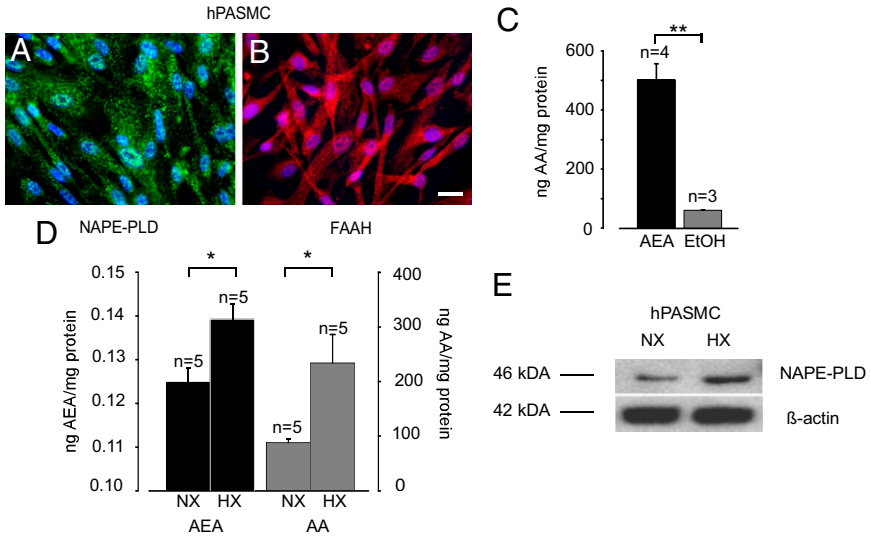

Fig. 5. Vascular smooth muscle cells display elevated AEA production under hypoxia. (*A* and *B*) Immunohistochemistry shows NAPE-PLD (green) and FAAH (red) expression in hPASMCs; nuclei are stained with hoechst (blue). (Scale bar, 20 μm.) (*C*) LC-MRM measurements reveal strongly elevated AA levels in hPASMCs after 1 h of AEA incubation (10 μM) compared with EtOH. $^*P < 0.05$, Student's *t* test. (*D*) AEA and AA levels in hPASMCs are strongly increased after 5 h of hypoxia (HX) compared with normoxia (NX). $^*P < 0.05$, Student's *t* test. (*E*) Western blot analysis shows enhanced NAPE-PLD protein expression after 5 h of HX compared with NX.

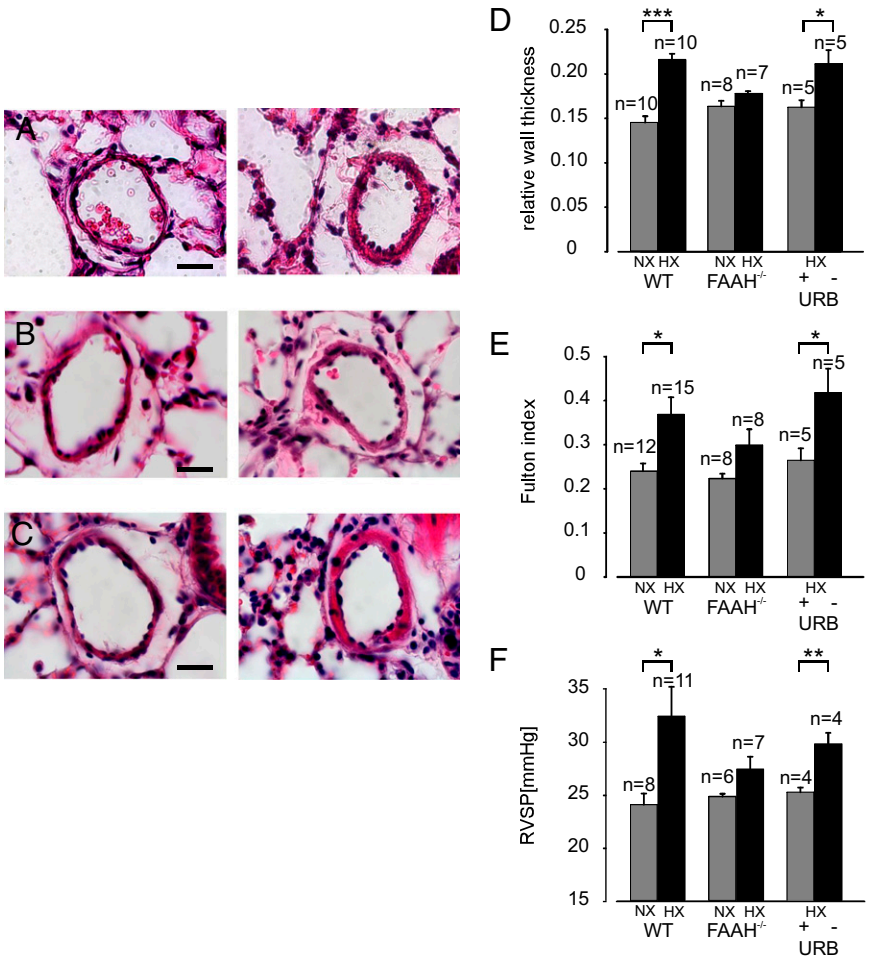

Fig. 6. The AEA/FAAH axis is involved in the generation of hypoxia-induced pulmonary hypertension. (*A*) H&E stainings of intrapulmonary arteries of WT mice demonstrate increased vascular wall thickness after 3 wk of HX (10% O_2) (*Right*) compared with NX (21% O_2) (*Left*). (Scale bar, 20 μm.) (*B*) H&E stainings of intrapulmonary arteries of *FAAH*$^{-/-}$ mice display no change in vascular wall thickness after 3 wk of HX (10% O_2) (*Right*) compared with NX (21% O_2) (*Left*). (Scale bar, 20 μm.) (*C*) H&E stainings of intrapulmonary arteries of WT mice after 3 wk of HX (10% O_2) 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.

pressure in wild-type mice after 3 wk of hypoxia (Fig. S6*A* and Fig. 6*F*), whereas in *FAAH*$^{-/-}$ mice, no obvious changes could be detected (Fig. S6*B* and Fig. 6*F*).

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. S6*C*).

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. 6 *C* and *D*), Fulton index (Fig. 6*E*), and RVSP (Fig. S6*D* and Fig. 6*F*), whereas treatment of mice for only 3 d did not have protective effects (Fig. S6*E*). 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

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 cytosolic phospholipase 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 glycerophosphodiesterse 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.

ACKNOWLEDGMENTS. We thank C. Schwitter for technical assistance with sample collection and preparation and Dr. V. Gieselmann for help with LC-MRM measurements, A. Zimmer and Dr. E. Schlicker for providing knockout mice, Dr. B. Cravatt for providing an FAAH antibody, and T. Baum for excellent technical assistance. We also thank Drs. I. Hall and M. Kotlikoff for helpful comments on the manuscript. The work was supported by the Deutsche Forschungsgemeinschaft Research Unit 926 (Projects SP3, SP8, CP1, and CP2) and by a junior research group of the state of North Rhine Westphalia (D.W.).

1. Járai Z, et al. (1999) Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. *Proc Natl Acad Sci USA* 96(24): 14136–14141.
2. Bátkai S, et al. (2004) Endocannabinoids acting at cannabinoid-1 receptors regulate cardiovascular function in hypertension. *Circulation* 110(14):1996–2002.
3. Herradón E, Martín MI, López-Miranda V (2007) Characterization of the vasorelaxant mechanisms of the endocannabinoid anandamide in rat aorta. *Br J Pharmacol* 152(5): 699–708.
4. Gauthier KM, et al. (2005) Endothelium-derived 2-arachidonylglycerol: An intermediate in vasodilatory eicosanoid release in bovine coronary arteries. *Am J Physiol Heart Circ Physiol* 288(3):H1344–H1351.
5. Neumann P, Hedenstierna G (2001) Ventilation-perfusion distributions in different porcine lung injury models. *Acta Anaesthesiol Scand* 45(1):78–86.
6. Hedenstierna G, Söderman C, Eriksson LS, Wahren J (1991) Ventilation-perfusion inequality in patients with non-alcoholic liver cirrhosis. *Eur Respir J* 4(6):711–717.
7. Maggiorini M, et al. (2001) High-altitude pulmonary edema is initially caused by an increase in capillary pressure. *Circulation* 103(16):2078–2083.
8. Ghofrani HA, et al. (2002) Sildenafil for treatment of lung fibrosis and pulmonary hypertension: A randomised controlled trial. *Lancet* 360(9337):895–900.
9. Wagner PD, Dantzker DR, Dueck R, Clausen JL, West JB (1977) Ventilation-perfusion inequality in chronic obstructive pulmonary disease. *J Clin Invest* 59(2):203–216.
10. Murray TR, Chen L, Marshall BE, Macarak EJ (1990) Hypoxic contraction of cultured pulmonary vascular smooth muscle cells. *Am J Respir Cell Mol Biol* 3(5):457–465.
11. Wahn H, Wolf J, Kram F, Frantz S, Wagner JA (2005) The endocannabinoid arachidonyl ethanolamide (anandamide) increases pulmonary arterial pressure via cyclooxygenase-2 products in isolated rabbit lungs. *Am J Physiol Heart Circ Physiol* 289(6): H2491–H2496.
12. Kozłowska H, et al. (2007) Identification of the vasodilatory endothelial cannabinoid receptor in the human pulmonary artery. *J Hypertens* 25(11):2240–2248.
13. Monteleone P, et al. (2005) Blood levels of the endocannabinoid anandamide are increased in anorexia nervosa and in binge-eating disorder, but not in bulimia nervosa. *Neuropsychopharmacology* 30(6):1216–1221.
14. Joosten MM, Balvers MG, Verhoeckx KC, Hendriks HF, Witkamp RF (2010) Plasma anandamide and other N-acylethanolamines are correlated with their corresponding free fatty acid levels under both fasting and non-fasting conditions in women. *Nutr Metab (Lond)* 7:49.
15. Voelkel NF, Morganroth M, Feddersen OC (1985) Potential role of arachidonic acid metabolites in hypoxic pulmonary vasoconstriction. *Chest* 88(4, Suppl):245S–248S.
16. Kiss L, et al. (2008) Direct and simultaneous profiling of epoxyeicosatrienoic acid enantiomers by capillary tandem column chiral-phase liquid chromatography with dual online photodiode array and tandem mass spectrometric detection. *Anal Bioanal Chem* 392(4):717–726.
17. Dorrington KL, et al. (1997) Time course of the human pulmonary vascular response to 8 hours of isocapnic hypoxia. *Am J Physiol* 273(3 Pt 2):H1126–H1134.
18. Okamoto Y, et al. (2005) Mammalian cells stably overexpressing N-acylphosphatidylethanolamine-hydrolysing phospholipase D exhibit significantly decreased levels of N-acylphosphatidylethanolamines. *Biochem J* 389(Pt 1):241–247.
19. Pacher P, et al. (2005) Hemodynamic profile, responsiveness to anandamide, and baroreflex sensitivity of mice lacking fatty acid amide hydrolase. *Am J Physiol Heart Circ Physiol* 289(2):H533–H541.
20. Mach F, Steffens S (2008) The role of the endocannabinoid system in atherosclerosis. *J Neuroendocrinol* 20(Suppl 1):53–57.
21. Zygmunt PM, et al. (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* 400(6743):452–457.
22. Grainger J, Boachie-Ansah G (2001) Anandamide-induced relaxation of sheep coronary arteries: The role of the vascular endothelium, arachidonic acid metabolites and potassium channels. *Br J Pharmacol* 134(5):1003–1012.
23. Raj JU, Chen P (1987) Role of eicosanoids in hypoxic vasoconstriction in isolated lamb lungs. *Am J Physiol* 253(3 Pt 2):H626–H633.
24. Pokreisz P, et al. (2006) Cytochrome P450 epoxygenase gene function in hypoxic pulmonary vasoconstriction and pulmonary vascular remodeling. *Hypertension* 47(4): 762–770.
25. Stenmark KR, et al. (1983) Leukotriene C4 and D4 in neonates with hypoxemia and pulmonary hypertension. *N Engl J Med* 309(2):77–80.
26. Wang L, et al. (2012) Hypoxic pulmonary vasoconstriction requires connexin 40-mediated endothelial signal conduction. *J Clin Invest* 122(11):4218–4230.
27. Simon GM, Cravatt BF (2008) Anandamide biosynthesis catalyzed by the phosphodiesterase GDE1 and detection of glycerophospho-N-acyl ethanolamine precursors in mouse brain. *J Biol Chem* 283(14):9341–9349.
28. Liu J, et al. (2006) A biosynthetic pathway for anandamide. *Proc Natl Acad Sci USA* 103(36):13345–13350.
29. Waypa GB, et al. (2013) Superoxide generated at mitochondrial complex III triggers acute responses to hypoxia in the pulmonary circulation. *Am J Respir Crit Care Med* 187(4):424–432.
30. Siegmund SV, Uchinami H, Osawa Y, Brenner DA, Schwabe RF (2005) Anandamide induces necrosis in primary hepatic stellate cells. *Hepatology* 41(5):1085–1095.
31. Clapper JR, et al. (2010) Anandamide suppresses pain initiation through a peripheral endocannabinoid mechanism. *Nat Neurosci* 13(10):1265–1270.