The endocannabinoid arachidonyl ethanolamide (anandamide) increases pulmonary arterial pressure via cyclooxygenase-2 products in isolated rabbit lungs

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In the lung, anandamide participates in the intrinsic control of airway responsiveness, exerting dual effects (inhibition and initiation of bronchospasm) depending on the state of contraction of the bronchial muscle. Anandamide could be shown to be synthesized in rat lung tissue on calcium ion stimulation (5). 2-AG has been detected in the rat lung as well (21) with no overt physiological significance. In guinea pigs, high doses of intravenous anandamide did not elicit bronchodilation but reduced airway epithelial injury and pulmonary leukocytosis (34). Thus cannabinoid actions on bronchial smooth muscle tone might depend on the route of application and the species used.

In several isolated organ models, cannabinoids act as strong vasodilators that relax arterial tone via activated CB₁ receptors (12), specific anandamide receptors (38, 18), VR₁ vanilloid receptors (45), or arachidonic acid products (14). In anesthetized rats, cannabinoids are potent coronary, cerebral, and renal vasodilator agents in vivo (39).

So far, the influence of endocannabinoids on the pulmonary circulation remains speculative. Substances with systemic vasodilator effects might act as pulmonary constrictors, e.g., bradykinin, and systemic vasoconstrictors may be pulmonary vasodilators, e.g., oxygen. Interestingly, in one early study the intravenous application of Δ⁹-tetrahydrocannabinol in anesthetized dogs resulted in a significant increase in total pulmonary vascular resistance sensitive to bilateral vagotomy (17). Long before cannabinoid receptors were cloned, this in vivo study was hampered by the unavailability of pharmacological tools targeting receptor-associated mechanisms.

On the other hand, treatment of patients with pulmonary hypertension is not an easy task. The use of calcium channel antagonists, prostacyclin analogs, or endothelin antagonists may lead to unsatisfactory results in some patients (11). Therefore, in an attempt to look for a possible new class of vasodilators in the lung circulation, we tried to characterize the biological effects of synthetic and endogenous cannabinoids using an isolated, ventilated, buffer-perfused rabbit lung model. Surprisingly, we found vasoconstriction rather than vasodilation after application of anandamide, which is degraded by the fatty acid amidohydrolase (FAAH) and further metabolized via cyclooxygenase-2 (COX-2).

METHODS

Isolated rabbit lung model. The preparation of isolated rabbit lungs was performed as described previously (40). All animal procedures
were in accordance with the guidelines of the independent and institutional Animal Care and Use Committee. In brief, male rabbits between 2.2 and 3.5 kg body weight were used. The animals were deeply anesthetized with 25 mg of ketamine and 80 mg of xylazine hydrochloride and then heparinized (1,000 U/kg). The rabbits were ventilated, after tracheotomy, with 4% CO2 (pH was held constant between 7.35 and 7.45), 17% O2, and 79% N2 (tidal volume, 9 ml/kg; frequency, 30 min⁻¹). After thoracotomy, a wide-bore cannula was inserted into the pulmonary artery. Ice-cold Krebs-Henseleit buffer was used for perfusion with an initial flow of 10–20 ml/min.

The lungs were placed in a 4°C equilibrated chamber, freely suspended from a force transducer. A second cannula was placed in the left ventricle. The left atrial appendage was ligated. The temperature of the perfusion fluid was increased to 37°C within 20 min. The definite flow rate was set at 100 ml/min with recirculation of the buffer medium (total circulating volume, 300 ml). Formaldehyde-sterilized perfusion circuit tubing and endotoxin-free buffer fluids were used. Pulmonary arterial pressure (PAP) and the weight gain of the isolated lungs were continuously registered. After a steady-state period of 45 min, in each isolated lung only one agonist and eventually one antagonist was used.

Detection of FAAH and COX-2. Expression of FAAH and COX-2 were assessed by RT-PCR using total RNA. cDNA was generated using Superscript II (Life Technologies). As a negative control, no reverse transcriptase was added. PCR primers were 5′-AGG TCA TCC ACC ACT TC and 5′-GTG AGT TTC CCG TTC AGC TC for rabbit GAPDH (202 bp); 5′-GTG GTG CTG ACC CCC ATG CTG G and 5′-TCC ACC TCC CGC ATG AAC CGC AG (24) for rabbit FAAH (301 bp); and 5′-TGT GCT CAA ACA GGA GCA TC and 5′-AAA AGC AGC TCT GGG TCA AA for rabbit COX-2 (159 bp). All PCR products were size fractionated by 2% agarose gel electrophoresis, and DNA bands were visualized by staining the gel with ethidium bromide.

Detection of endocannabinoids. For measuring endocannabinoid levels, native lung tissue was extracted with chloroform-methanol (2:1) containing 7 nmol of [3H]anandamide as internal standard. The upper layer was extracted two more times with ice-cold chloroform and deproteinated with acetone. The extract was dried under nitrogen and reconstituted in methanol for analysis by liquid chromatography/in-line mass spectrometry (LC/MS) with the use of an Agilent 1100 series LC-MSD equipped with a thermostated autosampler and column compartment. Anandamide and 2-AG in cellular extracts were quantified by LC/MS, as described previously (41).

Materials. Methyl arachidonyl fluorophosphonate (MAFP), HU-210 (−)-11-OH-Δ⁹-tetrahydrocannabinol dimethylheptyl, capsazepine, N-methylarachidonamide, noladin ether, AM-251 [N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide], and SC-19220 [8-chloro-dibenzo[b,f][1,4]oxazepine-10(11H)-carboxylic acid] were obtained from Tocris/Biotrend (Cologne, Germany) and dissolved in dimethyl sulfoxide or ethanol. Anandamide (arachidonyl ethanolamide) and Δ⁹-tetrahydrocannabinol were from Sigma (Deisenhofen, Germany). Nimesulide [N-(4-nitro-2-phenoxypyphenyl)methanesulfonamide], 2-AG, and SQ 29,548 [1S-[1a,2a(Z,3a,4a)-7-[3-[2-[(phenylamino)carbonyl]hydroxy]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptanoic acid] were from Alexis Biochemicals (Lausen, Switzerland).

Statistics. Values are expressed as means ± SE. Multiple comparisons to find differences between the various treatment groups were used (one-way ANOVA) and corrected using the Bonferroni post hoc test, with P values <0.05 considered statistically significant.

RESULTS

Effects of endogenous cannabinoids on PAP. The average baseline PAP in isolated lungs before drug application was 5.0 ± 0.2 mmHg (n = 88). Vehicle alone, dimethyl sulfoxide, or ethanol was without effect. Anandamide dose-dependently increased PAP (Fig. 1). 2-AG showed more pronounced effects at lower concentrations. Baseline PAP increased from 5.6 ± 0.6 mmHg in experiments with 0.2 μM 2-AG to 25.2 ± 8.3 mmHg (P < 0.05 vs. baseline, n = 5) and from 5.0 ± 0.4 to 44.5 ± 10.8 mmHg in experiments with 0.4 μM 2-AG (P < 0.01 vs. baseline, n = 4; P < 0.05 vs. 0.2 μM 2-AG). PAP increased within 1 min and reached maximum values at 8.8 ± 0.7 min with 5 μM anandamide (see original tracing in Fig. 1) and at 3.8 ± 0.4 min with 0.4 μM 2-AG. The effect lasted between 15 and 30 min, and PAP returned to baseline values in experiments with 0.5 and 1 μM anandamide and 0.2 and 0.4 μM 2-AG. Only the higher dose of anandamide showed a prolonged effect with a slightly increased PAP 45 min after anandamide application (8.6 ± 2.0 vs. 5.3 ± 0.9 mmHg before anandamide, P > 0.05).

Effects of endogenous cannabinoids on lung weight (edema). The average pure lung wet weight was 6.23 ± 0.11 g. In control rabbits, the increase of lung weight during the exper-
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Mechanism of anandamide-induced PAP increase. Because in the systemic arterial circulation cannabinoids cause vasodilation and hypotension mainly via CB1 cannabinoid receptors, we first antagonized with the specific and potent CB1 antagonist AM-251 (0.1 μM for specific CB1 antagonism and 5 μM for CB1 and CB2 receptor antagonism). That did not block the pressure increase after anandamide application (Fig. 2). Contrary, the 0.1 μM concentration of AM-251 further enhanced the effects of anandamide on PAP. Furthermore, the VR1 vanilloid receptor antagonist capsazepine (10 μM) could not reduce the pressure response after anandamide application (Fig. 2). AM-251 and capsazepine alone were without effect on PAP.

Anandamide is an arachidonic acid derivative and a substrate of FAAH. We tested whether structurally different synthetic or plant-derived cannabinoids that are not metabolized via the FAAH are effective. The polycyclic synthetic main metabolically stable endocannabinoid derivatives R-methanandamide (5 μM) and noladin ether (0.4 μM) as the 2-AG derivative noladin ether (0.4 μM, n = 4; P > 0.05 vs. baseline) did not show significant effects on pulmonary arterial pressure. For comparison, the maximum pressure effects of anandamide (5 μM, n = 6) and 2-AG (0.4 μM, n = 4) are included. The plant-derived Δ⁸-tetrahydrocannabinol (Δ⁸-THC, 5 μM, n = 4, P > 0.05) and the synthetic HU-210 (5 μM, n = 4, P > 0.05), which is structurally related to THC, show no significant effects. **P < 0.01 vs. baseline.

FAAH expression and effects of FAAH inhibition in rabbit lungs. We tried to detect FAAH, the main enzyme responsible for anandamide degradation, in rabbit lung tissue. FAAH RNA could readily be identified in lung (Fig. 4A) and kidneys (not shown). In our lung model, the FAAH inhibitor MAFP (100 nM) was without effect alone but eliminated any pressure effects after anandamide application (Fig. 4B).

Involvement of COX. Because arachidonic acid breakdown products of anandamide are potential substrates for COX, we blocked the latter with the unspecific COX inhibitor aspirin (100 μM) or the specific COX-2 inhibitor nimesulide (10 μM). Neither aspirin nor nimesulide alone showed effects when administered to the lungs. However, both treatments completely blocked the vasoconstrictor effect of anandamide (Fig. 5A). To evaluate our hypothesis that endocannabinoids elicit pulmonary vasoconstriction following enzymatic degradation through COX-2, we aimed to detect COX-2 messenger RNA in rabbit lungs. COX-2 RNA was found in either rat kidney, used as a positive reference (22), or rabbit lung (Fig. 5B). Pretreatment of isolated lungs with 0.5 μM SQ 29,548, a synthetic thromboxane receptor antagonist, failed to reduce the effects of anandamide. However, the effects of anandamide were significantly diminished after application of the specific prostanoid EP1 receptor antagonist SC-19220 (100 μM; Fig. 5A). SQ 29,548 and SC-19220 alone were without effect on PAP.

Endocannabinoids in native rabbit lung. Because application of endocannabinoids showed striking effects, we aimed to detect endogenous cannabinoids in untreated lungs. Either...
anandamide (99 ± 55 pmol/g wet tissue) or 2-AG (19.6 ± 8.4 nmol/g wet tissue) could be detected by LC/MS in each of five native rabbit lungs.

**DISCUSSION**

Our study demonstrates a marked increase of PAP after the application of endogenous cannabinoids in the isolated, ventilated, and buffer-perfused rabbit lung. Several lines of evidence led us to conclude that breakdown products of anandamide are further metabolized to COX-2 metabolites with vasoconstrictor properties in the pulmonary artery. EP1 prostanoid receptors are involved in the vasoconstriction.

First, either anandamide or 2-AG elicits pulmonary pressure increase. Both endocannabinoids are arachidonic acid metabolites that differ in their structure and their enzymatic degradation from plant-derived, such as Δ9-tetrahydrocannabinol, and synthetic, such as HU-210, cannabinoids. The latter two cannabinoids were ineffective in our model (Fig. 3), which is the first evidence against a specific cannabinoid receptor-mediated mechanism. Second, the metabolically stable endocannabinoid derivatives R-methanandamide and noladin ether were ineffective as well (Fig. 3). Third, the selective CB1 cannabinoid receptor antagonist AM-251 could not block the pulmonary vasoconstriction after anandamide application (Fig. 2). Contrarily, 0.1 μM AM-251 enhanced anandamide effects, which leaves open the possibility that the net effect of anandamide is pulmonary vasoconstriction via COX-2 metabolites, but activation of CB1 receptors located on rabbit pulmonary arteries might mediate vasodilation. Interestingly, AM-251 alone had no effects on PAP, and the 5 μM concentration of AM-251 did not enhance anandamide effects, which does not suggest a clear-cut mechanism. Comparing these findings with the well-known CB1 cannabinoid receptor-mediated vasodilatory effects of anandamide in several systemic arteries (12, 14, 18, 38, 39), we found a non-cannabinoid receptor-mediated vasoconstrictor effect in the pulmonary artery. Only a few reports of cannabinoid-induced vasoconstriction have been published. A recent report (33) shows that 2-AG elicits contraction of rat aortic rings independently of CB1 or CB2 cannabinoid receptor activation after conversion of 2-AG to thromboxane A2.
Anandamide loses its cannabimimetic activity when it is hydrolyzed to arachidonic acid and ethanolamine by the catalysis of an enzyme referred to as anandamide amidohydrolase or FAAH (for review, see Ref. 36). A nonspecific inhibitor is the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF). More specific is the most potent, irreversible FAAH inhibitor MAFP (9), which completely blocked any effects of anandamide in our model (Fig. 4B). The FAAH acts as an amidase for anandamide but also as an esterase for 2-AG (13). MAFP may also modulate 2-AG effects, but probably the main enzyme responsible for 2-AG degradation is a monoacylglycerol lipase blocked by a specific inhibitor (15). To ease interpretation of experiments, we focused on anandamide and not on 2-AG in the experiments designed to unmask the mechanism of action. In our study we were able to detect the RNA of the FAAH in native rabbit lungs (Fig. 4A). The distribution of FAAH was extensively investigated in the rat, with the highest RNA levels in the liver and intestine and only small amounts in the lung (7, 20). In mice, only negligible activity was found in the lung (42). To our knowledge, our results document for the first time physiologically relevant amounts of FAAH in rabbit lungs.

After enzymatic degradation of anandamide, free arachidonic acid may be further metabolized via lipoxygenases into leukotrienes or hydroxyeicosatetraenoic acids. On the other hand, anandamide-derived arachidonic acid may be substrate for cyclooxygenases, yielding prostaglandins, thromboxane, or prostacyclin (for review, see Ref. 4). Not only the unspecific inhibition of cyclooxygenases by aspirin but also the selective inhibition of COX-2 by nimesulide completely prevented the pulmonary pressure effect of anandamide (Fig. 5A). COX-1 is constitutively expressed in most tissues. In contrast, basal COX-2 expression is not observed in most tissues except those of the kidney and certain brain areas (32). COX-2 RNA has been found in rabbit pulmonary artery tissue (2), and we could now detect COX-2 RNA in rabbit lungs, yielding even stronger bands as for the reference organ kidney (Fig. 5B).

One important COX product is prostaglandin E2, and interaction with the EP1 receptor mediates algesia but also increases systemic blood pressure (35). In isolated rings of human pulmonary arteries, EP3 receptor activation induces contraction (30). Furthermore, thromboxane receptors may mediate pulmonary vasoconstriction as well (19). Conversely, prostacyclin and iLOprost are being used to treat severe pulmonary hypertension, and EP2 and EP4 receptors appear to be dominant mediators of vasodilation (44). We tried to antagonist receptors that are known to interact with COX metabolites, resulting in vasoconstriction. The synthetic thromboxane receptor antagonist SQ 29,548 failed to block the pressure increase after anandamide application. In contrast, the specific prostaglandin EP1 receptor antagonist SC-19220 significantly reduced the pressure effects after anandamide application (Fig. 5A). Prostaglandin E2 interacts with all four EP receptors (EP1, EP2, EP3, EP4). The EP1 receptor signals through increased calcium and mediates constriction in vas deferens and ileal smooth muscle (3, 6). In our rabbit lung model, EP1 antagonism significantly reduced the constrictive effects after anandamide application. However, mediators other than prostaglandin E2 and receptors other than EP1 may be involved in addition.

Endocannabinoid inactivation through oxygenation by COX-2 may represent a biologically meaningful and isoform-selective function for this enzyme (for review, see Ref. 22). Anandamide can be metabolized by COX-2 to produce prostaglandin E2 ethanolamide. This substance is able to contract guinea pig trachea (31). Prostaglandin E2 ethanolamide may partially be responsible for the anandamide-induced vasoconstriction in our model, which should be examined in future studies. In line with our results is a recent publication (1) reporting that in strips of lung parenchyma from guinea pigs, anandamide, but not HU-210, at high concentrations produced contractions that were unaffected by CB1 receptor antagonism but strongly inhibited by PMSF or indomethacin.

The biological meaning of endocannabinoids in the lung is unknown. Anandamide and 2-AG may participate in the intrinsic control of airway responsiveness (5) and further in the vascular tone of pulmonary arteries. Substantial amounts of 2-AG have been found in rat lung (21), but the only study investigating cannabinoids in rat lung tissue failed to identify anandamide by gas chromatography/mass spectrometry analysis (43). Using the same method, we detected both endocannabinoids in native rabbit lungs. 2-AG was abundant in a concentration 200 times higher than that of anandamide, which is in accordance with endocannabinoid contents in other tissues. Endocannabinoid tissue levels are best studied in rodent brain. In mouse forebrain, where endocannabinoids act as neurotransmitters, the specific 2-AG content is ~9 nmol/g tissue (29, 41) and the anandamide tissue concentration is 3.2 (41), or in another recent study, 52 pmol/g (29). The endocannabinoid content in native rabbit lungs in our study (99 ± 55 pmol/g for anandamide and 19.6 ± 8.4 nmol/g for 2-AG) is at least twofold higher and suggests biological relevance.

We aimed to look for a possible new class of vasodilators in pulmonary arteries. Surprisingly, we found vasoconstriction after the application of endocannabinoids that are degraded by FAAH. These findings may contribute to the understanding of the development of pulmonary hypertension under certain physiopathological circumstances. Bacterial lipopolysaccharide (LPS) may induce sepsis and acute inflammatory lung injury, and LPS activates endocannabinoid production in rat macrophages during septic shock (37). LPS induces the expression of prostaglandin G/H synthase in rabbit lung with increased production of thromboxane, resulting in pulmonary hypertension (8). Furthermore, COX-2-mediated oxygenation of endocannabinoids provides novel lipids that are structurally related to prostaglandins and extend the spectrum of prostaglandin actions (22), possibly also in the lung circulation.

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