Endothelial monocyte-activating polypeptide II causes NOS-dependent pulmonary artery vasodilation: a novel effect for a proinflammatory cytokine

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Tsai, Ben M., Meijing Wang, Matthias Clauss, Peichuan Sun, and Daniel R. Meldrum. Endothelial monocyte-activating polypeptide II causes NOS-dependent pulmonary artery vasodilation: a novel effect for a proinflammatory cytokine. Am J Physiol Regul Integr Comp Physiol 287: R767–R771, 2004. First published May 20, 2004; 10.1152/ajpregu.00248.2004.—Endothelial monocyte-activating polypeptide (EMAP) II is a novel proinflammatory cytokine that is released from apoptotic and hypoxic cells. The purpose of this study was to determine the effect of EMAP II on the pulmonary artery (PA) and to characterize its mechanism of action. To study this, isolated PA rings from adult male Sprague-Dawley rats were suspended on steel hooks connected to force transducers and immersed in 37°C organ baths containing modified Krebs-Henseleit solution. After equilibration, force displacement of phenylephrine-preconstricted PA was measured in response to EMAP II. Experiments were performed in endothelium-intact rings, endothelium-denuded rings, and in the presence of the NOS inhibitor Nω-nitro- L-arginine methyl ester (L-NAME). Pulmonary artery rings were then subjected to quantitative PCR analysis for inducible NOS (iNOS) mRNA. EMAP II caused a maximal vasodilation of 251 ± 30.7 mg in endothelium-intact PA. EMAP II caused no vasodilation in endothelium-denuded and L-NAME-treated PA (20 ± 14.0 mg and 17.5 ± 7.5 mg, respectively, P < 0.001 vs. endothelium intact). In addition to its vasoactive properties, EMAP II increased PA iNOS mRNA twofold compared with controls. These results demonstrate that 1) EMAP II causes PA vasodilation; 2) EMAP II-mediated PA vasodilation is endothelium dependent and NOS dependent; and 3) EMAP II upregulates iNOS mRNA expression in PA. This report constitutes the first demonstration of EMAP II’s effects on the pulmonary artery, its mechanism of action, and represents the identification of the first proinflammatory cytokine to cause PA vasodilation.

Acute lung injury; hypoxia; apoptosis; inflammation; endothelium

During acute lung injury and the adult respiratory distress syndrome, pulmonary vascular resistance is increased (8). This response is likely multifactorial and secondary to the effects of local hypoxia, inflammatory mediators, and endothelial dysfunction (32). During periods of systemic inflammation, activated resident macrophages release inflammatory mediators that likely disrupt endothelium-dependent vasorelaxation (15). Inflammatory cytokines have been shown to downregulate the expression and synthesis of endothelium-derived vasodilators such as constitutive nitric oxide synthase (eNOS) (38) and prostacyclin (7). However, under certain inflammatory conditions in which cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, or interferon-γ, are elevated, the expression of the inducible form of NOS (iNOS) is increased in pulmonary vascular smooth muscle (25). There is evidence that chronic hypoxia may also augment the expression of iNOS (5, 6, 36).

Endothelial monocyte-activating polypeptide (EMAP) II is a proinflammatory cytokine originally isolated from the supernatants of methylicholangthrene A (MetaA)-induced fibrosarcoma cells (14). EMAP II mRNA is ubiquitously expressed in a wide range of normal human tissue (24, 37), but it is upregulated by hypoxia and apoptosis (16, 18). EMAP II causes chemotactic migration of monocytes and polymorphonuclear leukocytes (PMNs) (2, 13) and is capable of inducing tissue factor in endothelial cells (13), which suggests procoagulant properties. EMAP II also has antiangiogenic activity and may be a negative regulator of lung vascular growth (2).

The hypertensive effect of proinflammatory cytokines on the pulmonary vasculature is well documented (30, 33). However, the direct effect of EMAP II on pulmonary vascular tone is unknown. Several lines of evidence led us to postulate that EMAP II augments pulmonary vasoconstriction: 1) EMAP II has proinflammatory effects (13), which may augment pulmonary hypertension (10, 30); 2) hypoxia is a potent stimulator of pulmonary vasoconstriction (34); and 3) EMAP II expression is increased by hypoxia (18). Surprisingly, we observed that EMAP II caused pulmonary artery vasodilation rather than vasoconstriction. We hypothesized that EMAP II caused PA vasodilation by an endothelium-dependent and NOS-dependent mechanism and that it upregulated iNOS mRNA expression in PA.

Materials and Methods

Animals. All animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” (NIH publication No. 85–23, revised 1985). All animal protocols are approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250–350 g were allowed ad libitum access to food and water up to the time of experimentation.

Isolated pulmonary artery ring preparation. Rats were anesthetized with pentobarbital sodium (150 mg/kg ip). Median sternotomy was performed and heparin sulfate (500 U) was injected into the right ventricular outflow tract. The heart and lungs were removed en bloc and placed in modified Krebs-Henseleit (KH) solution at 4°C. Under dissecting microscope, extralobar pulmonary artery (PA) branches were dissected out and cleared of surrounding tissue. Right and left main branch PA were cut into 2- to 3-mm-wide rings and suspended on steel hooks connected to force transducers (ADInstruments, Colorado Springs, CO) for measurement of vessel tension. Care was taken during this process to avoid endothelial injury. PA rings were immersed in individual water-jacketed organ chambers containing modified Krebs-Henseleit solution bubbled with 95% O2-5% CO2 at 37°C. Force displacement was recorded. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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cording using a PowerLab (ADInstruments) eight-channel data recorder on an Apple iMac PowerPC G4 Computer (Apple Computer, Cupertino, CA). Force displacement of PA rings is expressed as force in milligrams.

**Experimental protocol and groups.** Before the start of experimental protocols, PA rings were stretched to a predetermined optimal passive tension of 750 mg and allowed to equilibrate for 60 min, during which time KH solution was changed every 15 min. Viability of each PA ring was then checked by measuring contractile response to 80 mmol/l KCl. This dosage of KCl was determined to produce maximal contractile response in previous experiments. After washout of KCl, endothelial integrity of each PA ring was assessed with dilution to acetylcholine (1 μmol/l) after phenylephrine (PE; 1 μmol/l) preconstriction. Rings demonstrating <50% dilution to acetylcholine were discarded. After washout and PE preconstriction, a dose-response curve to EMAP II (0.1–20 μg/ml) was generated. The 10 μg/ml dose, which produced the maximal response in endothelium-intact PA, was the dose used in subsequent experiments. In parallel groups of experiments, the effects of removing PA endothelium and inhibiting NOS were, respectively, assessed. PA rings were mechanically denuded of endothelium by gently rubbing the endothelial surface with a roughened steel wire before organ bath suspension. Viability and endothelial integrity were checked as above, and PA rings demonstrating <200 mg vasoconstriction or >10% dilution were discarded. NOS inhibition was performed by incubating endothelium-intact PA rings with N•-nitro-l-arginine methyl ester (l-NAME, 100 μmol/l) for 30 min before PE preconstriction. At the conclusion of experiments, PA rings were immediately frozen in liquid nitrogen and stored at −80°C.

**RT-PCR.** Semiquantitative RT-PCR was used to assess iNOS gene expression in PA rings. After tissue homogenization of PA rings, total RNA was extracted from each PA segment using RNA STAT-60 (TEL-TEST, Friendswood, TX). Total RNA (0.1 μg) was subjected to cDNA synthesis using a cloned AMV first-strand cDNA synthesis kit (Maxim Biotech, South San Francisco, CA). cDNA from each sample was used for polymerase chain reaction of iNOS using message screen rat iNOS PCR kits (Maxim Biotech). PCR products were separated by electrophoresis on 1% agarose gel stained with ethidium bromide. Gels were photographed with a Polaroid Gelcam (Polaroid, Waltham, MA) under ultraviolet illumination (Spectronics, Westbury, NY). Gel photographs were scanned using an Epson Perfection 3200 Scanner (Epson America, Long Beach, CA) onto an iMac PowerPC G4 Computer and analyzed using ImageJ software (National Institutes of Health). Relative quantity of iNOS mRNA was represented as ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

**Chemicals and reagents.** All chemical reagents were obtained from Sigma (St. Louis, MO) unless otherwise specified. All drug concentrations are expressed as final molar concentration in the organ bath. All reagents were dissolved in deionized distilled water. Krebs-Henseleit solution is a physiological balanced salt solution containing (in mmol/l): 127 NaCl, 4.7 KCl, 17 NaHCO3, 1.17 MgSO4, 1.18 KH2PO4, 2.5 CaCl2, and 5.5 d-glucose. Final pH of all solutions was 7.35–7.45.

**EMAP II preparation.** To express EMAP II in the absence of endotoxin, a bacteria-free expression system was chosen. Therefore, mouse EMAP II was cloned into yeast using the Pichia expression kit pPICXcA (InVitrogen, Groningen, the Netherlands) for the expression of secreted proteins. EMAP II, cloned in pBluescript as described recently (16), was ligated in the pPICXcA linearized by Not I/Eco RI digestion. The resulting vector was amplified in Top 10 F’ bacteria and linearized with Bst XI for electro transformation in Pichia pastoris. The resulting yeast clones were selected with zeocin, and protein expression was induced by methanol containing media according to the instructions of the manufacturers. EMAP II protein was purified from the yeast supernatants after centrifugation and sterile filtration by cation exchange chromatography using HiTrap SP HP column (Amersham Biosciences 17–1151-01). EMAP II was eluted by buffer containing 1 M NaCl in 50 mM MES (pH 8.0).

**Statistical analysis.** All reported values are means ± SE (n = 4–8/group). Experimental groups were compared using two-way ANOVA with post hoc Bonferroni test (Prism 4, Graphpad Software, San Diego, CA). Differences at the 95% confidence interval (P < 0.05) were considered significant.

**RESULTS**

**Effect of EMAP II on endothelium-intact PA rings.** EMAP II (0.1–10 μg/ml) had no effect on resting pulmonary artery. However, in PE-preconstricted PA, the 10 μg/ml dose of EMAP II caused a significant vasodilation. Therefore, subsequent experiments were performed with PE preconstriction and used the 10 μg/ml dose of EMAP II. Preconstriction with 1 μmol/l PE caused a contraction of 485.0 ± 49.2 mg in endothelium-intact control PA and 536.2 ± 36.5 mg in endothelium-intact treatment PA before EMAP II treatment. Addition of 1 μmol/l acetylcholine caused a vasodilation of 315.0 ± 51.0 mg in control PA and 345 ± 34.0 mg in endothelium-intact treatment rings before EMAP II treatment. The addition of EMAP II (10 μg/ml) to endothelium-intact PA produced a maximum vasodilation of 243.7 ± 36.5 mg (P < 0.001 vs. control). Control PA rings were preconstricted with PE, but not EMAP II was added, and force displacement was measured over the corresponding time period of the EMAP II-treated PA rings (Fig. 1). Control PA exhibited no vasodilation during this time.

**Effect of EMAP II on endothelium-denuded PA rings.** Because other vasodilators such as acetylcholine act via a functional endothelium, we investigated the effect of EMAP II on PA rings mechanically denuded of endothelium. PA rings denuded of endothelium exhibited a PE-induced contraction of 412.8 ± 57.7 mg and a maximum vasodilation of 7.1 ± 4.7 mg (P < 0.0001 vs. endothelium-intact PA) in response to acetylcholine. EMAP II caused a maximum vasodilation of 20.0 ± 13.4 mg (P < 0.001 vs. endothelium-intact PA) in endothelium-denuded PA rings (Fig. 2).

**Effect of EMAP II in l-NAME-treated PA rings.** Because EMAP II required a functional endothelium to cause PA vasodilation, we hypothesized that its mechanism of action was...
EMAP II causes pulmonary artery vasodilation

Fig. 2. Effect of EMAP II on endothelium-denuded PA. EMAP II (10 µg/ml at time 0) is added to endothelium-intact (■; n = 8) and endothelium-denuded (○; n = 7) PA preconstricted with PE (1 µmol/l, not shown). Values are means ± SE. *P < 0.001 vs. endothelium intact.

dependent on NOS. Endothelium-intact PA rings treated with the nonspecific NOS inhibitor L-NAME had a vasoconstriction of 525.0 ± 76.6 mg in response to PE. Addition of EMAP II resulted in a maximum vasodilation of 22.5 ± 17.5 mg (Fig. 3; P < 0.001 vs. endothelium-intact PA).

PA expression of iNOS mRNA with EMAP II treatment. Proliferative cytokines and hypoxia increase the expression of iNOS; however, the effect of the proinflammatory and hypoxia-inducible cytokine EMAP II on iNOS expression is unknown. We measured iNOS expression in PA treated with EMAP II, which had twofold higher iNOS mRNA expression compared with control PA (20.2 ± 10.6% vs. 10.4 ± 6.4%, expressed as percentage of GAPDH mRNA). Figure 4 is a scanned gel photograph of representative bands from the two groups.

DISCUSSION

The results of this study revealed the following original findings: EMAP II itself causes pulmonary artery vasodilation;

EMAP II-mediated PA vasodilation is endothelium dependent and NOS dependent; and EMAP II upregulates iNOS mRNA expression in pulmonary artery. The proinflammatory effects of EMAP II have previously been demonstrated (2, 13), but little was known about the direct effects of EMAP II on the vasculature. This study demonstrates for the first time that EMAP II has direct pulmonary vasoactive properties. In addition to its vasodilative effects, EMAP II simultaneously demonstrates proinflammatory properties by increasing iNOS expression.

Endotoxemia imposes a systemic vasodilatory state, which is mediated in part by the effects of inflammatory cytokines on the systemic vasculature. Paradoxically, the pulmonary vasculature demonstrates increased resistance during endotoxemia and acute lung injury (19, 20, 28, 29). The pulmonary vascular endothelium is largely responsible for the low baseline pulmonary tone, and dysfunction or “endothelial stunning” results in pulmonary vasoconstriction. Proinflammatory cytokines such as TNF-α are known to contribute to pulmonary hypertension (30, 33). Therefore, our initial hypothesis was that EMAP II would cause pulmonary vasoconstriction in a manner similar to other inflammatory cytokines. Surprisingly, EMAP II caused PA vasodilation (Fig. 1). Although hypoxia is a known inducer of EMAP II expression, EMAP II caused vasodilation, whereas hypoxia has previously been shown to result in vasoconstriction (32). We speculate that hypoxia-induced EMAP II expression may be a counterregulatory mechanism that opposes the vasoconstriction caused by chronic hypoxia.

Because the vascular endothelium is intimately involved in the paracrine regulation of normal pulmonary vascular tone, we hypothesized that the mechanism of EMAP II-mediated pulmonary vasodilation was related to endothelial function. This was confirmed by the lack of vasodilation in endothelium-denuded PA exposed to EMAP II (Fig. 2). The vascular endothelium produces a number of vasodilating factors, including nitric oxide, prostaglandins, and endothelium-derived hyperpolarizing factor (EDHF). Nitric oxide (NO) is thought to be the key factor involved in the maintenance of low baseline pulmonary tone. NO is synthesized in endothelial cells by NOS from the substrate l-arginine (22, 23). Once formed, NO stimulates guanylate cyclase in vascular smooth muscle cells to produce cGMP. The mechanism of cGMP-mediated vasodilation is thought to involve activation of a protein kinase, inhibition of inositol triphosphate, inhibition of calcium influx, and myosin light chain dephosphorylation. The association between EMAP II-mediated vasodilation and pulmonary artery endothelium naturally led to the interrogation of NOS. Indeed, inhibition of NOS activity with l-NAME virtually abolished EMAP II-mediated vasodilation in endothelium-intact PA (Fig.

Fig. 3. Effect of EMAP II on endothelium-intact PA treated with l-NAME. EMAP II (10 µg/ml at time 0) is added to PE-preconstricted (1 µmol/l, not shown) endothelium-intact PA in the presence (○; n = 4) or absence (■; n = 8) of the nitric oxide synthase (NOS) inhibitor N-nitro-l-arginine methyl ester (l-NAME; 100 µmol/l). Values are means ± SE. *P < 0.01 and **P < 0.001 vs. endothelium intact without l-NAME.

Fig. 4. Gel photograph depicting inducible NOS (iNOS) and GAPDH mRNA bands in endothelium-intact PA with or without (control) EMAP II.
Thus the vasodilatory properties of EMAP II appear to be NO dependent. Under certain inflammatory conditions, iNOS production from vascular smooth muscle cells increases (3, 11, 25, 31) and is largely responsible for the hemodynamic sequelae of sepsis. Hypoxia is also capable of inducing iNOS expression (5, 6, 12, 36). Because EMAP II has proinflammatory properties and is upregulated by hypoxia, we measured iNOS expression in PA treated with EMAP II. In this regard, there was a twofold increase in iNOS expression in endothelium-intact PA treated with EMAP II compared with control PA. The time course of iNOS expression in PA treated with EMAP II (15 min) suggests that iNOS is not responsible for the immediate EMAP II-induced vasodilation we observed. The iNOS isofrom is induced by inflammatory stimuli and, therefore, requires time for active iNOS to be produced, eNOS, which is expressed under baseline conditions, is likely responsible for acute vasodilation. The upregulation of iNOS expression is consistent with the proinflammatory properties of EMAP II; however, the long-term sequelae of iNOS production in this scenario are unclear. The effects of NO, and the NO synthases, are clearly long-term sequelae of iNOS production in this scenario and tissue specific. The effects of NO, and the NO synthases, are clearly long-term sequelae of iNOS production in this scenario and tissue specific. For instance, NO is thought to mediate the sustained myocardial dysfunction caused by TNF-α (21). On the other hand, evidence suggests a cytoprotective role for NO in limiting inflammatory cytokine release from lung macrophages (23). Another possible explanation for the diurnometrically opposing effects of NO may be the source of iNOS (27), such that iNOS produced by vascular smooth muscle may be beneficial, whereas neutrophil-derived iNOS may be detrimental.

EMAP II was originally isolated from supernatants of MethA-induced fibrosarcoma cells (14). Since its discovery, it has been associated with a wide range of effects (13, 14), including induction of tissue factor in endothelial cells, endothelial E-selectin and P-selectin expression, PMN, monocyte chemotaxis, and inhibition of vasouclusion (1). It is now known that potent stimulants of EMAP II expression include apoptosis and hypoxia (16, 18). The time course of release ranges from 8 to 20 h after induction of programmed cell death and 20 to 28 h of hypoxia. A 34-kDa proform of EMAP II undergoes intracellular proteolytic cleavage to its mature form before release (13). There is significant amino acid homology with the mammalian p43 auxiliary protein of the multisynthase tRNA complex, which suggests that p43 is a putative precursor of EMAP II (17). In fact, human p43 itself has been shown to activate the inflammatory cascade and induce proinflammatory cytokine expression (17). Unfortunately, there are very little data on EMAP II expression in human tissues. Immunohistochemical analysis (24) has localized EMAP II expression in normal human tissues such as thyroid and colon, and there was also some staining in endothelial cells of the cardiovascular system, subsets of monocytes/macrophages, and epithelial cells of the pancreas, adrenal, and renal tubules. However, the extent that EMAP II influences the inflammatory process in humans is unknown, and the significance of EMAP II on human cardiovascular physiology is unclear.

Two aspects of our protocol make it difficult to correlate our findings with in vivo and clinical studies. First, the concentrations of EMAP II we used were much higher than isolated artery studies using TNF-α (9, 26). EMAP II levels in humans have not been quantitated, so it is not feasible to compare the potencies of EMAP II with other cytokines. However, recombinant human EMAP II has been used in studies on tumor biology (1), and the doses employed (50–250 µg/ml) in those studies were in the same magnitude as those in our protocol. Second, we demonstrated EMAP II vasoactivity in large conduit arteries, which may not have the same reactivity as more distal resistance vessels. Indeed, others have observed greater hypoxic vasoconstriction (35) and acetylcholine-induced relaxation (4) in small resistance PA compared with large PA branches. This leads us to believe that the response to EMAP II in smaller resistance vessels will be more pronounced.

Pulmonary hypertension during acute lung injury remains a challenging clinical dilemma. Circulating inflammatory mediators result in an overall decrease in systemic vascular resistance and an increase in PA vascular resistance. Indeed, this is the first proinflammatory cytokine to cause PA vasodilation. Although the proinflammatory effects of EMAP II, as a chemottractant for monocytes and PMNs, may exacerbate acute lung injury, mechanistic understanding of its vasoactive effects may lead to the development of therapeutic alternatives for the treatment of pulmonary hypertension during acute lung injury.

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