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Research Article **Vascular biology**

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β_2-Adrenergic agonists augment air pollution–induced IL-6 release and thrombosis

Sergio E. Chiarella,[1] Saul Soberanes,[1] Daniela Urich,[1] Luisa Morales-Nebreda,[1] Recep Nigdelioglu,[1] David Green,[2] James B. Young,[3] Angel Gonzalez,[1] Carmen Rosario,[1] Alexander V. Misharin,[4] Andrew J. Ghio,[5] Richard G. Wunderink,[1] Helen K. Donnelly,[1] Kathryn A. Radigan,[1] Harris Perlman,[4] Navdeep S. Chandel,[1] G.R. Scott Budinger,[1] and Gökhan M. Mutlu[1]

[1]Division of Pulmonary and Critical Care Medicine, [2]Division of Hematology and Oncology, [3]Division of Endocrinology, Metabolism, and Molecular Medicine, and [4]Division of Rheumatology, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA.
[5]United States Environmental Protection Agency, Research Triangle Park, North Carolina, USA.

Acute exposure to particulate matter (PM) air pollution causes thrombotic cardiovascular events, leading to increased mortality rates; however, the link between PM and cardiovascular dysfunction is not completely understood. We have previously shown that the release of IL-6 from alveolar macrophages is required for a prothrombotic state and acceleration of thrombosis following exposure to PM. Here, we determined that PM exposure results in the systemic release of catecholamines, which engage the β_2-adrenergic receptor (β_2AR) on murine alveolar macrophages and augment the release of IL-6. In mice, β_2AR signaling promoted the development of a prothrombotic state that was sufficient to accelerate arterial thrombosis. In primary human alveolar macrophages, administration of a β_2AR agonist augmented IL-6 release, while the addition of a beta blocker inhibited PM-induced IL-6 release. Genetic loss or pharmacologic inhibition of the β_2AR on murine alveolar macrophages attenuated PM-induced IL-6 release and prothrombotic state. Furthermore, exogenous β_2AR agonist therapy further augmented these responses in alveolar macrophages through generation of mitochondrial ROS and subsequent increase of adenylyl cyclase activity. Together, these results link the activation of the sympathetic nervous system by β_2AR signaling with metabolism, lung inflammation, and an enhanced susceptibility to thrombotic cardiovascular events.

Introduction

Based on air pollution exposure estimates in the US from the late 1970s to the early 2000s, Pope et al. projected that a 10 $\mu g/m^3$ fall in the mean levels of fine particulate air pollution ($PM_{2.5}$) would increase life expectancy by 0.61 years (1). At current levels in the developed world, these data suggest that PM exposure is associated with an average loss of 0.7 to 1.6 years of life, with a larger burden on urban dwellers. The public health impact of $PM_{2.5}$ exposure is greater in the developing world, where particle levels are often 10-fold higher than those observed in the US and Western Europe (2).

The mortality associated with acute exposure to ambient PM is largely due to an increased incidence of ischemic cardiovascular events; however, the mechanisms explaining this association are incompletely understood (3). We and others have suggested that exposure to PM induces a local inflammatory response in the lung, resulting in the release of proinflammatory cytokines, which enhance the systemic tendency toward thrombosis (3–9). Specifically, we discovered that exposure of mice to PM causes a prothrombotic state and accelerates vascular thrombosis by inducing the release of IL-6 from alveolar macrophages (4, 10). An additional mechanism linking PM exposure with cardiovascular events has been described by several groups of investigators who have observed changes in heart rate variability or peripheral vasoreactivity following exposure to PM and inferred from these data that PM-induced activation of the sympathetic nervous system might induce coronary vasoconstriction or arrhythmias (3, 11–15). However, the effect of PM on the sympathetic nervous system has not been directly investigated, and the consequences of sympathetic nervous system activation on lung inflammation and thrombosis are not known.

Here, we report that exposure to concentrated ambient PM at levels similar to those observed in the developing world induces the systemic and local release of catecholamines, which activate β_2-adrenergic receptors (β_2ARs) on human and murine alveolar macrophages to enhance PM-induced release of IL-6 and resulting thrombosis. In alveolar macrophages, exposure to PM induced the generation of ROS from the mitochondria, which primed adenylyl cyclase and consequently enhanced β_2AR-mediated generation of cAMP and phosphorylation of cAMP response element-binding protein (CREB) to augment *IL6* transcription. Consistent with these findings, the administration of formoterol, a selective β_2AR agonist widely used in clinical practice, augmented PM-induced IL-6 release and the resulting prothrombotic state and accelerated arterial thrombosis. These findings reveal an important adaptive mechanism by which systemic stress acting through catecholamines can enhance inflammation to promote thrombosis.

Results

Exposure to PM causes the release of catecholamines, which are required for PM-induced release of IL-6 and thrombosis. To directly evaluate the effects of PM on sympathetic nervous system activity, we measured tissue catecholamine levels in mice exposed to concentrated ambi-

Authorship note: Sergio E. Chiarella and Saul Soberanes, as well as G.R. Scott Budinger and Gökhan M. Mutlu, contributed equally to this work.

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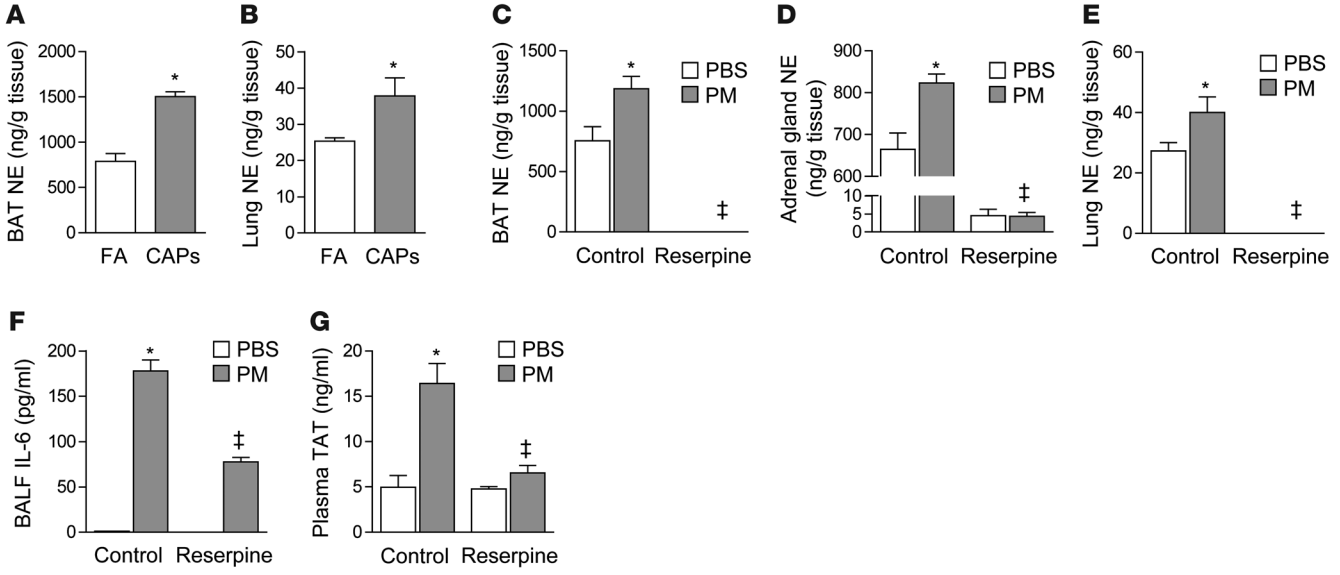

Figure 1

Catecholamines are required for PM-induced inflammation and thrombosis. We exposed wild-type (C57BL/6) mice contemporaneously to either CAPs ($PM_{2.5}$) or filtered air (FA) for 8 hours daily on 3 consecutive days and measured levels of norepinephrine (NE) in the (**A**) BAT and (**B**) lung tissue. We also performed intratracheal instillations of PM (200 μg/mouse) or control (PBS) in wild-type mice and gave reserpine (5 mg/kg 100 μl) or vehicle (20% ascorbic acid 100 μl) by gavage once 4 hours before treatment with PM or control (PBS). Twenty-four hours later, we measured levels of norepinephrine in the (**C**) BAT, (**D**) adrenal gland, and (**E**) lung. In identically treated mice, we measured (**F**) IL-6 in the BALF and (**G**) TAT complexes in the plasma. *$P < 0.05$, CAPs vs. FA, PM vs. PBS; ‡ $P < 0.05$, reserpine vs. control.

ent fine PM (<2.5 μm in diameter, $PM_{2.5}$) or filtered air, using a versatile aerosol concentration enrichment system (VACES) (ref. 16 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI75157DS1). In mice exposed to concentrated ambient particles (CAPs) for 8 hours daily on 3 consecutive days, we observed a significant increase in systemic and lung levels of catecholamines compared with mice exposed to filtered air (Figure 1, A and B). We observed similar changes in mice exposed to a well-characterized urban PM collected from the ambient air in Washington, DC (refs. 4, 10, and Figure 1, C–E). Both basal and PM-induced catecholamine levels were reduced after we induced a chemical sympathectomy by pretreating the mice with the vesicular monoamine transporter reserpine prior to the intratracheal instillation of PM (Figure 1, C–E, and ref. 17). We have previously shown that exposure of mice to PM via inhalation or intratracheal instillation results in an IL-6–dependent increase in thrombin-antithrombin (TAT) complexes in the plasma (4, 10). In mice treated with reserpine, we found that the PM-induced increases in BAL fluid (BALF) levels of IL-6 and plasma levels of TAT complexes were reduced (Figure 1, F and G). These data demonstrate that inhalational exposure of PM at levels seen in many world cities is sufficient to trigger the systemic release of catecholamines and suggest that these catecholamines contribute to PM-induced IL-6 release and thrombosis.

β_2ARs are required for catecholamine-induced upregulation of IL-6 and the resulting thrombosis after PM exposure. Signaling by catecholamines is mediated by αARs and/or βARs, which are variably expressed in different tissues and cell types, including the lung (18–20) and alveolar macrophages (21, 22). We treated mice with a nonselective βAR antagonist (propranolol 3 mg/kg i.p. q 8 hours for 48 hours) prior to the administration of PM and measured IL-6 release and thrombosis 24 hours later. Treatment with propranolol reduced the PM-induced increase in IL-6 levels in BALF (Figure 2A). We have previously reported that IL-6 is released from alveolar macrophages in response to PM and acts to increase the synthesis of clotting factors in the liver. We found that IL-6 release from macrophages is required for the PM-induced generation of intravascular thrombin and the reduced time to thrombosis after $FeCl_3$-induced carotid artery injury, a widely used murine model of stroke, following PM exposure (Supplemental Video 1, Supplemental Figure 2, and refs. 4, 10, 23). All of these effects were reduced in mice treated with propranolol (Figure 2, B–D).

Of the 3 subtypes of βARs, β_1AR and β_2AR are widely expressed, while expression of the β_3AR is largely restricted to the adipose tissue (24). To determine which βAR subtype is responsible for the catecholamine-induced augmentation of IL-6 release and thrombosis induced by PM, we performed intratracheal instillations of PM or PBS in global knockout mice singly deficient in β_1AR or β_2AR or doubly deficient in β_1AR and β_2AR and their wild-type littermate controls. Mice deficient in β_2AR or doubly deficient in β_1AR and β_2AR released less IL-6 into the BALF (Figure 2E). The effect of catecholamines and β_2AR was specific to IL-6, as the loss of β_2AR did not have an effect on the BALF levels of other cytokines induced by PM exposure (Figure 2, F and G; only TNF-α and MCP-1 are shown).

In addition to a reduction in IL-6, mice deficient in β_2AR or doubly deficient in β_1AR and β_2AR generated less intravascular thrombin (Figure 2H) and showed no acceleration of arterial thrombosis (Figure 2, I and J) in response to PM. We observed a similar reduction in IL-6 release and plasma TAT levels in β_2AR knockout mice (*Adrb2*$^{-/-}$) compared with their littermate controls 24 hours after the intratracheal administration of LPS (Supplemental Figure 3), suggesting that the role of catecholamines and β_2AR-mediated upregulation of IL-6 may not be limited to PM, but may represent a general response

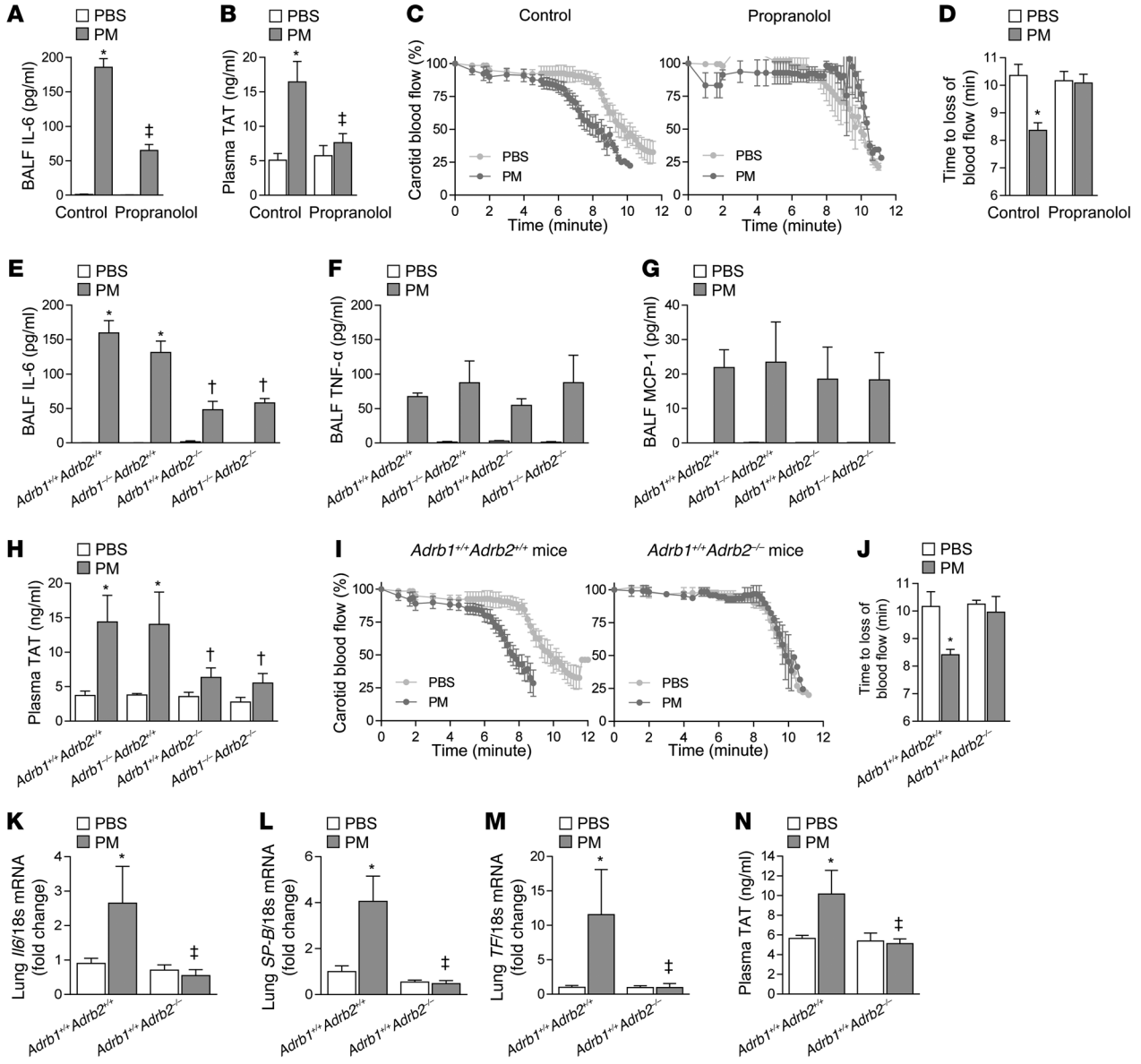

Figure 2

β_2ARs are required for the catecholamine-induced upregulation of IL-6 and the resulting thrombosis after PM exposure. We pretreated mice with propranolol (3 mg/kg in 100 μl i.p.) or vehicle (saline 100 μl i.p.) every 8 hours, followed 24 hours later by PM (200 μg/mouse) or control (PBS) and measured (**A**) the levels of IL-6 in the BALF, (**B**) the plasma levels of TAT complexes, and (**C**) the time to loss of blood flow after $FeCl_3$-induced injury to the carotid artery. (**D**) The time to complete (>75% reduction) loss of blood flow is shown. We performed intratracheal instillations in mice lacking either β_1AR (*Adrb1*$^{-/-}$*Adrb2*$^{+/+}$), β_2AR (*Adrb1*$^{+/+}$*Adrb2*$^{-/-}$), or both β_1AR and β_2AR (*Adrb1*$^{-/-}$*Adrb2*$^{-/-}$) and their wild-type littermate controls (*Adrb1*$^{+/+}$*Adrb2*$^{+/+}$) with PM or PBS and, 24 hours later, measured the levels of (**E**) IL-6, (**F**) TNF-α, and (**G**) MCP-1 in BALF, (**H**) the plasma levels of TAT complexes in the plasma, and (**I** and **J**) the time to loss of blood flow after $FeCl_3$-induced injury to the carotid artery. We exposed β_2AR deficient mice (*Adrb1*$^{+/+}$*Adrb2*$^{-/-}$) and wild-type littermate controls (*Adrb1*$^{+/+}$*Adrb2*$^{+/+}$) contemporaneously to either CAPs ($PM_{2.5}$) or filtered air for 8 hours daily on 3 consecutive days and measured levels of (**K**) *Il6*, (**L**) *SP-B*, and (**M**) *TF* mRNA in the lung tissue and (**N**) TAT complexes in the plasma. **P* < 0.05, PM vs. PBS or CAPs vs. FA; ‡*P* < 0.05, propranolol vs. control, *Adrb1*$^{+/+}$*Adrb2*$^{-/-}$ and *Adrb1*$^{-/-}$*Adrb2*$^{-/-}$ vs. *Adrb1*$^{-/-}$*Adrb2*$^{+/+}$ and *Adrb1*$^{+/+}$*Adrb2*$^{+/+}$.

during lung inflammation and injury. In wild-type mice exposed to concentrated ambient $PM_{2.5}$ via inhalation, we found an increase in the mRNA encoding *Il6* and its transcriptional targets surfactant protein B (SP-B) and tissue factor (TF) in whole-lung homogenates as well as an increase in plasma thrombin generation. None of these changes were observed in mice lacking β_2AR (Figure 2, K–N).

In human alveolar macrophages, stimulation of β_2*ARs increases and* β*AR blockade inhibits the release of IL-6 in response to PM.* We have previously shown that the IL-6 release from alveolar macrophages is required for the development of a PM-induced prothrombotic state sufficient to accelerate arterial thrombosis (4, 10). We cultured primary human macrophages from intubated patients undergoing non-

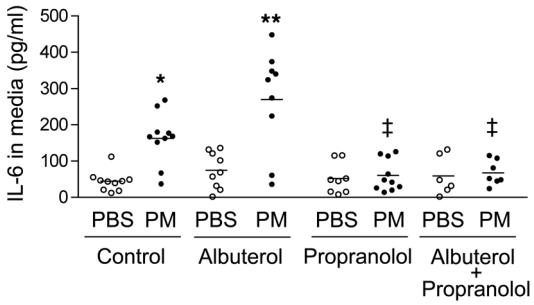

Figure 3
In human alveolar macrophages, stimulation of β_2AR augments and inhibition of βAR inhibits PM-induced IL-6 release. We treated primary human alveolar macrophages with PM (10 μg/cm^2) or control (medium) and with albuterol (10^{-7}M) or control (medium) in the presence or absence of propranolol (10 μM) or control (saline) and measured IL-6 levels in the medium 24 hours later. *P < 0.05, PM vs. PBS; ** P < 0.05, albuterol vs. control; ‡P < 0.05, propranolol, vs. control.

bronchoscopic alveolar lavage in the intensive care unit. After 24 hours in culture, we found that these cells released IL-6 into the medium in response to PM (Figure 3). To determine whether the stimulation of adrenergic receptors on the alveolar macrophages affects PM-induced IL-6 release, we treated primary human macrophages from the same patients with PM or control in the presence or absence of a β_2AR agonist, albuterol, or a βAR antagonist (beta blocker), propranolol, and measured the concentration of IL-6 released into the medium 24 hours later. Albuterol caused a doubling of IL-6 in the medium, whereas propranolol inhibited the PM-induced release of IL-6 into the medium (Figure 3).

β_2ARs on alveolar macrophages are required for catecholamine-induced upregulation of IL-6 and resulting thrombosis after PM exposure. Using intratracheal liposomal clodronate, we have previously reported that the majority of IL-6 released in response to PM originates from lung macrophages (4). Therefore, we sought to determine whether the activation of β_2ARs on these cells was directly responsible for the catecholamine-mediated augmentation of IL-6 release in response to PM. After confirming the expression of the β_2AR in primary alveolar macrophages from wild-type mice (Supplemental Figure 4 and ref. 25–27), we generated *Lysm-Cre Adrb2$^{flox/flox}$* mice and validated macrophage-specific deletion of β_2AR in cells isolated from homogenized lungs (Figure 4, A and B). We then exposed *Lysm-Cre Adrb2$^{flox/flox}$* and control mice (*Adrb2$^{flox/flox}$*) to concentrated ambient $PM_{2.5}$ via inhalation. Compared with the control mice, the *Lysm-Cre Adrb2$^{flox/flox}$* mice had reduced levels of IL-6, generated fewer TAT complexes in the plasma, and exhibited slower thrombosis after carotid injury 24 hours after PM exposure (Figure 4, C–F).

Treatment with β_2 agonists worsens PM-induced release of IL-6 and resulting thrombosis. To determine whether activation of β_2AR is sufficient to augment PM-induced IL-6 release and thrombosis, we treated wild-type mice and *Lysm-Cre Adrb2$^{flox/flox}$* mice with a long-acting β_2AR agonist, formoterol (1×10^{-5} M via inhalation, every 12 hours), beginning 24 hours before exposure to concentrated

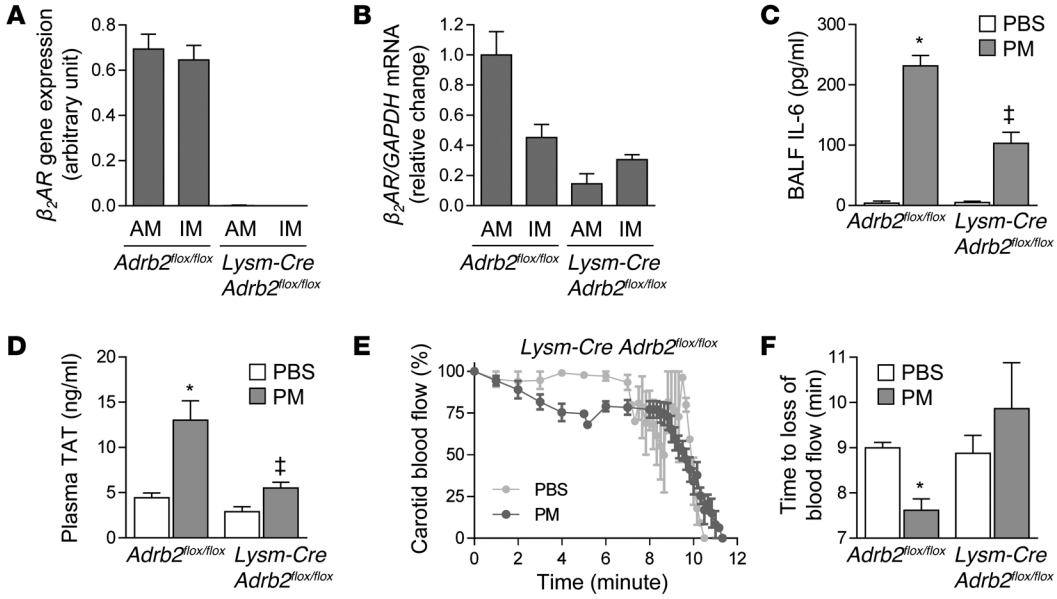

Figure 4
β_2ARs on alveolar macrophages are required for catecholamine-induced upregulation of IL-6 and resulting thrombosis after PM exposure. (**A** and **B**) Mice deficient in β_2AR specifically in monocytes and macrophages were generated by crossing *Lysm-Cre* mice with *Adrb2$^{flox/flox}$* mice. Inflammatory cells from lung homogenates of *Adrb2$^{flox/flox}$* and *Lysm-Cre Adrb2$^{flox/flox}$* mice were sorted, and deletion of the *Adrb2* gene was confirmed by assessment of (**A**) gene expression and (**B**) mRNA. We performed intratracheal instillations in *Adrb2$^{flox/flox}$* and *Lysm-Cre Adrb2$^{flox/flox}$* mice with PM (200 μg/mouse) or vehicle (PBS) and, 24 hours later, measured (**C**) the levels of IL-6 in BALF, (**D**) TAT complexes in the plasma, and (**E** and **F**) the time to loss of blood flow after $FeCl_3$-induced injury to the carotid artery (the time to complete loss of blood flow [>75% reduction]). *P < 0.05, PM vs. PBS or CAPs vs. FA; ‡P < 0.05, *Adrb2$^{flox/flox}$* vs. *Lysm-Cre Adrb2$^{flox/flox}$*.

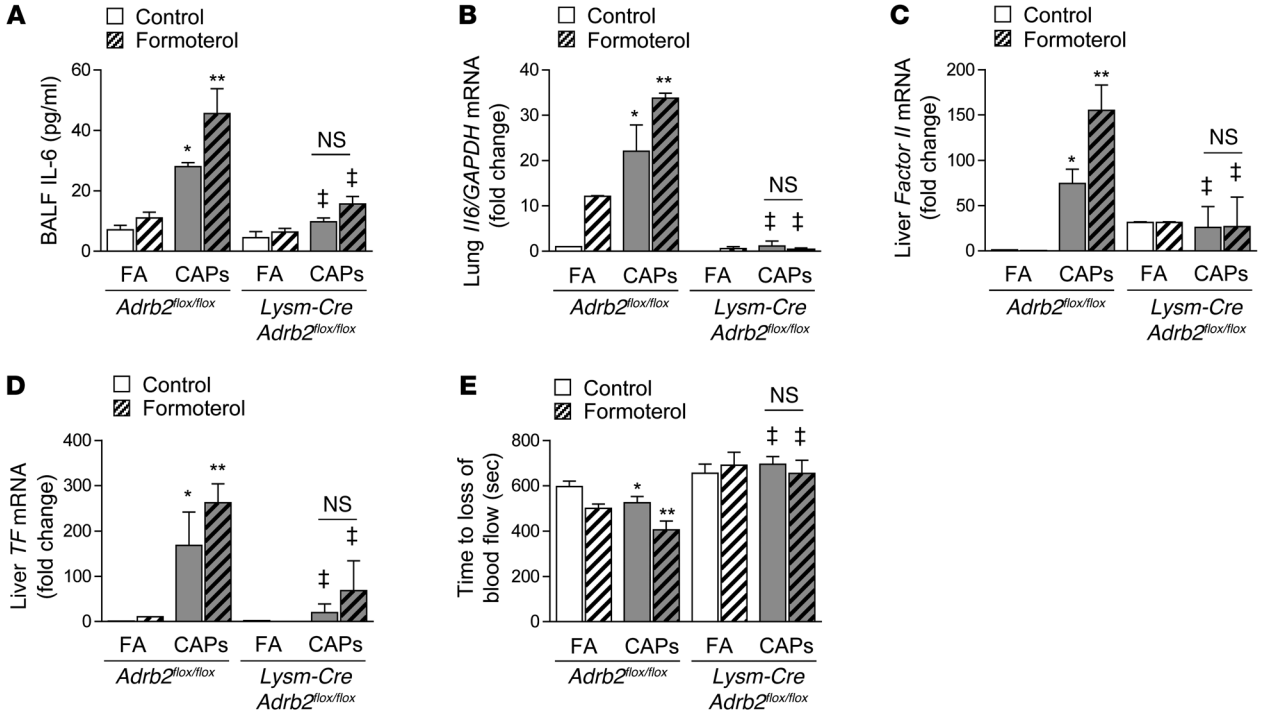

Figure 5

Treatment with β_2 agonists worsens PM-induced release of IL-6 and resulting thrombosis. We treated wild-type mice with formoterol (10^{-5}M) or vehicle control (ethanol 2%), both with 1 ml via inhalation over 30 minutes every 12 hours, and exposed them contemporaneously to CAPs ($PM_{2.5}$) or filtered air for 8 hours daily on 3 consecutive days. We measured levels of (**A**) IL-6 in BALF, (**B**) *Il6* mRNA in lung tissue, (**C**) prothrombin (factor II), and (**D**) *TF* mRNA in liver and (**E**) the time to loss of blood flow after $FeCl_3$-induced injury to the carotid artery (the time to complete loss of blood flow [>75% reduction]). **P* < 0.05, PM vs. PBS or CAPs vs. FA; ***P* < 0.05, formoterol vs. control; ‡*P* < 0.05, *Adrb2*$^{flox/flox}$ vs. *Lysm-Cre Adrb2*$^{flox/flox}$.

ambient PM. In wild-type mice treated with formoterol, the levels of IL-6 in the BALF were twice that of the sham-treated controls (Figure 5A), and the levels of *Il6* mRNA in whole-lung homogenates were increased after inhalational exposure to concentrated ambient PM (Figure 5B). In contrast, formoterol did not affect the IL-6 levels in *Lysm-Cre Adrb2*$^{flox/flox}$ mice (Figure 5, A and B). The enhanced PM-induced release of IL-6 in response to formoterol in wild-type (*Adrb2*$^{flox/flox}$) mice was associated with increased expression of *prothrombin* (factor II) and *TF* mRNA in the liver (Figure 5, C and D) and a further acceleration of carotid artery thrombosis after $FeCl_3$-induced injury (Figure 5E). These effects were absent in *Lysm-Cre Adrb2*$^{flox/flox}$ mice (Figure 5, C–E). Similar effects of formoterol were seen after the intratracheal administration of PM (Supplemental Figure 5). Exposure to concentrated ambient PM with or without formoterol did not cause a significant change in the number or differential of immune cells in the lungs in *Adrb2*$^{flox/flox}$ and *Lysm-Cre Adrb2*$^{flox/flox}$ mice (Supplemental Figure 6).

Engagement of β_2AR augments PM-induced release of IL-6 from alveolar macrophages in vitro. We conducted a series of experiments to determine the mechanism by which the activation of β_2ARs on macrophages augments PM-induced IL-6 release and thrombosis. We isolated primary alveolar macrophages from *Adrb2*$^{-/-}$ mice and their wild-type littermate controls and compared the release of IL-6 in response to PM. Medium levels of IL-6 measured 24 hours after treatment with PM were lower in the *Adrb2*$^{-/-}$ compared with the wild-type macrophages. Treatment of primary alveolar macrophages from wild-type, but not *Adrb2*$^{-/-}$, animals with the β_2AR agonist albuterol (10^{-7} M) augmented the PM-induced release of IL-6 (Figure 6A). To examine the molecular mechanisms of this response, we used an alveolar macrophage cell line (MH-S), which expresses many of the markers seen in wild-type macrophages (Supplemental Figure 7). MH-S cells treated with PM 1 hour earlier showed augmented IL-6 release in response to treatment with epinephrine, and the (R), but not the (S), enantiomer of albuterol (Figure 6, B and C, and Supplemental Figure 8).

β_2 agonist therapy and forskolin augment PM-induced release of IL-6 only when they are administered following exposure to PM. We treated MH-S cells with PM or control in the presence or absence of a β_2AR agonist, albuterol (10^{-7} M), or a direct activator of adenylyl cyclase, forskolin (50 μM), administered either simultaneously or 1 hour after treatment with PM, and measured IL-6 release into the medium. While albuterol did not have an effect on IL-6 when it was administered simultaneously with PM, it doubled the PM-induced IL-6 release when it was administered 1 hour after the PM (Figure 7A). Similarly, forskolin induced a modest increase in PM-induced IL-6 release, but doubled the IL-6 release when it was administered 1 hour after PM treatment (Figure 7B).

PM-induced mitochondrial ROS release primes adenylyl cyclase to augment the release of IL-6 in response to β_2 agonists. Stimulation of β_2AR initiates a signaling pathway that increases the activity of adenylyl cyclase, which converts ATP to 3′,5′cAMP (21). When we directly activated adenylyl cyclase with forskolin or inhibited it with SQ2253, the augmentation and inhibition of PM-induced IL-6 release in MH-S cells was similar to that observed after treatment

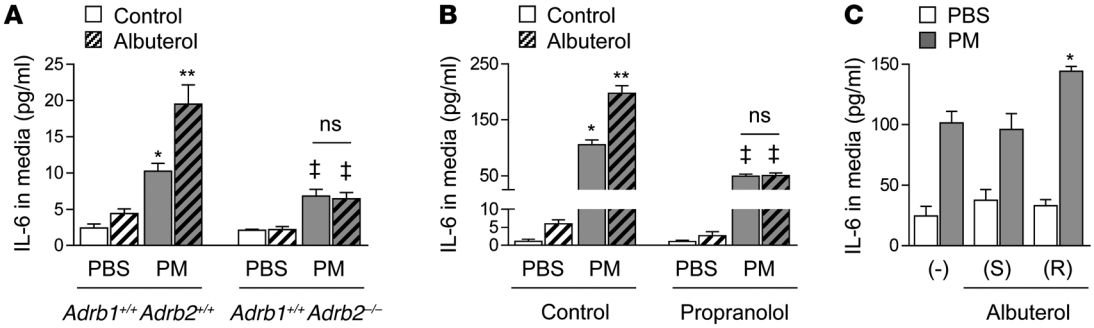

Figure 6

Engagement of β_2AR augments PM-induced release of IL-6 from alveolar macrophages in vitro. (**A**) We treated primary alveolar macrophages isolated from *Adrb2*$^{-/-}$ mice (*Adrb1*$^{+/+}$*Adrb2*$^{-/-}$) and their wild-type littermate controls (*Adrb1*$^{+/+}$*Adrb2*$^{+/+}$) with PM (10 μg/cm^2) or control (medium) and measured IL-6 levels in the medium 24 hours later. (**B**) We treated murine alveolar macrophages (MH-S cells) with PM (10 μg/cm^2) or control (medium) and with albuterol (10^{-7}M) or control (medium) in the presence or absence of propranolol (10 μM) (or control [PBS]) and measured IL-6 levels in the medium 24 hours later. (**C**) We treated MH-S cells with PM (10 μg/cm^2) or control (medium) and with the (S) or (R) enantiomer of albuterol (10^{-7}M) or control. *P < 0.05 for comparison between PM-treated cells with or without albuterol or forskolin; *P < 0.05, PM vs. PBS; **P < 0.05, albuterol vs. control, and simultaneous vs. control; ‡P < 0.05, *Adrb1*$^{+/+}$*Adrb2*$^{-/-}$ vs. *Adrb1*$^{+/+}$*Adrb2*$^{+/+}$, propranolol vs. control.

with albuterol or propranolol, respectively (Figure 7C). Consistent with our findings using albuterol, the PM-induced augmentation of cAMP levels after forskolin administration was only observed when the PM was administered 1 hour before the forskolin (Figure 7, A and B). We observed a similar augmentation of forskolin-induced cAMP by PM in the presence of 2 phosphodiesterase inhibitors (3-isobutyl-1-methylxanthine [IBMX] and aminophylline), suggesting the increase in cAMP did not result from an inhibition of phosphodiesterases (Figure 7D).

To explore the mechanism by which PM exposure enhances the activity of adenylyl cyclase to augment IL-6 release, we treated MH-S cells and primary alveolar macrophages isolated from wild-

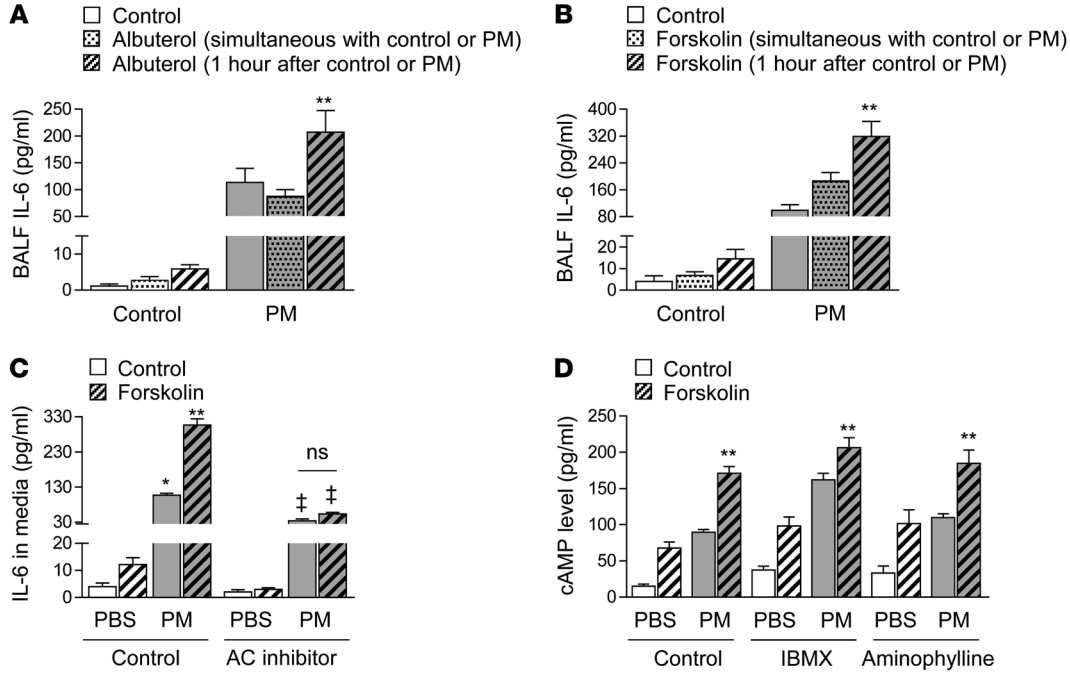

Figure 7

β_2 Agonist therapy and forskolin augment PM-induced release of IL-6 only when administered following exposure to PM. We treated MH-S cells with PM (10 μg/cm^2) or control (medium) and with (**A**) albuterol (10^{-7}M) or (**B**) forskolin (50 μM) and control administered simultaneously with the PM or 1 hour after the PM. (**C**) We treated MH-S cells with PM (10 μg/cm^2) or control (medium) and with forskolin (50 μM) or control (saline) 1 hour after PM treatment in the absence of presence of an adenylyl cyclase (AC) inhibitor (SQ22536, 300 μM) (or control [H_2O]) and measured IL-6 levels in the medium 24 hours later. (**D**) We treated MH-S cells with PM (10 μg/cm^2) or control (medium) and with forskolin (50 μM) or control 1 hour after PM treatment in the absence or presence of IBMX (1 μM) and aminophylline (10 μM) and measured cAMP levels in cell lysates 1 minute later. *P < 0.05, PM vs. PBS; **P < 0.05, albuterol or forskolin (1 hour after PM) vs. control and albuterol or forskolin (simultaneous with PM); ‡ P < 0.05, AC inhibitor vs. control.

type mice with the mitochondrially targeted antioxidant Mito-Q (Figure 8, A and B) or the nonselective combined superoxide dismutase/catalase mimetic Eukarion 134 (EUK-134) (Figure 8C). Inhibition of mitochondrial oxidant generation with these compounds prevented the PM-induced augmentation of cAMP after forskolin treatment (Figure 8D), suggesting that PM-induced ROS are required for increased adenylyl cyclase activity. To determine whether mitochondrial ROS alone were sufficient to augment adenylyl cyclase activity, we measured forskolin-induced cAMP generation in cells treated 1 hour earlier with antimycin A, an electron transport chain inhibitor that increases mitochondrial ROS generation (Supplemental Figure 9 and Figure 8E). The addition of stigmatellin, which also blocks electron transport but inhibits mitochondrial ROS generation (Supplemental Figure 9), prevented the augmentation of IL-6 release by PM or antimycin A (Figure 8, E and F). These results demonstrate an unexpected link between mitochondrial metabolism and the stress response mediated by catecholamines.

We then examined the consequences of the enhanced cAMP production in response to ligation of the β_2AR on IL-6 release. Increases in cAMP activate PKA to induce the phosphorylation and nuclear translocation of the transcription factor CREB (21). We observed an oxidant-dependent increase in nuclear levels of CREB following forskolin treatment in cells treated 1 hour earlier with PM as compared with vehicle (Figure 8G). Similar to our findings in cells with genetic loss or pharmacologic inhibition of β_2AR, the administration of albuterol 1 hour after PM failed to augment PM-induced IL-6 release in MH-S cells with a stable knockdown of CREB (Figure 8H). Consistent with reports that CREB augments the NF-κB–mediated transcription of *Il6* (28), we found that PM-induced IL-6 release was completely inhibited in MH-S cells with a stable knockdown of p65 (Figure 8I) (29). Collectively, these results show that activation of β_2AR following exposure to PM augments adenylyl cyclase activity, which acts through CREB to augment NF-κB–mediated transcription of *Il6*.

Discussion

We describe an important mechanism linking PM air pollution to the activation of the sympathetic nervous system and consequently the release of catecholamines, which act through β_2ARs on macrophages to augment IL-6 release and accelerate thrombosis. Our findings reveal an elegant system for augmenting inflammation-induced thrombosis in response to systemic stress exclusively in the presence of a concomitant inflammatory stimulus. In the absence of an inflammation-induced metabolic signal (mitochondrial ROS generation), catecholamines have no effect on IL-6 release from murine or human lung macrophages or the resulting thrombosis in mice. Similarly, in the absence of catecholamines, IL-6 release in response to particles is not sufficient to augment thrombosis. However, mitochondrial ROS released in response to PM primed adenylyl cyclase such that a subsequent catecholamine signal enhances IL-6 release to levels sufficient to promote thrombosis. The excess cardiovascular mortality associated with urban PM air pollution exposure, which is not seen with other pollutants, may be explained by their ability to activate both pathways simultaneously.

IL-6 is unique among the cytokines in its ability to induce an acute phase response and enhance the tendency toward thrombosis (30). Exposure of normal humans to ambient PM has often been associated with increases in IL-6 and its transcriptional targets fibrinogen, factor VIII, and C-reactive protein as well as alterations in the levels of platelet activation and coagulation factors (7, 8, 31). For example, in a recent observational study of healthy young people exposed to marked reductions in the levels of ambient PM during the Beijing Olympics, the number of subjects with detectable levels of C-reactive protein, which is transcriptionally regulated by IL-6, decreased from 55% in the pre-Olympic period to 36% in the post-Olympic period (31). The relevance of our findings to human exposure is supported by our observation that the release of IL-6 from human alveolar macrophages was augmented by the β_2 agonist albuterol and inhibited by the nonselective βAR antagonist propranolol.

We found that activation of β_2AR in alveolar macrophages augmented the NF-κB–mediated transcription of *Il6* via a mechanism that required adenylyl cyclase and CREB. However, in the absence of an inflammatory stimulus (PM), activation of β_2AR had no detectable effect on *Il6* transcription. These data reveal an important mechanism by which activation of the systemic nervous system can augment the innate immune response. The selectivity of this system is explained by a common requirement for mitochondrial ROS generation in the signaling pathways linking PM with the activation of NF-κB and the activation of CREB via β_2ARs and adenylyl cyclase. These findings suggest that mitochondrial ROS not only participate directly in the activation of NF-κB but also "prime" the cell to respond to catecholamines with enhanced inflammation. This mechanism is attractive, as the IL-6–mediated activation of coagulation will occur when stress and injury coexist, but will not occur in response to stress in the absence of injury. This mechanism also explains why this effect may have been missed in other studies examining the effect of β_2AR agonists on inflammatory cytokine levels; we only observed an effect of β_2AR agonists on IL-6 release when they were administered after PM exposure, allowing time for priming of adenylyl cyclase (22).

In our studies, we limited our examination to the effects of β_2AR agonists on PM-induced IL-6 release. However, our limited studies using LPS suggest this mechanism might be generalized to other stimuli that result in lung inflammation. If so, our findings suggest a potential mechanism for explaining the excess mortality observed in patients with inflammatory lung diseases treated with β_2AR agonists. In observational studies conducted in patients with asthma, investigators reported an association between the use of these drugs and a 4-fold increase in the risk of asthma-related mortality, prompting the US FDA to issue a black box warning regarding the safety of these agents (32–38). In contrast, the use of βAR blockers in patients with chronic obstructive pulmonary disease has been associated with a reduced risk of exacerbation (~30%–50%) and improved survival (39–42), despite the prediction that these agents might worsen bronchoconstriction through their effects on airway smooth muscle cells. In 2 large randomized trials of β_2 agonist therapy for patients with acute lung injury, treatment with the β_2 agonist of acute lung injury showed no benefit and a trend toward harm (43–45).

Using a murine model of acid aspiration and influenza A–induced lung injury, Imai and colleagues found that the release of IL-6 required the oxidation of plasma membrane phospholipids, which activated TLR4 to induce the TIR domain–containing adapter-inducing IFN-β–mediated (TRIF-mediated) and TNF receptor–associated factor–mediated (TRAF-mediated) activation

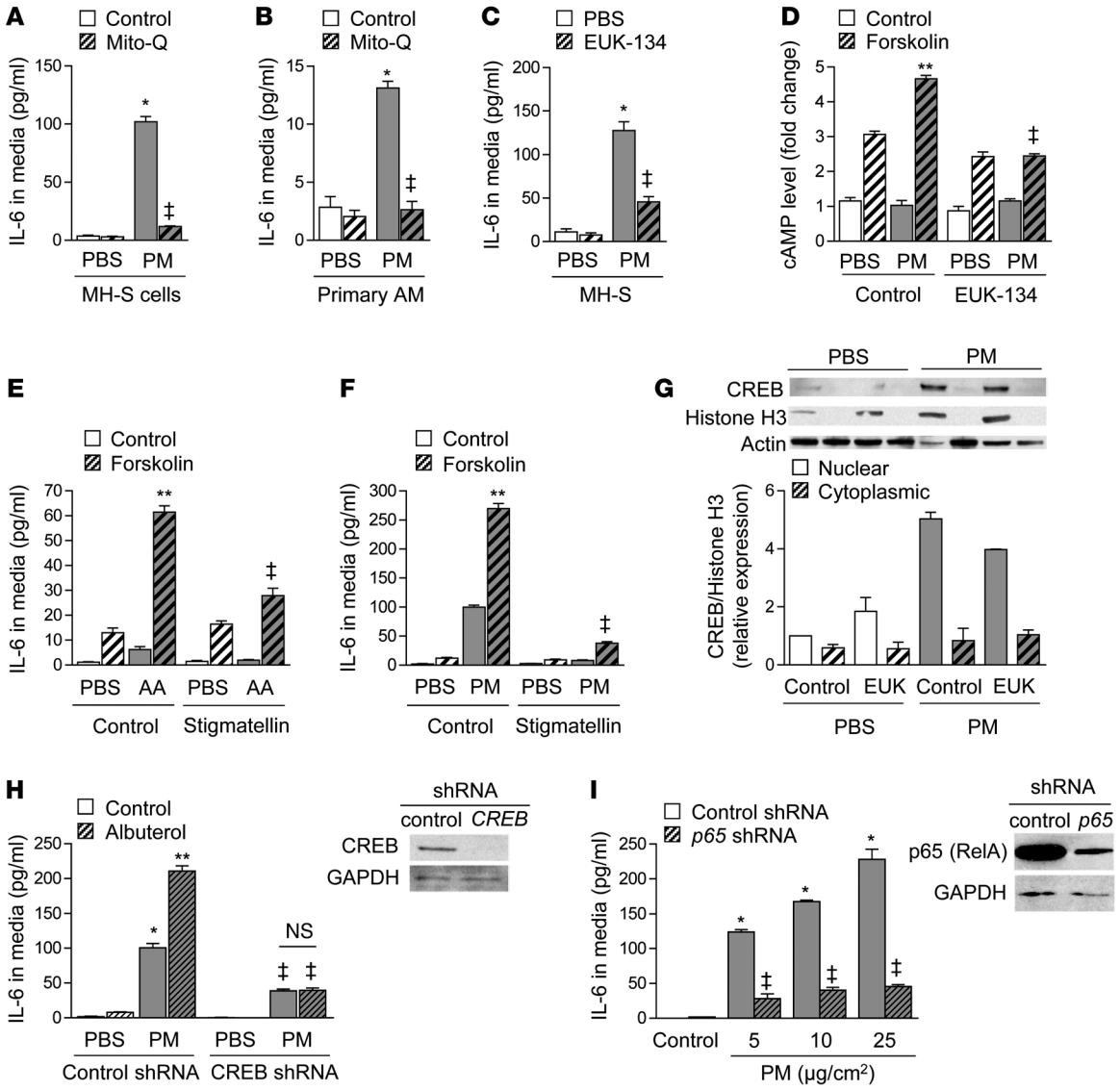

Figure 8

PM-induced ROS generation and priming of adenylyl cyclase are required for β_2 agonist–mediated worsening of IL-6 release. We treated (**A**) MH-S cells and (**B**) primary alveolar macrophages (AM) with PM (10 μg/cm²) or control (medium) and measured IL-6 levels in the absence or presence of a mitochondrially targeted antioxidant (Mito-Q or control [TPP]) or (**C**) a nontargeted antioxidant (EUK-134) (20 μM). (**D**) We treated MH-S cells with PM or control and with forskolin (50 μM) or control and measured cAMP levels 1 minute after forskolin treatment. (**E**) We treated MH-S cells with antimycin A (AA) (1 μM) or vehicle and with forskolin (50 μM) and measured IL-6 in the medium 24 hours later in the absence or presence of stigmatellin (1 μM). (**F**) We treated MH-S cells with PM or control and with forskolin (50 μM) or control and measured IL-6 levels 24 hours later in the absence or presence of stigmatellin. (**G**) We treated MH-S cells with PM or control and with EUK-134 or control and performed immunoblotting against cAMP CREB in the nuclear and cytoplasmic fractions 4 hours later. (**H**) We measured IL-6 levels in control and CREB shRNA–transfected MH-S cells after treatment with PM or PBS. (**I**) We treated control and p65-shRNA–transfected cells with PM or control and with albuterol or control and measured IL-6 levels. **P* < 0.05, PM vs. PBS; ***P* < 0.05, albuterol or forskolin vs. control; ‡*P* < 0.05, *Adrb1*$^{+/+}$*Adrb2*$^{-/-}$ vs. *Adrb1*$^{+/+}$*Adrb2*$^{+/+}$, AC inhibitor, mito-Q, EUK-134, or stigmatellin vs. control, CREB or p65 vs. control shRNA.

of NF-κB (46). We also found that the release of IL-6 in response to PM required mitochondrial ROS generation and NF-κB, suggesting it might occur via a similar pathway. An important finding of our study is that the NF-κB–mediated increase in IL-6 was not sufficient to activate systemic coagulation in the absence of a parallel activation of macrophage β_2ARs.

There are some limitations to our study. The *Lysm-Cre* system results in the expression of Cre recombinase in neutrophils in addition to monocytes and macrophages (47). As we and others have reported that PM exposure is associated with an influx of neutrophils into the lung, we cannot exclude the possibility that the loss of β_2ARs in neutrophils contributes

to the diminished thrombosis we observed in these animals (4, 48). In addition, our in vitro work raises important questions regarding the mechanisms by which particle exposure increases the generation of ROS from the mitochondria and the molecular targets of these ROS, which are responsible for the priming of adenylyl cyclase. In addition, we have previously shown that PM exposure increases the number of platelets via a mechanism that requires the release of IL-6 from alveolar macrophages. (4) While other groups have shown that PM can cause the activation of platelets (49), we have not been able to detect this in our model (4). In mice, epinephrine alone is unable to promote platelet shape change or aggregation (50–53), but it may potentiate the effect of known activators of platelets such as adenosine diphosphate (ADP) (51, 54, 55). Therefore, while a PM-induced increase in platelet number or activity may contribute to the development of ischemic cardiovascular events in humans, catecholamine-induced changes in platelet numbers or activation alone are unlikely to explain the effects of catecholamines in our model (56).

We did not investigate the mechanism or mechanisms by which PM leads to the activation and the release of catecholamines. In this regard, nerve fibers in the lung have been shown to be activated in response to acrolein (a component of cigarette smoke) or capsaicin through the activation of transient receptor potential (TRP) channels, a superfamily of cation channels that are present on the plasma membrane of many cells including nerve fibers (57, 58). Over 26 TRP channels have been described, and at least 2 of these channels, TRPA1 and TRPV1, have been reported to be activated by PM and play a role in PM-induced lung inflammation; however, it is not known whether they play a role in the PM-induced activation of the sympathetic nervous system (59, 60).

In conclusion, we have discovered a new molecular link between activation of the sympathetic nervous system and the innate immune response in the lung following exposure to PM air pollution. The systemic increase in catecholamines induced by PM air pollution exposure augments the release of IL-6 from lung macrophages via a pathway that requires mitochondrial ROS, adenylyl cyclase, and CREB. The resulting increase in IL-6 release contributes to the development of a hypercoagulable state. These results provide an important mechanism by which activation of the sympathetic nervous system following PM air pollution exposure can increase the risk of thrombotic cardiovascular events. Furthermore, these data provide a possible mechanism explaining the observed association between the use of β_2AR agonists and mortality in patients with inflammatory lung diseases and provide a rationale for the use of βAR blocking agents in these patients.

Methods

Human subjects

Nonbronchoscopic BALF was obtained for analysis as part of the clinical care of patients in an intensive care unit. Nonbronchoscopic BALF specimens were collected by trained respiratory therapists using a BAL-Cath (Kimberly-Clark Corporation). The therapist instilled 60 ml of nonbacteriostatic into the lung via the wedged catheter followed by aspiration of returned fluid (typically 10–30 ml). The intensivist caring for the patient chose the lung to be sampled, and the design of the catheter directed it to the dependent lobes of the lung. After the fluid was sent for clinical testing, 1 ml of the residual fluid was placed on ice and brought to the laboratory. The fluid was centrifuged at 600 *g* for 10 minutes, then washed and resuspended in 10 ml of RPMI medium with 10% FBS supplemented with penicillin, streptomycin, and amphotericin B. The cells were then counted (hemacytometer; Trypan Blue), and 100,000 cells were plated on Primaria Cell Culture 12-well plates (Corning). The cells were used 24 hours after plating.

Animals

We used 8- to 12-week-old male mice lacking either *Adrb1* or *Adrb2* or both *Adrb1* and *Adrb2* and their wild-type littermates as controls. Conditional deletion of *Adrb2* was achieved by crossing *Adrb2*$^{flox/flox}$ mice (a gift of Florent Elefteriou, Vanderbilt University, Nashville, Tennessee, USA, and Gerard Karsenty, Columbia University, New York, New York, USA) (61, 62) with *Lysm-Cre* mice (Jackson Laboratories strain B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J), both on the C57BL/6 background.

Antibodies and reagents

Propranolol was purchased from Hikma Farmaceutica/West Ward Pharmaceuticals. Albuterol was purchased from Nephron Pharmaceuticals. EUK-134 was purchased from Eukarion Inc. TRIzol was purchased from Life Technologies. CREB and p65 antibodies were purchased from Cell Signaling. All other chemicals were purchased from Sigma-Aldrich.

Exposure of mice to PM

Inhalational exposure to CAPs. We exposed mice to $PM_{2.5}$ CAPs (concentrated from ambient air in downtown Chicago) 8 hours per day for 3 days in a chamber connected to VACES (refs. 10, 63–65, and Supplemental Figure 1). The VACES system draws approximately 100 l/min of ambient air from an elevation of 9 m from which the PM is condensed and then resuspended for delivery to a chamber designed specifically to ensure uniform distribution of the particles. We exposed control mice to filtered air in an identical chamber connected to the VACES in which a Teflon filter was placed on the inlet valve to remove all particles. We estimated ambient $PM_{2.5}$ concentrations as the mean of reported values from the 4 EPA monitoring locations closest to our location (66). Particle counts in the chamber were measured with a TSI 3775 particle counter (Shoreview) and used to determine the enrichment in the chamber compared with the ambient air as previously described (10). The mean daily ambient $PM_{2.5}$ concentration in Chicago was 11.99 ± 0.68 μg/m^3 during the study period, and the mean concentration in the PM exposure chamber was 109.1 ± 6.18 μg/m^3. Chicago is the third largest city in the US, with approximately 2.7 million and 9.5 million residents in the city and metropolitan area, respectively. Interstate highways, railroads, and 2 major airports connect the city to other urban areas in the region. Major point sources of particulate air pollution include 2 coal-fired power plants and metal processing, paint, and solvent factories (the last being in the southern and southeast parts of the city) (67). Mobile source emissions account for a majority of atmospheric nitrogen compounds, while refineries, coal burning, and steel manufacturing are responsible for sulfur compounds (67). The composition of airborne PM is primarily sulfate and organic carbons and secondary nitrates (68). Particulate NO_3^-, SO_4^{2-}, and elemental carbon concentrations (2.5, 2.9, and 1.5 μg/m^3, respectively) approximate those in other major American cities (69).

Intratracheal instillation of PM. For intratracheal exposure experiments in mice, we used an urban PM collected from ambient air in Washington, DC (National Institute of Standards and Technology standard reference material, SRM 1649a). The characteristics of PM have been previously described (70, 71). We anesthetized the mice with isoflurane

and intubated them orally with a 20-gauge angiocath (4, 10, 72). We instilled either PM suspended in 50 μl of sterile PBS (vortexed prior to instillation) or PBS (control).

Collection of BALF and plasma and measurement of IL-6 and plasma TAT levels

We collected BALF and plasma 24 hours after exposure to PM or PBS or 3 days after exposure to CAPs or filtered air, as we have previously described (4, 10, 72). A 200 μl aliquot of the BALF was placed in a cytospin and centrifuged at 1,200 *g* for 5 minutes at 4°C. The supernatant was used to measure IL-6 and other cytokine levels using the Mouse Inflammation Kit and BD Cytometric Bead Array (BD Biosciences) as we previously described (4). We measured plasma TAT levels as previously described (1, 2).

$FeCl_3$ model of arterial thrombosis

After mice were anesthetized, the left carotid artery was dissected and isolated from the surrounding tissue with paraffin; the adventitia of the artery was treated with Whatman filter paper precisely cut to 1 mm in diameter and soaked in 10% $FeCl_3$. The carotid blood flow was continuously measured using Transonic TS420 Transit-Time Perivascular Flowmeter (Transonic Systems) until blood flow was reduced by more than 75% from baseline, which was used to define the time to loss of blood flow. The application of $FeCl_3$ led to a 2- to 3 mm-long carotid thrombus (Supplemental Figure 2 and Supplemental Video 1).

Cell culture and generation of MH-S cells with stable knockdown of CREB

The murine alveolar macrophage cell line (MH-S, ATCC) was cultured in RPMI medium in 10% FBS. Stably transfected cell lines were generated by infection with lentiviruses encoding a scrambled shRNA or shRNA constructs against CREB and selected with puromycin (5 μg/ml). The lentiviruses were generated by cotransfecting HEK293 cells with pLKO.1-puro plasmids using the Mission Lentiviral Packaging DNA according to the manufacturer's recommendations (SHP001, SHCLNG NM_1333828 [*CREB*], and SHC312 [scrambled control]; Sigma-Aldrich). Primary alveolar macrophages were obtained as previously described by repeated bronchoalveolar lavage with PBS containing 0.6 mM ethylenediaminetetraacetic acid (4). The primary alveolar macrophages were plated and cultured in RPMI medium in 10% FBS.

Tissue homogenization

The lung, heart, adrenal glands, and uterus were removed after the right ventricle was perfused in situ with sterile PBS until the lungs and liver were clear. Brown fat was isolated from the midscapular region of the back. All organs were kept on ice prior to and during homogenization (Tissue Tearor, 30 s) in a flow cytometry tube with 1 ml of PBS. An additional 2 ml of PBS was added to the resulting homogenate and subjected to Dounce homogenization (20 strokes).

Assessment of CREB nuclear translocation

Approximately 10^7 cells per condition (10 cm dishes) were removed from the plate with trypsin and washed twice in PBS (200 *g*, 10 minutes). The cell pellet was suspended in 2 ml of NP-40 buffer (100 ml of Chelsky buffer [0.01M Tris-HCl, 0.01M NaCl, 0.003 M $MgCl_2$, 0.03 M sucrose, pH 7.0] and 500 μl NP-40), then centrifuged twice (HB-40 rotor, 1500 *g*, 10 minutes). The supernatants from the washes were used for immunoblotting (cytosolic fraction). The nuclear pellet was washed twice in 2 ml $CaCl_2$ buffer (100 ml Chelsky buffer, 1 ml 10 mM $CaCl_2$) and centrifuged (HB-40 rotor, 1500 *g*, 10 minutes). The resulting pellet was resuspended in 1 ml of buffer (20 mM Tris-HCl, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 M EDTA, pH 7.9) and centrifuged (SS43 rotor, 25,000 *g*, 30 minutes), and the supernatant (nucleoplasm) was used for immunoblotting (nuclear fraction).

Measurement of IL-6

Cell supernatants or BALF was centrifuged at 300 *g* for 10 minutes, and IL-6 levels were measured using a commercially available ELISA according to the manufacturer's instructions (Invitrogen).

Quantitative real-time RT-PCR (qRT-PCR)

We isolated total RNA from mouse lung using a commercially available system (TRIzol; Invitrogen) and performed qRT-PCR reactions for *Il6*, SP-B, TF, *Creb*, and *Adrb2* using IQ SYBR Green superscript analyzed on a Bio-Rad IQ5 Real-Time PCR Detection System using previously described primer sequences and the Pfaffl method (10). The following primer sequences were used: IL-6, forward: CCGGAGAACCTGCTGGCAATCC, IL-6, reverse: TTCCATCCAGTTGCCTTCTTGG; SP-B, forward: GCCTTGTCCTCGGATGTTC, SP-B, reverse: TAGCCTGTTCACTGGTGTTC; β_2AR, forward: ATCTGAAGGAAGATTCCACGCCCA, β_2AR, reverse: AGAGGGTGAATGTGCCCATGATGA.

Measurement of cAMP

cAMP levels in cell lysates were measured using a cAMP immunoassay kit according to the manufacturer's directions (Assay Designs). Treatment with forskolin, a direct activator of adenylyl cyclase (100 μM, 1 minute) (Ascent Scientific) or control vehicle (2% ethanol) was used as a positive control.

Measurement of catecholamine levels

Catecholamine levels in plasma, BALF, homogenized lung, brown adipose tissue (BAT), and adrenal gland were measured using high-pressure liquid chromatography with an electrochemical detector (LC-4C Detector; Bioanalytical Systems Inc.) as we previously described (73, 74).

Measurement of inflammatory cell populations in dispase-digested lungs

After the mice were euthanized, lungs were perfused through the right ventricle with 5 ml of PBS. The lungs were removed, and the large airways were dissected from the peripheral lung tissue. The peripheral lung tissue was cut into small pieces with scissors, transferred into C-tubes (Miltenyi Biotec), and processed in digestion buffer (1 mg/ml of collagenase D and 0.1 mg/ml DNase I, both from Roche in HBSS) and a gentleMACS dissociator (Miltenyi Biotec), as we have recently described (75).

Cells were stained with viability dye Aqua (Invitrogen) or eFluor 506 (eBioscience), incubated with FcBlock (BD Biosciences), and stained with a mixture of fluorochrome-conjugated antibodies. Data were acquired on a BD LSR II Flow Cytometer using BD FACSDiva software (BD Biosciences), and compensation and data analyses were performed offline using FlowJo software (TreeStar). Cell sorting was performed on a FACSAria II instrument (BD Biosciences) with the same configuration as the LSR II. Cell populations were identified using sequential gating strategy (see Results), and the percentage of cells in the live/singlets gate was multiplied by the number of live cells (after Trypan blue exclusion) to obtain an absolute live-cell count.

Statistics

We report all data as mean ± SEM. We subjected all data to 1-way ANOVA. When ANOVA indicated a significant difference, we explored individual

differences with 2-tailed Student's *t* test using Bonferroni's correction for multiple comparisons (Prism 6; Graphpad). Statistical significance was defined as $P < 0.05$.

Study approval

All animal experiments and procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at Northwestern University. All studies using samples obtained from human subjects were approved by the Northwestern University Institutional Review Board with a waiver of consent.

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Address correspondence to: Gökhan M. Mutlu, Pulmonary and Critical Care Medicine, Northwestern University Feinberg School of Medicine, 240 E. Huron Street, McGaw M-300, Chicago, Illinois 60611, USA. Phone: 312.908.8163; Fax: 312.908.4650; E-mail: g-mutlu@northwestern.edu.

Gökhan M. Mutlu's present address is: Section of Pulmonary and Critical Care Medicine, University of Chicago, Chicago, Illinois, USA.

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