Metformin Targets Mitochondrial Electron Transport to Reduce Air-Pollution-Induced Thrombosis

Graphical Abstract

Highlights
- Metformin prevents IL-6-dependent thrombosis induced by urban particulate matter.
- Metformin inhibits mitochondrial complex I to prevent ROS-mediated IL-6 release.
- Metformin inhibits mitochondrial ROS to prevent CRAC channel activation.
- Mitochondrial ROS and CRAC channel inhibition in vivo prevent thrombosis.

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In Brief
Air pollution exposure has been linked to a variety of poor health outcomes, including an increased risk of death attributable to ischemic cardiovascular events. Soberanes et al. find that metformin may hold promise as a therapy, as its capacity to act as a mitochondrial complex I inhibitor prevents accelerated thrombosis in a murine model of particulate-matter inhalation.
Metformin Targets Mitochondrial Electron Transport to Reduce Air-Pollution-Induced Thrombosis

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SUMMARY

Urban particulate matter air pollution induces the release of pro-inflammatory cytokines including interleukin-6 (IL-6) from alveolar macrophages, resulting in an increase in thrombosis. Here, we report that metformin provides protection in this murine model. Treatment of mice with metformin or exposure of murine or human alveolar macrophages to metformin prevented the particulate matter-induced generation of complex III mitochondrial reactive oxygen species, which were necessary for the opening of calcium release-activated channels (CRAC) and release of IL-6. Targeted genetic deletion of electron transport or CRAC channels in alveolar macrophages in mice prevented particulate matter-induced acceleration of arterial thrombosis. These findings suggest metformin as a potential therapy to prevent some of the premature deaths attributable to air pollution exposure worldwide.

INTRODUCTION

Exposure to particulate matter air pollution is a major public health concern. In the developed world, it is estimated that lifespan would be extended for 0.8 years for every 10 μg/m³ fall in the mean levels of particulate matter (PM) less than 2.5 μm in size, and exposure to PM was recently estimated to increase all-cause mortality in the United States Medicare Population by 7% (Di et al., 2017; Pope et al., 2009). In the developing world, the large number of urban dwellers and the very high levels of PM suggest effects of PM air pollution exposure on health are even more substantial (WHO Regional Office for Europe, 2013). While air pollution exposure has been linked to a variety of poor health outcomes, the major driver of mortality is an increased risk of death attributable to ischemic cardiovascular events, primarily heart attacks and ischemic/thrombotic strokes (Pope et al., 2009).

Urban PM air pollution consists of a core of ash or carbon decorated by organic molecules and metals that condense onto their surface during the combustion of fossil fuels (Nel, 2005). These latter features distinguish urban particulates from desert dust, volcanic ash, and wood smoke. To cope with life in dusty or smoky environments, mammals have evolved efficient mechanisms to clear ambient particles. For example, alveolar macrophages and mucociliary clearance efficiently clear carbon-based nanomaterials from the lungs to the larynx/feces with minimal or no inflammation (Duch et al., 2011; Semmler-Behnke et al., 2007). In contrast, we and others have shown that urban PM air pollution induces the release of pro-inflammatory cytokines, including interleukin-6 (IL-6) from alveolar macrophages before the particles are cleared. Alveolar macrophage-produced IL-6 enters the circulation to induce the transcription of several coagulation factors in the liver, and augments the tendency toward arterial thrombosis in a murine model of stroke—findings lacking in mice deficient in IL-6 (Chiarella et al., 2014; Mutlu et al., 2007). Key findings of this model were recently confirmed in humans in an interventional trial of filtered air compared with ambient air conducted in a region of China with high levels of ambient PM (Li et al., 2017). Accordingly, small molecules with acceptable risk profiles that can attenuate IL-6 release in response to PM in this model are predicted to lower the risk of arterial thrombosis in exposed populations.
RESULTS

Metformin Prevents Increased Susceptibility to Arterial Thrombosis after Injury Induced by Exposure to PM

We treated mice with a therapeutic dose of metformin in their drinking water (100 mg/kg/day) beginning 24 hr before exposure to concentrated ambient PM (CAPs, PM_{2.5}) air pollution via inhalation (8 hr per day on three consecutive days). Metformin reduced the PM-induced acceleration of the time to carotid occlusion after ferric chloride-induced injury (a model of ischemic stroke) to a level that was similar to those measured in mice exposed to filtered air (Figures 1A and 1B). We have previously reported that the release of IL-6 from alveolar macrophages is necessary for this response (Mutlu et al., 2007). Accordingly, we measured the expression of the Il6 gene in alveolar macrophages, which was reduced in the metformin-treated mice compared to those treated with filtered air (Figures 1C and 1D). These results suggest that metformin may reduce the risk of PM-induced thrombosis in response to exposure to PM, highlighting the potential therapeutic benefits of metformin in reducing the risk of cardiovascular events associated with PM exposure.
animals (Figure 1C). Metformin inhibited PM-induced IL-6 protein release in response to PM in alveolar macrophages ex vivo (Figure 1D). Metformin also inhibited the PM-induced increase in IL-6 in bronchoalveolar lavage (BAL) fluid, in alveolar macrophages obtained from mice 24 hr after PM was administered intratracheally, and in a murine alveolar macrophage cell line (MHS) (Figures S1A–S1D).

Metformin Inhibits Electron Transport and PM-Induced Mitochondrial ROS Generation in Alveolar Macrophages

We assessed the ability of systemically administered metformin to inhibit mitochondrial respiration in alveolar macrophages by measuring the ratio of oxidized to reduced nicotinamide adenine dinucleotide (NAD+/NADH) in alveolar macrophages immediately after their isolation. The ratio of NAD+/NADH was significantly reduced in primary alveolar macrophages from mice treated with metformin in their drinking water when measured by mass spectroscopy or a colorimetric test (Figures 1E and S1E). To determine the role of metformin on the generation of mitochondrially derived ROS, we continuously measured the fluorescence of an oxidant-sensitive mitochondrially localized dye (MitoSOX) in primary murine alveolar macrophages on the stage of an epifluorescent microscope. Oxidation of the dye was observed within 2 min of adding PM to the perfusate and was reduced in cells pretreated with metformin before PM exposure (Figures 1F and 1G).

Metformin Prevents the Generation of Mitochondrial ROS to Reduce PM-Induced IL-6 Release

To determine whether metformin inhibits PM-induced mitochondrial ROS generation through its effects on complex I of the mitochondrial electron transport chain, we generated an alveolar macrophage cell line (MHS) stably transfected with lentiviral vectors encoding a yeast protein ND11, which can transfer electrons from NADH to the ubiquinone pool without blocking electron transport and increases ROS generation at complex III by reducing electron flow from the upstream complex I. Accordingly, we treated cells with a suppressor of superoxide production from mitochondrial complex III, which were necessary but not sufficient for PM-induced IL-6 release.

Metformin Inhibits PM-Induced Opening of Calcium-Release-Activated Ca²⁺ Channels

The rapidity of the oxidant response to PM prompted us to examine changes in intracellular calcium in response to PM. When primary alveolar macrophages were perfused with calcium-free medium and treated with PM, we observed a small increase in cytosolic calcium suggestive of endoplasmic reticulum calcium store depletion. Subsequent perfusion with calcium-replete medium was associated with a substantial increase in cytosolic calcium. Both of these responses were inhibited by metformin (Figure 3A). The increase in cytosolic calcium suggested that PM might promote opening of calcium-release-activated Ca²⁺ channels (CRAC). Indeed, the PM-induced increase in cytosolic calcium was inhibited by the CRAC channel inhibitors, Synta-66 and 2-2-aminoethoxydi-phenyl borate (2-ABP) as well as the calcium chelator, 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA) (Figure 3B). Treatment of primary murine alveolar macrophages with Synta-66 also attenuated PM-induced release of IL-6 (Figure 3C). Metformin did not inhibit store depletion or CRAC channel activation in response to thapsigargin (Figure S3A). Consistent with these findings, MHS cells stably transfected with lentiviruses encoding shRNAs targeting Strn1 or Orai1, both components of the CRAC channel, showed reductions in PM-induced increases in intracellular calcium in calcium-replete medium, and reductions in PM-induced IL-6 release, compared with control transfected cells (Figures 3D–3F, S3C, and S3D).
We have previously shown that PM-induced IL-6 release requires NF-κB activation and is augmented by cAMP response element binding protein (Chiarella et al., 2014). In other systems, CRAC channel activation enhances inflammatory gene expression through the activation of nuclear factor of activated T cells (NFAT) (Jairaman et al., 2015, 2016). Consistent with this mechanism, pretreatment of MHS cells with the calcineurin inhibitor cyclosporine A attenuated PM-induced IL-6 release without affecting PM-induced ROS generation or calcium release (Figures S3E–S3G).

Exposure to Particulate Matter Air Pollution Induces the Opening of CRAC Channels Downstream of Mitochondrial ROS Generation

Treatment of MHS cells with Synta-66 before PM administration or knockdown of Orai1 did not affect PM-induced MitoSOX oxidation, suggesting the effects of PM on mitochondrial ROS generation are independent of CRAC channel opening (Figure 4A). Metformin inhibited the PM-induced increase in cytosolic calcium in MHS cells transfected with a control vector, but not in MHS cells that stably expressed NDI1 (Figures 4B

Figure 2. Metformin Inhibits Mitochondrial Electron Transport Complex I to Limit PM-Induced ROS Generation from Complex III

(A–C) A murine alveolar macrophage cell line (MHS) was stably transfected with a lentivirus encoding GFP and NDI1, a yeast protein capable of transferring electrons from NADH to complex II/III but incapable of ROS generation, or GFP alone. These cells were exposed to PM (10 µg/m³) in the presence or absence of metformin and oxygen consumption (Seahorse XF Analyzer) (A) and the oxidation of MitoSOX (B) were measured 4 hr later; (C) the release of IL-6 into the medium was measured 24 hr later (minimum of eight replicates per measurement, *p < 0.05).

(D and E) MHS cells were treated with a suppressor of superoxide production from complex III (S3QEL) or complex I (S1QEL) (both at 5 mM) and mitochondrial ROS generation was measured (D) immediately after PM exposure as in (F); (E) IL-6 release was measured 4 hr later (n = 3, *p < 0.05).

(F and G) MHS cells were treated with a selective activator of AMPK A-79662 (2 mM) or vehicle and phosphorylation of AMPK (F) and PM-induced IL-6 release (G) were measured.

(H and I) MHS cells were treated with a dose of antimycin A (10 µM) or mitochondrially targeted paraquat (5 µM) selected to restore mitochondrial ROS to levels similar to PM alone, or in the presence of low dose PM, for measurement of MitoSOX oxidation (H, as in F) and IL-6 release (I) after 4 hr (n = 3–6 per condition, *p < 0.01).

See also Figure S2.
and 4C). These results suggest that metformin prevents CRAC channel opening in response to PM via its ability to inhibit mitochondrial ROS generation. Indeed, administration of S3QEL or the complex I inhibitor piercidin A prevented the PM-induced store depletion, CRAC channel activation, and IL-6 release, while the inactive control compound had no effect (Figures 4D, 4E, and S4A). These results suggest that metformin prevents CRAC channel opening in response to PM via its ability to inhibit mitochondrial ROS generation. Indeed, administration of S3QEL or the complex I inhibitor piercidin A prevented the PM-induced store depletion, CRAC channel activation, and IL-6 release, while the inactive control compound had no effect (Figures 4D, 4E, and S4A). Further, the complex I inhibitor piercidin A prevented the PM-induced store depletion, CRAC channel activation, and IL-6 release, while the inactive control compound had no effect (Figures 4D, 4E, and S4A).

**Genetic Deletion of Mitochondrial Electron Transport Prevents the Increase in IL-6 in Response to PM**

The intratracheal administration of PM did not induce the recruitment of inflammatory cells to the lung, suggesting tissue-resident alveolar macrophages are responsible for the acute effects of PM in vivo (Figures S5A and S5B). This allows us to determine the importance of mitochondrial ROS generation in the response to PM in vivo by generating mice lacking the nuclear-encoded mitochondrial transcription factor A (TFAM) in macrophages (CrtCD11c/Tfamflox/flox). TFAM is a nuclear-encoded gene that is required for the transcription of genes encoded by mitochondrial DNA, including 13 genes that encode necessary components of most complexes within the electron transport chain (Fisher et al., 1992). Flow sorting of myeloid cell populations of 8- to 12-week-old CrtCD11c/Tfamflox/flox and Tfamflox/flox mice showed similar numbers of alveolar macrophages and other myeloid cell populations in the lung and no obvious morphologic changes (Figure S5C). Flow-sorted alveolar macrophages from CrtCD11c/Tfamflox/flox exhibited reduced levels of Tfam mRNA while these levels were normal in neutrophils (Figure S5A). When we treated CrtCD11c/Tfamflox/flox mice with PM intratracheally, the acceleration of carotid thrombosis in response to PM was attenuated, and BAL fluid levels of IL-6 were reduced compared with...
Figure 4. PM-Induced CRAC Channel Activation Occurs Downstream of Mitochondrial ROS Generation

(A) MHS cells were stably transfected with lentiviruses encoding shRNA against Stim1 or Orai1 or a scrambled shRNA and treated with vehicle or PM (10 μg/cm²) and the oxidation of MitoSOX dye was measured (n = 3, *p < 0.05).

(B) MHS cells transfected with GFP or GFP-NDI1 were treated with PM with or without metformin (1 mM) and changes in intracellular calcium upon the addition of calcium-replete medium were measured. A representative tracing is shown. Thapsigargin (TG) (25 nM) was added to the cells at the end of each experiment. See also Figure S4.

(C) Peak calcium measures after the addition of calcium-replete medium in cells treated with PM with or without metformin (1 mM) (n = 3, *p < 0.05); refers to (B).

(D) MHS cells were treated with S3QEL (5 μM) 1 hr before treatment with PM (10 μg/cm²) and immediate changes in intracellular calcium and IL-6 release into the media after 24 hr were measured. A representative tracing is shown. Thapsigargin (TG) (25 nM) was added to the cells at the end of each experiment. See also Figure S4.

(E) Peak calcium measurements after the addition of calcium-replete medium (n = 3, *p < 0.05); refers to (D).

(F) MHS cells were pretreated for 1 hr with the PLC inhibitor U73122 or the inactive control compound U73343 (both 200 nM), and changes in intracellular calcium upon the addition of calcium-replete medium were measured. A representative tracing is shown. Thapsigargin (TG) (25 nM) was added to the cells at the end of each experiment.

(G) Peak calcium measurements after the addition of calcium-replete medium (n = 3, *p < 0.05); refers to (F).

(H) MHS cells were pretreated for 1 hr with the PLC inhibitor U73122 or the inactive control compound U73343 (both 200 nM), and IL-6 release (24 hr) was measured. (n = 3, *p < 0.05).

(I) MHS cells were pretreated for 1 hr with the PLC inhibitor U73122 or the inactive control compound U73343 (both 200 nM), and MitoSOX oxidation was measured. (n = 3, mean results with SEM are shown).
Tfam<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> controls (Figure 5B). To confirm our in vitro findings suggesting mitochondrial ROS were required for the generation of IL-6 in response to PM, we treated mice with Mito-TEMPO subcutaneously beginning 1 day before PM exposure. Treatment with Mito-TEMPO also prevented PM-induced thrombosis (Figure 5C).

Consistent with these findings, IL-6 levels were reduced in primary alveolar macrophages isolated by BAL from Cre<sup>CD11c</sup>/Tfam<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> mice compared with those from Tfam<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> mice (Figure 5D).

Similarly, alveolar macrophages from Cre<sup>CD11c</sup>/Tfam<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> mice failed to generate ROS or increase intracellular calcium in response to PM (Figures 5E–5H).

**Genetic Deletion of Orai1 in Alveolar Macrophages Prevents the Increase in IL-6 in Response to PM**

We used a similar strategy to examine the importance of CRAC channel activation in the response to PM in vivo.
Orai1 was efficiently deleted in alveolar macrophages from CD11cCre/Orai1flox/flox animals compared with Orai1flox/flox controls (Figure 6A). The acceleration of carotid thrombosis induced by the intratracheal administration of PM in Orai1flox/flox controls was absent in CD11cCre/Orai1flox/flox mice (Figure 6B). Primary alveolar macrophages from CreCD11c/Orai1flox/flox mice showed reduced levels of IL-6 production and intracellular calcium release in response to PM compared with those from Orai1flox/flox mice (Figures 6C–6E). Consistent with these findings, the intratracheal administration of Synta-66 to mice simultaneously with the intratracheal instillation of PM (10 μg/mouse) and the levels of IL-6 in the BAL were measured 6 hr later (n = 3, *p < 0.05).

Metformin Prevents PM-Induced ROS Generation, CRAC Channel Activation, and IL-6 Production in Human Macrophages

Our data suggest that PM induces the generation of mitochondrial ROS, which augment IL-6 release by activating CRAC channels. To confirm the potential importance of these findings in humans, we measured mitochondrial ROS generation and calcium levels in human alveolar macrophages after treatment with metformin and/or Synta-66. Consistent with our findings in murine alveolar macrophages, metformin inhibited mitochondrial ROS and calcium-mediated activation of IL-6, we reasoned it would likely affect other processes in alveolar macrophages triggered by exposure to PM. Therefore, mice were treated in the drinking water with metformin for 24 hr before we instilled PM intratracheally. Alveolar macrophages were flow sorted from whole-lung homogenates 24 hr later for transcriptomic analysis (RNA-seq) and metabolomic analysis. Metformin treatment resulted in significant changes in the measured metabolites in alveolar macrophages, including reductions in glycolytic and tricarboxylic acid cycle intermediates, as well as high-energy phosphates, with little change in amino acids (Figure S6). While the overall change in metabolites was highly significant (p < 0.001 by ANOVA), individual metabolites did not reach significance after corrections for multiple comparisons.

**Figure 6. CRAC Channel Activation Is Necessary for PM-Induced Acceleration of Carotid Thrombosis**

(A) Levels of Orai1 mRNA in flow-sorted alveolar macrophages and neutrophils from mice deficient in Orai1, a necessary component of CRAC channels, in alveolar macrophages. (n = 3, *p < 0.05).

(B) Mice were treated with PBS or PM (10 μg, intratracheally), and 24 hr later, the time to cessation of carotid artery blood flow after a standardized ferric chloride injury was measured in Orai1flox/flox mice and CreCD11c/Orai1flox/flox mice (n = 4, *p < 0.05).

(C) Primary alveolar macrophages were treated with PM and the levels of IL-6 were measured 24 hr later (n = 3, *p < 0.05).

(D) Primary alveolar macrophages were loaded with Fura-2 for measurement of intracellular calcium levels after PM exposure in calcium-free medium followed by calcium-replete medium. Representative tracings are shown. Thapsigargin (TG) (25 nM) was added to the cells at the end of each experiment.

(E) Peak calcium measurements after the addition of calcium-replete medium (n = 3, *p < 0.05); refers to (D).

(F) Wild-type mice were treated with Synta-66 (10 μM) intratracheally or vehicle simultaneous with the intratracheal instillation of PM (10 μg/mouse) and the levels of IL-6 in the BAL were measured 6 hr later (n = 3, *p < 0.05).
(false discovery rate [FDR] p < 0.05) (Table S1). Metformin treatment alone had a relatively minor effect on gene expression in alveolar macrophages in the steady state (476 differentially expressed genes, FDR p < 0.05), but the response to PM exposure differed substantially when compared with untreated animals (1,313 differentially expressed genes, FDR p < 0.05) (Figures 7E and 7F, Table S1). To better understand the changes induced by PM and metformin, we performed k-means clustering of the 1,285 differentially expressed genes identified by an ANOVA-like test, FDR p < 0.001 (Figure 7G, Table S1). Genes downregulated after PM exposure (cluster 1) include those related to signal transduction and the cellular developmental process. Consistent with our findings, the genes upregulated in response to PM and relatively downregulated by metformin (cluster 2) included ROS biosynthetic process, neutrophil chemotaxis, and the acute phase response. Genes that were downregulated upon administration of PM and metformin (cluster 4) were associated with platelet aggregation and cell division (spindle assembly).

Surprisingly, many of the genes that were upregulated in response to PM and metformin treatment compared with PM treatment alone (cluster 3) were related to the unfolded protein response, negative regulation of intracellular signal transduction, and regulation of the response to stress. These findings suggest metformin induces a stress response that could indirectly suppress inflammatory signaling. To explore this hypothesis further, we examined the expression of a curated list of chaperone proteins in our dataset, which showed that many genes identified as human chaperones were upregulated in alveolar macrophages from metformin- and PM-treated animals (Figure 7H).

We confirmed upregulation of chaperone genes using gene set enrichment analysis (enrichment score 0.32, FDR p = 0.017, Figure 4I) (Subramanian et al., 2005; Brehme et al., 2014). Using MHS cells transfected with ND1, we showed that the increase in the selected chaperones in metformin- and PM-treated cells was dependent on the ability of metformin to inhibit complex I (Figure 7J). These data suggest the intriguing hypothesis that complex I inhibition by metformin may also attenuate inflammatory signaling pathways indirectly by triggering global cellular stress response pathways that protect against macromolecular damage.

**DISCUSSION**

We found that the commonly used drug metformin can attenuate PM-induced IL-6 release from alveolar macrophages and reduce the resulting increase in the risk of arterial thrombosis after injury. Metformin acted as a complex I inhibitor in alveolar macrophages to reduce mitochondrial ROS from complex III of the mitochondrial electron transport chain in response to PM. In alveolar macrophages from mice and humans, PM-induced mitochondrial ROS generation caused endoplasmic reticulum calcium store depletion, and the opening of store-operated calcium channels, which augmented IL-6 release. Mitochondrial ROS alone were insufficient to induce store depletion or IL-6 release but acted in concert with particles to augment these responses. Our results provide genetic evidence in vivo to support the importance of signaling by mitochondrial ROS and CRAC channels in the release of IL-6 from alveolar macrophages and in the accelerated thrombosis after carotid artery injury induced by PM air pollution exposure.

In a murine alveolar macrophage-like cell line, we could restore PM-induced signaling events by overexpressing the yeast protein ND11, which can transfer electrons from NADH to the ubiquinone pool but cannot generate ROS and is insensitive to metformin (Seo et al., 1998). Using small molecules that suppress superoxide production specifically at complex III or complex I, we show that ND11 restores electron flux to complex III to restore mitochondrial ROS generation. These data are consistent with previous studies showing that metformin inhibits the generation of ROS induced by reverse electron transport (Bantandier et al., 2006; Bridges et al., 2014). An interesting aspect
of our study is the relationship between mitochondrial ROS and calcium release. Our findings using inhibitors implicate the activation of PLC in the store depletion induced by mitochondrial ROS upon PM exposure, but additional studies are required to better understand these pathways.

Inhibition of electron transport by metformin activates AMPK, which is proposed as a mechanism for its antidiabetic effects. Our finding that mitochondrially targeted antioxidants mimicked the effects of metformin in vitro and in vivo argue against a direct role for electron transport inhibition or AMPK activation in this protection. Furthermore, when we treated primary alveolar macrophages or mice with a direct activator of AMPK, PM-induced IL-6 release was unaffected. Unbiased transcriptional profiling of alveolar macrophages from metformin-treated animals using RNA-seq showed only small differences between metformin-treated and untreated animals, however, there were marked differences in their response to PM. Specifically, alveolar macrophages from metformin-treated animals showed a significant upregulation of chaperone genes involved in proteostasis in response to PM when compared with untreated control animals. Using an alveolar macrophage cell line, we showed that this chaperone response could be attributed to metformin’s ability to inhibit complex I. It is possible that this response is further attributable to the activation of AMPK, although others have reported that AMPK activation results in downregulation of proteostasis genes (Dai et al., 2015).

Our findings may have implications for humans. For example, in a recent study of people residing in an area of China with high levels of PM exposure, treatment with a respiratory filter to reduce PM exposure resulted in decreased levels of oxidative stress, C-reactive protein (CRP) and fibrinogen (both transcriptional targets of IL-6), and catecholamines, thereby validating key components of the mouse model we used in this study (Li et al., 2017). Our finding that metformin prevented PM-induced IL-6 release from human alveolar macrophages supports a similar trial with metformin in high-risk individuals, particularly given its low cost and safety. In addition, the finding that metformin reduces PM-induced IL-6 release may partially explain the association between metformin use and reduced levels of CRP and cardiovascular risk observed in patients with type 2 diabetes (Maruthur et al., 2016). Consistent with this hypothesis, IL-6 and CRP have both been identified as independent risk factors for the development of ischemic cardiovascular disease, and the administration of an inhibitor of IL-1β reduced IL-6 levels, CRP, and cardiovascular risk in a recent randomized clinical trial (Ridker, 2016; Ridker et al., 2017). Furthermore, the unexpected finding that metformin activated proteostasis genes and inhibited inflammation might explain some of the protection against age-related phenotypes in mice administered metformin and provide an additional rationale for proposed studies to administer metformin to humans to prevent the accumulation of age-related phenotypes (Barzilai et al., 2016).

Limitations of Study

First, the dose of metformin we used in mice achieves plasma/tissue concentrations similar to those measured in humans on standard treatment doses for type 2 diabetes (Chandel et al., 2016), but it is not known if metformin is effectively taken up by human alveolar macrophages in vivo. Second, we used urban PM obtained from the air around our laboratories in Chicago, and from the National Institute of Standards and Technology from air in Washington, DC, both of which have been characterized (Mutlu et al., 2018; Poster et al., 1999). As the epidemiologic link between PM exposure and cardiovascular disease is not dependent on geography, our results are likely to be broadly applicable (Lelieveld et al., 2015). Nevertheless, it is possible PM from some regions may act via additional mechanisms. Third, the Cre driver we used to delete Tfam and Orai1 in alveolar macrophages also targets dendritic cells in the lung. This is perhaps less important for Tfam, which only affects mitochondrial biogenesis, as tissue-resident alveolar macrophages persist for months without input from the bone marrow, while both CD11b+ and CD103+ dendritic cells turn over relatively rapidly (1–2 weeks) in the lung (Misharin et al., 2014, 2017). Fourth, the loss of TFAM in macrophages will impair ATP production, depolarize the mitochondrial membrane, and reduce the supply of metabolic intermediates, among other effects. Therefore, our results using these animals should be interpreted in the context of our in vitro and in vivo studies. Fifth, our data using the inhibitor U73122 implicate activation of PLC by mitochondrial ROS when PM are present in the depletion of calcium from ER stores and activation of CRAC channels. The precise molecular targets of mitochondrial ROS and PM in this pathway, however, are not known. Finally, while we show the administration of PM results in rapid (<2 min) generation of mitochondrial ROS, dissecting the precise molecular mechanisms by which this occurs will require further study.

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Supplemental Information includes six figures and one table and can be found with this article online at https://doi.org/10.1016/j.cmet.2018.09.019.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

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**STAR METHODS**

### KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact: Scott Budinger (s-buding@northwestern.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human Subjects
All studies using samples obtained from human subjects were approved by the Northwestern University Institutional Review Board. All human subjects provided written informed consent prior to enrolment into the study. The following inclusion criteria were applied: donor lung is suitable for transplant, recipient provided written informed consent, sufficient amount of tissue was provided for isolation of alveolar macrophages. Information about donor’s sex, health status, previous exposure to metformin or other drugs is not available.

Mouse Models
All animal experiments and procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at Northwestern University. C57BL/6J mice were bred in our facility and our colonies are refreshed yearly with mice purchased from the Jackson Laboratory. CreCD11c mice were purchased from the Jackson Laboratory and bred in house. Tfam<sup>fl/fl</sup> mice were generated by Ozgene as we have previously described (Hamanaka et al., 2013). Orai1<sup>fl/fl</sup> mice were provided by Amgen and generated as described (Somasundaram et al., 2014). All experiments were performed with littermate controls. Number of animals per group was determined based on our previous publications. Ten- to sixteen-week-old male mice were used for experiments. Investigators were not blinded to the group allocation. Mice were housed at the Center for Comparative Medicine at Northwestern University, in microisolator cages, with standard 12 hr light/darkness cycle, ambient temperature 23°C14°C and were provided standard rodent diet (Envigo/Teklad LM-485) and water <i>ad libitum</i>.

Murine Model of PM Exposure
Inhalational exposure to PM<sub>2.5</sub> CAPs was performed as previously described (Chiarella et al., 2014). Briefly, mice were housed 8 hr per day for 3 consecutive days in a chamber connected to a Versatile Aerosol Concentration and Exposure System (VACES). 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<sub>2.5</sub> concentrations as the mean of reported values from the 4 EPA monitoring locations closest to our location. The mean concentration in the PM exposure chamber was 118.3 ± 5.21 μg/m<sup>3</sup>. 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). We instilled either PM suspended in 50 μl of sterile PBS (vortexed prior to instillation) or PBS (control).

Murine Model of Arterial Thrombosis Induced by FeCl<sub>3</sub> Injury to the Carotid Artery
This technique has been previously described in detail (Chiarella et al., 2014). Briefly, mice were anesthetized and 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 of a standard size (generated with a mouse ear punch device) soaked in freshly prepared 10% FeCl<sub>3</sub>. The carotid blood flow was continuously measured using Transonic TS420 Transit-Time Perivascular Flowmeter (Transonic Systems). The application of FeCl<sub>3</sub> led to a 2- to 3 mm-long carotid thrombus.

Murine Alveolar Macrophages and Cell Lines
The murine alveolar macrophage cell line (MH-S, ATCC CRL-2019, originating from male BALB/c mouse) was cultured in RPMI medium in 10% FBS supplemented with 10 μM β-mercaptoethanol at 37°C and 5% CO<sub>2</sub>. Cell culture and generation of MHS cells with stable knockdown of Orai1, Stim1 or a scrambled shRNA control were performed using the Sigma mission lentiviral packaging mix (Sigma-Aldrich) with the following catalog numbers: ORAI1 (TRCN0000125405), STIM1 (TRCN0000193400), non-target control (SHC312). Transformed cells were cultured with 10 μg/ml puromycin for 2 passages and reduction in the expression of the target gene was assessed using western blot or RT-PCR.

Mouse primary alveolar macrophages were isolated by bronchoalveolar lavage performed in euthanized mice with 3 ml of PBS with 1 mM EDTA. Only male mice were used as a source of alveolar macrophages. The lavage was centrifuged at 300 g for 10 min and resuspended in RPMI supplemented with 10% FBS and plated in a density of 100,000 cells/cm<sup>2</sup>. Cell purity was analyzed by flow cytometry and was confirmed to be >95%.

METHOD DETAILS

IL-6 Measurements
The production of IL-6 in bronchoalveolar lavage fluid on the lung of mice exposed to particulate matter, and <i>In vitro</i> IL-6 production in human alveolar macrophages was determined by ELISA using a kit (ThermoFisher cat# KHC0061 and KMC0061) as previously described (Mutlu et al., 2007).
Measurement of the NAD+/NADH Ratio and Metabolomics in Primary Alveolar Macrophages

The NAD+/NADH ratio in alveolar macrophages was measured using two methods. For both methods, mice were treated with metformin in their drinking water for three days and BAL macrophages were harvested by gently BAL through a surgically placed tracheostomy tube (6 sequential lavages) in PBS containing (0.5 mM EDTA). The cell pellet was washed twice in PBS and cells were counted (Cellometer K2, Nexcelom Bioscience). In the first method, 100,000 cells were used for measurement of the NAD+/NADH using an assay kit from Abcam (ab65348) according to the manufacturer’s instructions. In the second method, 100,000 cells were resuspended in 75 µl in PBS solution and snap frozen in liquid nitrogen. The cell solution was then thawed and centrifuged for 15 min at 20,000g, 4°C, and filtered using a 10-kDa-molecular weight cutoff filter and then split into two. The first half was used to determine total NAD+ (NAD+/NADH), and the second half to determine NADH after heating the samples at 333 K for 30 min. The supernatant was collected for LCMS analysis by High-Performance Liquid Chromatography and High-Resolution Mass Spectrometry and Tandem Mass Spectrometry (HPLC-MS/MS). Specifically, system consisted of a Thermo Q-Exactive in line with an electrospray source and an Ultimate3000 (Thermo) series HPLC consisting of a binary pump, degasser, and autosampler outfitted with a Xbridge Amide column (Waters; dimensions of 4.6 mm × 100 mm and a 3.5 µm particle size). The mobile phase A contained 95% (vol/vol) water, 5% (vol/vol) acetonitrile, 20 mM ammonium hydroxide, 20 mM ammonium acetate, pH = 9.0; B was 100% Acetonitrile. The gradient was as following: 0 min, 15% A; 3 min, 45% A; 10 min, 60% A; 10.1-11 min, 75% A; 11.1 min, 15% A; 11.1-15 min, 15% A with a flow rate of 400 µL/min. The capillary of the ESI source was set to 275°C, with sheath gas at 45 arbitrary units, auxiliary gas at 5 arbitrary units and the spray voltage at 4.0 kV. In positive/negative polarity switching mode, a selective ion monitoring method for target ions was used and MS1 data were collected at a resolution of 70,000. The automatic gain control (AGC) target was set at 1 × 10⁶ and the maximum injection time was 200 ms. The target ions were subsequently fragmented for confirmation purpose, using the higher energy collisional dissociation (HCD) cell set to 30% normalized collision energy in MS2 at a resolution power of 17,500. Sample volumes of 25 µl were injected. Data acquisition and analysis were carried out by Xcalibur 4.0 software and Tracefinder 2.1 software, respectively (both from Thermo Fisher Scientific).

Measurement of Mitochondrial ROS Using MitoSOX and Intracellular Calcium Imaging with Fura-2

Live cell imaging was used to determine changes in intracellular calcium concentration and mitochondrial superoxide production, using the fluorescent probes MitoSOX (2 µM) and Fura-2 (10 µM). Cells were plated in glass plates for 24 hr in HBSS medium, then washed and incubated in Hank’s buffer containing 2 µM MitoSOX-Red and 1 µg/ml of Fura-2 for 30 min at 37°C in a 5% CO₂ atmosphere. Cells were washed and incubated in Hank’s buffer for 30 min in the dark, then mounted in the Olympus DSU Spinning Disc Confocal microscope attached to a perfusion system and perfused with 1 ml/min of HBSS media in a 5% CO₂ atmosphere at 37°C for the duration of the experiment. Investigators were not blinded to the group allocation and treatment. Single-cell [Ca²⁺]cyt measurements were done according to the protocol described previously (McNally et al., 2012). Image acquisition and analysis was performed using Slidebook (Derby, CO). For data analysis, regions of interest were drawn around single cells, background subtracted, and the F340/F380 intensity ratios were determined for each time point. The F340/F380 intensity ratios were converted to [Ca²⁺]cyt using the formula:

\[ [\text{Ca}^{2+}]_{\text{cyt}} = \beta \cdot K_d \cdot (R / R_{\text{max}}) / (R_{\text{max}} - R) \]

Where R is the F340/F380 fluorescence intensity ratio and \( R_{\text{max}} = 9.645 \) and \( R_{\text{min}} = 0.268 \) were determined by in vitro calibration of Fura-2 pentapotassium salt. \( \beta = 20.236 \) was determined from the Fmin/Fmax ratio at 380 nm, and Kd is the apparent dissociation constant of Fura-2 binding to Ca²⁺ (135 nmol).

Isolation of Human Alveolar Macrophages

A small biopsy of donor lung tissue was obtained at the time of lung transplantation surgery and used for isolation via FACSorting (Bharat et al., 2016). Briefly, lung tissue was infiltrated with mixture of collagenase and DNAse I and digested at 37°C for 30 min, chopped into 2-3 mm pieces with fine scissors and digested for another 15 min. The resulting single cell suspension was passed through the 40 µm filter, centrifuged, and red blood cells were lysed using BD Pharm Lyse buffer (BD Pharmingen). Following live/dead staining with eFluor506 viability dye (eBioscience/Affymetrix) and incubation with Fc-blocking reagent (Biolegend) cells were incubated with mixture of fluochrome conjugated antibodies for 30 min. Alveolar macrophages were sorted on BD SORP FACS Aria III instrument in RHLCCC Flow Cytometry Core facility with 100 um nozzle, at 40 psi using MACS buffer as a capture media. Alveolar macrophages were identified as singlets/CD45+/live/CD15–/HLA-DR++/CD206++/CD169+. The cell pellet was 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 hr after plating.

Transcriptome Profiling via mRNA-Seq

Mouse alveolar macrophages were isolated via FACSorting at indicated time points. Approximately 100,000 cells were sorted into MACS buffer, immediately pelleted and lysed in RLT Plus buffer supplemented with 2-mercaptoethanol (Qiagen). RNA was isolated using RNeasy Plus kit with genomic DNA removal step. RNA quality was assessed on TapeStation 4200 instrument (Agilent), all samples had RNA integrity number (RIN) over 7. RNA-seq libraries were prepared from 100 ng of total RNA, starting with poly(A) enrichment and followed by NEB Next RNA Ultra I chemistry. Libraries were quantified and assessed on Qubit fluorimeter (Invitrogen) and TapeStation 4200, correspondingly, multiplexed and sequenced on NextSeq 500 instrument (illumina), 75 bp, single end reads,
to the average sequencing depth of 6x10⁶ reads per sample. Over 94% of reads had Q score over 30. Reads were demultiplexed and mapped to mm10 version of the mouse genome using TopHat2 aligner and mapped to the genomic features using HTSeq and counts processed using edgeR package to estimate differentially expressed genes. FDR p value less than 0.05 was used to identify differentially expressed genes. K-means clustering was performed using GENE-E. Gene ontology analysis was performed using GOrilla on two unranked gene lists. The RNA-seq dataset is available at GEO: GSE98731.

**Metabolic Analysis**

Mice were treated with metformin in their drinking water for three days and BAL macrophages were harvested by gently BAL through a surgically placed tracheostomy tube (6 sequential lavages) in PBS containing (0.5mM EDTA). The cell pellet was washed twice in PBS and cells were counted (Cellometer K2, Nexcelon Bioscience). The samples were dried using SpeedVac. 50 μl of 50% acetonitrile was added to the tube for reconstitution following by overtaxing for 30 sec. Samples solution was then centrifuged for 15 min @ 20,000g, 4 °C. Supernatant was collected for LCMS analysis. Samples were analyzed by High-Performance Liquid Chromatography and High-Resolution Mass Spectrometry and Tandem Mass Spectrometry (HPLC-MS/MS). Specifically, system consisted of a Thermo Q-Exactive in line with an electrospray source and an Ultimate3000 (Thermo) series HPLC consisting of a binary pump, degasser, and auto-sampler outfitted with a Xbridge Amide column (Waters; dimensions of 4.6 mm × 100 mm and a 3.5 μm particle size). The mobile phase A contained 95% (vol/vol) water, 5% (vol/vol) acetonitrile, 20 mM ammonium hydroxide, 20 mM ammonium acetate, pH = 9.0; B was 100% Acetonitrile. The gradient was as following: 0 min, 15% A; 2.5 min, 30% A; 7 min, 43% A; 16 min, 62% A; 16.1-18 min, 75% A; 18-25 min, 15% A with a flow rate of 400 μL/min. The capillary of the ESI source was set to 275 °C, with sheath gas at 45 arbitrary units, auxiliary gas at 5 arbitrary units and the spray voltage at 4.0 kV. In positive/negative polarity switching mode, an m/z scan range from 70 to 850 was chosen and MS1 data were collected at a resolution of 70,000. The automatic gain control (AGC) target was set at 1 × 10⁶ and the maximum injection time was 200 ms. The top 5 precursor ions were subsequently fragmented, in a data-dependent manner, using the higher energy collisional dissociation (HCD) cell set to 30% normalized collision energy in MS2 at a resolution power of 17,500. The sample volumes of 25 μL were injected. Data acquisition and analysis were carried out by Xcalibur 4.0 software and Tracefinder 2.1 software, respectively (both from Thermo Fisher Scientific).

**Phenotyping and Isolation of the Immune Cells in the Murine Lungs via Flow Cytometry and Cell Sorting**

Identification and isolation of the immune cells by flow cytometry and cell sorting were performed as described previously (Misharin et al., 2013). Briefly, the mice were euthanized and the lungs were perfused through the right ventricle with 10 ml of HBSS with Ca²⁺ and Mg²⁺, dissected and infiltrated with collagenase and DNase I, chopped into 2-3 mm fragments, transferred into C-tubes (Miltenyi) and subjected to mechanical disintegration using GentleMACS instrument (Miltenyi). The resulting single cell suspension was filtered through 40 um filter, and subjected to CD45-enrichment using corresponding magnetic microbeads (Miltenyi), stained with eFluor560 viability dye, followed by the mixture of fluorescently labeled antibodies. Cell counts were obtained on K2 cell counter (Nexcelom) using acridine orange to discriminate nucleated cells from debris and propidium iodide to discriminate dead cells. Data were acquired on BD LSR II instrument, cell sorting was performed BD SORP FACS Aria III instrument, 100 um nozzle, 40 psi pressure.

**Quantitative RT-PCR**

We isolated total RNA from mouse lungs, sorted cells or cell cultures using a commercially available system (TRIzol; Invitrogen) and performed qRT-PCR reactions using IQ SYBR Green superscript analyzed on a Bio-Rad IQ5 Real-Time PCR Detection System using the following primer sequences:

- IL-6 (5’-TTCCATCCAGTTGCTTCTTG-3’, 5’-TTCTCATTTCCACGATTTCAG-3’);
- TFAM (5’-CCAAAAAGACCTCGTTCAGC-3’, 5’-ATGTCCTCCGGATCGTTCAC-3’);
- mRPL19 (5’-GAAGGTCAAAAAGGAAATGTGTTCAA-3’, 5’-TTTCGTGCTTCCITGGTCTTAGA-3’).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**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 methods for RNA-seq analysis above. For metabolomics, 154 detected metabolites in all samples were analyzed using two way ANOVA followed by multiple comparisons using the two stage linear step up procedure of Benjamini, Krieger and Yekutieli with a FDR q value of 0.1. For some assays (RNA-seq, quantitative RT-PCR, murine model of arterial thrombosis) power and sample size were estimated based on our previous work, the observed data met or exceeded these criteria. The statistical parameters and criteria for significance can be found in the figure legends.

**DATA AND SOFTWARE AVAILABILITY**

The RNA-seq dataset, containing raw and processed data, is available at GEO: GSE98731.