Elsevier Editorial System(tm) for Free Radical Biology and Medicine
Manuscript Draft

Manuscript Number: FRBM-D-14-01087R1

Title: Targeting the isoprenoid pathway to abrogate progression of pulmonary fibrosis

Article Type: Original Research/ Original Contribution

Keywords: macrophage, reactive oxygen species, mitochondria, pulmonary fibrosis, Rac1, isoprenoid pathway

Corresponding Author: Dr. A. Brent Carter,

Corresponding Author's Institution: University of Iowa

First Author: Heather L Osborn-Heaford, MD

Order of Authors: Heather L Osborn-Heaford, MD; Shubha Murthy, PhD; Linlin Gu, PhD; Jennifer L Larson-Casey, PhD; Alan J Ryan, PhD; Lei Shi, BA; Michael Glogauer, DDS, PhD; Jeffrey D Neighbors, PhD; Raymond Hohl, MD, PhD; A. Brent Carter

Abstract: Fibrotic remodeling in lung injury is a major cause of morbidity. The mechanism that mediates the ongoing fibrosis is unclear, and there is no available treatment to abate the aberrant repair. Reactive oxygen species (ROS) have a critical role in inducing fibrosis by modulating extracellular matrix deposition. Specifically, mitochondrial hydrogen peroxide (H2O2) production by alveolar macrophages is directly linked to pulmonary fibrosis as inhibition of mitochondrial H2O2 attenuates the fibrotic response in mice. Prior studies indicate that the small GTP-binding protein, Rac1, directly mediates H2O2 generation in the mitochondrial intermembrane space. Geranylgeranylation of the C-terminal cysteine residue (Cys189) is required for the for Rac1 activation and mitochondrial import. We hypothesized that impairment of geranylgeranylation would limit mitochondrial oxidative stress, and, thus, abrogate progression of pulmonary fibrosis. By targeting the isoprenoid pathway with a novel agent, digeranyl bisphosphonate (DGBP), which impairs geranylgeranylation, we demonstrate that Rac1 mitochondrial import, mitochondrial oxidative stress, and progression of the fibrotic response to lung injury are significantly attenuated. These observations reveal that targeting the isoprenoid pathway to alter Rac1 geranylgeranylation halts the progression of pulmonary fibrosis after lung injury.

Division of Pulmonary, Allergy and Critical Care Medicine
The University of Alabama at Birmingham

April 7, 2015

Dr. Regina Brigelius-Flohe
Associate Editor
Free Radical Biology and Medicine

Dear Brigelius-Flohe,

Enclosed you will find a revised manuscript entitled “Targeting the isoprenoid pathway to abrogate progression of pulmonary fibrosis” that we would like considered for publication in Free Radical Biology and Medicine. Pulmonary fibrosis is a disease with high morbidity and mortality, and there are currently no therapeutic means to halt development or progression of the disease. Prior studies indicate that the small GTP-binding protein, Rac1, directly mediates H_2O_2 generation in the mitochondrial intermembrane space. Geranylgeranylation of the C-terminal cysteine residue (Cys^{189}) is required for the for Rac1 activation and mitochondrial import. We hypothesize that impairment of geranylgeranylation would limit mitochondrial oxidative stress, and, thus, abrogate progression of pulmonary fibrosis. By targeting the isoprenoid pathway with a novel agent, digeranyl bisphosphonate (DGBP), which impairs geranylgeranylation, we demonstrate that Rac1 mitochondrial import, mitochondrial oxidative stress, and progression of the fibrotic response to lung injury are significantly attenuated. We have addressed all of the reviewers concerns with the responses included as a separate file. These revisions have resulted in 13 new figures and 4 revised figures.

Thank you for the opportunity to improve the manuscript.

Sincerely,

A. Brent Carter, M.D.
Professor

Response to Reviewers

Reviewer #1:

Comment 1: Data Figure 1 [Panel D] are compelling, but do not provide evidence to support the conclusion that geranylgeranylation of Rac1 is also inhibited under these conditions. These data suggest that geranylgeranylation of Rap1 is inhibited following exposure to chrysotile. Additional data are needed to demonstrate that geranylgeranylation of Rac1 is inhibited under these conditions. This can be done using labeled mevalonate [panel A], or by demonstrating that the subcellular distribution of Rac1 is altered under these conditions [i.e., increase in the cytosolic distribution]. This can be done using Triton X-114 phase partition assay. Furthermore, these data provide evidence for expression of significant amounts of Rap1 in these cells and that its geranylgeranylation is significant impaired.

Does this contribute to pathology?

Response 1: We agree that although we show that Rap 1A geranylgeranylation is impaired with DGBP, we need to show that geranylgeranylation of Rac1 is likewise impaired. To determine if DGBP modulates Rac1 geranylgeranylation, we exposed macrophages to vehicle or DGBP overnight followed by chrysotile exposure. Lysates were separated into an aqueous phase (hydrophilic), which contain non-prenylated proteins, and a detergent phase (hydrophobic), which retains the prenylated proteins. Both Rac1 and non-geranylated Rap 1A were absent in the vehicle-exposed aqueous phase; whereas DGBP increased Rac1 and Rap 1A in the aqueous phase, which indicates they are non-geranylgeranylated. In contrast, Rac1 increased in the detergent phase with chrysotile exposure, but it was not present with DGBP treatment. In the revised manuscript, see Figure 1D.

Comment 2: Data in Figure 1 [Panel E]. How is Rac1 activity quantified [y-axis]? These details are missing in the Methods section as well.

Response 2: Rac1 and Rac2 activity were determined using a bead pull-down kit (Cytoskeleton Inc.) or Rac1 activity was determined using the G-LISA kit (Cytoskeleton Inc.), according to manufacturer's protocols. During PAK-binding domain-GST pull-down bound protein was eluted and separated by SDS-PAGE (note that only active Rac1 or Rac2 binds to the PAK-binding domain). Immunoblots were probed with an antibody specific to Rac1 or Rac2, and GST expression was determined by Coomassie staining, as a loading control. Active Rac1 was also determined by the binding of Rac1 to PAK-PBD beads immobilized in a 96-well plate using G-LISA. The bound active Rac1 was detected with a Rac1 specific antibody. Absorbance was read at 490 nm and normalized to protein concentration in the lysate sample. In the revised manuscript, see Methods.

Comment 3: Figure 2 [Panel A]. These findings suggest that mitochondrial localization of Rac1 is reduced by DGBP treatment. How about total Rac1?

Response 3: We agree that further evidence is needed to show that the localization of Rac1 changes with chrysotile exposure. Because mitochondrial Rac1 has been linked to H_2O_2 generation and the fibrotic phenotype, we determined if DGBP modulated mitochondrial Rac1 and localization. Macrophages were cultured in vehicle or DGBP overnight followed by chrysotile exposure. Chrysotile increased mitochondrial Rac1 content, whereas immunoreactive Rac1 was below control levels in DGBP-treated cells. Rap 1A was not seen in isolated mitochondria. In contrast, chrysotile exposure decreased Rac1 in the cytoplasm, while DGBP treatment increased cytoplasmic Rac1 expression. Non-geranylgeranylated Rap 1A was present in the cytoplasm of DGBP-treated cells, suggesting the geranylgeranylation of Rac1 is necessary for mitochondrial import. Thus, DGBP does not change total cellular Rac1; it changes the localization of Rac1. These findings were observed in vitro and in vivo. In the revised manuscript, see Figure 1E, Figure 2A, and Figure 3J.

Comment 4: Figure 2 [Panels B-E]. It is necessary to provide control data, not just chrysotile-induced effects in the absence or presence of DGBP. Figure 3 [Panels E and F]. Please

provide control values as well. Such data would help in assessing the effects of DGBP.
Response 4: We agree that control data is necessary to validate the effects of DGBP on chrysotile- and bleomycin-induced fibrosis. Because the manuscript focuses on the effect of DGBP in macrophages, we have replaced the GSH assay with a pHPA assay in alveolar macrophages obtained from mice. In the revised manuscript, see Figure 2C-E, Figure 2H, Figure 3G-H, Figure 4B-C, and Figure 4F.
Comment 5: Figure 3 [Panels H]. In the upper Panel, please provide WB for other Rho GTPases [Cdc42 or Rap1] to demonstrate that the effects are only specific for Rac1. In the lower panel, same criticisms, stated above, would apply here since geranylgeranylation of Rap1, but not Rac1, is attenuated under these conditions. It may be necessary to conclusively demonstrate that geranylgeranylation of Rac1 is significantly impaired under these conditions.
Response 5: We agree that we should provide specificity for Rac1 by showing other Rho GTPases. Because Rac1 is linked to pulmonary fibrosis and mitochondrial oxidative stress *in vivo* after bleomycin, we evaluated if bleomycin modulates Rac1 mitochondrial import in alveolar macrophages from mice. Bleomycin increased Rac1 mitochondrial localization compared to vehicle-treated saline-exposed mice, whereas mice treated with DGBP showed complete absence of immunoreactive Rac1 in mitochondria. In contrast, Rap 1A is not present in the BAL cell mitochondria in any condition. Compared to the mitochondrial fraction, there was less Rac1 in the cytoplasm in vehicle-treated mice suggesting that bleomycin induces the post-translational modification of Rac1 necessary for mitochondrial import. Furthermore, an immunoblot analysis showed that non-geranylgeranylated Rap 1A was increased in the cytoplasm of BAL cells obtained from mice treated with DGBP. In aggregate, these data strongly suggest that Rac1-mediated mitochondrial H_2O_2 is linked to pulmonary fibrosis, and disruption of geranylgeranylation and Rac1 activation with DGBP in alveolar macrophages provides a novel therapeutic target for preventing fibrotic development. In the revised manuscript, see Figure 1D and Figure 3J.
Comment 6: Figure 5 [Panels B-E]. These data are interesting, but it is unclear how these assays are done. Panel B represents data using a pull-down assay. Does this assay quantify activation of Rac2 as well? It may be necessary to show that total Rac1 and Rac2 are not changed under these conditions. Altogether, these data indicate that in IPF subjects the mitochondrial Rac1 is activated without significant changes in whole cell and mitochondrial Rac1 abundance. If so, what is the explanation for Rac1 activation? The authors need to discuss those points.
Response 6: We agree that the Rac1 and Rac2 activation assays were left out of the methods. Both Rac1 and Rac2 activation was performed in this Figure 5. Rac1 and Rac2 activity were determined using a bead pull-down kit (Cytoskeleton Inc.). During PAK-binding domain-GST pull-down bound protein was eluted and separated by SDS-PAGE (note that only active Rac1 or Rac2 bind to the PAK-binding domain). Immunoblots were probed with an antibody specific to Rac1 or Rac2, and GST expression was determined by Coomassie staining, as a loading control. Densitometry of multiple experiments were performed to compile the data in a graph. Geranylgeranylation of Rac1 and Rac2 is a post-translational modification that is necessary for activation, interaction with other proteins, and mitochondrial import.
Comment 7: Lastly, how is mitochondrial Rac1 activity measured in Panel E. Does increase in mitochondrial Rac1 in IPF imply Rac1 activation, which is independent of its geranylgeranylation? These points need to be discussed.
Response 7: Rac1 activity was determined using the G-LISA kit (Cytoskeleton Inc.), according to manufacturer's protocols. Active Rac1 was determined by the binding of Rac1 to PAK-PBD beads immobilized in a 96-well plate using G-LISA. The bound active Rac1 was detected with a Rac1 specific antibody. Absorbance was read at 490 nm and normalized to protein concentration in the mitochondrial lysate sample. In this experiment, we measured whole cell Rac1 activity and mitochondrial Rac1 activity and expressed the y-axis as mitochondrial Rac1

activity as a % of whole cell Rac1 activity. Rac1 activity in IPF mitochondria represented approximately 100% of the whole cell activity and thus, was significantly higher compared to the normal subjects. In the revised manuscript, see Figure 5E.

Reviewer #2:

Major points/suggestions:

Comment 1: The proposed mechanism of DGBP's effects in lung macrophages is that the drug reduces the geranylgeranylation of Rac1. At this point you only show indirect evidence of this. It would be important to demonstrate directly that Rac1 is un-prenylated, for example with metabolic labeling or mass spec.

Response 1: We agree that although we show that Rap 1A geranylgeranylation is impaired with DGBP, we need to show that geranylgeranylation of Rac1 is likewise impaired. To determine if DGBP modulates Rac1 geranylgeranylation, we used Triton X-114 separation. We exposed macrophages to vehicle or DGBP overnight followed by chrysotile exposure. Lysates were separated into an aqueous phase (hydrophilic), which contain non-prenylated proteins, and a detergent phase (hydrophobic), which retains the prenylated proteins. Both Rac1 and non-geranylated Rap 1A were absent in the vehicle-exposed aqueous phase; whereas DGBP increased Rac1 and Rap 1A in the aqueous phase, which indicates they are non-geranylgeranylated. In contrast, Rac1 increased in the detergent phase with chrysotile exposure, but it was not present with DGBP treatment. In the revised manuscript, see Figure 1D.

Comment 2: The results conflict with those of the Swedish group (Khan et al) who showed that knockout of GGTase type I blocks prenylation of Rac1 and causes the protein to accumulate in the active GTP-bound form in macrophages. Because you hypothesize that DGBP blocks Rac1 prenylation, you should determine Rac1-GTP and total Rac1 levels in whole macrophage lysates after treatment (and show this data side-by-side with anaylses of mitochondrial Rac1).

Response 2: We agree that our results are in conflict with the previous study; however, we found that DGBP has no effect on pro-inflammatory gene expression in macrophages stimulated with LPS (Supplemental Figure 2), which suggests that inhibition of isoprenylation of Rac1 does not alter the inflammatory response of macrophages. This difference may be based on the stimulus, their use of bone marrow derived macrophages, or the difference in localization of the macrophages in mice. The localization of Rac1 to the mitochondria, however, was not investigated in that study. Because mitochondrial Rac1 has been linked to H_2O_2 generation and the fibrotic phenotype, we determined if DGBP modulated mitochondrial Rac1 and localization. Macrophages were cultured in vehicle or DGBP overnight followed by chrysotile exposure. Chrysotile increased mitochondrial Rac1 content, whereas immunoreactive Rac1 was below control levels in DGBP-treated cells. Rap 1A was not seen in isolated mitochondria. In contrast, chrysotile exposure decreased Rac1 in the cytoplasm, while DGBP treatment increased cytoplasmic Rac1 expression. Non-geranylgeranylated Rap 1A was present in the cytoplasm of DGBP-treated cells, suggesting the geranylgeranylation of Rac1 is necessary for mitochondrial import. To examine if DGBP modulates Rac1 activity, cells were exposed to vehicle or DGBP. Rac1 activation increased significantly after chrysotile exposure, whereas the activity in DGBP-treated cells was reduced to control levels. In aggregate, these results demonstrate that inhibition of geranylgeranylation by altering GGPP synthase activity is an effective way to abrogate Rac1 activation. In the revised manuscript, see Discussion, page 18, paragraph 3 and page 19, paragraph 1; see Figure 1D-F.

Comment 3: You should also determine whether the drug increases LPS-induced cytokine production (Il-1b, Tnfa, Il-6). The latter experiment would be important if you plan to test the compound in humans.

Response 3: We agree that showing the effect of DGBP on pro-inflammatory gene expression is important with regard to considering treatment in humans. We found that DGBP has no effect

on pro-inflammatory gene expression in macrophages stimulated with LPS, which suggests that inhibition of isoprenylation of Rac1 does not alter the inflammatory response of macrophages. In the revised manuscript, see Supplemental Figure 2.

Comment 4: You should also test the impact of incubating your macrophages with a GGTase type I-inhibitor and quantify Rac1-GTP levels and Rac1 import into mitochondria. These results could either strengthen your current conclusions or be informative for point 4 below.

Response 4: We agree that the GGTase transfers the geranylgeranyl moiety to the Rho GTPase. Because Rac1-mediated mitochondrial H_2O_2 generation requires Rac1 geranylgeranylation and Rac1 null mice are protected from pulmonary fibrosis, we investigated the role of DGBP in modulating chrysotile-induced pulmonary fibrosis. WT mice with subcutaneous osmotic pumps delivering vehicle or DGBP were exposed to chrysotile. We first determined if DGBP altered mitochondrial Rac1 localization in alveolar macrophages 21 days after chrysotile exposure. Mitochondria isolated from mice exposed to chrysotile had greater Rac1 content in mitochondria than saline-exposed mice, whereas mitochondrial Rac1 content was markedly reduced in the mice treated with DGBP. The opposite changes were seen in the cytoplasmic fraction indicating that Rac1 geranylgeranylation is necessary for mitochondrial Rac1 import. Similar findings were found *in vitro* using the geranylgeranyl transferase type I inhibitor (GGTI). Chrysotile increased mitochondrial localization of Rac1 in vehicle-treated cells, while macrophages treated with GGTI had decreased Rac1 in the mitochondria and an increase in the cytoplasmic fraction. Rac1 activity was also increased in the macrophages exposed to chrysotile, whereas activity was significantly reduced in cells treated with GGTI. In the revised manuscript, see Figure 1D-F and Supplemental Figure 1.

Comment 5: There is still a possibility that DGBP increases Rac1-GTP levels but prevents mitochondrial import and thereby restricts the ability of Rac1 to contribute to disease. You should consider this possibility and show clear data that support or negate it.

Response 5: We agree that Rac1-GTP may not be altered by DGBP, but, in addition to GTP, the isoprenylation of Rac1 is also required for activation. We have shown in vitro, in vivo, and ex vivo that mitochondrial Rac1 activity (Murthy et al, JBC 2010, Osborn-Heaford et al, JBC 2012) and the ability of Rac1 to generate H2O2 are linked to disease development. DGBP inhibits Rac1 activation by reducing GGPP levels. Although GTP must bind at the appropriate sites (10-17, 57-61, 115-118), the geranylgeranylation of C189 must also occur. We have also shown that a C189S mutant of Rac1 lacks activity and the ability to mediate H2O2 production. It is beyond the scope in this manuscript to evaluate the GTP binding regions. Our observations show that DGBP impairs geranylgeranylation and activation of Rac1. In the revised manuscript, see Figure 1D-F.

Comment 6: Statins reduce GGPP production and has been shown to reduce the prenylation of Rho family proteins. Please discuss in more detail why you think statins do not produce the same effect on the lung as your drug.

Response 6: We agree that statins, theoretically, would provide a novel therapeutic agent in fibrosis. Although statins reduce GGPP levels and activation of RhoGTPases, the use of statins has been associated with interstitial lung abnormalities in smoking individuals likely due to inhibition of several intermediates in the isoprenoid pathway. Because HMG-CoA is a proximal enzyme in the isoprenoid pathway it may alter cell membrane integrity and reduce the *N*-glycosylation of growth-factor receptors. Statins also activate Akt, and multiple studies show that Akt is linked to fibrosis development. Moreover, statins are potent anti-inflammatory agents, so it is plausible that statins have a role in the polarization of macrophages to an M2 phenotype, which are anti-inflammatory and repair injured tissue; however, an imbalance of macrophages with a predominance of an M2 phenotype can promote fibrosis. In the revised manuscript, see Discussion, page 17, paragraph 2.

Comment 7: Has anyone proposed to test, or actually tested, any of the available Rac1 inhibitors in treatment of these lung disorders? Wouldn't that be a more direct approach, considering your convincing evidence that Rac1 is involved in the pathogenesis?
Response 7: No, inhibitors of Rac1 have not been used in any other disease except cancer (GGTI). This manuscript is planned to lead to consideration of DGBP as a therapy for pulmonary fibrosis in humans.
Minor points:
Comment 8: The methods sections contains too little detail and too much "as described". for example, please describe, at least briefly, Rac1 activity assays (both pull-downs and GLSIAs) and which Rac1 and Rac2 knockout mice were used (conditional or conventional gene targeting; if conditional, which Cre)?
Response 8: We agree that the methods section contained abbreviated information in several areas. The methods have been revised to include Rac1 activity assays, Triton X-114 separation, and generation of the mice used in the study. In the revised experiment, see Methods section.

Highlights

- Pulmonary fibrosis is a devastating disease with high morbidity and mortality
- Alveolar macrophages have a key role in modulating development of pulmonary fibrosis
- The post-translational modification of Rac1 mediates mitochondrial oxidative stress in macrophages
- Impairment of the isoprenoid pathway prevent Rac1 activation, mitochondrial oxidative stress, and abrogates development and progression of pulmonary fibrosis

Targeting the isoprenoid pathway to abrogate progression of pulmonary fibrosis

Heather L. Osborn-Heaford[1*], Shubha Murthy[1*], Linlin Gu[6*], Jennifer L. Larson-Casey[2,6*], Alan J. Ryan[1], Lei Shi[3], Michael Glogauer[9], Jeffrey D. Neighbors[4], Raymond Hohl[1,5], and A. Brent Carter[1,2,3,6,7,8]

[1]Department of Internal Medicine, [2]Free Radical and Radiation Biology Program, [3]Human Toxicology Program, [4]Department of Chemistry, [5]Department of Pharmacology, University of Iowa, [6]Deparment of Medicine, University of Alabama at Birmingham, AL, [7]Iowa City VA Healthcare System, Iowa City, IA, [8]Birmingham VAMC, Birmingham, AL, and [9]Canadian Institutes of Health Research Group in Matrix Dynamics, University of Toronto, Toronto, Ontario, Canada
*These authors contributed equally.

Running Title: Isoprenoid pathway and pulmonary fibrosis

Corresponding Author: A. Brent Carter, M.D.
1918 University Blvd.
404 MCLM
Department of Medicine
University of Alabama at Birmingham
Birmingham VAMC
Birmingham, Alabama 35294

ABSTRACT

Fibrotic remodeling in lung injury is a major cause of morbidity. The mechanism that mediates the ongoing fibrosis is unclear, and there is no available treatment to abate the aberrant repair. Reactive oxygen species (ROS) have a critical role in inducing fibrosis by modulating extracellular matrix deposition. Specifically, mitochondrial hydrogen peroxide (H_2O_2) production by alveolar macrophages is directly linked to pulmonary fibrosis as inhibition of mitochondrial H_2O_2 attenuates the fibrotic response in mice. Prior studies indicate that the small GTP-binding protein, Rac1, directly mediates H_2O_2 generation in the mitochondrial intermembrane space. Geranylgeranylation of the C-terminal cysteine residue (Cys^{189}) is required for the for Rac1 activation and mitochondrial import. We hypothesized that impairment of geranylgeranylation would limit mitochondrial oxidative stress, and, thus, abrogate progression of pulmonary fibrosis. By targeting the isoprenoid pathway with a novel agent, digeranyl bisphosphonate (DGBP), which impairs geranylgeranylation, we demonstrate that Rac1 mitochondrial import, mitochondrial oxidative stress, and progression of the fibrotic response to lung injury are significantly attenuated. These observations reveal that targeting the isoprenoid pathway to alter Rac1 geranylgeranylation halts the progression of pulmonary fibrosis after lung injury.

INTRODUCTION

Pulmonary fibrosis is a devastating lung disease that is increasing in incidence, and no current therapeutic modalities are available to halt its progression. In particular, idiopathic pulmonary fibrosis (IPF), which is the most common form, has a median survival of 3-5 years after the diagnosis (1-3). The factors that regulate the process of tissue remodeling in pulmonary fibrosis are poorly understood. Defining the molecular mechanisms that mediate pulmonary fibrosis is urgently needed to prevent the development and/or halt the progression of the disease.

Reactive oxygen species (ROS) have a crucial role in inducing a fibrotic response to lung injury by modulating extracellular matrix deposition. Alveolar macrophages are critical in regulating host responses to lung injury, and H_2O_2 production by macrophages is directly linked to pulmonary fibrosis (4,5). The primary source of H_2O_2 in alveolar macrophages in the setting of fibrosis is the mitochondria (4-6). Moreover, inhibition of mitochondrial H_2O_2 or administration of catalase attenuates the fibrotic phenotype in mice (4,5,7).

The Rho GTP-binding proteins, including Rac1, play an important role in host defense. Rac1 regulates several cellular functions in macrophages, such as cell adhesion, actin polymerization and migration, and phagocytosis (8-10). Rac1 activation also increases the generation of H_2O_2 in nearly every cell type (7,11-14). In macrophages, Rac1 directly mediates H_2O_2 generation in the mitochondrial intermembrane space (6). Rac1 is biologically relevant in that mice harboring a conditional deletion of Rac1 in macrophages are protected from developing asbestos-induced pulmonary fibrosis (6,7).

The C-terminal cysteine residue in Rho GTPases, such as Cys^{189} in Rac1, can be modified by geranylgeranylation with the requisite geranylgeranyl moiety derived from the isoprenoid pathway. This post-translational modification is necessary for activation, interaction with other proteins, and mitochondrial import (6,15). Because mitochondrial Rac1 activity is linked to the development of the fibrotic phenotype in mice, we sought to target the isoprenoid pathway to inhibit Rac1 mitochondrial import as a therapeutic maneuver to prevent the fibrotic response to lung injury. Statins, which block the rate-limiting enzyme, HMG-CoA reductase, of the isoprenoid pathway, have been associated with interstitial lung abnormalities in smoking individuals likely due to inhibition of several intermediates in the isoprenoid pathway (16). Thus, we chose to use a more specific inhibitor of geranylgeranylation by inhibiting geranylgeranylpyrophosphate (GGPP) synthase, the enzyme that catalyzes the next to the last step in the post-translational modification of Rac1. Our novel observations reveal that targeting the isoprenoid pathway to alter Rac1 geranylgeranylation halts progression of pulmonary fibrosis after lung injury.

MATERIALS AND METHODS

Materials

Bleomycin was obtained from the University of Iowa Hospital and Clinics hospital stores. Chrysotile was provided Dr. Peter S. Thorne, College of Public Health. University of Iowa, Iowa City, IA. *p*-hydroxylphenyl acetic acid (*p*HPA), horseradish peroxidase (HRP), α-ketoglutarate and NADPH were purchased from Sigma Chemical Company (St. Louis, MO).

Human subjects

The Human Subjects Review Board of the University of Iowa Carver College of Medicine approved the protocol of obtaining alveolar macrophages from normal volunteers and patients with IPF and asbestosis. Normal volunteers had to meet the following criteria: (1) age between 18 and 55 years; (2) no history of cardiopulmonary disease or other chronic disease; (3) no prescription or nonprescription medication except oral contraceptives; (4) no recent or current evidence of infection; and (5) lifetime nonsmoker. Alveolar macrophages were also obtained from patients with IPF. Patients with IPF had to meet the following criteria: (1) FVC and DLCO at least 50% predicted; (2) current nonsmoker; (3) no recent or current evidence of infection; and (4) evidence of restrictive physiology on pulmonary function tests and interstitial fibrosis on chest computed tomography. Fiberoptic bronchoscopy with bronchoalveolar lavage was performed after subjects received intramuscular atropine (0.6 mg) and local anesthesia. Three sub-segments of the lung were lavaged with five 20-ml aliquots of normal saline, and the first aliquot in each was discarded. The percentage of alveolar macrophages was determined by Wright-Giemsa stain and varied from 90 to 98%.

Mice

Wild-type C57Bl/6 mice were from Jackson Laboratories (Bar Habor, Maine). The University of Iowa Institutional Animal Care and Use Committee approved all protocols. After equilibration, osmotic pumps (Alzet, Cupertino, CA) containing either vehicle (water) or DGBP (0.2 mg/kg/day) were implanted subcutaneously, as describe previously (17). Rac1 null and Rac2 knockout mice (a generous gift from Dr. Michael Glogauer, University of Toronto, Toronto, CA) have been previously described (5,18). Briefly, Rac1 null mice are conditional and were generated using *LysM*^{cre} to selectively delete Rac1 from cells of the granulocyte/monocyte lineage. The Rac2 knockout mice were generated using conventional gene targeting to delete the Rac2 gene as Rac2 is only expressed in cells of the granulocyte/monocyte lineage. Bleomycin (1.3—2.0 U/kg) or chrysotile (100 μg) was administered intratracheally. Mice were euthanized and fibrosis determined as previously described (6,19).

Cell culture

THP-1 macrophages were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI-1640 media supplemented with fetal bovine serum and penicillin/streptomycin. All experiments were performed with media supplemented with 0.5% serum.

Synthesis of digeranyl bisphosphonate (DGBP)

DGBP (U.S. Patent: 7,268,164) was synthesized as previously described (20).

Determination of H_2O_2 generation.

Extracellular H_2O_2 production was determined fluorometrically, as previously described (4). Mitochondrial H_2O_2 was measured by suspending mitochondria in

phenol-red free Hanks' balanced salt solution supplemented with 6.5 mM glucose, 1 mM HEPES, 6 mM sodium bicarbonate, 1.6 mM *p*HPA, 0.95 μg/ml HRP and 5mM α-ketoglutarate.

Isolation of mitochondria and membrane fractions

Mitochondria and cytoplasm were isolated as previously described (4,7).

Rac1 and Rac2 GTPase activation assays

Rac1 and Rac2 activity were determined using a bead pull-down kit (Cytoskeleton Inc.) or Rac1 activity was determined using the G-LISA kit (Cytoskeleton Inc.), according to manufacturer's protocols. Negative and positive lysate controls were incubated with GTPγS or GDP, respectively, during PAK-binding domain-GST pull-down for Rac1 and Rac2. Bound protein was eluted and separated by SDS-PAGE. Immunoblots were probed with an antibody specific to Rac1 or Rac2, and GST expression was determined by Coomassie staining, as a loading control. Active Rac1 was also determined by the binding of Rac1 to PAK-PBD beads immobilized in a 96-well plate using G-LISA. The bound active Rac1 was detected with a Rac1 specific antibody. Absorbance was read at 490 nm and normalized to protein concentration in the lysate sample.

Hydroxyproline assay

Lung tissue was dried to stable weight and acid hydrolyzed with 6N HCl for 24 h at 120 °C. Hydroxyproline concentration normalized to dry weight of the lung was determined as described previously (5).

Immunoblot analysis

Whole cells lysates and sub-cellular fractions were separated by SDS-PAGE and transferred to PVDF membranes. Immunoblot analyses on the membranes were performed with the designated antibodies followed by the appropriate secondary antibody cross-linked to HRP.

ELISA

Active TGF-β in BAL fluid was measured by ELISA (R&D, Minneapolis, MN), according to manufacturer's instructions.

Triton X-114 Separation

The separation of geranylgeranylated and non-geranylgeranylated Rac1 was prepared according to a previously published protocol (21). Briefly, cells were lysed in ice-cold Triton X-114 lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, and 1% Triton X-114). Cell lysates were sonicated and cleared by centrifugation. The supernatant was incubated at 37 °C for 10 min and centrifuged at room temperature for 2 min at 12,000 x g. The detergent, or lower phase, was diluted with buffer that did not contain Triton X-114, and the aqueous, or upper phase, was transferred to a new tube.

Statistical analysis

Statistical comparisons were performed using an unpaired, two-tailed *t* test or one-way ANOVA followed by Tukey's post-test to compare columns. Values in figures are expressed as means with standard errors and $p < 0.05$ was considered to be significant.

RESULTS

Impaired geranylgeranylation of Rac1 by DGBP attenuates Rac1 activation and H_2O_2 generation

The C-terminal cysteine residues of Rho GTPases, including Rac1, are known to undergo geranylgeranylation, a post-transcriptional modification that is required for activation, interaction with other proteins, and mitochondrial import (6,15). Geranylgeranylation is catalyzed by geranylgeranyltransferase (GGTase), which transfers the geranylgeranyl moiety to the GTPase (Figure 1A). Because previous data demonstrate that the absence of Rac1 in macrophage mitochondria attenuates fibrosis development (6), we utilized a potent inhibitor of geranylgeranylpyrophosphate (GGPP) synthase, digeranyl bisphosphonate (DGBP), to inhibit geranylgeranylation of Rac1 (Figure 1B). DGBP was synthesized as previously described (20) and contains two polar groups that mimic the pyrophosphate and bind to the active site of GGPP synthase, the enzyme that catalyzes the conversion of farnesylpyrophosphate to GGPP (Figure 1C).

To determine if DGBP modulates Rac1 geranylgeranylation, we exposed macrophages to vehicle or DGBP overnight followed by chrysotile exposure. Lysates were separated into an aqueous phase (hydrophilic), which contain non-prenylated proteins, and a detergent phase (hydrophobic), which retains the prenylated proteins. An immunoblot analysis for Rac1 and Rap 1A, using a Rap 1A antibody that only recognizes the non-geranylgeranylated protein and is indicative of reduced GGPP levels (21,22), was performed. Both Rac1 and non-geranylated Rap 1A were absent in the vehicle-exposed aqueous phase; whereas DGBP increased Rac1 and Rap 1A in the

aqueous phase, which indicates they are non-geranylgeranylated (Figure 1D). In contrast, Rac1 increased in the detergent phase with chrysotile exposure, but it was not present with DGBP treatment.

Because mitochondrial Rac1 has been linked to H_2O_2 generation and the fibrotic phenotype (6), we determined if DGBP modulated mitochondrial Rac1 and localization. Macrophages were cultured in vehicle or DGBP overnight followed by chrysotile exposure. Chrysotile increased mitochondrial Rac1 content, whereas immunoreactive Rac1 was below control levels in DGBP-treated cells (Figure 1E, upper panel). Rap 1A was not seen in isolated mitochondria. In contrast, chrysotile exposure decreased Rac1 in the cytoplasm, while DGBP treatment increased cytoplasmic Rac1 expression (Figure 1E, lower panel). Non-geranylgeranylated Rap 1A was present in the cytoplasm of DGBP-treated cells, suggesting the geranylgeranylation of Rac1 is necessary for mitochondrial import.

To examine if DGBP modulates Rac1 activity, cells were exposed to vehicle or DGBP. Rac1 activation increased significantly after chrysotile exposure, whereas the activity in DGBP-treated cells was reduced to control levels (Figure 1F). DGBP also decreased H_2O_2 generation in chrysotile-exposed macrophages (Figure 1G). In aggregate, these results demonstrate that inhibition of geranylgeranylation by altering GGPP synthase activity is an effective way to abrogate Rac1 activation and oxidative stress in macrophages.

Geranylgeranylation of Rac1 is required for chrysotile-induced pulmonary fibrosis

Because Rac1-mediated mitochondrial H_2O_2 generation requires Rac1 geranylgeranylation and Rac1 null mice are protected from pulmonary fibrosis (6), we investigated the role of DGBP in modulating chrysotile-induced pulmonary fibrosis. WT mice with subcutaneous osmotic pumps delivering vehicle or DGBP were exposed to chrysotile. We first determined if DGBP altered mitochondrial Rac1 localization in alveolar macrophages 21 days after chrysotile exposure. Mitochondria isolated from mice exposed to chrysotile had greater Rac1 content in mitochondria than saline-exposed mice, whereas mitochondrial Rac1 content was markedly reduced in the mice treated with DGBP (Figure 2A). The opposite changes were seen in the cytoplasmic fraction indicating that Rac1 geranylgeranylation is necessary for mitochondrial Rac1 import (Figure 2B). Similar findings were found *in vitro* using the geranylgeranyl transferase type I inhibitor (GGTI). Chrysotile increased mitochondrial localization of Rac1 in vehicle-treated cells, while macrophages treated with GGTI had decreased Rac1 in the mitochondria (Supplemental Figure S1A) and an increase in the cytoplasmic fraction (Supplemental Figure S1B). Rac1 activity was also increased in the macrophages exposed to chrysotile, whereas activity was significantly reduced in cells treated with GGTI (Supplemental Figure S1C).

To determine if BAL cell mitochondrial H_2O_2 production was modulated by DGBP, we measured the rate of H_2O_2 generation and found that vehicle-treated mice exposed to chrysotile had more than 3-fold greater H_2O_2 production than mice exposed to saline, and DGBP treatment reduced the rate to control levels (Figure 2C).

DGBP protects mice from developing chrysotile-induced pulmonary fibrosis

To further evaluate the effect of DGBP in protecting mice from chrysotile-induced pulmonary fibrosis, the mice were administered vehicle or DGBP subcutaneously in osmotic pumps, and exposed to saline or chrysotile the following day. Mice exposed to saline had normal lung architecture with vehicle (Figure 2D) and DGBP treatment (Figure 2E). Chrysotile-exposed mice that received vehicle had significant architectural changes in their lung parenchyma and large amounts of collagen deposition (Figure 2F), whereas the lungs of the DGBP-treated mice were essentially normal (Figure 2G). The histological findings were confirmed biochemically measuring hydroxyproline content in lung tissue (Figure 2H). In aggregate, these observations suggest that geranylgeranylation of Rac1 has a critical role in development of a fibrotic phenotype after chrysotile-induced lung injury. Moreover, these data suggest that macrophage-derived mitochondrial H_2O_2 plays an important role in mediating the development of pulmonary fibrosis.

Bleomycin-induced oxidative stress is attenuated by DGBP

To determine if DGBP had similar effects on other forms of lung injury, we measured mitochondrial H_2O_2 levels in alveolar macrophages obtained after bleomycin exposure. WT mice were treated with saline or bleomycin at a dose of 1.3 or 2.0 U/kg. H_2O_2 levels were significantly elevated in alveolar macrophages after bleomycin exposure. Further, bleomycin at 2.0 U/kg induced more mitochondrial H_2O_2 compared to the lower dose (Figure 3A).

To investigate the effect of DGBP in bleomycin-induced fibrosis, osmotic pumps containing either vehicle or DGBP were implanted subcutaneously in WT mice. Mice

were exposed to saline or bleomycin the following day. Mice exposed to saline had normal lung architecture and no collagen deposition with vehicle (Figure 3B) and DGBP treatment (Figure 3C). Bleomycin treatment resulted in widespread lung architectural destruction and collagen deposition in animals that received vehicle (Figure 3D), whereas the lungs of the DGBP-treated mice showed normal lung architecture and no significant collagen deposition (Figure 3E). The histological observations were verified biochemically by a hydroxyproline assay. DGBP-treated mice showed significantly less hydroxyproline compared to vehicle-treated mice exposed to bleomycin (Figure 3F). Taken together, these data suggest that GGPP synthase is a novel therapeutic target to limit the fibrotic response to bleomycin- induced lung injury.

Because DGBP-treated mice showed reduced pulmonary fibrosis following bleomycin, we determined if mitochondrial H_2O_2 production was modulated by DGBP. After 21 days, BAL cell mitochondrial H_2O_2 production rate showed that vehicle-treated mice exposed to bleomycin had more than 4-fold greater H_2O_2 production than mice exposed to saline, and DGBP treatment reduced the rate to control levels (Figure 3G). These data strongly suggest that the increase flux through the isoprenoid pathway in alveolar macrophages is, in part, accountable for the mitochondrial oxidative stress.

Based on our prior data linking mitochondrial oxidative stress to the development of pulmonary fibrosis (4,6,7,19), we determined if DGBP treatment would limit the fibrotic response to bleomycin-induced lung injury. We measured the pro-fibrotic cytokine, active TGF-β, in BAL fluid. Mice treated with vehicle following bleomycin exposure showed significantly more active TGF-β in BAL fluid than vehicle-treated mice exposed to saline, and DGBP treatment reduced active TGF-β below control levels after

bleomycin exposure (Figure 3H). These data suggest that the reduction in macrophage mitochondrial H_2O_2 production limits the development of a pro-fibrotic environment.

The post-translational modification of geranylgeranylation is common to all Rho GTPases. The two most common GTPases in macrophages are Rac1 and Rac2 (23,24). To determine if modulation of Rac1 and/or Rac2 was linked to the development of pulmonary fibrosis, we exposed WT, Rac2 KO, and Rac1 null mice to saline or bleomycin. WT and Rac2 KO mice showed significant increases in hydroxyproline in the lung tissue after bleomycin exposure, whereas the hydroxyproline content in lungs of conditional Rac1 null mice was not altered by bleomycin (Figure 3I). Moreover, there was no significant difference between the hydroxyproline levels in WT and Rac2 KO mice, while the lungs of Rac1 null mice had a substantial reduction in hydroxyproline content compared to the other two strains of mice. The data demonstrate that Rac1 expression in alveolar macrophages has a critical role in the development of a fibrotic phenotype after bleomycin.

Because Rac1 is linked to pulmonary fibrosis and mitochondrial oxidative stress *in vivo* after bleomycin, we evaluated if bleomycin modulates Rac1 mitochondrial import in alveolar macrophages from mice. Bleomycin increased Rac1 mitochondrial localization compared to vehicle-treated saline-exposed mice, whereas mice treated with DGBP showed complete absence of immunoreactive Rac1 in mitochondria (Figure 3J). In contrast, Rap 1A is not present in the BAL cell mitochondria in any condition. Compared to the mitochondrial fraction, there was less Rac1 in the cytoplasm in vehicle-treated mice suggesting that bleomycin induces the post-translational modification of Rac1 necessary for mitochondrial import. Furthermore, an immunoblot

analysis showed that non-geranylgeranylated Rap 1A was increased in the cytoplasm of BAL cells obtained from mice treated with DGBP (Figure 3J). In aggregate, these data strongly suggest that Rac1-mediated mitochondrial H_2O_2 is linked to pulmonary fibrosis, and disruption of geranylgeranylation and Rac1 activation with DGBP in alveolar macrophages provides a novel therapeutic target for preventing fibrotic development.

Inhibition of GGPP synthase with DGBP halts progression of fibrosis

To investigate the therapeutic potential of arresting progression of pulmonary fibrosis by impairing geranylgeranylation, we first exposed mice to bleomycin and then installed osmotic pumps seven days after bleomycin exposure (Figure 4A). Lung injury was present seven days after bleomycin (data not shown). Vehicle- (Figure 4B) and DGBP-treated mice (Figure 4C) exposed to saline had normal lungs without collagen deposition. As expected, bleomycin exposure in vehicle-treated mice showed wide spread lung destruction and collagen deposition compared to the saline-exposed mice (Figure 4D). In contrast, lungs of DGBP-treated mice showed small patches of collagen, but there were significantly less collagen compared to the vehicle-treated mice (Figure 4E). The quantitative measure of lung collagen content by hydroxyproline assay confirmed these histological findings (Figure 4D). Taken together, these observations suggest that the isoprenoid pathway may be a novel target for halting pulmonary fibrosis following lung injury.

Alveolar macrophages from IPF patients show increased mitochondrial oxidative stress and Rac1 activation

Lungs of patients with IPF reveal an oxidant/antioxidant imbalance resulting from increased oxidant production (25-27); however, the source(s) and type(s) of ROS has

not been determined. Because mitochondria-derived H_2O_2 production in alveolar macrophages contributes to pulmonary fibrosis (4-7,19,28), we measured mitochondrial H_2O_2 production in alveolar macrophages from IPF patients. Isolated mitochondria from IPF patients showed significantly greater H_2O_2 levels than normal subjects (Figure 5A). In contrast, there was no difference in H_2O_2 generation from isolated membrane fractions, which was significantly less than the mitochondrial fraction (data not shown).

Rac1 and Rac2 are associated with $O_2^{\cdot -}$ and H_2O_2 generation (6,7,23,24). We found that alveolar macrophages from IPF patients showed significantly greater Rac1 and lower Rac2 activation compared to values obtained for normal subjects (Figure 5B). Because a greater fraction of Rac1 is activated in alveolar macrophages from IPF patients, we determined if localization of Rac1 in the mitochondria is increased in IPF macrophages. Immunoblot analysis showed similar amounts of Rac1 in mitochondria of normal subjects and IPF patients (Figure 5C). This was confirmed by densitometry of immunoblot analyses among multiple normal subjects and patients (data not shown). In contrast, Rac2 content was decreased in alveolar macrophage mitochondria from IPF patients compared to normal subjects. Whole cell Rac1 and Rac2 expression was similar in normal subjects and IPF patients (Figure 5D).

Because mitochondrial Rac1 activity has a direct effect on mitochondrial H_2O_2 levels (6), we measured Rac1 activity in whole cell lysates and isolated mitochondria. Rac1 activity in IPF mitochondria represented approximately 100% of the whole cell activity and thus, was significantly higher compared to the normal subjects (Figure 5E). In aggregate, these *in vivo* and *ex vivo* observations indicate that geranylgeranylation is

required for Rac1-mediated oxidative stress in alveolar macrophages, and GGPP synthase is a novel target to attenuate fibrotic remodeling after lung injury.

DISCUSSION

Pulmonary fibrosis is a devastating lung disease that is increasing in incidence. In particular, IPF has a grim prognosis, and supportive care is the primary means of treatment as no current therapeutic modalities are available to halt its progression. The goal in this study was to abrogate the development and progression of pulmonary fibrosis by focusing on the modulation of mitochondrial H_2O_2 generation in alveolar macrophages, which is a critical determinant of the fibrotic response to lung injury (4,6,7,19). By disrupting the isoprenoid pathway as a therapeutic target, we found that inhibiting geranylgeranylation attenuated Rac1-mediated activation and the progression of pulmonary fibrosis.

The isoprenoid pathway is a target for drug therapy in multiple conditions. Statins are the most widely prescribed drug in the United States and are used to inhibit HMG-CoA reductase, which is the rate-limiting enzyme that converts HMG-CoA to mevalonate. Statins are clearly important in the management of hypercholesterolemia as well as the prevention of stroke (29,30). Although statins reduce GGPP levels and activation of RhoGTPases, the use of statins has been associated with interstitial lung abnormalities in smoking individuals likely due to inhibition of several intermediates in the isoprenoid pathway (16). Because HMG-CoA is a proximal enzyme in the isoprenoid pathway it may alter cell membrane integrity and reduce the *N*-glycosylation of growth-factor receptors. Statins also activate Akt (31), and multiple studies show that Akt is linked to fibrosis development (32-37). Moreover, statins are potent anti-inflammatory agents (38-40), so it is plausible that statins have a role in the polarization of macrophages to an M2 phenotype, which are anti-inflammatory and repair injured

tissue (41-43); however, an imbalance of macrophages with a predominance of an M2 phenotype can promote fibrosis (19,44).

In osteoporosis, the bisphosphonates adsorb to bone mineral and reduce bone resorption by inhibition of farnesyl diphosphate synthase, which synthesizes farnesyl diphosphate through successive condensations of isopentenyl pyrophosphate with dimethylallyl pyrophosphate and geranyl pyrophosphate (45-47). Agents that disrupt the isoprenoid pathway have been used for cancer therapeutically. Farnesyl transferase (FTase), which catalyzes the farnesylation of the Ras proteins, and geranylgeranyltranferase I (GGTase I), which catalyzes the final step in the lipid post-translational modification of Rho GTPases, have been studied because Ras and Rho GTPases have been shown to be essential for cell growth and proliferation (48-51). To date, the isoprenoid pathway has not been targeted as a treatment strategy for pulmonary fibrosis.

DGBP inhibits GGPP synthase by mimicking the substrate farnesyl pyrophosphate with its two polar groups that bind to the active site of GGPP synthase. The hydrophobic chains bind to the interior of the enzyme at the site where GGPP would be released. One study showed that GGTase I deficiency induces pro-inflammatory gene expression in macrophages, and Rac1 and other Rho GTPases were localized to the plasma membrane (52), which contrasts from our observations. In fact, we found that DGBP has no effect on pro-inflammatory gene expression in macrophages stimulated with LPS (Supplemental Figure 2), which suggests that inhibition of isoprenylation of Rac1 does not alter the inflammatory response of macrophages. This difference in their study may be based on the stimulus, their use of bone marrow derived macrophages, or

the difference in localization of the macrophages in mice. The localization of Rac1 to the mitochondria, however, was not investigated in that study (52). Our results demonstrate that the inhibition of GGPP synthase reduces the activation of Rac1. Although DGBP has the potential to limit the isoprenylation of other Ras and Rho GTPases, the DGBP concentrations used in our studies had no apparent toxicity *in vitro* or *in vivo*. Furthermore, the predominant Rho GTPase in macrophages, Rac2, is not activated in IPF patients. These results suggest that Rac1 is preferentially activated in the mitochondria, there is a larger pool of inactive Rac1 in normal subjects, or other factors are involved increasing Rac1 activity in the mitochondria of alveolar macrophages.

Mitochondrial-derived oxidants are linked to TGF-β activation and Smad signaling in multiple tissues (28,53). This association is critical because a reduction in oxidative stress decreases TGF-β activation. In addition, mitochondrial complex III-mediated $O_2^{\cdot -}$ generation is directly associated with TGF-β-mediated Smad signaling in human fibroblasts (28), which results in fibrotic remodeling. To our knowledge, the isoprenoid pathway has not been directly associated with TGF-β activation; however, our results indicate that DGBP treatment *in vivo* significantly reduces the level of active TGF-β in BAL fluid. In aggregate, these observations indicate that increased flux through the isoprenoid pathway promotes the development of a fibrotic phenotype.

The lungs of IPF patients are considered to have an oxidant/antioxidant imbalance (25-27), but the source of oxidative stress in IPF is not known. We discovered that IPF alveolar macrophages have increased mitochondrial H_2O_2 levels. The primary source of H_2O_2 in macrophages is the mitochondria in inflammatory and fibrotic states (19,54),

and Rac1, at least in part, regulates mitochondrial H_2O_2 levels in macrophages (6). A conditional deletion of Rac1 in macrophages significantly attenuates development of pulmonary fibrosis (4,6,54) and highlights the importance of macrophages in aberrant lung repair following injury. Studies show that the alveolar epithelium and fibroblasts have a critical role in pulmonary fibrosis (55-58); however, our findings demonstrate that alveolar macrophage-derived oxidative stress is linked to fibrotic repair. Moreover, these observations uncover a mechanism that mediates pulmonary fibrosis and provide a novel therapy that abrogates progression of the fibrotic phenotype by targeting the isoprenoid pathway.

ACKNOWLEDGEMENTS

This work was supported, in whole or in part, by National Institutes of Health Grants 2R01ES015981-07 and R01ES014871. This work was also supported by a Merit Review from the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development Grant 1BX001135-01.

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FIGURE LEGENDS

Figure 1. Digeranyl bisphosphonate attenuates Rac1 activity and H_2O_2 production. (A) Schematic flow diagram of isoprenoid pathway. (B) Chemical diagram of digeranyl bisphophonate (DGBP). U.S. Patent: 7,268,164. (C) Schematic flow diagragm of the isopresnoid pathway showing the location of DGBP inhibition of GGPP synthase. (D) Macrophages were cultured overnight with vehicle (water) or DGBP (10 μM). Cells were exposed to chrysotile (10 μg/cm^2) for 30 min. Cells were fractionated into aqueous or detergent phases. Lysates were subjected to immunoblot analysis for Rap 1A and Rac1. (E) Macrophages were cultured overnight with vehicle or DGBP (10 μM) and exposed to chrysotile (10 μg/cm^2) for 30 min. Immunoblot analysis was performed for Rap 1A and Rac1 in isolated mitochondria (upper panel) and cytoplasm (lower panel). (F) Macrophages were cultured overnight with vehicle or DGBP (10 μM) and exposed to chrysotile (10 μg/cm^2 for 30 min. Rac1 activity was measured by G-LISA and normalized to protein concentration. n = 4, * p < 0.018 vs. - chrysotile (vehicle) and ** p < 0.022 vs. chrysotile (vehicle). (G) Macrophages were cultured in the presence vehicle or DGBP (10 μM) overnight and exposed to chrysotile (10 μg/cm^2) for 30 min. H_2O_2 was measured by pHPA assay and is expressed in pmoles/mg. n = 5, * p < 0.0001 vs. all other conditions.

Figure 2. DGBP abrogates macrophage H_2O_2 generation and development of chrysotile-induced pulmonary fibrosis. Osmotic pumps containing vehicle or DGBP were implanted subcutaneously in C57Bl/6 WT mice. DGBP was administered at 0.2 mg/kg/day. Mice were exposed to saline or chrysotile (100 μg/50 ml NS) intratracheally. After 21 days, alveolar macrophages were isolated by BAL. An immunoblot analysis for

Rac1 was performed in isolated (A) mitochondria or (B) cytoplasm. (C) Mitochondria were isolated from alveolar macrophages obtain from saline+vehicle (*n* = 5), saline+DGBP (n = 6) and chrysotile+vehicle (*n* = 6), and chrysotile+DGBP (n = 7) mice. The rate of H_2O_2 generation was performed by pHPA assay. * $p < 0.0001$ vs. all other conditions. Lungs were removed and processed for Masson's trichrome staining. Micrographs are representative of (D) saline+vehicle (*n* = 8), (E) saline+DGBP (*n* = 8), (F) chrysotile+vehicle (*n* = 6), and (G) chrysotile+DGBP (*n* = 6) mice. (H) Lungs were extracted and homogenized for hydroxyproline assay. * $p < 0.0004$ vs. all other conditions.

Figure 3. DGBP abrogates macrophage H_2O_2 generation and development of bleomycin-induced pulmonary fibrosis. (A) C57Bl/6 WT mice were administered saline (*n* = 6) or bleomycin (1.3 (*n* = 6) or 2.0 (*n* = 4) U/kg) intratracheally. Alveolar macrophages were isolated 21 days later by BAL. Mitochondria were isolated, and H_2O_2 generation was measured by pHPA assay and is expressed in pmol/mg. * $p < 0.0001$ vs. saline; ** $p < 0.0001$ vs.1.3 U/kg. Osmotic pumps containing vehicle (B) and (D) or (C) and (E) DGBP were implanted subcutaneously. DGBP was administered at 0.2 mg/kg/day. Saline or bleomycin (2.0 U/kg) was administered intratracheally. Lungs were extracted and processed for Masson's trichrome staining. Micrographs are representative of saline+vehicle (*n* = 4), bleomycin+vehicle (*n* = 6), saline+DGBP (*n* = 5), and bleomycin+DGBP (*n* = 6). (F) Lungs were extracted and homogenized for hydroxyproline assay and is expressed in mg/g dry lung weight. Saline+vehicle (*n* = 6), bleomycin+vehicle (*n* = 8) and saline+DGBP (*n* = 5), bleomycin+DGBP (*n* = 7). ***** $p < 0.008$ vs. saline groups and ** $p < 0.014$ vs. bleomycin (vehicle). (G) Mitochondria were

isolated from alveolar macrophages obtain from saline+vehicle (*n* = 5) saline+DGBP (n = 6) and bleomycin+vehicle (*n* = 5), bleomycin+DGBP n = 6) mice. The rate of H_2O_2 generation was performed by pHPA assay. * $p < 0.0001$ vs. all other conditions. (H) Active TGF-β in BAL fluid was measured by ELISA. *n* = 4 in all. * $p < 0.0001$ vs. all other groups and ** $p < 0.0001$ vs. bleomycin (vehicle). (I) WT, Rac2 KO, and Rac1 null mice were exposed to saline (*n* = 6, *n* = 4, *n* = 6) or bleomycin (*n* = 4, *n* = 6, *n* = 6). After 21d lungs were extracted and homogenized for hydroxyproline assay and is expressed in mg/g. * $p < 0.0125$ vs. Rac1 null (bleomycin); ** $p < 0.0045$ vs. Rac1 null (bleomycin). (J) WT mice with osmotic pumps containing vehicle or DGBP were exposed to saline or bleomycin. Alveolar macrophages were obtained after 21 days by BAL. An immunoblot analysis was perfomed for Rac1 and Rap 1A in isolated mitochondria and in isolated cytoplasm.

Figure 4. DGBP attenuates progression of bleomycin-induced pulmonary fibrosis. (A) Schematic diagram of experimental design. C57Bl/6 WT mice were administered saline or bleomycin (2.0 U/kg) intra-tracheally. Osmotic pumps containing vehicle or DGBP were implanted subcutaneously seven days later. DGBP was delivered at 0.2 mg/kg/day. Mice were euthanized 21 days after bleomycin. Lungs were removed and processed for Masson's trichrome staining. Micrographs are representative of (B) saline+vehicle (*n* = 4), (C) saline+DGBP (*n* = 4), (D) bleomycin+vehicle (*n* = 5), and (E) bleomycin+DGBP (*n* = 7). (E) Lungs were extracted and homogenized for hydroxyproline assay. * $p < 0.036$ vs. all other groups and ** $p < 0.004$ vs. bleomycin+vehicle.

Figure 5. Alveolar macrophages from IPF patients have increased mitochondrial

H_2O_2 production and mitochondrial Rac1 activity. Alveolar macrophages (AM) were obtained by BAL and mitochondria were isolated. (A) Normal subjects (*n* = 5) and IPF patients (*n* = 7). H_2O_2 was measured by pHPA assay and is expressed as nmoles/mg. * *p* < 0.019 normal vs. IPF mitochondria. (B) Rac1 and Rac2 activity was determined by PAK-binding domain pull-down followed with immunoblot analysis in normal subjects (n = 6) and IPF patients (n = 6). A representative immunoblot analysis of Rac1 and Rac2 activity is shown. Densitometry was performed of Rac1 and Rac2 normalized to GST and is expressed graphically in arbitrary units. * *p* < 0.0340 compared to normal. Normal subjects (*n* = 3) and IPF patients (*n* = 4). (C) Mitochondria and (D) cytoplasm were isolated and immunoblot analyses for Rac1 and Rac2 were performed. Normal subjects (*n* = 5) and IPF patients (*n* = 7). Representative immunoblot is shown. (E) Rac1 activity was measured by GLISA in whole cell lysates and isolated mitochondria and is expressed as mitochondrial Rac1 activity as a % of whole cell activity. * *p* < 0.016. Normal subjects (*n* = 8) and IPF patients (*n* = 6).

Supplemental Figure 1. GGTI inhibits mitochondrial localization of Rac1 and Rac1 activity in macrophages. Macrophages were cultured overnight with vehicle (DMSO) or GGTI (2 μM). Cells were exposed to chrysotile (10 μg/cm^2) for 30 min. (A) Mitochondria and (B) cytoplasm were isolated, and an immunoblot analysis was performed for Rac1. (C) Macrophages were cultured overnight with vehicle (DMSO) or GGTI (2 μM). Cells were exposed to chrysotile (10 μg/cm^2) for 30 min. Rac1 activity assay was performed by G-LISA and normalized to protein concentration. *n* = 3; * *p* < 0.05 vs. all groups and ** *p* < 0.05 vs. all groups.

Supplemental Figure 2. Inhibition of GGPP synthase with DGBP does not alter

pro-inflammatory cytokine expression. Macrophages were cultured overnight with vehicle or DGBP (10 μM). After 24h, cells were stimulated LPS (1 μg/mL) overnight. (A) TNF-α and (B) IL-6 production were measured by ELISA. $n = 6$ for all groups. * $p < 0.05$ vs. untreated controls.

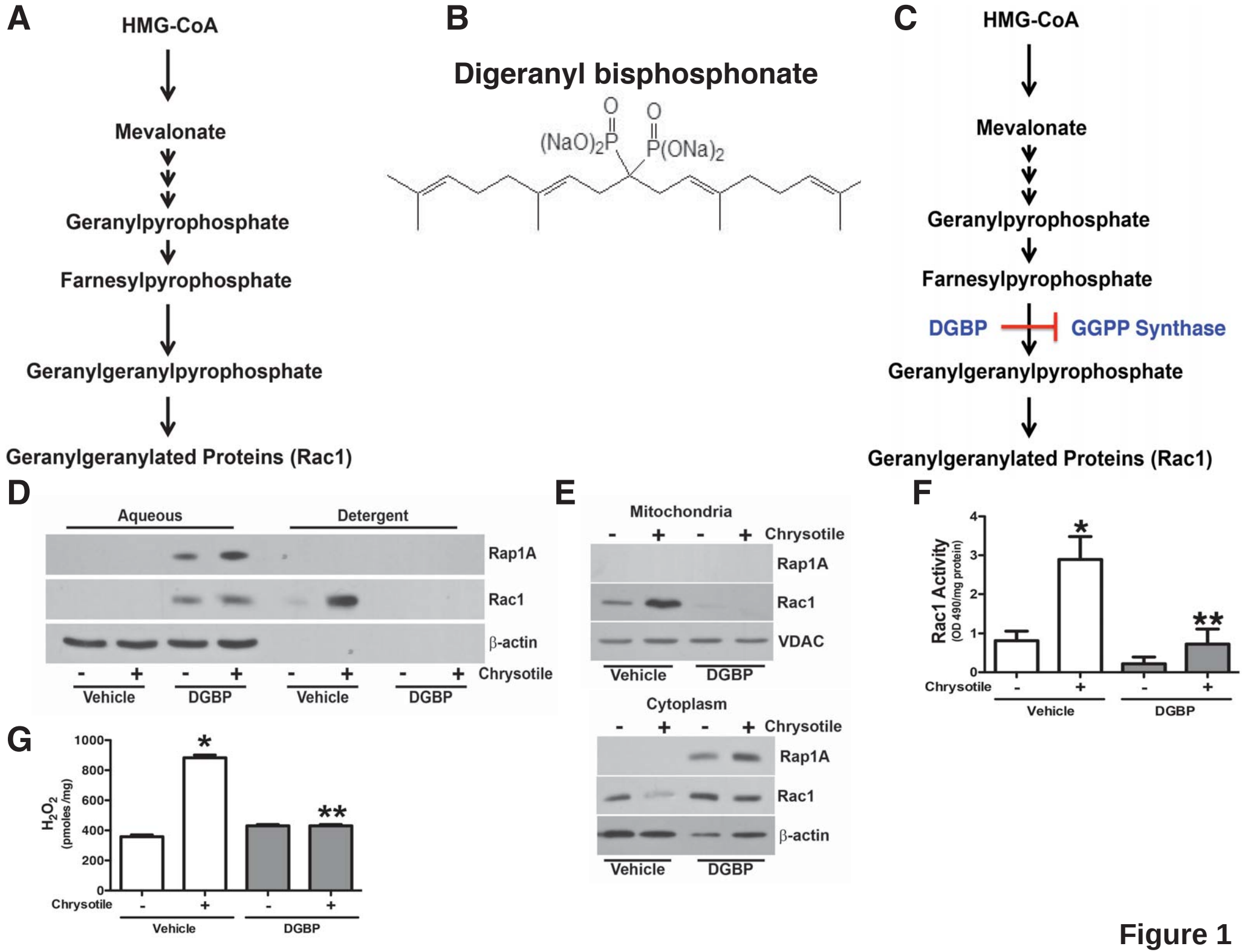

A
HMG-CoA
Mevalonate
Geranylpyrophosphate
Farnesylpyrophosphate
Geranylgeranylpyrophosphate
Geranylgeranylated Proteins (Rac1)
B
Digeranyl bisphosphonate
$(NaO)_2P$
$P(ONa)_2$
C
HMG-CoA
Mevalonate
Geranylpyrophosphate
Farnesylpyrophosphate
DGBP
GGPP Synthase
Geranylgeranylpyrophosphate
Geranylgeranylated Proteins (Rac1)
D
Aqueous
Detergent
Rap1A
Rac1
β-actin
Chrysotile
Vehicle
DGBP
E
Mitochondria
Chrysotile
Rap1A
Rac1
VDAC
Vehicle
DGBP
Cytoplasm
Chrysotile
Rap1A
Rac1
β-actin
Vehicle
DGBP
F
Rac1 Activity
(OD 490/mg protein)
Chrysotile
Vehicle
DGBP
G
H_2O_2
(pmoles /mg)
Chrysotile
Vehicle
DGBP

Figure 1

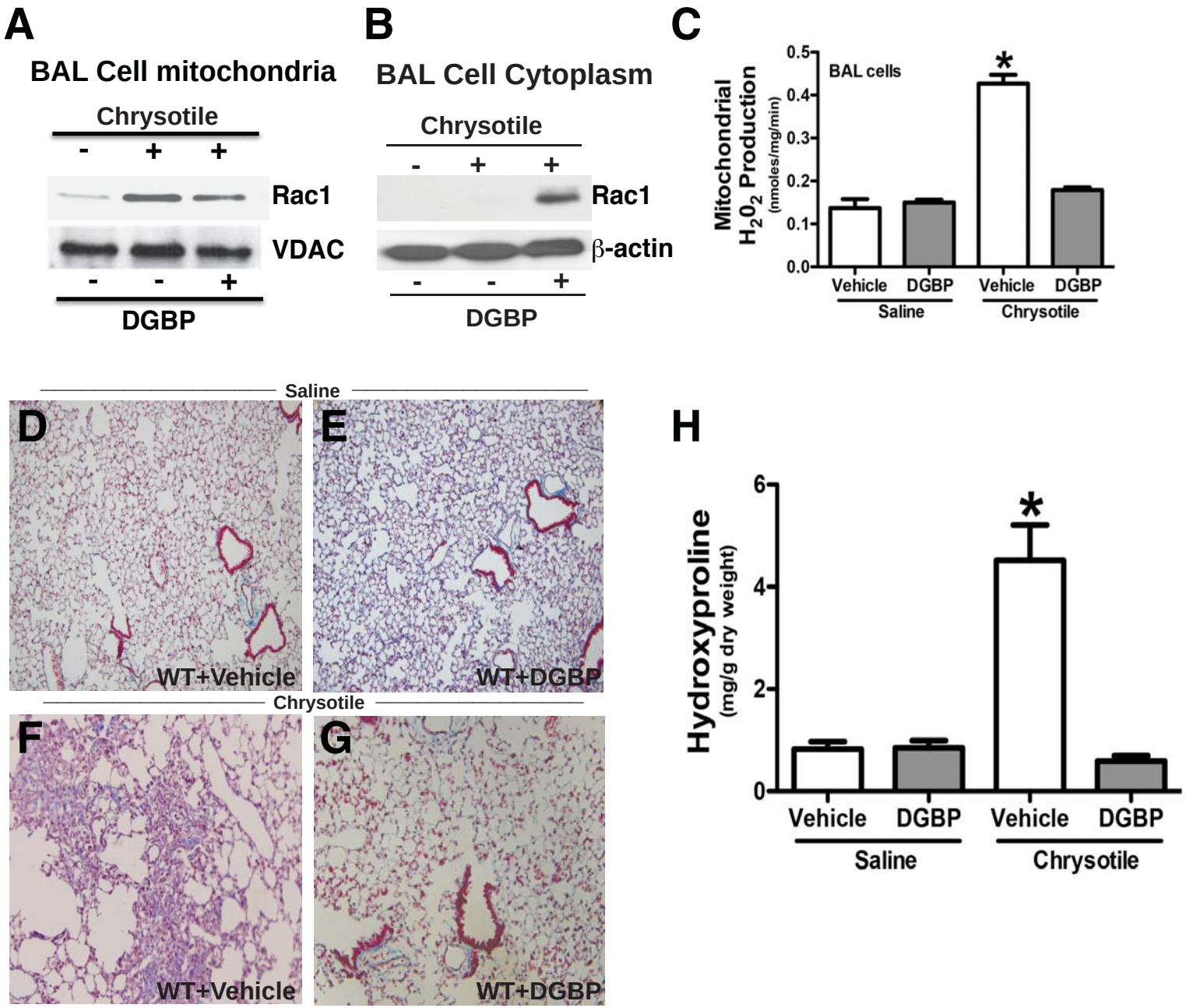

A
BAL Cell mitochondria
Chrysotile
- + +
Rac1
VDAC
- - +
DGBP
B
BAL Cell Cytoplasm
Chrysotile
- + +
Rac1
β-actin
- - +
DGBP
C
BAL cells
Mitochondrial H_2O_2 Production (nmoles/mg/min)
0.5
0.4
0.3
0.2
0.1
0.0
*
Vehicle DGBP Vehicle DGBP
Saline
Chrysotile
Saline
D
WT+Vehicle
E
WT+DGBP
Chrysotile
F
WT+Vehicle
G
WT+DGBP
H
Hydroxyproline (mg/g dry weight)
6
4
2
0
*
Vehicle DGBP Vehicle DGBP
Saline
Chrysotile

Figure 2

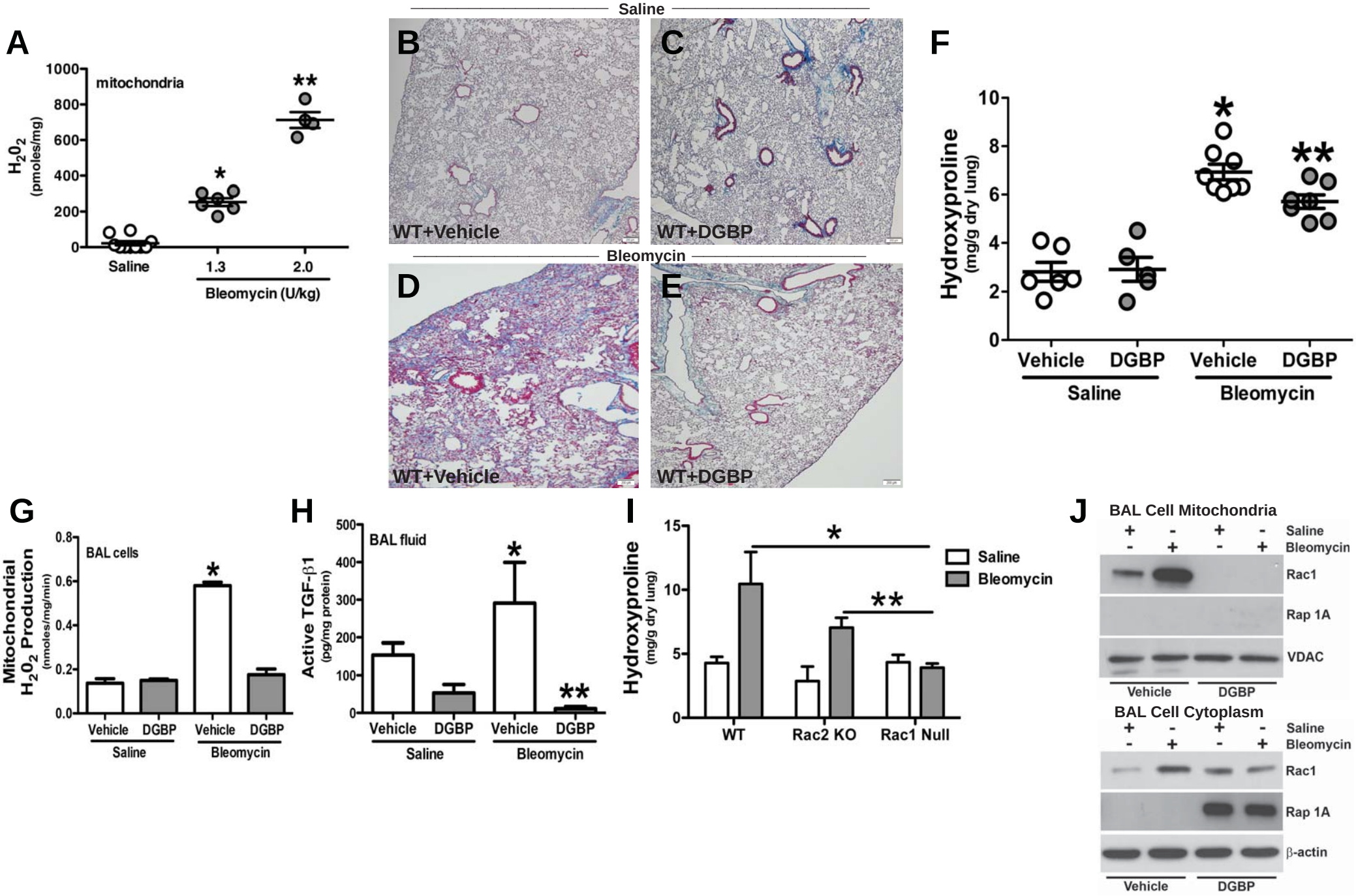

A
mitochondria
H_2O_2 (pmoles/mg)
1000
800
600
400
200
0
Saline
1.3
2.0
Bleomycin (U/kg)
Saline
B
WT+Vehicle
C
WT+DGBP
Bleomycin
D
WT+Vehicle
E
WT+DGBP
F
Hydroxyproline (mg/g dry lung)
10
8
6
4
2
0
Vehicle
DGBP
Vehicle
DGBP
Saline
Bleomycin
G
BAL cells
Mitochondrial H_2O_2 Production (nmoles/mg/min)
0.8
0.6
0.4
0.2
0.0
Vehicle
DGBP
Vehicle
DGBP
Saline
Bleomycin
H
BAL fluid
Active TGF-β1 (pg/mg protein)
500
400
300
200
100
0
Vehicle
DGBP
Vehicle
DGBP
Saline
Bleomycin
I
Hydroxyproline (mg/g dry lung)
15
10
5
0
Saline
Bleomycin
WT
Rac2 KO
Rac1 Null
J
BAL Cell Mitochondria
Saline
Bleomycin
Rac1
Rap 1A
VDAC
Vehicle
DGBP
BAL Cell Cytoplasm
Saline
Bleomycin
Rac1
Rap 1A
β-actin
Vehicle
DGBP

Figure 3

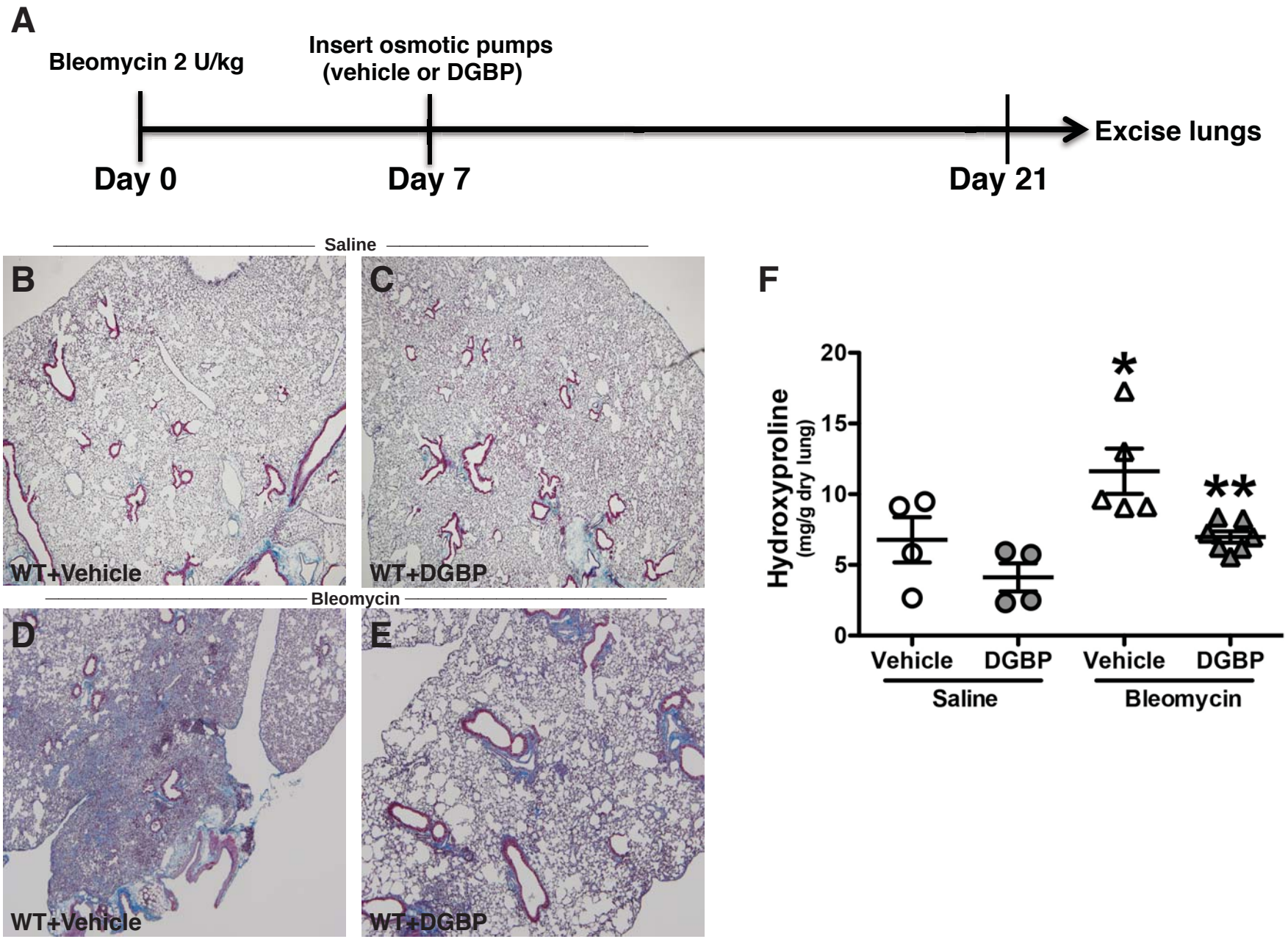

A
Bleomycin 2 U/kg
Insert osmotic pumps
(vehicle or DGBP)
Excise lungs
Day 0
Day 7
Day 21
Saline
B
C
WT+Vehicle
WT+DGBP
Bleomycin
D
E
WT+Vehicle
WT+DGBP
F
Hydroxyproline
(mg/g dry lung)
20
15
10
5
0
*
**
Vehicle
DGBP
Vehicle
DGBP
Saline
Bleomycin

Figure 4

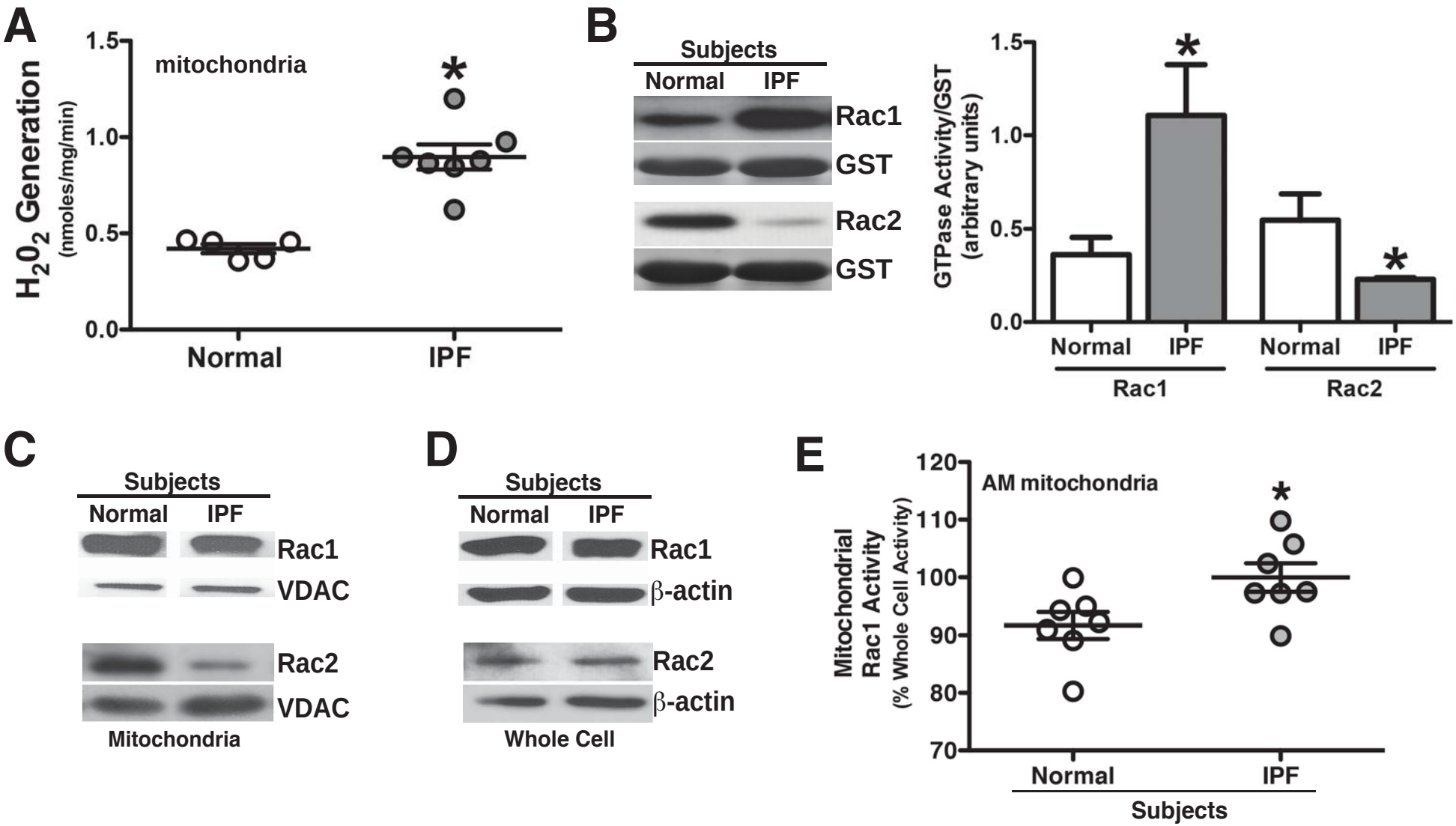
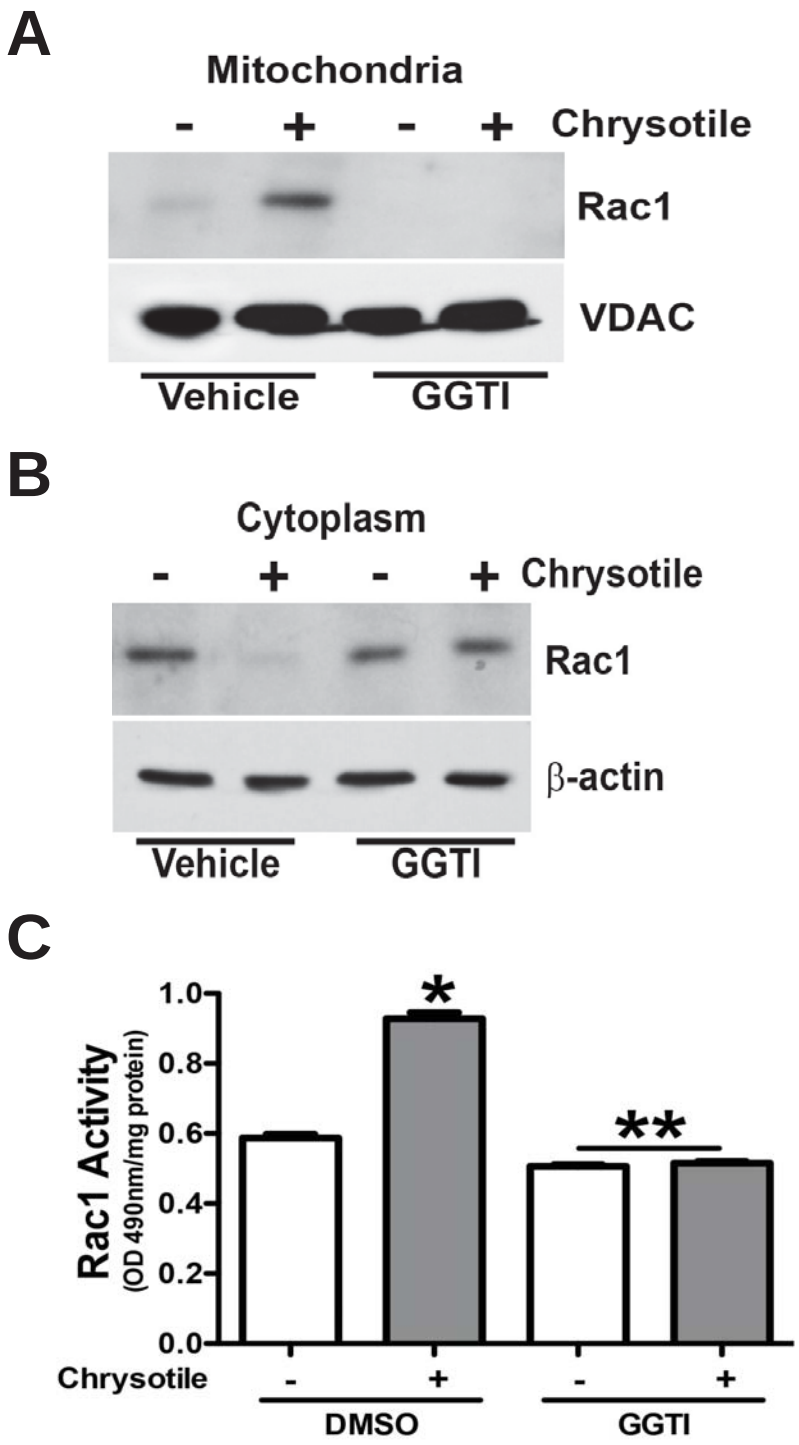
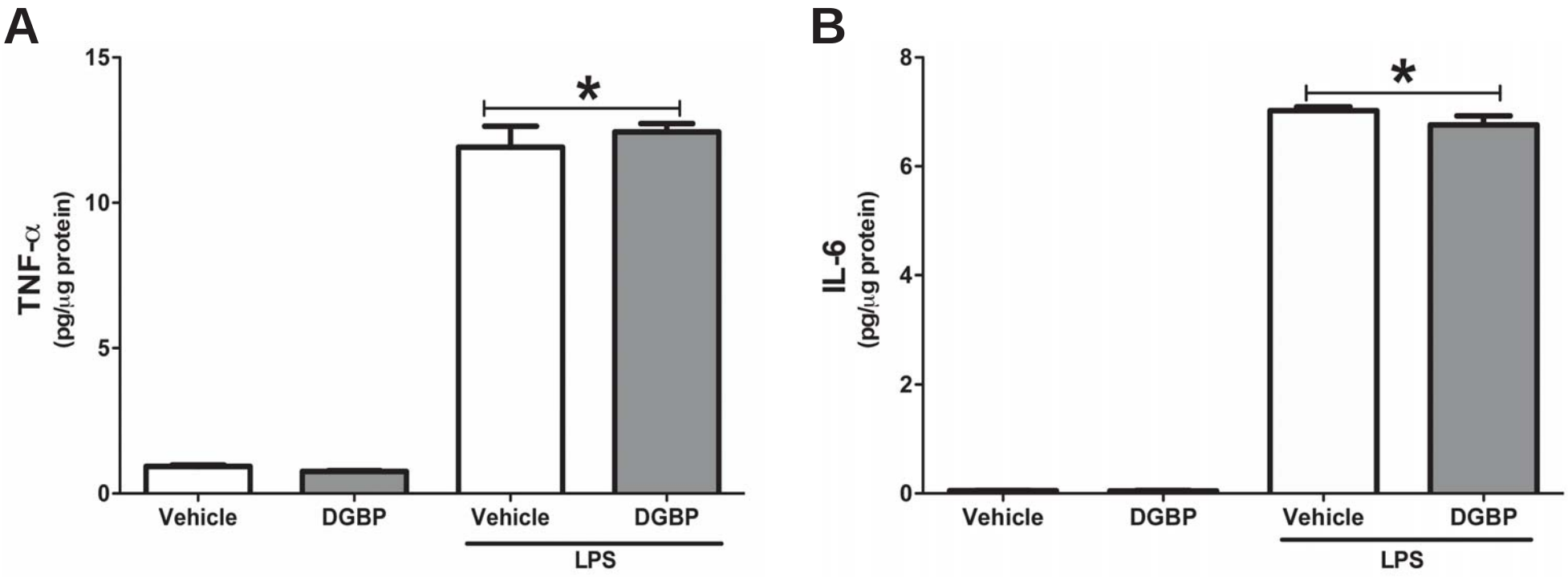

A
mitochondria
H_2O_2 Generation
(nmoles/mg/min)
1.5
1.0
0.5
0.0
Normal
IPF
*
B
Subjects
Normal
IPF
Rac1
GST
Rac2
GST
GTPase Activity/GST
(arbitrary units)
1.5
1.0
0.5
0.0
*
*
Normal
IPF
Normal
IPF
Rac1
Rac2
C
Subjects
Normal
IPF
Rac1
VDAC
Rac2
VDAC
Mitochondria
D
Subjects
Normal
IPF
Rac1
β-actin
Rac2
β-actin
Whole Cell
E
AM mitochondria
Mitochondrial
Rac1 Activity
(% Whole Cell Activity)
120
110
100
90
80
70
*
Normal
IPF
Subjects

Figure 5



Supplemtal Figure 1



Supplemental Figure 2