

IL-4 promotes asymmetric dimethylarginine accumulation, oxo-nitrative stress, and hypoxic response-induced mitochondrial loss in airway epithelial cells

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Background: Obesity is known to increase asthma risk and severity. Increased levels of asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor, are associated with mitochondrial toxicity, asthma, and metabolic syndrome. IL-4 upregulates the expression of protein arginine methyltransferases, which are essential for ADMA formation. Importantly, cross-talk between IL-4, ADMA, and mitochondrial dysfunction could explain how obesity and IL-4 can synergize to exacerbate allergic inflammation.

Objective: We sought to investigate how IL-4, a key asthma-associated cytokine, can influence ADMA-related effects on lungs.

Methods: BEAS2B (bronchial epithelial) cells were treated with IL-4 followed by ADMA and investigated for oxo-nitrative stress and resultant mitochondrial toxicity after 48 hours by using flow cytometry, confocal imaging, immunoblotting, and fluorimetric assays.

Results: IL-4-induced mitotoxicity in BEAS2B cells was significantly higher in the presence of exogenous ADMA. IL-4 treatment led to proteolytic degradation of dimethylarginine dimethylaminohydrolase 2, which catabolizes ADMA. IL-4 pretreatment was associated with increased intracellular ADMA accumulation and increased ADMA-induced mitotoxicity. Airway epithelial cells treated with IL-4 followed by ADMA showed exaggerated oxo-nitrative stress and potent induction of the cellular hypoxic response, despite normoxic conditions. The hypoxic response was associated with reduced mitochondrial function but was reversible by overexpression of

the mitochondrial biogenesis factor, mitochondrial transcription factor A.

Conclusion: We conclude that IL-4 promotes intracellular ADMA accumulation, leading to mitochondrial loss through oxo-nitrative stress and hypoxic response. This provides a novel understanding of how obesity, with high ADMA levels, and asthma, with high IL-4 levels, might potentiate each other and highlights the potential of mitochondrial-targeted therapeutics in obese subjects with asthma. (*J Allergy Clin Immunol* 2016;■■■:■■■-■■■.)

Key words: IL-4, ADMA, hypoxic response, mitochondrial transcription factor A, mitochondria

Mitochondrial dysfunction is now considered an important aspect of asthma pathogenesis. Mitochondrial dysfunction can lead to asthma-like features, and transfer of healthy mitochondria to airway epithelium leads to resolution of airway inflammation and remodeling in mouse models of allergic asthma.^{1,2} We have shown previously that increased asymmetric dimethylarginine (ADMA) levels contribute to asthma pathogenesis through oxo-nitrative stress.^{3,4} ADMA is generated endogenously during the proteolysis of methylated proteins through the action of protein arginine methyl transferases (PRMTs),^{5,6} and increased ADMA levels correlate with the pathophysiology of a variety of diseases, including asthma.⁷ Increased ADMA production, high inducible nitric oxide synthase (iNOS) levels, and increased peroxynitrite ion formation are observed in the murine model of allergic airway inflammation (AAI) resembling asthma,⁷⁻⁹ which is also characterized by increased mitochondrial dysfunction, as depicted by decreased mitochondrial activity and ATP production.¹⁰ In mice with metabolic syndrome or obesity, increased circulating or lung ADMA levels were associated with airway hyperresponsiveness (AHR) in the absence of any cellular inflammation.¹¹ Ultrastructural evidence of mitochondrial pathology was seen in these models.¹² Recently, ADMA has been suggested to be a biomarker, as well as a therapeutic target, in obese human subjects with asthma.¹³

Two important questions merit further consideration. How does allergic inflammation lead to accumulation of ADMA? Does accumulation of ADMA trigger any established cellular programs that regulate mitochondrial function? Understanding this cross-talk between classical inflammation, arginine metabolism, and cellular bioenergetics is necessary for developing more effective therapeutic strategies. This is an important health issue because of the twin epidemics of obesity and asthma, with strong clinical evidence of bidirectional links, as well as a combined obese asthma phenotype that is often resistant to usual treatment.¹⁴

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Abbreviations used

AAI:	Allergic airway inflammation
ADMA:	Asymmetric dimethylarginine
AHR:	Airway hyperresponsiveness
DAB:	Diaminobenzidine
DAPI:	4',6-Diamidino-2-phenylindole
DDAH:	Dimethylarginine dimethylaminohydrolase
eNOS:	Endothelial nitric oxide synthase
HFA:	High fat
HFR:	High fructose
HIF-1 α :	Hypoxia-inducible factor 1 α
HRP:	Horseradish peroxidase
iNOS:	Inducible nitric oxide synthase
mtROS:	Mitochondrial reactive oxygen species
OVA:	Ovalbumin
PGC-1 α :	Peroxisome proliferator-activated receptor γ coactivator 1 α
PRMT:	Protein-arginine methyl transferase
ROS:	Reactive oxygen species
TFAM:	Mitochondrial transcription factor A

Previous reports have shown that IL-4, a well-known mediator of allergic asthma, upregulates expression of protein arginine methyl transferases (PRMTs), which leads to arginine methylation, an essential step in the formation of ADMA.^{15,16} The effect of IL-4 on dimethylarginine dimethylaminohydrolase (DDAH), which degrades ADMA, is not known. DDAH-1 protein is degraded by calpain, a calcium-dependent cysteine protease.^{17,18} Calpain binding sites are also predicted to be present on DDAH-2 protein, which is found in airway epithelial cells (see the [Methods](#) section). If IL-4 activates calpain-mediated proteolysis of DDAH-2, it could synergize with upregulation of PRMT and enhance the effects of ADMA.

We have previously reported that the airway epithelium in mice with allergic inflammation shows marked upregulation of hypoxia-inducible factor 1 α (HIF-1 α).¹⁹ Activated HIF-1 α , which is the primary effector of the hypoxic response, inhibits transcription of peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α),^{20,21} which is the transcription factor for mitochondrial transcription factor A (TFAM).²² TFAM is a candidate gene responsible for mitochondrial biogenesis,²³⁻²⁵ and loss of TFAM induces mitochondrial dysfunction.²⁶ The hypoxic response can be triggered in normoxic conditions by oxidative stress.²⁷ Therefore we investigated whether IL-4 promotes intracellular ADMA accumulation through altered expression of PRMT and DDAH, accentuating the cellular hypoxic response and leading to the mitochondrial dysfunction observed in asthmatic patients. This conceptual model integrates allergic and metabolic aspects of asthma, corresponding to what is increasingly seen in clinical practice.

METHODS

Cell culture

Human bronchial epithelial cells (BEAS2B) were purchased from the American Type Culture Collection, cultured in bronchial epithelial cell growth medium (Lonza, Basel, Switzerland) medium, and maintained at 37°C in a humidified atmosphere with 5% CO₂/95% air. The cytokines IL-4, IL-5, IL-13, IL-1 β , and TNF- α were purchased from BD Biosciences (San Jose, Calif) and used at 10, 10, 10, 2, and 20 ng/mL, respectively. Cells were first stimulated with cytokines for 24 hours followed by ADMA at 100 μ mol/L (Cayman Chemicals, Ann Arbor, Mich) dose induction for another 24 hours.

Measurement of mitochondrial reactive oxygen species, peroxynitrite levels, and apoptosis

Reactive oxygen species (ROS) measurement was done by using a mitochondrial-specific ROS measuring dye, MitoSOX red (Life Technologies, Grand Island, NY). BEAS2B cells were grown in 6-well plates, and the respective treatments were given. After 48 hours of induction, the cells were stained with MitoSOX red for 15 minutes, and measurement was done by using flow cytometry (BD FACSCalibur). For peroxynitrite ion measurement, dihydrorhodamine 123 (Life Technologies) was used. Cells were incubated with dihydrorhodamine 123 for 30 minutes and then analyzed by means of flow cytometry. Apoptosis was measured in BEAS2B cells by means of Annexin V (BD Biosciences) staining. After appropriate induction, cells were incubated with Annexin V for 20 minutes, and fluorescence was measured by using flow cytometry (BD FACSCalibur).

Measurement of mitochondrial shape and mass

Mitochondrial shape was checked by using mitochondrial green fluorescent protein and mitochondrial red fluorescent protein (Life Technologies). BEAS2B cells were grown in 6-well plates with cover slips, and respective treatments were given. The cells were transfected with mitochondrial green fluorescent protein and mitochondrial red fluorescent protein for 16 hours. Then cells were fixed with 2% paraformaldehyde, and 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, Calif) was used for nuclear staining. The cells were viewed under a Leica DMI6000 microscope (Leica, Wetzlar, Germany) at $\times 63$ magnification. For measurement of mitochondrial mass, BEAS2B cells were grown in 6-well plates, and respective treatments were given. The cells were stained with mitotracker green and mitotracker red for 15 minutes, and measurement was done by using flow cytometry (BD FACSCalibur).

Flow cytometry

The number of iNOS-, endothelial nitric oxide synthase (eNOS)-, PGC-1 α -, and TFAM-producing cells was quantified by means of flow cytometry in IL-4 plus ADMA-treated BEAS2B cells. After induction of BEAS2B cells with IL-4 and ADMA, cells were trypsinized and washed with PBS. These cells were fixed with 2% paraformaldehyde for 15 minutes and then permeabilized with 0.1% triton X100 (Sigma, St Louis, Mo) for another 15 minutes. Blocking was done with 1.5% FBS (Life Technologies), and then cells were incubated with primary antibodies against iNOS, eNOS, PGC-1 α , and TFAM (rabbit polyclonal antibody; Abcam, Cambridge, United Kingdom) and DDAH-2 (mouse mAb, Abcam) for 3 hours. Cells were then washed with PBS and stained with anti-rabbit and anti mouse (donkey anti-rabbit and donkey anti-mouse, respectively) secondary antibody conjugated with Alexa Fluor 488 (Life Technologies). Cells were washed and resuspended in PBS and subjected to flow cytometric analysis on a BD FACSCalibur (BD Biosciences).

Immunofluorescence

BEAS2B cells were plated in duplicate chambers of a 6-well plate and cultured in bronchial epithelial cell growth medium. After appropriate induction, cells were fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton-X100 (Sigma). Blocking was done with 1.5% FBS. These cells were labeled with primary antibodies against iNOS, eNOS, HIF-1 α , PGC-1 α , and TFAM, followed by Alexa Fluor 488 (Life Technologies)-conjugated secondary antibodies. DAPI (Invitrogen) was used for nuclear staining. Cells were viewed under a Leica DMI6000 microscope at $\times 63$ magnification. Morphometry was performed by using ImageJ software (National Institutes of Health, Bethesda, Md).

ELISA

Total cell lysate was prepared from human recombinant IL-4-treated BEAS2B cells. Total ADMA levels were measured in the cell lysate and supernatant by using an ELISA (ADMA ELISA kit; DLD Diagnostika, Hamburg, Germany) and normalized to total protein content and supernatant volume, respectively, as per the manufacturer's protocol.

Immunoblotting

Immunoblotting was done, as mentioned earlier.⁴ Total or nuclear lysate was prepared from BEAS2B cells after respective induction, run on 10% SDS-PAGE, and then transferred onto a polyvinylidene difluoride membrane (MDI, Ambala Cantt, India). Blots were incubated with primary mAbs for PRMT-2, DDAH-2, HIF-1 α , TFAM and PGC-1 α . Anti-rabbit or anti-mouse secondary antibodies (Sigma) conjugated with horseradish peroxidase (HRP) were used, and diaminobenzidine (DAB) along with H₂O₂ was used as a substrate. TATA-binding protein and α -tubulin were used as loading controls for nuclear and total cell lysate, respectively (both from Santa Cruz Biotechnology, Dallas, Tex). Densitometry was performed with ImageJ software.

Calcium assay

The intracellular calcium load was measured by using flow cytometry and imaging. BEAS2B cells were treated with human recombinant IL-4 for 3 hours. Intracellular calcium was stained with Fluo-3AM for 15 minutes, and measurement was done by using flow cytometry (BD FACSCalibur). In brief, cells were grown on cover slips and given respective treatments followed by Fluo-3AM staining for 15 minutes. Then the cells were fixed with 2% paraformaldehyde, and DAPI (Invitrogen) was used for nuclear staining. Cells were viewed under a Leica DMI6000 microscope at $\times 63$ magnification.

Calpain activity

Calpain activity was measured with a calpain activity assay kit (BioVision, Mountain View, Calif). Cell lysate was prepared by using extraction buffer after treatment of BEAS2B cells with human recombinant IL-4. Thirty micrograms of protein was used, and calpain activity was measured according to the manufacturer's instructions.

Calpain site prediction

The calpain binding site in the DDAH-2 sequence was predicted by using a multiple kernel learning algorithm-based online tool,²⁸ which is available at <http://calpain.org>.

Plasmid and transfection

HIF-1 α small interfering RNA and TFAM-cDNA plasmid along with untargeted scramble control and cDNA plasmid were procured from Dharmacon (Lafayette, Colo) and OriGene (Rockville, Md). Transfection experiments were done in BEAS2B cells after respective induction with LTX Plus (Invitrogen), X-tremeGENE transfection reagent (Roche, Basel, Switzerland) per the manufacturer's protocol.

Statistical analysis

Representative data are shown as means $\pm$ SEMs of at least 3 experiments. Variations in data between the different groups were tested by using a 2-tailed unpaired *t* test. A *P* value of less than .05 was considered significant.

RESULTS

IL-4 augments ADMA-induced mitochondrial ROS and peroxynitrite levels

To better understand the cross-talk between IL-4 and ADMA, we analyzed mitochondrial reactive oxygen species (mtROS) and peroxynitrite ion formation in BEAS2B cells treated with different cytokines that are known to play an important role in airway disease (IL-5, IL-1 β , IL-13, TNF- α , and IL-4),²⁹⁻³¹ either alone or followed by exogenous ADMA addition after 24 hours (Fig 1, A and B). When used at their standard published doses, cytokines were not significantly different in their capacity to induce mtROS or peroxynitrite by themselves, but a significant

synergistic effect was seen for IL-4, followed by ADMA treatment. Such a synergistic effect was also seen for TNF- α and IL-13 but not for IL-5 or IL-1 β . Higher doses of IL-1 β , followed by ADMA, led to greater induction of mtROS, but this was still lower than the induction by IL-4 followed by ADMA (see Fig E1 in this article's Online Repository at www.jacionline.org). Having confirmed that IL-4 and ADMA act synergistically in inducing mtROS, we focused on the mechanisms of this interaction. The temporal sequence in which ADMA and IL-4 were added was critical (Fig 2, A and B). ADMA added along with IL-4 or before IL-4 did not lead to greater mtROS or peroxynitrite production than ADMA alone, whereas ADMA after IL-4 was about twice as effective (henceforth IL-4 plus ADMA refers to this sequence). IL-4 plus ADMA treatment caused alterations in mitochondrial shape (Fig 2, C; ie, an increase in donut/blob-shaped mitochondria depicting dysfunctional mitochondria).³² This alteration in mitochondrial shape correlated with a significant decrease in mitochondrial mass, complex I/IV activity, ATP levels, and an increase in iNOS levels and apoptosis of BEAS2B cells (Fig 2, C-E, and see Figs E2 and E3 in this article's Online Repository at www.jacionline.org). In contrast, neither IL-4 nor ADMA had a notable effect when given individually.

IL-4 alters ADMA metabolism

To determine whether IL-4 treatment might contribute to alteration in ADMA metabolism, sensitizing the cell to exogenous ADMA, we measured the effect of IL-4 on the known airway epithelial ADMA-metabolizing enzymes PRMT-2 (synthesis) and DDAH-2 (degradation).⁴ IL-4 treatment in BEAS2B cells led to significantly increased PRMT-2 and diminished DDAH-2 expression (Fig 3, A-D). IL-4 administration alone could induce a greater increase in intracellular ADMA levels than treatment with exogenous ADMA. A marked synergistic effect was observed for IL-4 plus ADMA treatment (Fig 3, E). IL-4 also induced an increase in extracellular ADMA levels in IL-4 plus ADMA-treated cells, confirming that the observed differences in intracellular ADMA levels are related to changes in ADMA degradation and not just partitioning across compartments (Fig 3, F). Together, this fits a model in which IL-4, by preventing ADMA degradation, sensitizes the cell to external circulating ADMA. The mechanisms were investigated further.

IL-4 promotes calpain-mediated DDAH-2 degradation

An earlier report shows that IL-4 significantly increased calpain activity in BEAS2B cells (Fig 4, A). This increase was accompanied by an increase in intracellular calcium levels on IL-4 treatment (Fig 4, B-D). Cotreatment with the calpain inhibitor calpeptin-I prevented the IL-4-induced reduction in DDAH-2 levels (Fig 4, E-G).

Intracellular ADMA accumulation triggers a hypoxic response and alters expression of mitochondrial turnover proteins

To determine whether the marked oxo-nitrative stress associated with IL-4 plus ADMA is sufficient to induce the

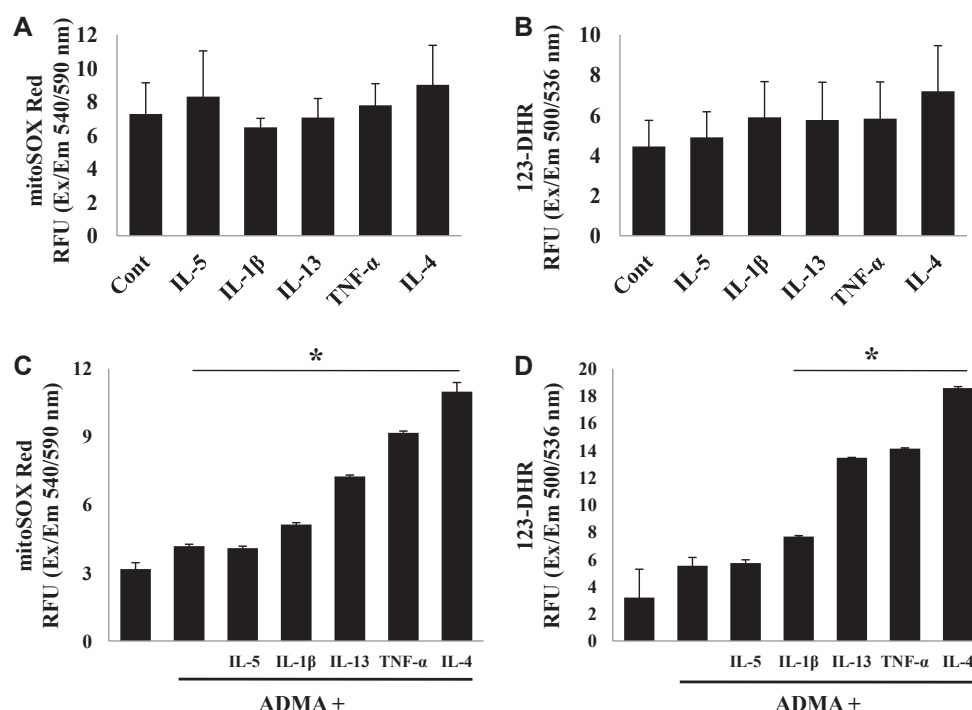


FIG 1. Treatment of BEAS2B cells with inflammatory cytokines followed by ADMA triggers oxo-nitrative stress. **A** and **B**, mtROS and peroxynitrite ion formation in BEAS2B cells treated with different cytokines. **C** and **D**, Treatment of BEAS2B cells with cytokines (IL-4, IL-5, IL-13, IL-1 β , and TNF- α) plus ADMA showed a significant increase in mtROS and peroxynitrite ion formation, with the most prominent increase being in the IL-4 plus ADMA group. Data represent means $\pm$ SEs ($n = 4$). * $P < .05$. Cont, Control; RFU, relative fluorescence units.

hypoxic response despite normoxic conditions, we measured HIF-1 α expression in BEAS2B cells. IL-4 plus ADMA treatment led to a significant multifold increase in HIF-1 α expression compared with vehicle treatment (Fig 5, A-D). The increased hypoxic response in IL-4 plus ADMA-treated cells was associated with significantly decreased PGC-1 α and TFAM expression (Fig 5, E-J). To determine whether the observed changes in cultured airway epithelial cells were relevant to diseased lungs, we measured IL-4, ADMA, HIF-1 α , and TFAM expression in lungs of mice with diet-induced metabolic syndrome (high-fructose [HFR] and high-fat [HFA] diet-fed mice) and AAI (ovalbumin [OVA]-treated mice) (see the Methods section in this article's Online Repository at www.jacionline.org). Each of the models, whether diet or allergen induced, showed increased IL-4, ADMA, and HIF-1 α and diminished TFAM expression (see Figs E4 and E5, and the Methods section in this article's Online Repository at www.jacionline.org).

Pharmacologic inhibition of iNOS or HIF-1 α knockdown partially inhibits IL-4 plus ADMA-induced mitotoxicity

We next determined whether attenuation of oxo-nitrative stress through iNOS inhibition could restore the IL-4 plus ADMA-mediated decrease in mitochondrial mass. Pharmacologic inhibition of iNOS by 1400W was modestly effective in attenuating the IL-4 plus ADMA-induced decrease in mitochondrial mass (Fig 6, A) whereas external induction of peroxynitrite was sufficient to induce a decrease in mitochondrial

mass (Fig 6, B). HIF-1 α knockdown also attenuated IL-4 plus ADMA/peroxynitrite-mediated inhibition of mitochondrial mass (Fig 6, B, and see Fig E6 in this article's Online Repository at www.jacionline.org). This was associated with partial restoration of PGC-1 α and TFAM expression in IL-4 plus ADMA- or peroxynitrite-treated cells (Fig 6, C and D).

TFAM overexpression inhibits IL-4 plus ADMA-induced mitotoxicity

We next overexpressed TFAM (see the Methods section in this article's Online Repository) in IL-4 plus ADMA-treated BEAS2B cells (see Figs E7 and E8 in this article's Online Repository at www.jacionline.org) to determine whether restoring normal TFAM levels would reverse the IL-4 plus ADMA-mediated mitochondrial injury. Overexpression of TFAM inhibited IL-4 plus ADMA-mediated decrease in mitochondrial complex I and IV activity and ATP production (see Fig E8, A-C). Normal mitochondrial shape and mass were restored in TFAM-overexpressing IL-4 plus ADMA-treated cells (Fig 6, E and F). IL-4 plus ADMA-induced decrease in mitochondrial membrane potential and cytochrome c leakage were significantly inhibited by TFAM overexpression, and mtROS levels were restored to normal (see the Methods section in this article's Online Repository and Figs E7, G and H, and E8, D and F).

DISCUSSION

There is emerging evidence of the important role of ADMA in the pathogenesis of cardiometabolic and chronic lung diseases,

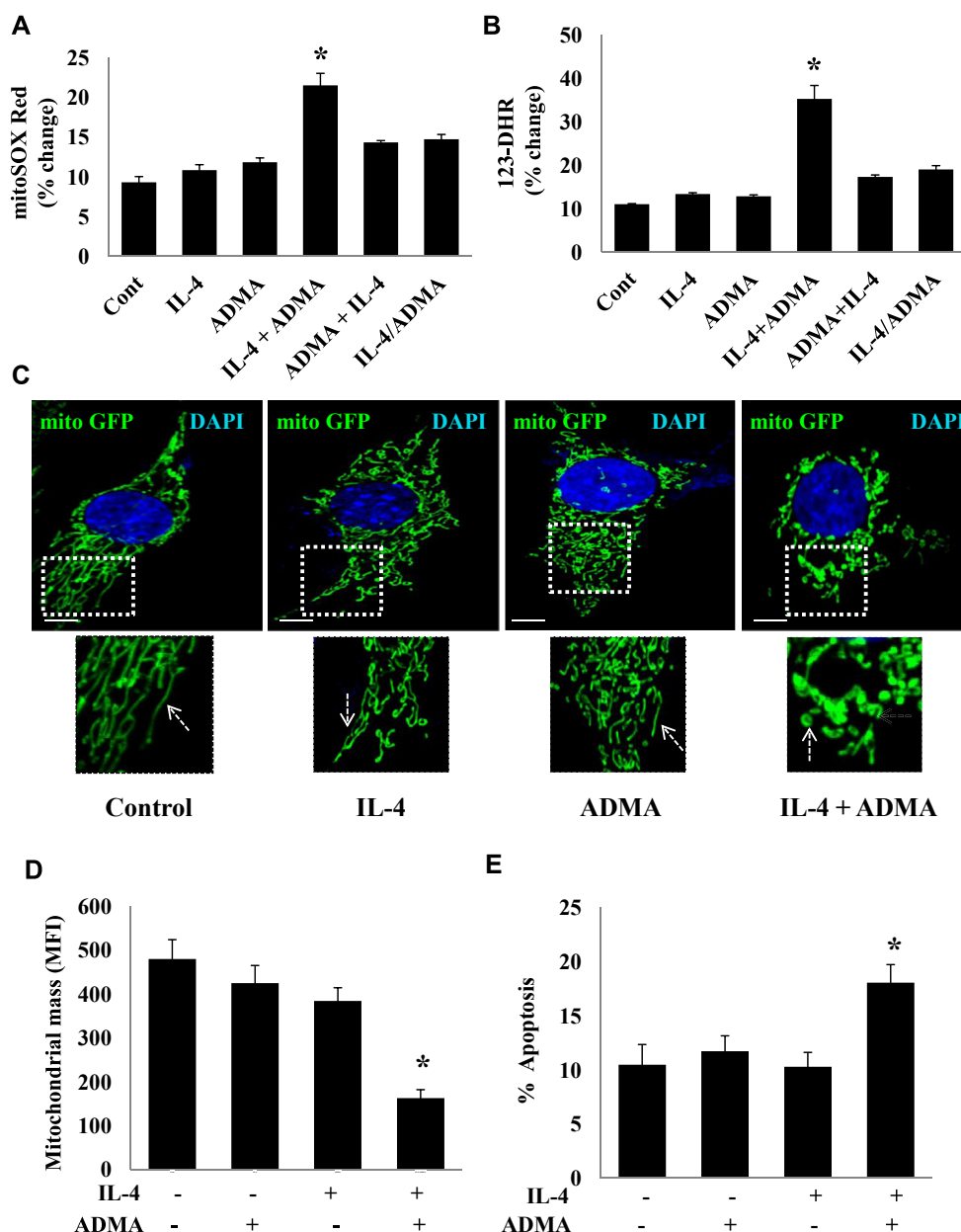


FIG 2. IL-4 plus ADMA treatment causes mitochondrial dysfunction and apoptosis in BEAS2B cells. Increased mtROS levels and peroxynitrite ion formation were measured in BEAS2B cells treated with IL-4, ADMA, or both in different treatment sequences. **A** and **B**, IL-4 followed by ADMA had a severe effect on mitochondrial dysfunction. **C**, Donut-shaped mitochondria were observed in IL-4 plus ADMA-treated BEAS2B cells stained with mitochondrial green fluorescent protein plasmid (magnification $\times 63$). Scale = 20 μ m. **D**, Decrease in mitochondrial mass (mitotracker green staining) was observed in BEAS2B cells treated with IL-4 plus ADMA by using flow cytometry. **E**, IL-4 plus ADMA-induced apoptosis (Annexin V) in BEAS2B cells was measured by using flow cytometry. Data represent means $\pm$ SEs ($n = 3$). * $P < .05$. Cont, Control; MFI, geometric mean fluorescence intensity.

providing a mechanistic basis to the earlier observations of a bidirectional relationship between obesity and asthma.^{33,34} Here, for the first time, we show that IL-4 promotes ADMA accumulation. This promotes oxo-nitrate stress and hypoxic response, which is further associated with reduced expression of mitochondrial biogenesis factors, especially TFAM, in cultured airway epithelial cells. These changes are mirrored in lungs of mice with either metabolic syndrome or allergic

inflammation, further confirming the strong mechanistic links between cardiometabolic and lung disease.

The role of ADMA in asthma is well studied. We have previously reported increased ADMA levels in the lungs and sera of mice with AAI.⁴ Increase in ADMA levels correlated with increased mitochondrial oxo-nitrate stress in the lung and strategies to reduce ADMA attenuated airway inflammation and remodeling.^{4,35-37} Expression of PRMT and DDAH, which

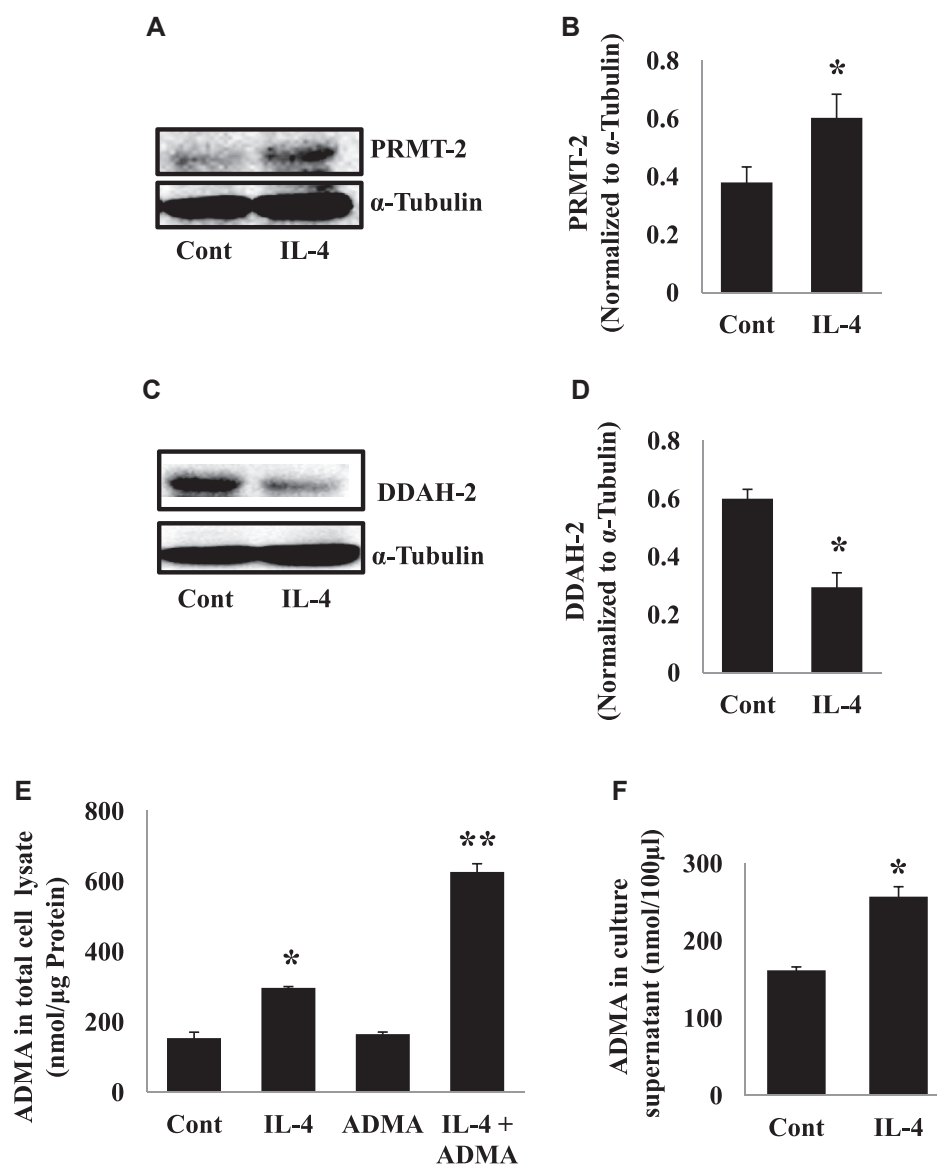


FIG 3. IL-4 causes accumulation of ADMA through increased expression of PRMT-2 and decreased expression of DDAH-2. Expression of PRMT-2 was increased (**A** and **B**) and expression of DDAH-2 was decreased (**C** and **D**) in IL-4-treated BEAS2B cells. Levels of extracellular and intracellular ADMA were measured in culture supernatant and total cell lysate of IL-4-treated BEAS2B cells, respectively (**E** and **F**). BEAS2B cells accumulated more ADMA in IL-4-treated followed by ADMA-treated conditions. Data represent means $\pm$ SEs (n = 3). * P < .05, as compared to control and ** P < .05, as compared to IL-4 alone. Cont, Control.

mediate ADMA synthesis and degradation, respectively, is known to be altered in asthmatic patients, and overexpression of DDAH attenuates asthma features in experimental models.¹³ Sun et al¹⁵ have previously shown that IL-4 induces PRMT1 in a rat model of asthma but did not investigate its effects on ADMA or its downstream effects. The interplay between IL-4 and DDAH has not been reported previously. In this context our finding that IL-4 increases PRMT, reduces DDAH, and multiplies manifold the mitotoxic effects of additional ADMA exposure gains significance. We propose that a complex interplay between inflammatory cytokines might exist *in vivo*, which could modulate the PRMT-ADMA-DDAH axis, leading to abnormally

high intracellular ADMA levels and mitotoxicity. Our *in vitro* data using BEAS2B cells reveal that IL-4 has a pronounced deleterious effect on mitochondrial health in the presence of exogenously added ADMA (Figs 1, C and D, and 2). Although comparable effects were also seen for TNF- α and IL-13, IL-13 can bind to IL-4 receptor, and TNF- α was previously shown to induce mitotoxicity. We chose to add exogenous ADMA in our *in vitro* experiments to mimic the *in vivo* effects of increased ADMA levels in multiple body fluids, ranging from blood to exhaled breath condensate.¹¹ This also mimics the cross-talk between metabolic syndrome with increased circulating ADMA levels and allergic lung inflammation. Although IL-4 signaling

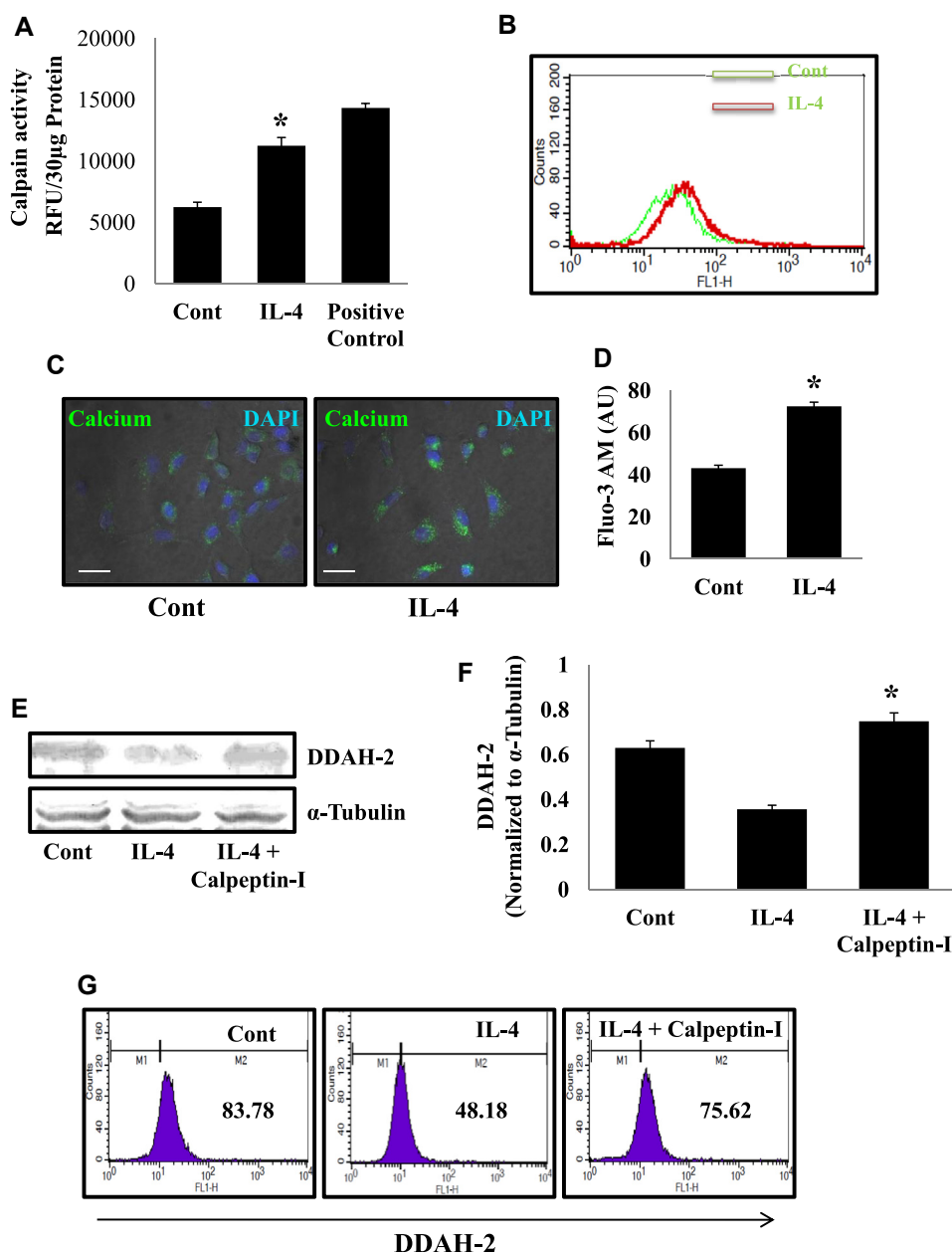


FIG 4. IL-4 treatment reduces DDAH-2 expression through calpain activation in BEAS2B cells. **A**, IL-4-treated BEAS2B cells showed significantly increased calpain activity. **B–D**, Calcium flux was checked in IL-4-treated BEAS2B cells stained with fluo3AM by means of imaging and flow cytometry to determine the mechanism behind the activation of calpain. **E–G**, IL-4-mediated decrease in DDAH-2 expression was rescued by calpeptin-I, a calpain inhibitor. Cont, Control; RFU, relative fluorescence units. * $P < .05$.

is known to induce total ROS,³⁸ it was not sufficient to induce mtROS alone in our model (Fig 1, A and B). This might be because IL-4-induced ROS is generated through cell membrane-localized NOX-1/5-mediated systems that are not predominant in the mtROS-generating machinery. By altering the sequence of IL-4 and ADMA addition, we found that pretreatment with IL-4 followed by exogenous ADMA treatment led to a much greater increase in mtROS and peroxynitrite levels than other combinations (Fig 2, A and B). Intracellular ADMA levels after exogenous ADMA exposure were about 5-fold higher in IL-4-pretreated cells. Because intracellular ADMA levels were

not increased by exogenous ADMA, in the absence of IL-4 treatment (Fig 3, E, third bar), it can be inferred that under normal conditions, ADMA is rapidly degraded inside cells. Importantly, intracellular and extracellular ADMA levels were 2-fold increased by IL-4 in the absence of any ADMA treatment (Fig 3, E and F). Thus the combined effect of IL-4 and exogenous ADMA on intracellular ADMA is highly synergistic, related to ADMA metabolism, and not simply due to entry of exogenous ADMA. We have shown in lungs that intracellular ADMA is synthesized by PRMT-2 and degraded by DDAH-2,⁴ although other isoforms regulate ADMA metabolism in other systems.^{39,40}

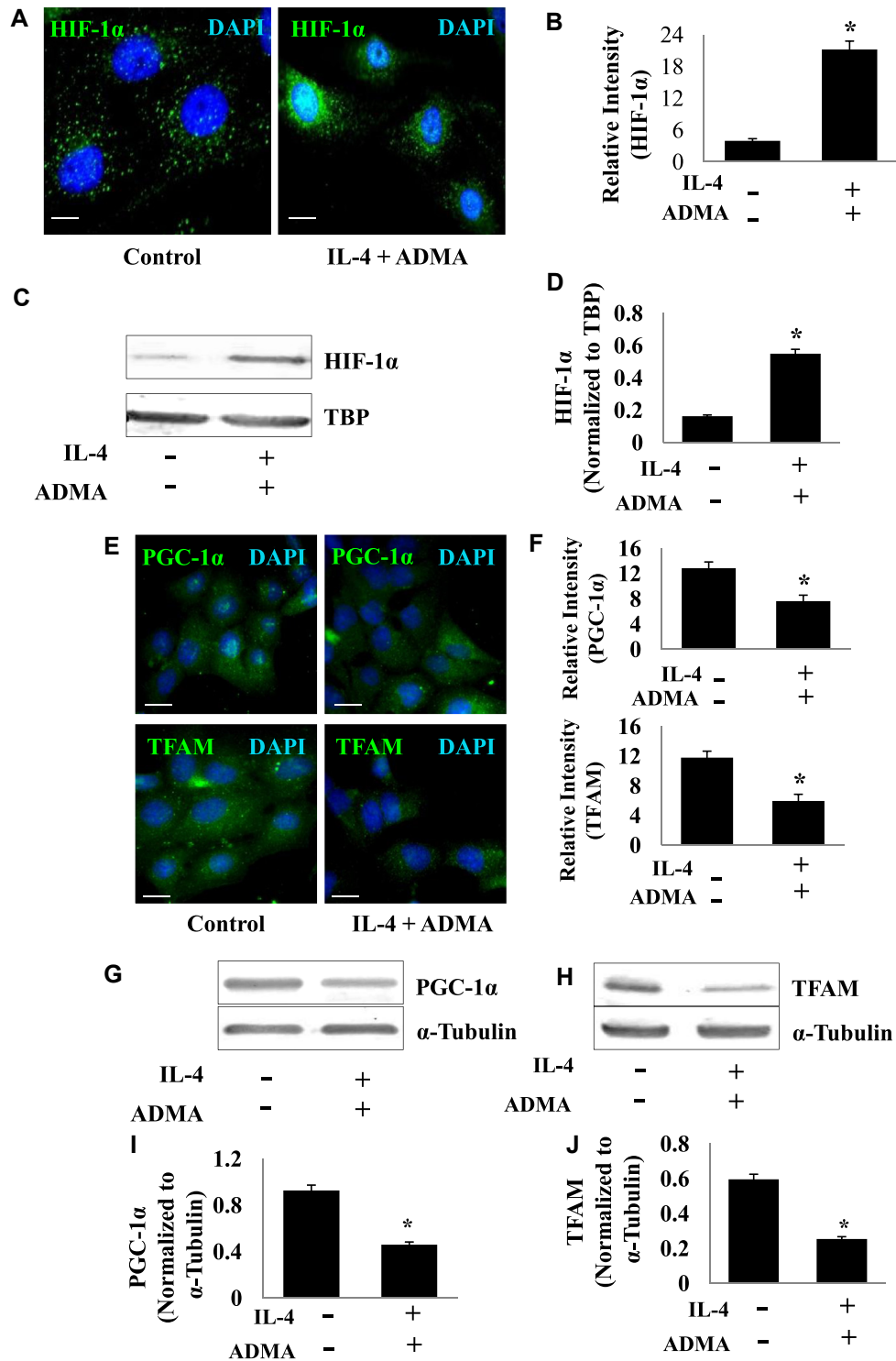


FIG 5. BEAS2B cells treated with IL-4 plus ADMA show an increase in hypoxic response and diminished mitochondrial biogenesis factors. **A** and **B**, Increased expression of HIF-1α in BEAS2B cells treated with IL-4 plus ADMA (immunocytochemistry). **C-J**, IL-4 plus ADMA treatment significantly increased HIF-1α protein expression (Fig 5, **C** and **D**), conversely decreasing PGC-1α and TFAM expression (Fig 5, **E-J**). Fig 5, **F**, presents the summary data of immunocytochemical imaging, and Fig 5, **I** and **J**, summarize the Western blot data (magnification $\times 63$). Scale = 20 μ m. These data imply that IL-4 plus ADMA treatment inhibits mitochondrial biogenesis through the HIF-1α–PGC-1α–TFAM signaling axis. Data represent means $\pm$ SEs ($n = 3$). * $P < .05$. Cont, Control.

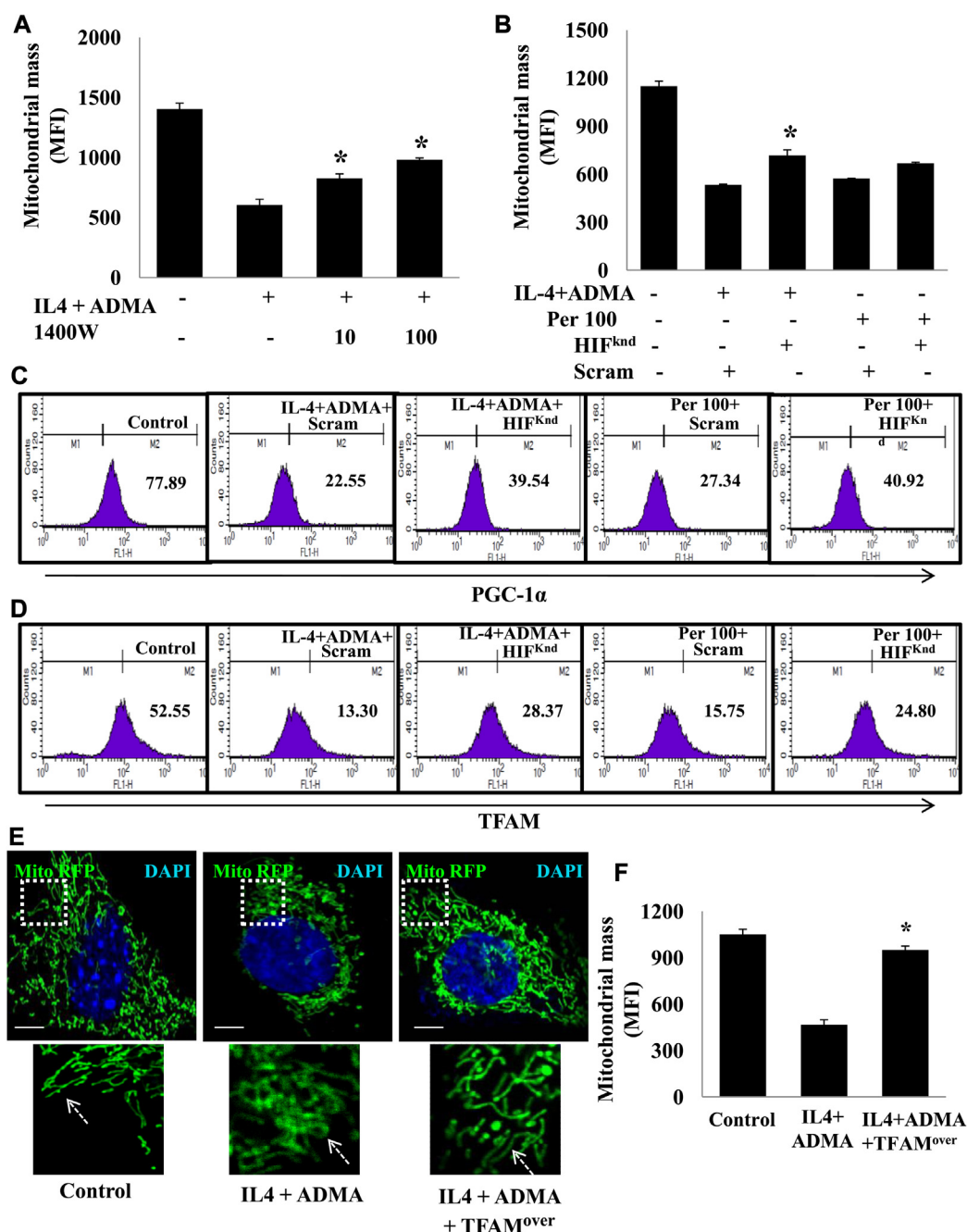


FIG 6. Pharmacologic inhibition of iNOS or HIF-1 α knockdown (HIF-1 α^{knd}) could not attenuate mitochondrial functions. **A**, IL-4 plus ADMA-mediated decrease in mitochondrial mass was partially rescued by pharmacologic inhibition of iNOS (by 1400W). **B**, HIF-1 α knockdown partially restored mitochondrial mass in IL-4 plus ADMA-induced BEAS2B cells. **C** and **D**, IL-4 plus ADMA- or peroxynitrite-induced decrease in PGC-1 α and TFAM expression is partially restored by HIF-1 α knockdown. **E** and **F**, IL-4 plus ADMA-induced alterations in mitochondrial shape (Fig 6, **E**) and mass (Fig 6, **F**) are significantly restored by TFAM overexpression (TFAM^{over}). Data represent means $\pm$ SEs ($n = 5$). * $P < .05$. MFI, Geometric mean fluorescence intensity; Per 100, Peroxynitrite; Scram, scrambled small interfering RNA.

Here, IL-4 significantly increased PRMT-2 and decreased DDAH-2 expression in BEAS2B cells (Fig 3). Therefore we infer that IL-4 inhibits ADMA degradation capacity of airway epithelial cells by reducing DDAH-2 and thereby makes them more susceptible to exogenous ADMA-induced oxo-nitrosative stress and mitotoxicity. To further elucidate the mechanism

of IL-4-mediated degradation of DDAH-2, we quantified DDAH-2 RNA levels after IL-4 treatment, which remained unchanged (data not shown). We next verified whether IL-4 posttranslationally modulates DDAH-2 levels. An earlier report states that DDAH-1 (another isoform of DDAH) is degraded by calpain, a calcium-dependent cysteine protease.⁴¹ Further

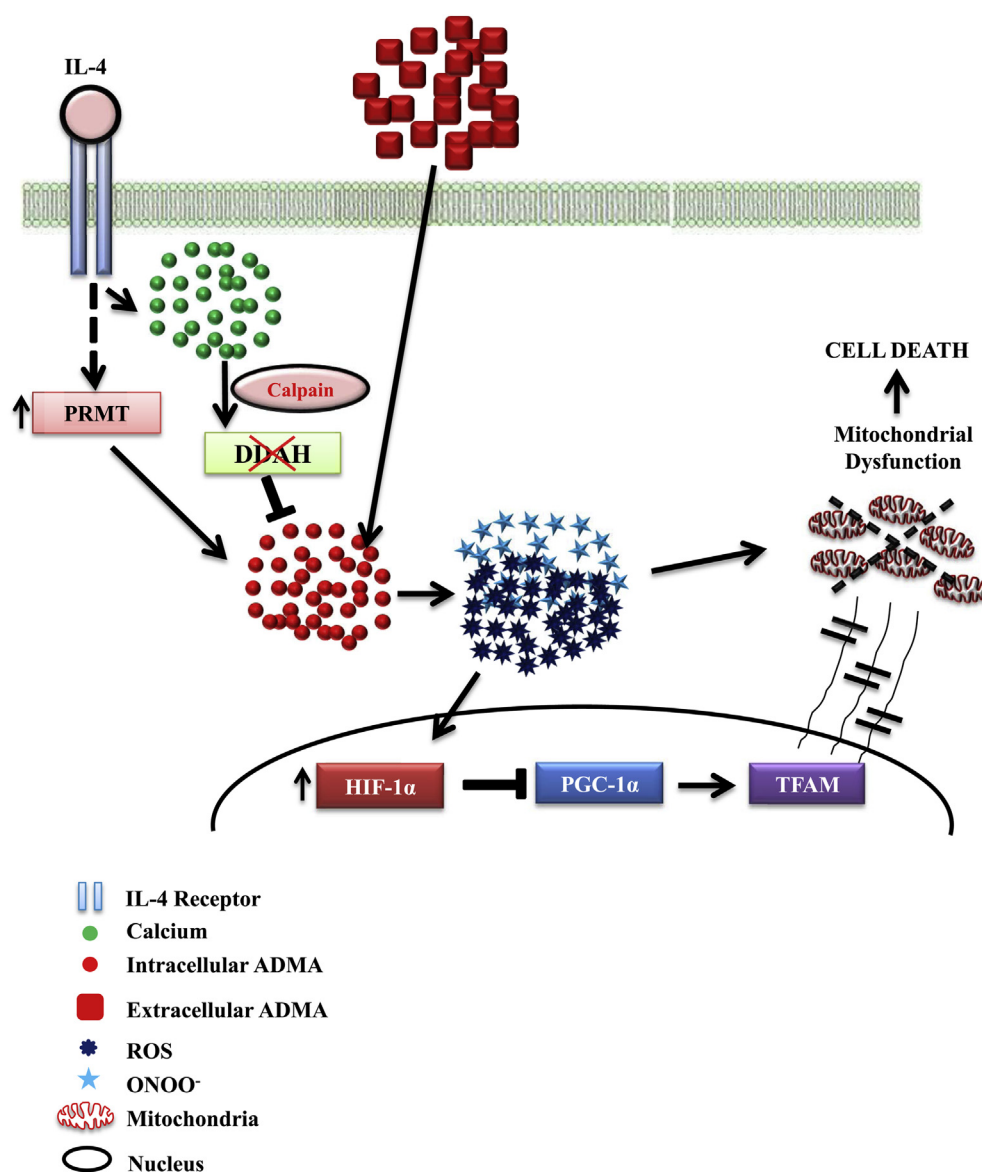


FIG 7. Schematic showing the mechanism of IL-4-mediated intracellular ADMA accumulation. Binding of IL-4 to IL-4 receptor leads to increased PRMT-2 expression and decreased DDAH-2 expression through calpain activation. Thus ADMA starts accumulating in the epithelial cells. Exogenous ADMA generated because of the complex interplay of inflammatory cytokines enters the epithelial cells, but its degradation machinery is hampered, thus further exaggerating the ADMA levels. This overaccumulation of ADMA causes oxo-nitrative stress and leads to hypoxic response and HIF-1 α activation in the nucleus. Activated HIF-1 α decreases mitochondrial biogenesis by the HIF-1 α -PGC-1 α -TFAM axis. Moreover, oxo-nitrative stress can also directly cause mitochondrial injury, and mitochondrial biogenesis is also diminished in the cell. This collectively leads to alteration of mitochondrial turnover and eventually cell death.

bioinformatic analysis with an online prediction tool (<http://calpain.org>) based on a multiple kernel learning algorithm²⁸ revealed a number of potential amino acid residues in DDAH-2 susceptible to calpain-mediated cleavage. Experimental validation proved that IL-4 increases calpain activity in BEAS2B cells (Fig 4, A), which is known to act through an increase in intracellular calcium levels, as depicted in Fig 4, B-D. Calpeptin-I, a calpain inhibitor, was able to block the IL-4-mediated degradation of DDAH-2 (Fig 4, E-G). Although DDAH-1 is generally described as the major DDAH isoform that regulates ADMA levels,⁴² the role of DDAH-2 in modulating

ADMA degradation has been documented in patients with lung disease.⁴³ We focused on DDAH-2 based on our previous findings in a murine model of AAI.⁴

We further extended our study to evaluate the role of IL-4 plus ADMA treatment in induction of the hypoxic response. The mechanism by which a hypoxic response can develop even in normoxic conditions and its relevance to allergic asthma have been previously discussed.¹⁹ A modest hypoxic response can have protective effects on airway epithelial cells, whereas an exaggerated response might be highly deleterious and proasthmatic.¹⁹ We found that IL-4 plus ADMA treatment leads

to induction of HIF-1 α (Fig 5, A-D), a key transcription factor regulating the hypoxic response. To determine the *in vivo* relevance, we measured IL-4, ADMA, HIF-1 α , and TFAM expression in lungs of mice with diet-induced metabolic syndrome (HFR and HFA diet-fed mice) and AAI (OVA-treated mice). Interestingly, each of these showed increased IL-4, ADMA, and HIF-1 α and diminished TFAM expression (see Figs E4 and E5). We have recently shown that an HFR diet in mice leads to AHR and peribronchial collagen deposition.¹¹ Previously, we found exaggerated peribronchial collagen deposition in mice with experimentally induced exuberant hypoxic response¹⁹ and recently showed that airway epithelial mitochondrial health importantly determines myofibroblast proliferation.² Putting this disparate evidence together, we speculate that airway epithelium is susceptible to ADMA-induced hypoxic response and that this drives airway remodeling in metabolic models of AHR through direct and/or mitochondrial effects. We do not believe that there is true hypoxia in airway epithelial cells of such mice because they have normal tidal volumes and respiratory rates during single-chamber plethysmography, as well as near-normal baseline airway resistance, suggesting patency of the major airways. The effect of ADMA and IL-4 in mice with a genetically impaired hypoxic response is not known but merits exploration. The requirement of HIF-1 α for proper lung maturation makes this technically complex.

The observed mitotoxicity in our model cannot be entirely attributed to either hypoxic response or oxo-nitrative stress. First, HIF-1 α is shown to mediate mitochondrial dysfunction, mainly by altering mitochondrial functions but not shape and integrity,⁴⁴ as seen by us.

Second, our rescue experiments with iNOS inhibitor (1400W) and HIF-1 α knockdown were only modestly effective in recovering the IL-4 plus ADMA-induced decrease in mitochondrial mass (Fig 6, A and B) and, similarly, the levels of PGC-1 α and TFAM (Fig 6, C and D). The rescue might have been improved by simultaneously targeting HIF-1 α and oxo-nitrative stress. However, previous studies have shown that overexpression of TFAM alone is sufficient to improve mitochondrial biogenesis and reduce oxidative stress-mediated mitochondrial damage.⁴⁵ Therefore we used TFAM overexpression as a strategy to rescue the IL-4 plus ADMA-mediated mitochondrial turnover. TFAM overexpression significantly restored mitochondrial mass and function in comparison with HIF-1 α knockdown or 1400W (Fig 6 and see Fig E8).

Taken together, our results suggest that intracellular ADMA accumulation, when ADMA and IL-4 levels are both increased, causes airway epithelial injury and remodeling (Fig 7). We speculate that this contributes to the pathogenesis of difficult-to-treat allergic asthma in obese patients. Exposure to IL-4 sensitizes airway epithelial cells to ADMA exposure because of calpain-mediated degradation of DDAH-2, an ADMA-catabolizing enzyme. ADMA induces mitotoxicity through multiple mechanisms, including oxidative stress-mediated HIF-1 α upregulation causing mitochondrial dysfunction and downregulation of PGC-1 α and TFAM, critical factors associated with mitochondrial biogenesis. Restoration of TFAM returned mitochondrial health to near-normal levels. Eriocitrin, a compound isolated from lemons, is shown to increase TFAM expression and ATP production and can be tested in experimental models of metabolic syndrome and asthma.⁴⁶

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Key messages

- IL-4 promotes accumulation of ADMA in lung cells and potentiates ADMA-related mitochondrial toxicity.
- In the presence of IL-4, ADMA potently leads to oxo-nitrative stress-induced hypoxic response in the airway epithelium despite normoxic conditions.
- The hypoxic response is associated with mitochondrial loss and can be reversed by overexpression of mitochondrial biogenesis factors, such as TFAM.

REFERENCES

1. Agrawal A, Prakash YS. Obesity, metabolic syndrome, and airway disease: a bioenergetic problem? *Immunol Allergy Clin North Am* 2014;34:785-96.
2. Ahmad T, Mukherjee S, Pattnaik B, Kumar M, Singh S, Rehman R, et al. Miro1 regulates intercellular mitochondrial transport & enhances mesenchymal stem cell rescue efficacy. *EMBO J* 2014;33:994-1010.
3. Mabalirajan U, Ahmad T, Leishangthem GD, Dinda AK, Agrawal A, Ghosh B. L-arginine reduces mitochondrial dysfunction and airway injury in murine allergic airway inflammation. *Int Immunopharmacol* 2010;10:1514-9.
4. Ahmad T, Mabalirajan U, Ghosh B, Agrawal A. Altered asymmetric dimethyl arginine metabolism in allergically inflamed mouse lungs. *Am J Respir Cell Mol Biol* 2010;42:3-8.
5. Tran CT, Leiper JM, Vallance P. The DDAH/ADMA/NOS pathway. *Atheroscler Suppl* 2003;4:33-40.
6. Fiedler L. The DDAH/ADMA pathway is a critical regulator of NO signalling in vascular homeostasis. *Cell Adh Migr* 2008;2:149-50.
7. Klein E, Weigel J, Buford MC, Holian A, Wells SM. Asymmetric dimethylarginine potentiates lung inflammation in a mouse model of allergic asthma. *Am J Physiol Lung Cell Mol Physiol* 2010;299:L816-25.
8. Mabalirajan U, Ahmad T, Leishangthem GD, Joseph DA, Dinda AK, Agrawal A, et al. Beneficial effects of high dose of L-arginine on airway hyperresponsiveness and airway inflammation in a murine model of asthma. *J Allergy Clin Immunol* 2010;125:626-35.
9. Wells SM, Buford MC, Miglaccio CT, Holian A. Elevated asymmetric dimethylarginine alters lung function and induces collagen deposition in mice. *Am J Respir Cell Mol Biol* 2009;40:179-88.
10. Mabalirajan U, Ghosh B. Mitochondrial dysfunction in metabolic syndrome and asthma. *J Allergy (Cairo)* 2013;2013:340476.
11. Singh VP, Aggarwal R, Singh S, Banik A, Ahmad T, Pattnaik BR, et al. Metabolic syndrome is associated with increased oxo-nitrative stress and asthma-like changes in lungs. *PLoS One* 2015;10:e0129850.
12. Leishangthem GD, Mabalirajan U, Singh VP, Agrawal A, Ghosh B, Dinda AK. Ultrastructural changes of airway in murine models of allergy and diet-induced metabolic syndrome. *ISRN Allergy* 2013;2013:261297.
13. Scott JA, Grasmann H. Arginine metabolism in asthma. *Immunol Allergy Clin North Am* 2014;34:767-75.
14. Diaz J, Farzan S. Clinical implications of the obese-asthma phenotypes. *Immunol Allergy Clin North Am* 2014;34:739-51.
15. Sun Q, Yang X, Zhong B, Jiao F, Li C, Li D, et al. Upregulated protein arginine methyltransferase 1 by IL-4 increases eotaxin-1 expression in airway epithelial cells and participates in antigen-induced pulmonary inflammation in rats. *J Immunol* 2012;188:3506-12.
16. Scharte M, Jurk K, Kehrel B, Zarbock A, Van Aken H, Singbartl K. IL-4 enhances hypoxia induced HIF-1 α protein levels in human transformed intestinal cells. *FEBS Lett* 2006;580:6399-404.
17. Li J, Kil C, Considine K, Smarkucki B, Stankewich MC, Balgley B, et al. Intrinsic indicators for specimen degradation. *Lab Invest* 2013;93:242-53.
18. Armandis T, Ferrer-Vicens I, Garcia-Trevijano ER, Miralles VJ, Garcia C, Torres L, et al. Calpains mediate epithelial-cell death during mammary gland involution: mitochondria and lysosomal destabilization. *Cell Death Differ* 2012;19:1536-48.
19. Ahmad T, Kumar M, Mabalirajan U, Pattnaik B, Aggarwal S, Singh R, et al. Hypoxia response in asthma: differential modulation on inflammation and epithelial injury. *Am J Respir Cell Mol Biol* 2012;47:1-10.

20. Zhu L, Wang Q, Zhang L, Fang Z, Zhao F, Lv Z, et al. Hypoxia induces PGC-1 α expression and mitochondrial biogenesis in the myocardium of TOF patients. *Cell Res* 2010;20:676-87.
21. Krishnan J, Suter M, Windak R, Krebs T, Felley A, Montessuit C, et al. Activation of a HIF1 α -PPAR γ axis underlies the integration of glycolytic and lipid anabolic pathways in pathologic cardiac hypertrophy. *Cell Metab* 2009;9:512-24.
22. Dillon LM, Rebelo AP, Moraes CT. The role of PGC-1 coactivators in aging skeletal muscle and heart. *IUBMB Life* 2012;64:231-41.
23. Larsson NG, Wang J, Wilhelmsson H, Oldfors A, Rustin P, Lewandoski M, et al. Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat Genet* 1998;18:231-6.
24. Ekstrand MI, Falkenberg M, Rantanen A, Park CB, Gaspari M, Hultenby K, et al. Mitochondrial transcription factor A regulates mtDNA copy number in mammals. *Hum Mol Genet* 2004;13:935-44.
25. Takamatsu C, Umeda S, Ohsato T, Ohno T, Abe Y, Fukuoh A, et al. Regulation of mitochondrial D-loops by transcription factor A and single-stranded DNA-binding protein. *EMBO Rep* 2002;3:451-6.
26. Viader A, Golden JP, Baloh RH, Schmidt RE, Hunter DA, Milbrandt J. Schwann cell mitochondrial metabolism supports long-term axonal survival and peripheral nerve function. *J Neurosci* 2011;31:10128-40.
27. Movafagh S, Crook S, Vo K. Regulation of hypoxia-inducible factor-1 α by reactive oxygen species: new developments in an old debate. *J Cell Biochem* 2015;116:696-703.
28. DuVerle DA, Ono Y, Sorimachi H, Mamitsuka H. Calpain cleavage prediction using multiple kernel learning. *PLoS One* 2011;6:e19035.
29. Kips JC. Cytokines in asthma. *Eur Respir J Suppl* 2001;34:24s-33s.
30. Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* 2008;118:3546-56.
31. Townley RG. Interleukin 13 and the beta-adrenergic blockade theory of asthma revisited 40 years later. *Ann Allergy Asthma Immunol* 2007;99:215-24.
32. Ahmad T, Aggarwal K, Pattnaik B, Mukherjee S, Sethi T, Tiwari BK, et al. Computational classification of mitochondrial shapes reflects stress and redox state. *Cell Death Dis* 2013;4:e461.
33. Agrawal A, Mabalirajan U, Ahmad T, Ghosh B. Emerging interface between metabolic syndrome and asthma. *Am J Respir Cell Mol Biol* 2011;44:270-5.
34. Serafino-Agrusa L, Spatafora M, Scichilone N. Asthma and metabolic syndrome: current knowledge and future perspectives. *World J Clin Cases* 2015;3:285-92.
35. Staab EB, Weigel J, Xiao F, Madayiputhiya N, Wyatt TA, Wells SM. Asymmetric dimethyl-arginine metabolism in a murine model of cigarette smoke-mediated lung inflammation. *J Immunotoxicol* 2015;12:273-82.
36. Carraro S, Giordano G, Piacentini G, Kantar A, Moser S, Cesca L, et al. Asymmetric dimethylarginine in exhaled breath condensate and serum of children with asthma. *Chest* 2013;144:405-10.
37. Wells SM, Holian A. Asymmetric dimethylarginine induces oxidative and nitrosative stress in murine lung epithelial cells. *Am J Respir Cell Mol Biol* 2007;36:520-8.
38. Sharma P, Chakraborty R, Wang L, Min B, Tremblay ML, Kawahara T, et al. Redox regulation of interleukin-4 signaling. *Immunity* 2008;29:551-64.
39. Boger RH. Asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase, explains the "L-arginine paradox" and acts as a novel cardiovascular risk factor. *J Nutr* 2004;134:2842S-53S.
40. Vallance P, Leiper J. Cardiovascular biology of the asymmetric dimethylarginine: dimethylarginine dimethylaminohydrolase pathway. *Arterioscler Thromb Vasc Biol* 2004;24:1023-30.
41. Chua BT, Guo K, Li P. Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. *J Biol Chem* 2000;275:5131-5.
42. Liu X, Fassett J, Wei Y, Chen Y. Regulation of DDAH1 as a potential therapeutic target for treating cardiovascular diseases. *Evid Based Complement Alternat Med* 2013;2013:619207.
43. Aggarwal S, Gross CM, Kumar S, Dimitropoulou C, Sharma S, Gorshkov BA, et al. Dimethylarginine dimethylaminohydrolase II overexpression attenuates LPS-mediated lung leak in acute lung injury. *Am J Respir Cell Mol Biol* 2014;50:614-25.
44. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metab* 2006;3:187-97.
45. Chakrabarty S, D'Souza RR, Kabekkodu SP, Gopinath PM, Rossignol R, Satyamoorthy K. Upregulation of TFAM and mitochondria copy number in human lymphoblastoid cells. *Mitochondrion* 2014;15:52-8.
46. Hiramitsu M, Shimada Y, Kuroyanagi J, Inoue T, Katagiri T, Zang L, et al. Eriocitrin ameliorates diet-induced hepatic steatosis with activation of mitochondrial biogenesis. *Sci Rep* 2014;4:3708.

METHODS

Development of an *in vivo* model of metabolic syndrome

C57BL/6 mice (4-5 weeks old) were obtained from the National Institute of Nutrition (Hyderabad, India) and acclimatized for a week before the start of the experiments. All animals were maintained per Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines, and protocols were approved by the institutional animal ethics committee. After 1 week of acclimatization, the mice were separated into 3 groups ($n = 6$) based on the diets they would be provided, namely control, HFA, and HFR groups, and were housed in individually ventilated cages ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and 50% humidity and 12/12-hour dark/light cycle) with enrichment facilities. The control mice had free access to standard rodent chow (5.5% fat and nil refined fructose). HFA mice had a high-fat diet, with 60% of energy from fat, and HFR mice had an HFR diet, with 70% energy from fructose. Mice were kept on this diet for 18 weeks and killed for collection of lung tissue. The HFA and HFR diets were obtained from Research Diet (New Brunswick, NJ).

Development of an *in vivo* model of allergic asthma

Male BALB/c mice (8-10 weeks old) were obtained from the National Institute of Nutrition and acclimatized for 1 week, according to animal ethics guidelines provided by the institute animal ethical committee. Mice were divided into 2 groups: sensitization/challenge (sham/PBS [normal control animals]) and OVA/OVA (chicken egg OVA, grade V; Sigma-Aldrich, St Louis, Mo). An allergic asthmatic mice model was developed, as described previously.^{E1}

Immunohistochemistry

Formalin-fixed, paraffin-embedded lung tissue sections were prepared for immunohistochemical analysis. Sections were stained with the primary antibodies for HIF-1 α (mouse mAb, Abcam) and TFAM (rabbit polyclonal, Abcam), followed by HRP-conjugated secondary antibodies (Bang Genei). The signal was detected by using DAB (Sigma), and hematoxylin was used as a counterstain.

Immunoblotting

Immunoblotting was done, as mentioned earlier.^{E2} Total cell lysate and cytosolic fractions were prepared from BEAS2B cells and run on 10% SDS-PAGE, followed by transfer onto a polyvinylidene difluoride membrane (MDI). The blots were incubated with a primary mAb for HIF-1 α , TFAM, and cytochrome c, respectively. Anti-rabbit or anti-mouse secondary antibodies (Sigma) conjugated with HRP were used, and DAB along with H_2O_2 was

used as a substrate. α -Tubulin was used as loading controls for total cell lysate and cytosolic fractions, respectively (both from Santa Cruz Biotechnology).

ELISA

Total lung lysate was prepared from the AAI and metabolic syndrome murine models (HFA and HFR). Total ADMA levels were measured in the lung lysate by means of ELISA and normalized to total protein content, per the manufacturer's protocol (DLD Diagnostika).

ATP measurement

The ATP assay was measured with the ATP Bioluminescence Assay Kit (Sigma or Invitrogen), according to the manufacturer's instructions. Briefly, 100 $\mu\text{g/mL}$ total cell lysate of BEAS2B cells was prepared and used for the assay, and measurements were taken with a Bioluminometer (Berthold AG model). The amount of bioluminescent signal was recorded and expressed as relative light units.

Measurement of complex I and IV activity

Mitochondrial protein extraction was done, and total mitochondrial protein was used for measurement of complex I and complex IV activity (Sigma-Aldrich), as described earlier.^{E3}

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured by using JC-1 staining. BEAS2B cells, treated with IL-4 plus ADMA alone, or IL-4 plus ADMA followed by TFAM overexpression, were stained with JC-1 (Life Technologies) for 15 minutes and measured by using flow cytometry (BD FACSCalibur).

REFERENCES

- E1. Mabalirajan U, Ahmad T, Leishangthem GD, Joseph DA, Dinda AK, Agrawal A, et al. Beneficial effects of high dose of L-arginine on airway hyperresponsiveness and airway inflammation in a murine model of asthma. *J Allergy Clin Immunol* 2010;125:626-35.
- E2. Ahmad T, Mabalirajan U, Ghosh B, Agrawal A. Altered asymmetric dimethyl arginine metabolism in allergically inflamed mouse lungs. *Am J Respir Cell Mol Biol* 2010;42:3-8.
- E3. Mabalirajan U, Ahmad T, Leishangthem GD, Dinda AK, Agrawal A, Ghosh B. L-arginine reduces mitochondrial dysfunction and airway injury in murine allergic airway inflammation. *Int Immunopharmacol* 2010;10:1514-9.

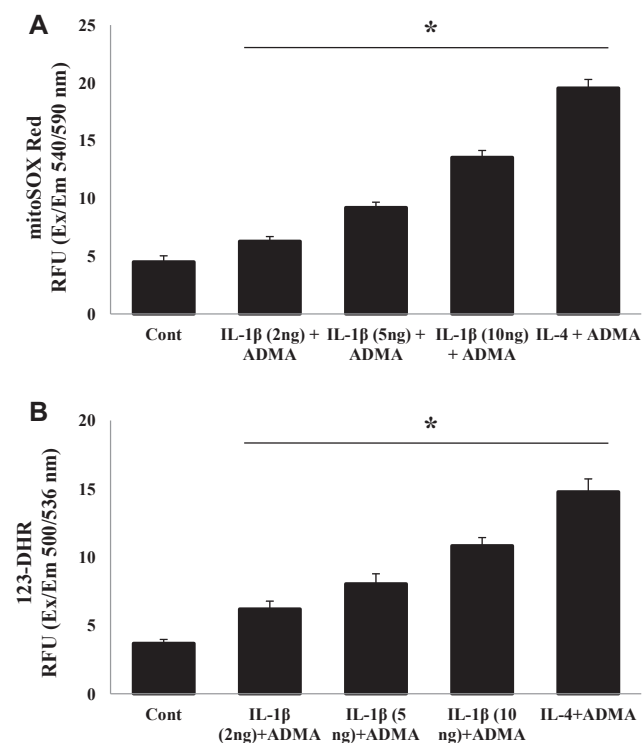


FIG E1. IL-1 β , followed by ADMA, leads to dose-dependent induction of mtROS but is less potent than IL-4 followed by ADMA. **A** and **B**, mtROS and peroxynitrite ion formation in BEAS2B cells treated with different doses of IL-1 β followed by ADMA and IL-4 followed by ADMA. Treatment of BEAS2B cells with different doses of IL-1 β (2, 5, and 10 ng) plus ADMA showed an increase in mtROS and peroxynitrite ion formation in a dose-dependent manner. However, the most prominent increase was found in the IL-4 plus ADMA group. Data represent means $\pm$ SEs ($n = 3$). $*P < .05$. Cont, Control; RFU, relative fluorescence units.

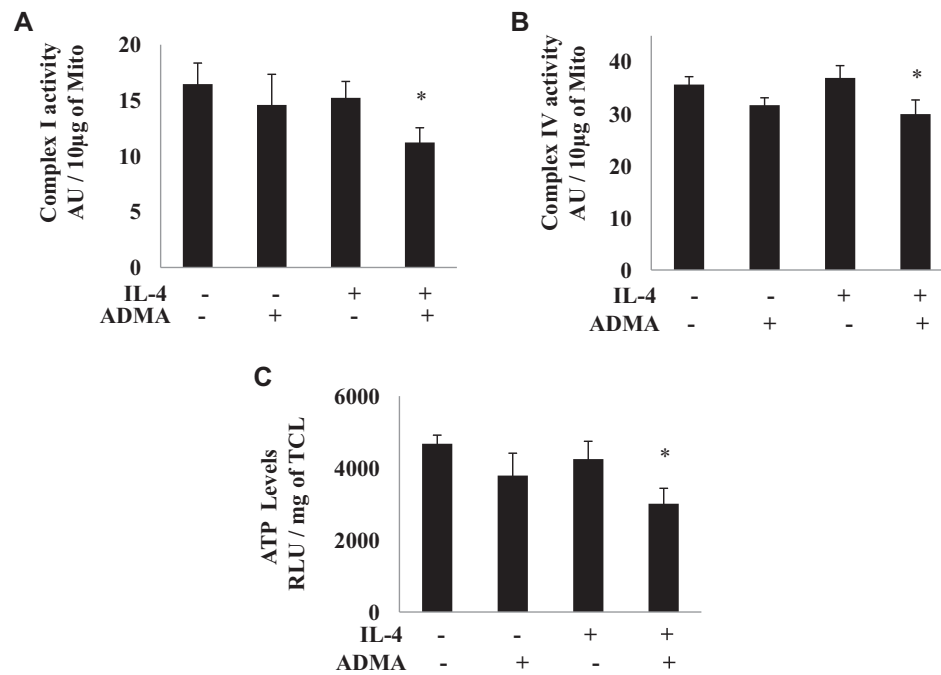


FIG E2. Altered mitochondrial functions in IL-4 plus ADMA-treated BEAS2B cells. significant decreases in mitochondrial complex I (**A**), complex IV (**B**), and ATP (**C**) levels in BEAS2B cells treated with IL-4 plus ADMA were shown. Data represent means $\pm$ SEs ($n = 3$). * $P < .05$. *Mito*, Mitochondrial extract; *TCL*, total cell lysate.

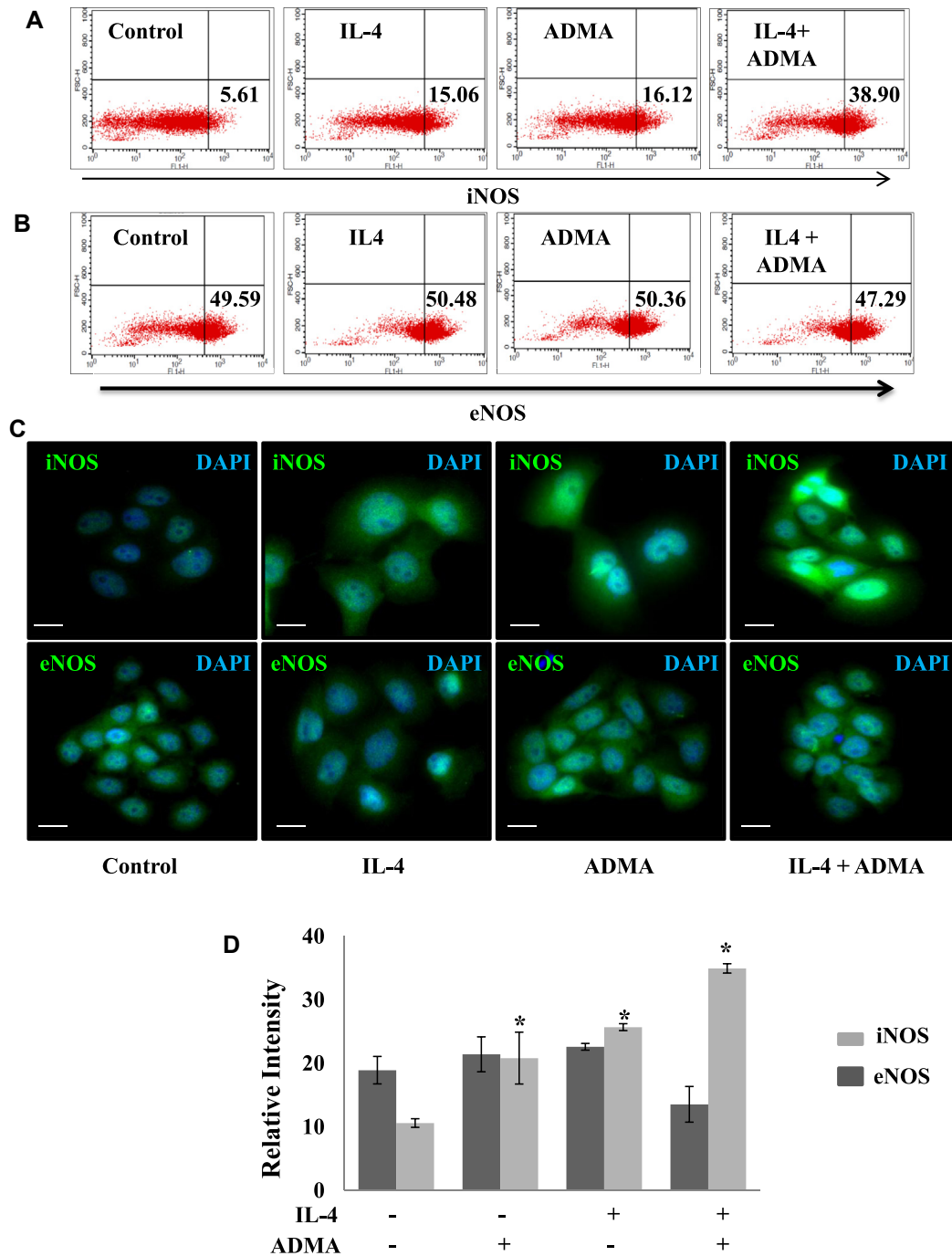


FIG E3. IL-4 plus ADMA treatment leads to iNOS activation. **A** and **B**, A significant increase in iNOS (Fig E3, A) but not eNOS (Fig E3, B) levels is observed in BEAS2B cells induced with IL-4 plus ADMA. Treatment with IL-4 plus ADMA shows a significant increase in nuclear translocation of iNOS but not eNOS (magnification $\times 63$) (**C**) and the total fluorescence intensity of the images was determined and quantified (**D**). Scale = 20 μ m. Data represent mean $\pm$ SE ($n = 3$). * $P < .05$.

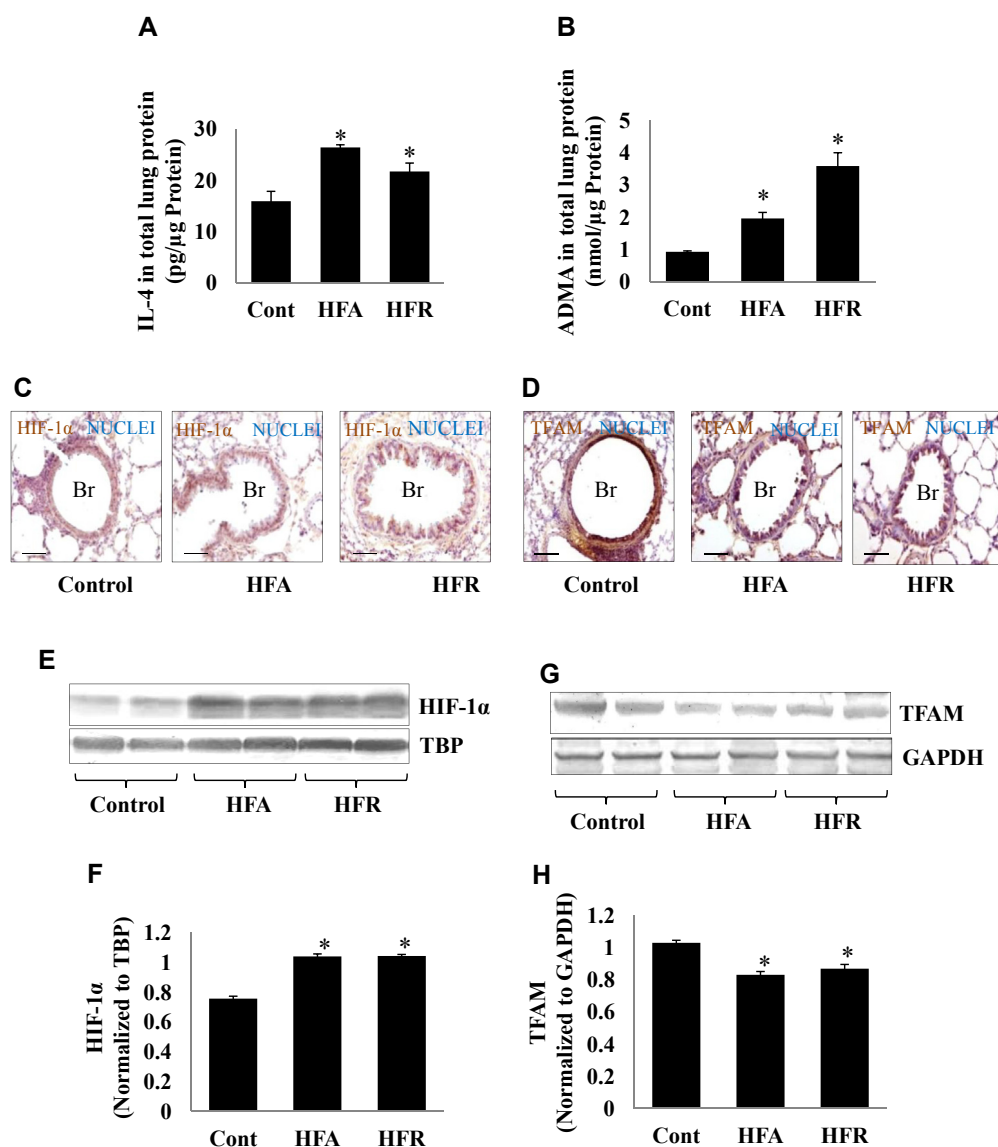


FIG E4. Increased HIF-1 α and decreased TFAM expression correlate with augmented ADMA production in murine model of metabolic syndrome. **A** and **B**, IL-4 and ADMA levels were measured in total lung lysates of HFA/HFR mice. **C** and **D**, Expression of HIF-1 α and TFAM in lung sections, respectively. **E** and **G**, Immunoblots for HIF-1 α in nuclear extract, and TFAM in total lung lysate of mice fed an HFA and HFR diet compared with control mice. Parts **F** and **H** summarize the immunoblots, magnification $\times 20$. Scale = 20 μ m. Data represent mean $\pm$ SE (n = 6). Br, Bronchi; Cont, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA-binding protein. * $P < .05$.

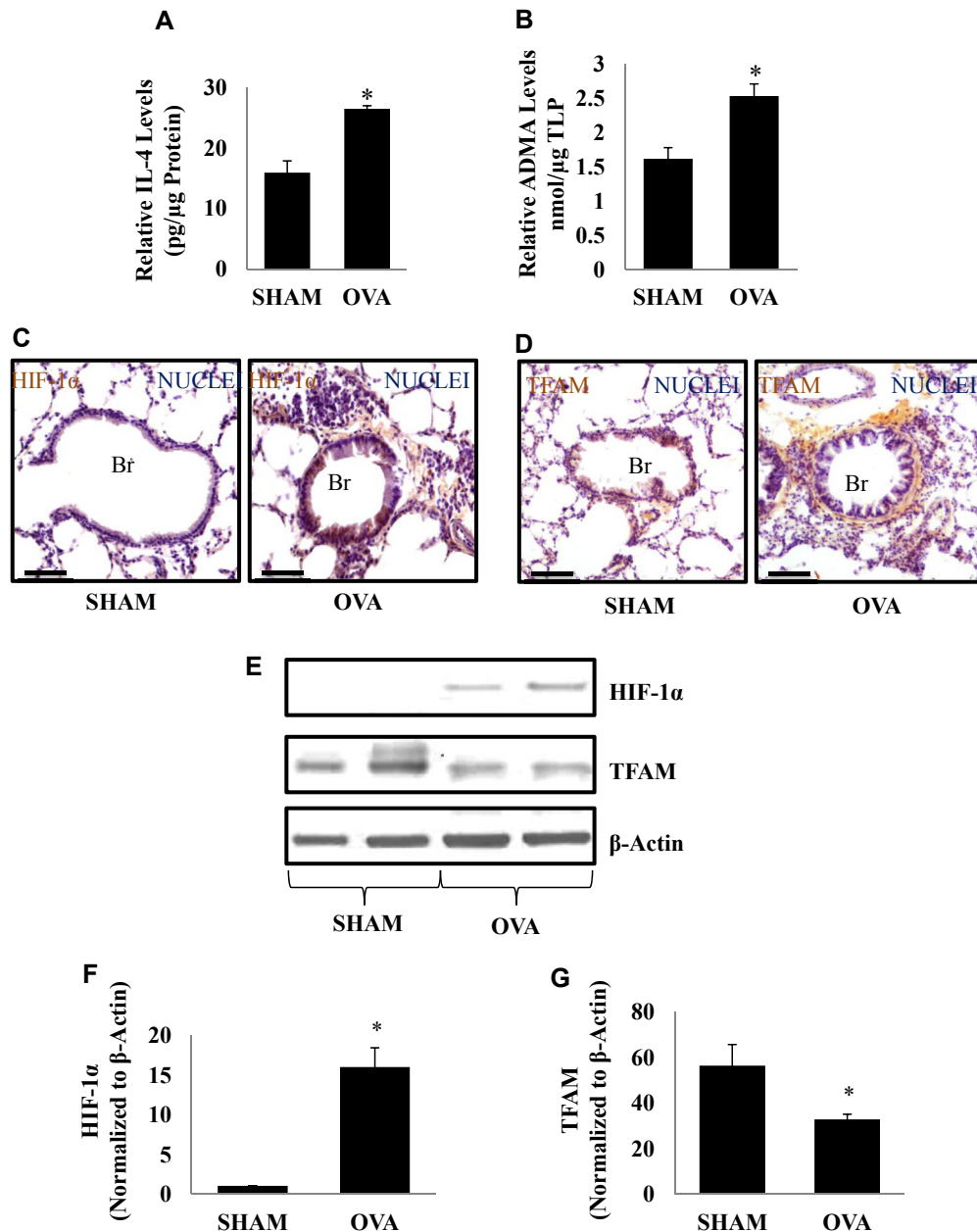


FIG E5. Increased HIF-1 α and decreased TFAM expression correlate with augmented ADMA production in a mouse model of AAI. **A** and **B**, Increased IL-4 and ADMA levels were measured in total lung lysates of OVA-challenged mice. **C** and **D**, Expression of HIF-1 α and TFAM in lung sections, respectively. **E**, Immunoblots for HIF-1 α and TFAM in total lung lysates of allergic asthmatic mice. Parts **F** and **H** summarize the immunoblot data. The data from Fig E5, C-G, indicate that a significant increase in HIF-1 α correlates with decreased TFAM expression (magnification $\times 20$). Scale = 20 μ m. Data represent means $\pm$ SEs (n = 6). * P < .05. Br, Bronchi TLP, total lung protein.

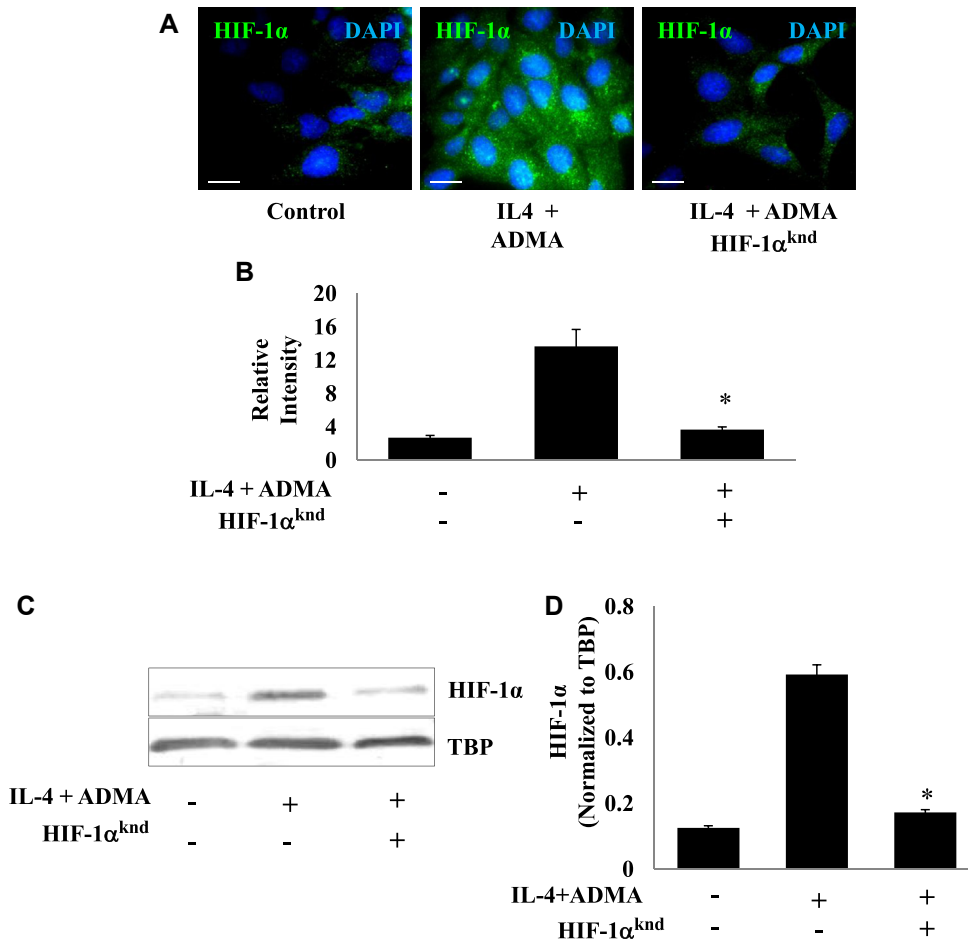


FIG E6. Validation of HIF-1 α knockdown. HIF-1 α knockdown (HIF-1 α^{knd}) was validated by means of immunocytochemistry (**A**) and immunoblotting with HIF-1 α antibody (**C**). The fluorescence intensity of the images and the densitometry of the bands in the immunoblot were determined and quantified in **B** and **D** respectively. It was significantly suppressed after transfection of small interfering RNA compared with induced conditions. Data represent mean $\pm$ SE ($n = 3$). * $P < .05$. *TBP*, TATA-binding protein.

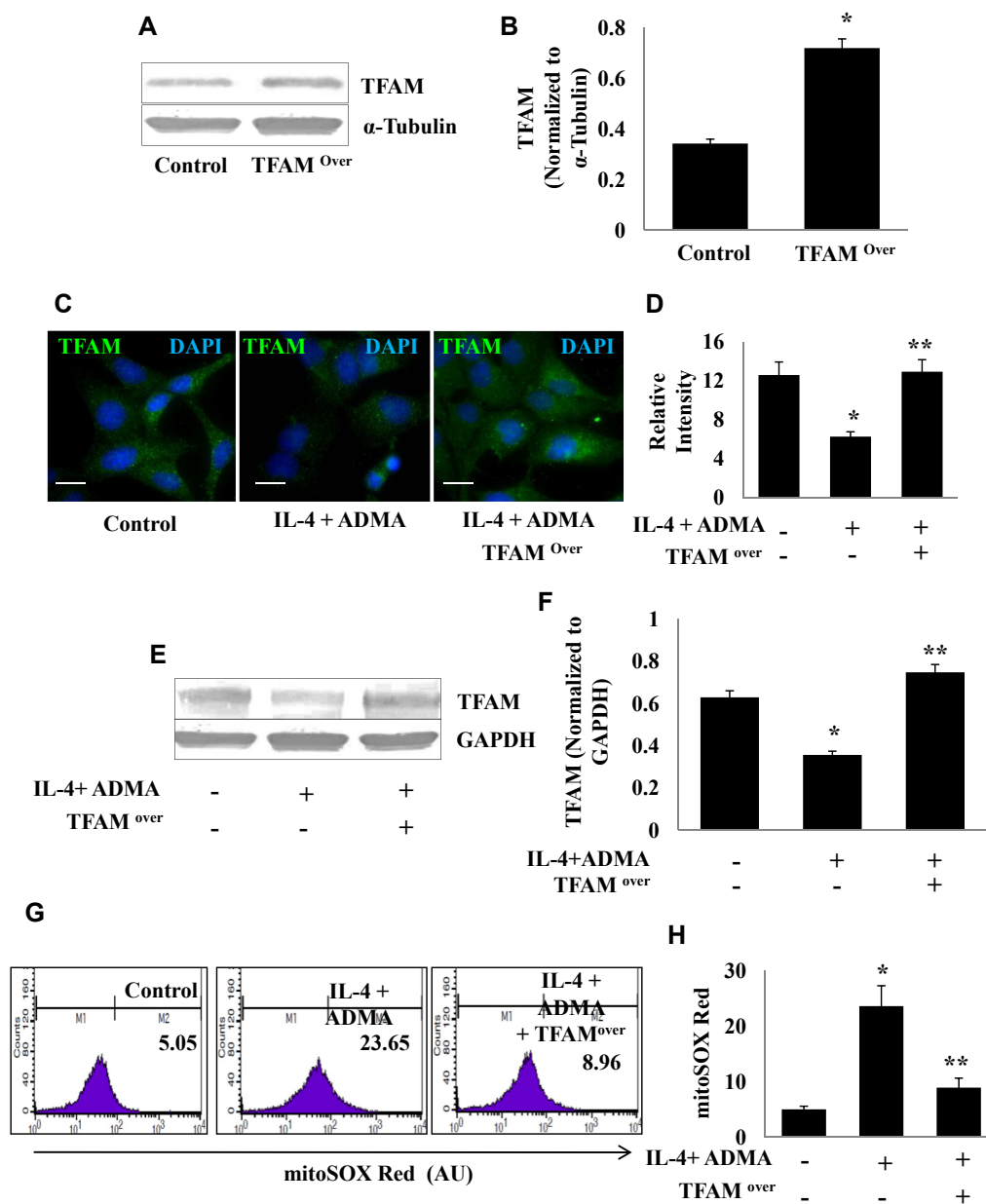


FIG E7. Validation of TFAM overexpression. **A-F**, TFAM overexpression (TFAM^{over}) was confirmed by means of immunocytochemistry and immunoblotting with TFAM antibody. **G** and **H**, TFAM expression was significantly increased after transfection of cDNA plasmid compared with induced conditions. We further checked the mtROS, which was found to be decreased in TFAM-overexpressed conditions. Data represent means $\pm$ SEs ($n = 3$). * $P < .05$, as compared to control and ** $P < .05$, as compared to IL-4 plus ADMA. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

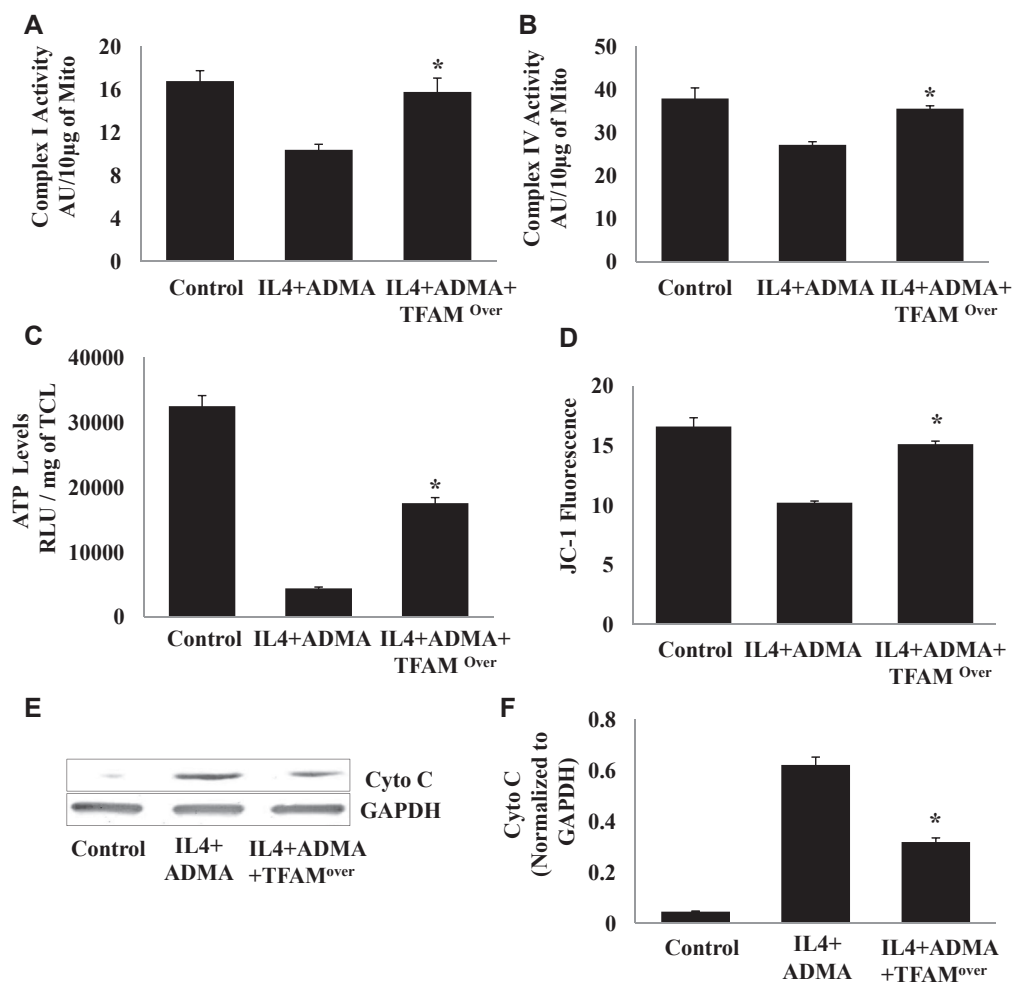


FIG E8. TFAM overexpression restored mitochondrial biogenesis and hence function. **A** and **B**, TFAM overexpression restored IL-4 plus ADMA-induced decrease in mitochondrial complex I/IV activity in BEAS2B cells. **C** and **D**, IL-4 plus ADMA treatment leads to decreased ATP levels (Fig E8, C) and mitochondrial membrane potential (JC-1 staining) that was significantly restored by TFAM overexpression (Fig E8, D). **E** and **F**, Immunoblotting for cytochrome c in cytosolic extract confirmed that overexpression of TFAM restored cytochrome c (Cyto c) release from mitochondria. Data represent means $\pm$ SEs ($n = 5$). * $P < .05$. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; Cyto C, cytochrome C; Mito, mitochondrial extract; TCL, total cell lysate.