

Metformin protects the heart against chronic intermittent hypoxia through AMPK-dependent phosphorylation of HIF-1 α

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Chronic intermittent hypoxia (IH), a major feature of obstructive sleep apnea syndrome (OSA), is associated with greater severity of myocardial infarction. In this study, we performed RNA sequencing of cardiac samples from mice exposed to IH, which reveals a specific transcriptomic signature of the disease, relative to mitochondrial remodeling and cell death. Corresponding to its activation under chronic IH, we stabilized the Hypoxia Inducible Factor-1 α (HIF-1 α) in cardiac cells *in vitro* and observed its association with an increased autophagic flux. In accordance, IH induced autophagy and mitophagy, which are decreased in HIF-1 α ^{+/-} mice compared to wild-type animals, suggesting that HIF-1 plays a significant role in IH-induced mitochondrial remodeling. Next, we showed that the AMPK metabolic sensor, typically activated by mitochondrial stress, is inhibited after 3 weeks of IH in hearts. Therefore, we assessed the effect of metformin, an anti-diabetic drug and potent activator of AMPK, on myocardial response to ischemia–reperfusion (I/R) injury. Daily administration of metformin significantly decreases infarct size without any systemic beneficial effect on insulin resistance under IH conditions. The cardioprotective effect of metformin was lost in AMPK α 2 knock-out mice, demonstrating that AMPK α 2 isoform promotes metformin-induced cardioprotection in mice exposed to IH. Mechanistically, we found that metformin inhibits IH-induced mitophagy in myocardium and decreases HIF-1 α nuclear expression in mice subjected to IH. *In vitro* experiments demonstrated that metformin induced HIF-1 α phosphorylation, decreased its nuclear localization, and HIF-1 transcriptional activity. Collectively, these results identify the AMPK α 2 metabolic sensor as a novel modulator of HIF-1 activity. Our data suggest that metformin could be considered as a cardioprotective drug in OSA patients independently of their metabolic status.

Abbreviations

AMPK, AMP-activated protein kinase; CoCl₂, cobalt chloride; ER, endoplasmic reticulum; HIF-1, hypoxia inducible factor-1; I/R, ischemia–reperfusion; IH, intermittent hypoxia; MI, myocardial infarction; N, normoxia; OSA, obstructive Sleep Apnea.

Introduction

Obstructive sleep apnea syndrome (OSA) is a highly prevalent chronic disease that affects nearly 1 billion people worldwide [1]. OSA is associated with the development or aggravation of cardiovascular and metabolic diseases [2]. Among cardiovascular diseases, OSA is linked to an increase in infarct size [3] recognized to impair post-myocardial infarction (MI) structural and functional recovery [4] and to accelerate the development of heart failure in apneic patients [5].

OSA is characterized by repetitive pharyngeal collapses leading to chronic intermittent hypoxia (IH). IH is now well recognized to increase infarct size in several rodent models [6–8]. In the myocardium, IH induces oxidative stress [8,9] and sustained Hypoxia Inducible Factor-1 (HIF-1) activation [6,7,10], leading to cardiomyocyte apoptosis through convergent mechanisms such as endoplasmic reticulum (ER) stress [7,11,12] and mitochondrial dysfunction [13,14]. Specifically, we previously demonstrated that chronic IH activates HIF-1, which is responsible for IH-induced ER stress [13–15], calcium homeostasis alteration [7], mitochondria-associated ER-membrane disruption, mitochondrial dysfunction [13], and cardiomyocyte death [6,7].

Maintenance of mitochondrial function and integrity is primordial for cardiomyocyte survival, requiring a highly dynamic network that can alter metabolic function and the response to ischemia–reperfusion (I/R) injury [16]. Mitochondrial stress is linked to mitochondrial remodeling particularly through mitophagy [17,18]. This process enables the selective clearance of dysfunctional mitochondria, correlating with beneficial effects for cardiac protection [19]. However, along with severe stress, mitophagy is inefficient, leading to the release of pro-apoptotic factors and cell death [20]. This illustrates that, depending on the context, short-term and long-term upregulation of mitophagy exerts a differential impact on mitochondrial homeostasis and cell viability [21].

Among the proteins activated independently by both mitochondrial stress and IH [22], the AMP-activated Protein Kinase (AMPK) has been demonstrated to prevent cardiomyocyte death in several contexts [23]. However, the impact of chronic IH on myocardial AMPK activity remains unclear, depending on the depth and duration of IH, as well as the model and the tissue studied [24–27].

Because chronic IH alters mitochondrial function and leads to cardiomyocyte apoptosis [13–15], we first investigated if IH induced mitochondrial remodeling by evaluating autophagy and mitophagy. Since HIF-1

is recognized to activate genes encoding the autophagic process [28,29], we also questioned if IH-induced mitochondrial remodeling depends on HIF-1. Then, considering that AMPK activation has been demonstrated to be involved in the myocardial response to mitochondrial stress, we questioned its myocardial activation state under chronic IH. We also investigated the impact of metformin treatment (an oral anti-diabetic drug and AMPK activator) on (a) myocardium response to I/R by measuring infarct size and (b) mitochondrial stress under IH. Finally, we explored how AMPK activation can modulate HIF-1 activity.

Results

Intermittent hypoxia exposure

Chronic IH was induced in mice as illustrated by the design in Fig. 1A. Chronic IH allowing 60 cycles (5–21% FiO₂) *per* hour is a unique and recognized model reproducing many cardiometabolic consequences of OSA. It was validated through systematic body weight assessment every week, as well as hematocrit measurement at the end of exposure. As expected, the body weight of hypoxic mice decreased during the first days of exposure (Fig. 1B) before rising normally, and hematocrit was significantly increased at the end of exposure (Fig. 1C).

Intermittent hypoxia induces cardiac stress and mitochondrial remodeling

We investigated whether chronic IH alters the myocardial transcriptome (Fig. 2A). RNA sequencing and bioinformatic analysis revealed that 86 genes were dysregulated, with 42 upregulated and 44 down-regulated (FDR < 0.05) (Fig. 2B,C). Among the top 20 differentially expressed genes, Panther process analyses highlighted changes in erythrocytes O₂/CO₂ exchanges and oxidative stress, which are hallmark of IH (Fig. 2D). Gene Ontology biological process analysis revealed changes in response to stress and metabolic processes (Fig. 2E), consistent with the previously demonstrated impact of IH on myocardial mitochondrial dysfunction. Of particular importance, Angiopoietin 1 (*Angpt1*), Thioredoxin-interacting protein (*Txnip*), Nicotinamide riboside kinase 2 (*Nmrk2*), NUA family SNF1-like kinase 1 (*Nuak1*), or 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1 (*Pfkfb1*) genes belonging to the top 20 dysregulated genes are hallmark genes of hypoxia and glycolysis

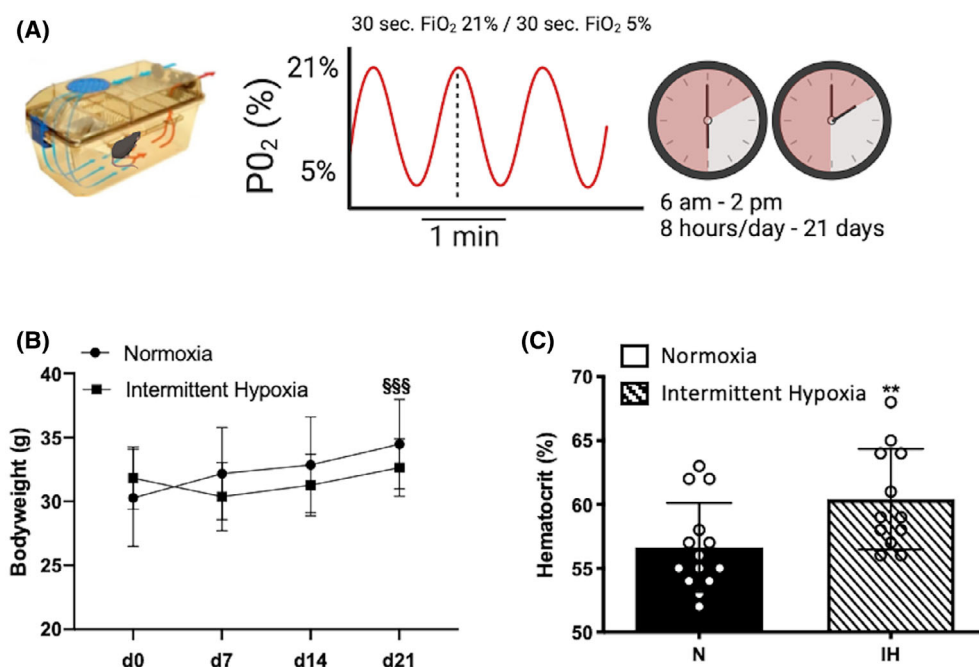


Fig. 1. Intermittent hypoxia model validation. Scheme of intermittent hypoxia (IH) exposition; animals were exposed in their own cages to 1-min cycles of IH (30 s of inspired O_2 fraction (FiO_2) 21%/30 s FiO_2 5%) or Normoxia (N, same noises generated by cycles without dropping FiO_2), 8 h per day (D), from 6 am to 2 pm (A). Bodyweight at day (d) 0, d7, d14, and d21 of N or IH ($n = 18$) (B), data are represented as mean $\pm$ standard deviation; $***P < 0.001$ d21 vs. d0, two-way ANOVA, Šidák *post-hoc* tests. Hematocrit after 21 days of N or IH ($n = 12$ –14) (C), data are presented as individual plots and expressed as mean $\pm$ standard deviation; $**P < 0.01$ IH vs. N, *t*-test. Scheme was made with [Biorender.com](https://biorender.com).

(Fig. 2F). Finally, Gene Set Enrichment Analysis performed on the dysregulated genes revealed changes in cardiac stress, cell death, and mitochondrial remodeling biological processes (Fig. 2G). Considering the pathways highlighted by our analysis and the impact of chronic IH on HIF-1-dependent mitochondrial dysfunction and cardiomyocyte death, we then investigated the impact of IH and HIF-1 on mitochondrial remodeling.

Intermittent hypoxia-induced mitophagy is dependent on HIF-1

First, we investigated the link between HIF-1 chemical activation and autophagic flux in *in vitro* H9c2 cardiomyoblast (Fig. 3A). HIF-1 stabilization by cobalt chloride ($CoCl_2$) increased HIF-1 α nuclear localization (Fig. 3B,C) and this was associated with a marked increase in autophagic flux. Indeed, chloroquine strongly increased LC3II/I ratio in $CoCl_2$ -treated cells compared with control cells (Fig. 3D).

Next, to demonstrate the role of HIF-1 in IH-induced mitochondrial remodeling *in vivo*, we exposed HIF-1 $^{+/+}$ and HIF-1 $^{-/-}$ mice to normoxia (N)

or IH (Fig. 4A). The partial decrease in HIF-1 mRNA transcripts (Fig. 4B) was sufficient to decrease HIF-1 activity in HIF-1 $^{+/+}$ mice compared to HIF-1 $^{+/+}$ upon IH (Fig. 4C). In HIF-1 $^{+/+}$ and HIF-1 $^{-/-}$ mice exposed to chronic IH, we investigated the expression of myocardial autophagic and mitophagic markers (Fig. 4D). Twenty-one days of IH induced autophagy and mitophagy, as revealed by a significant increase in the LC3II/I ratio, a significant decrease in p62 (Fig. 4E,F) as well as a significant increase in BNIP3 and Parkin (Fig. 4G,H) protein expressions in HIF-1 $^{+/+}$ (Fig. 4I). In HIF-1 $^{-/-}$, IH does not increase LC3II/I ratio and p62 expressions compared to N mice (Fig. 4E,F). Moreover, only under IH, BNIP3 and Parkin expressions were significantly decreased in HIF-1 $^{-/-}$ mice compared to HIF-1 $^{+/+}$ mice, suggesting that mitophagy under IH is driven by HIF-1 (Fig. 4G–I). After 21 days of chronic IH, there was no significant difference in mitochondrial dynamics markers expressions such as Mfn2, OPA1 and Drp1 between HIF-1 $^{+/+}$ and HIF-1 $^{-/-}$ (Fig. 4J–O). Images acquired by transmission electronic microscopy displayed characteristic mitophagy marks primarily in HIF-1 $^{+/+}$ cardiomyocytes from mice exposed to IH, revealing an abundance of

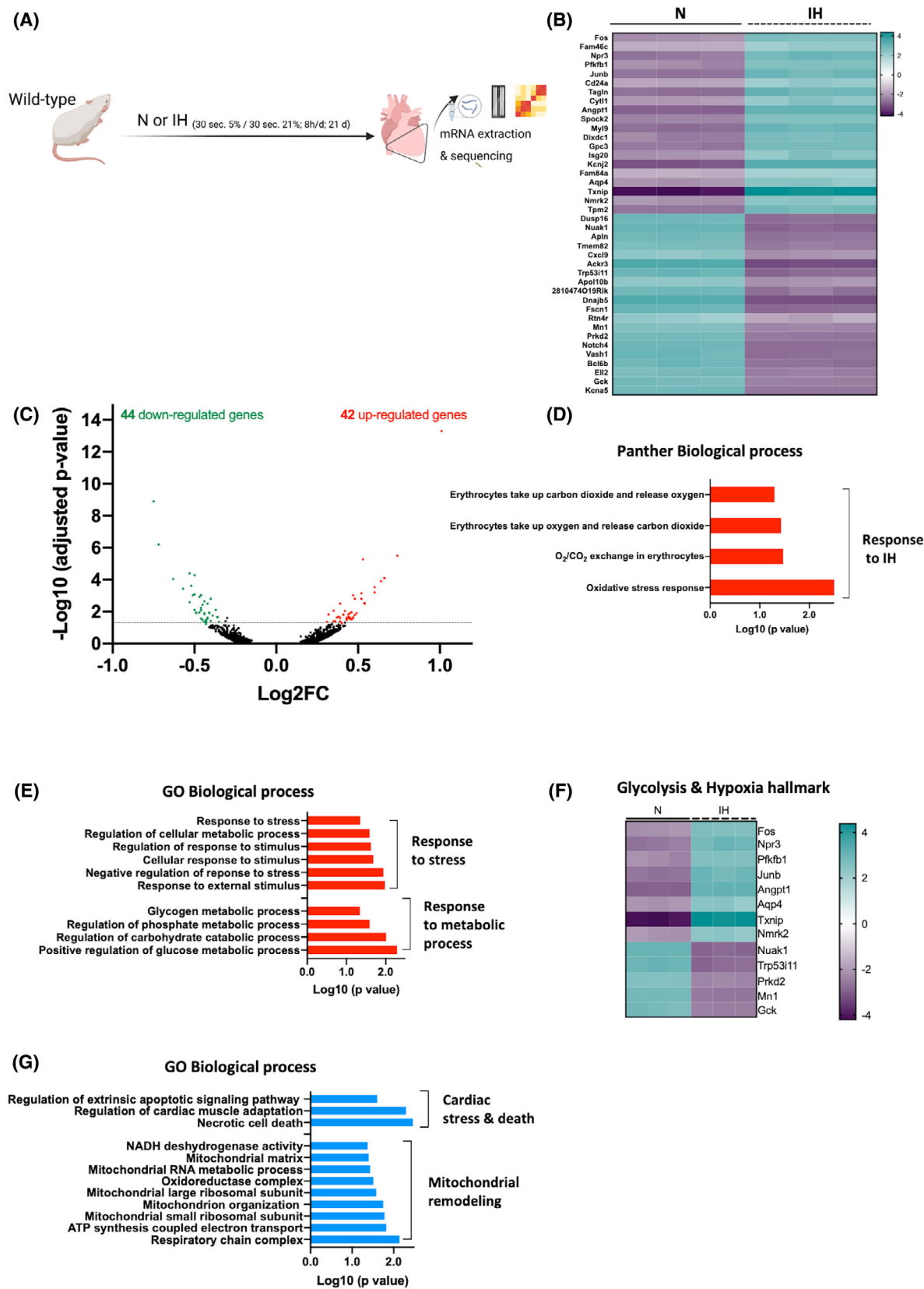


Fig. 2. Intermittent Hypoxia uncovers cardiac stress and mitochondrial remodeling in the heart through RNA sequencing analysis. Flow chart of the RNA sequencing from mice exposed to Normoxia (N) or Intermittent Hypoxia (IH) (A). Heatmap showing differentially expressed genes ($n = 3$ biological replicates per group, $FDR < 0.05$) (B). Volcano plot of differentially expressed genes according to fold change and significance ($FDR < 0.05$) (C). Enrichment analysis of biological process using Panther (D) and gene ontology (E) terms for top 20 differentially regulated genes. Heatmap showing glycolysis and hypoxia hallmark for top 20 differentially expressed genes ($FDR < 0.05$) (F). Enrichment analysis of biological process using gene ontology terms for all differentially expressed genes (G). Schema was made with [Biorender.com](https://biorender.com).

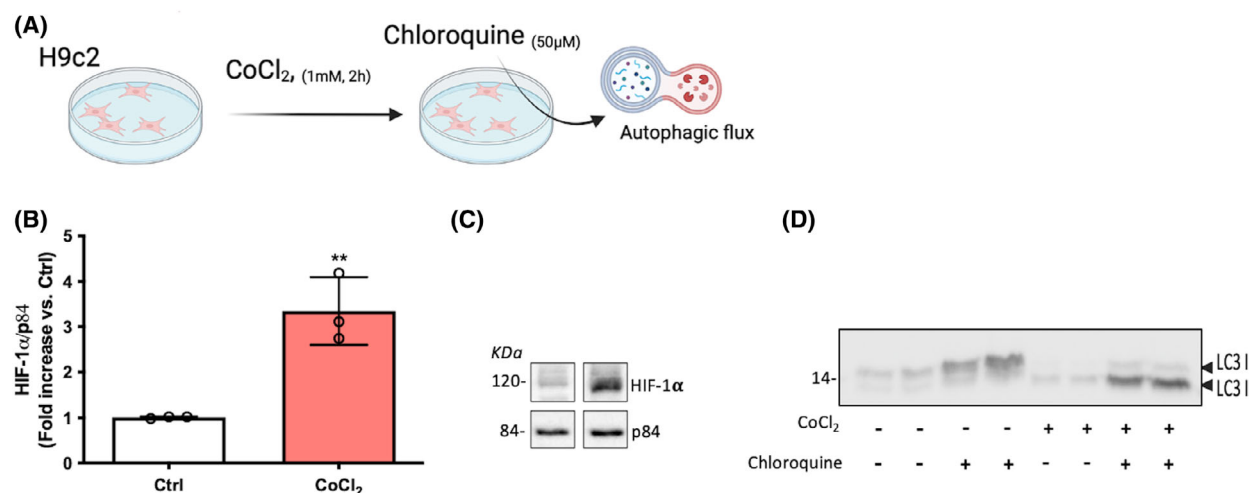


Fig. 3. HIF-1 activation is associated with an increase in autophagic flux *in vitro*. H9c2 cells were treated with CoCl₂ (1 mM, 2 h) with or without chloroquine (50 μM, 1 h) (A), nuclear HIF-1 expression relative to p84 (B) and representative image of western blot ($n = 3$) (C), data are presented as individual plots and expressed as mean $\pm$ standard deviation; ** $P < 0.01$, t -test. LC3 II and I western blot ($n = 2$) (D). Schema was made with [Biorender.com](https://biorender.com).

mitochondria with abnormal forms and the detection of autophagosomal membranes surrounding the mitochondria (Fig. 4P). All these results indicate that IH stresses mitochondria and induces mitophagy which depends on HIF-1.

Metformin reverses IH-increased infarct size through AMPK activation

IH was previously demonstrated to activate AMPK in *gastrocnemius* skeletal muscle of mice [22]. Considering the mitochondrial stress induced by IH in hearts, we then investigated AMPK activation in hearts from IH mice. Surprisingly, we observed that AMPK phosphorylation on Thr172, its activation site, was not increased but rather decreased in myocardium from IH mice (Fig. 5A–D). Thus, we used metformin to activate AMPK during N or IH exposure to explore its impact on cardiomyocyte death after an *in vivo* ischemia–reperfusion procedure (Fig. 5E). Metformin, at the concentration of 300 mg·kg^{−1}, has already been shown to activate AMPK signaling in mouse heart

[30]. Twenty-four hours after the last administration, such treatment successfully increased the phosphorylation of ACC on Ser79, the *bona fide* substrate of AMPK, under both IH (Fig. 5A,E–H) and N (Fig. 5E,I–K) conditions. Remarkably, despite equivalent areas at risk (AAR) in all groups (Fig. 5L), IH significantly increased the area of necrosis (AAN) which was significantly reduced by metformin treatment in IH mice only (Fig. 5M,N). These results indicate that metformin specifically protects hypoxic hearts against ischemia–reperfusion. Surprisingly, while metformin improved systemic insulin sensitivity in normoxic mice, it failed to improve it in IH mice, indicating that the cardioprotective effect of metformin does not come from a systemic metabolic effect in IH conditions (Fig. 5O,P).

Finally, we sought to determine whether the cardioprotective effect of metformin depends on AMPK activation by using AMPK $\alpha 2^{-/-}$ (the main AMPK isoform in cardiomyocyte) mice exposed to IH and treated or not with metformin (Fig. 6A). AMPK $\alpha 2$ expression is highly decreased in AMPK $\alpha 2^{-/-}$ as

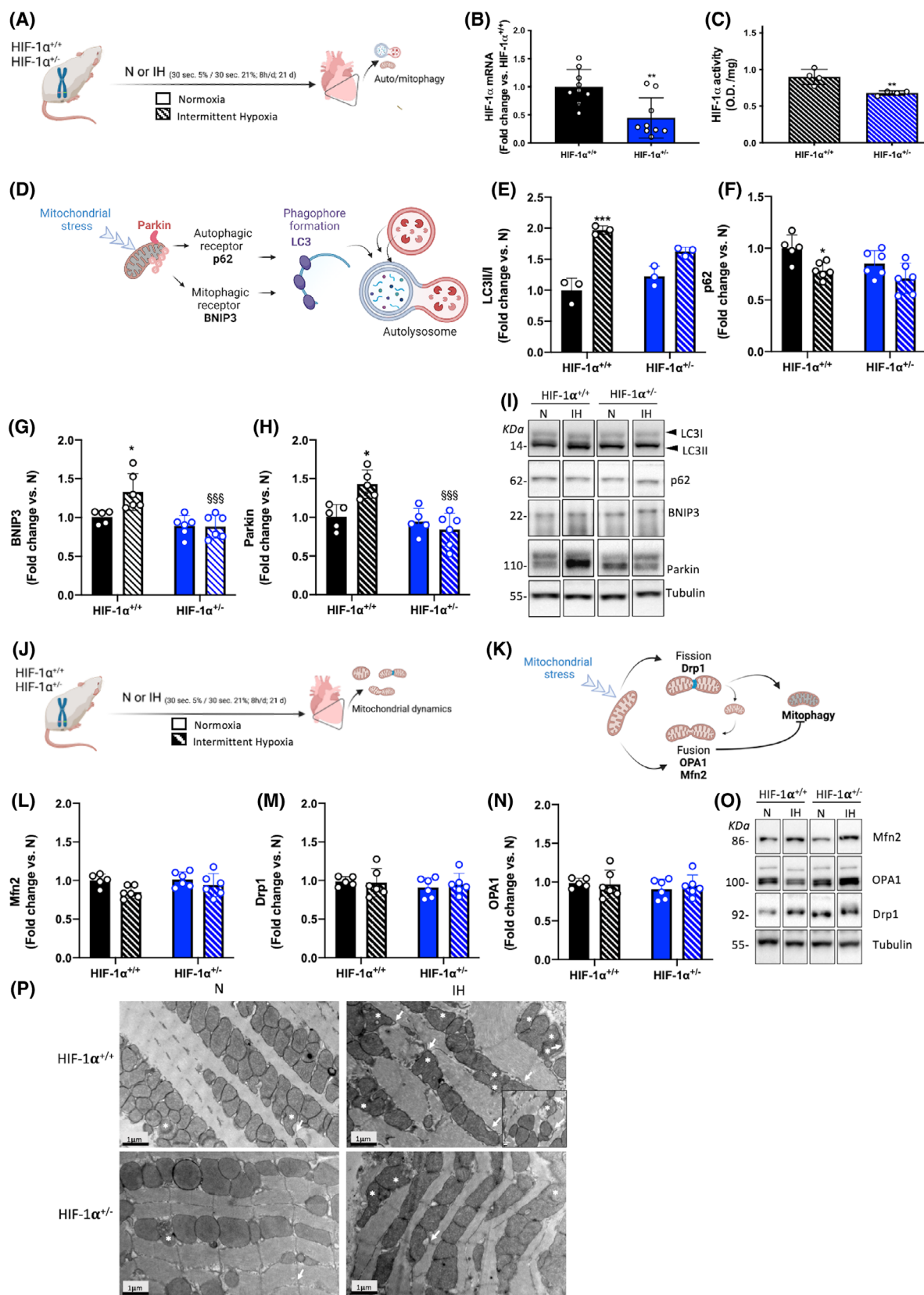


Fig. 4. Intermittent hypoxia induces mitophagy through HIF-1 activation. HIF-1^{+/+} and HIF-1^{+/-} mice were exposed to 21 days of Normoxia (N) or IH (1-min cycle of FiO₂ 5–21%) (A); *HIF-1^{+/-} model validation*: HIF-1 mRNA expression in HIF-1^{+/+} and, HIF-1^{+/-} (*n* = 9) (B) and HIF-1 activity measured by ELISA in HIF-1^{+/+} and HIF-1^{+/-} exposed to Intermittent Hypoxia (IH) (*n* = 4) (C), data are presented as individual plots and expressed as mean ± standard deviation; ***P* < 0.01, *t*-test; Autophagy, mitophagy: HIF-1^{+/+} and HIF-1^{+/-} mice were exposed to 21 days N or IH (A), scheme of protein explored and involved in mitophagy (D), LC3 II relative to LC3 I ratio (E), p62 (F), BNIP3 (F), and Parkin (H) expression relative to tubulin, representative image of western blots (*n* = 3–6) (I); *Mitochondrial dynamics*: HIF-1^{+/+} and HIF-1^{+/-} mice exposed to 21 days of N or IH (J), scheme of protein explored and involved in mitochondrial dynamics (K), Mfn2 (L), OPA (M), Drp1 (N) expression relative to tubulin; representative images of western blot (*n* = 5–6) (O), all data are presented as individual plots and expressed as mean ± standard deviation; **P* < 0.05, ****P* < 0.001 IH vs. N, §§§*P* < 0.001 HIF-1^{+/-} vs. HIF-1^{+/+}, two-way ANOVA, Sidák *post-hoc* tests; representative images of cardiac mitochondrial remodeling acquired by transmission electron microscopy (stars for mitochondria with abnormal forms; arrows for autophagosomal membrane formation) (×2900), the inset image represents another example of abnormal mitochondrial form and autophagosomal formation in IH condition, (×2900) (*n* = 4) (P). Scheme were made with [Biorender.com](https://biorender.com).

compared to AMPKα2^{+/+} (Fig. 6B,C). Importantly, metformin failed to decrease infarct size in AMPKα2^{-/-} mice subjected to IH (Fig. 6D,E). Taken together, these data indicate that metformin rescues IH-induced increase in infarct size through AMPKα2 activation.

Metformin reverses IH-induced mitophagy and decreases HIF-1α nuclear expression

To comprehend how metformin specifically protects from IH-induced cardiomyocyte death, we investigated its effect on IH-induced auto/mitophagy (Fig. 7A). Metformin significantly decreased the LC3II/I ratio (Fig. 7B), abolished the IH-induced decrease in p62 expression (Fig. 7C), and significantly decreased BNIP3 and Parkin expressions (Fig. 7D–F) in the hearts of mice exposed to IH. Collectively, these results indicate that metformin reverses IH-induced mitochondrial stress. As done previously, we also checked the impact of metformin on other proteins involved in mitochondrial dynamics (Fig. 7G,H). Except for an increase in OPA1 expression in both N and IH mice, metformin did not exert any effect on Mfn2 and Drp1 expressions (Fig. 7I–L). Because we demonstrated that IH-induced mitophagy is dependent on HIF-1, we also explored HIF-1 nuclear expression, a hallmark of its activation (Fig. 7M). In the hearts, IH significantly increased HIF-1 nuclear expression and this is significantly abolished by metformin (Fig. 4N,O). Thus, our results suggest that the beneficial effect of AMPK activation by metformin could be explained by a decrease in HIF-1 activation.

Metformin increases HIF-1 phosphorylation and decreases HIF-1 activity

As HIF-1 phosphorylation is described to regulate its activity, we investigated the impact of metformin on HIF-1α phosphorylation in H9c2 cells exposed to

CoCl₂, in which overexpression is associated with a decrease in cell viability (Fig. 8A–E). As expected, CoCl₂ increased HIF-1 expression (Fig. 8E,F) and tended to increase HIF-1 phosphorylation (Thr/Ser-P-HIF-1) in H9c2 control cells (Fig. 8E,G,H). Interestingly, metformin significantly increased HIF-1 phosphorylation only in the presence of CoCl₂ revealing that metformin-induced HIF-1α phosphorylation occurs when HIF-1α is stabilized (Fig. 8E,F–H). In parallel, in H9c2 transfected with the WT-HIF-1α plasmid where HIF-1 is over-activated (Fig. 8I,J), metformin significantly decreased HRE-GFP fluorescence (Fig. 8K). This indicates that metformin-induced HIF-1 phosphorylation is associated with a decrease in HIF-1 activity. Finally, we treated AMPKα2^{+/+} and AMPKα2^{-/-} mice with CoCl₂ in order to corroborate the role of AMPKα2 on HIF-1 phosphorylation (Fig. 8L). CoCl₂ tended to increase HIF-1α expression in AMPKα2^{+/+} and AMPKα2^{-/-} mice (Fig. 8M). However, HIF-1α phosphorylation increased in AMPKα2^{+/+} but not in AMPKα2^{-/-} mice (Fig. 8N, O), suggesting that AMPKα2 plays a role in HIF-1α phosphorylation. Taken together, these results indicate that metformin could phosphorylate HIF-1α through AMPKα2 leading to a decrease in HIF-1 activity.

Discussion

This study demonstrates that chronic IH induces an increase in infarct size through mitochondrial stress, which is dependent on HIF-1 sustained activation. Our study also provides a rationale for a drug repositioning of metformin as a potential drug to specifically prevent cardiovascular risk in OSA.

Using an unbiased transcriptomic analysis, we uncovered that IH is associated with cardiac, oxidative, and metabolic stress processes. Among the top 20 dysregulated genes by IH, HIF-1-related genes such as *Fos*, *Junb*, *Angpt1*, *Txnip*, *Nmrk2*, *Nuak1*, and *Pfkfb1* implicated in proliferation, oxidative stress, or

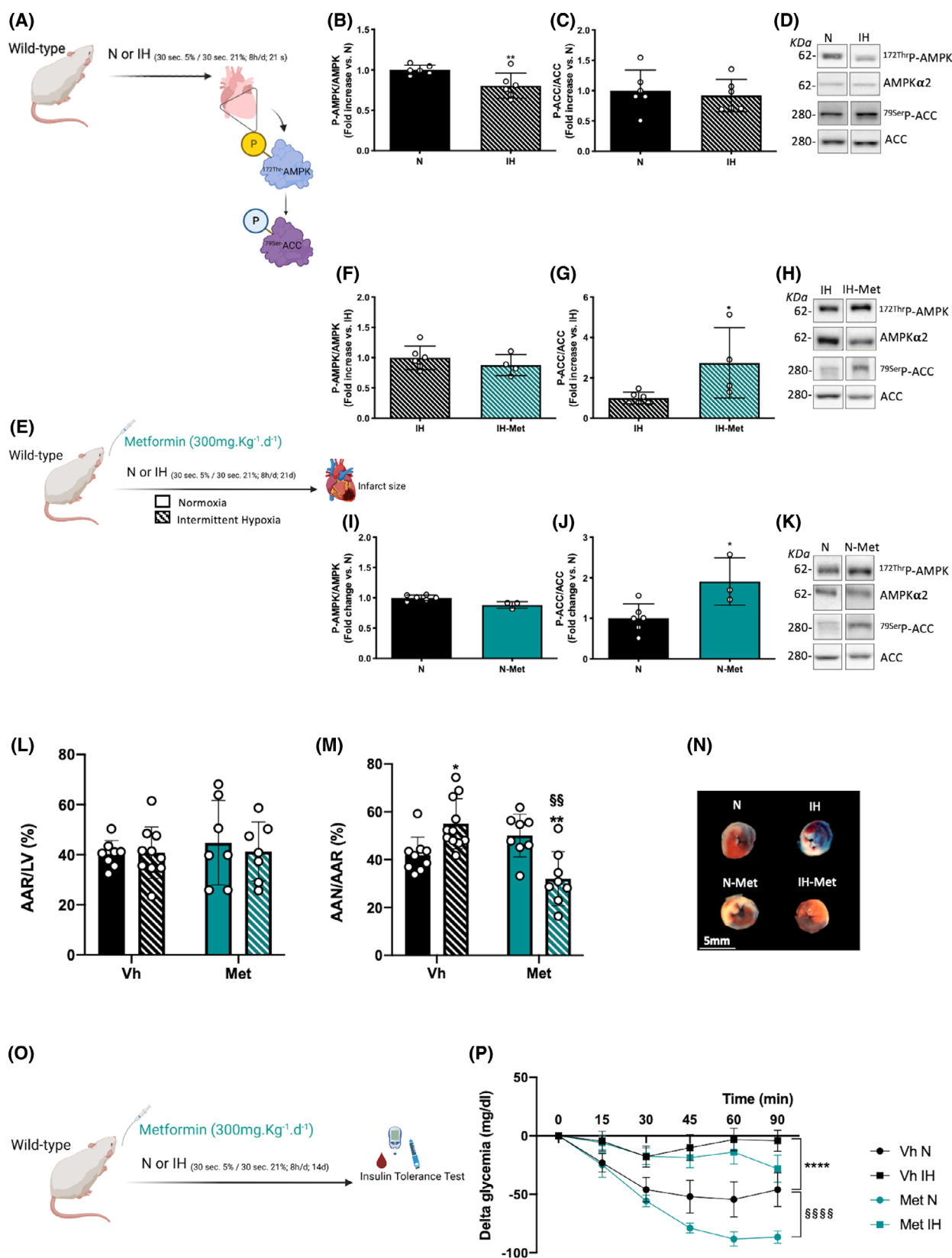


Fig. 5. Metformin specifically reverses intermittent hypoxia-induced increase in infarct size. (A–D) AMPK activation (A) is decreased in mice exposed to intermittent hypoxia (IH). ^{125}I -P-AMPK/AMPK (B) and ^{75}S -P-ACC/ACC (C) ratio and representative image of western blot ($n = 6$) (D), data are presented as individual plots and expressed as mean $\pm$ standard deviation; $^{**}P < 0.01$, t -test. (E–N) Metformin reverses IH increase in infarct size (E, K). Wild-type mice were treated with vehicle (Vh, CmCNa 0.01%, 0.1 mL $\cdot$ 10 g $^{-1}$) or metformin (Met, 300 mg $\cdot$ kg $^{-1}$ $\cdot$ day $^{-1}$) during exposure to 21 days of Normoxia (N) or IH (1-min cycle of FiO $_2$ 5–21%) (E); ^{125}I -P-AMPK/AMPK (F, I) ^{75}S -P-ACC/ACC (G, J) ratio and representative image of western blot (H, K) in IH and N mice treated with metformin, respectively ($n = 3$ –6), data are presented as individual plots and expressed as mean $\pm$ standard deviation; $^{*}P < 0.05$, t -test. (E, L–N) Mice were submitted to an ischemia–reperfusion protocol (E), area at risk (AAR) relative to Left ventricle (LV) (L) and area at necrosis (AN) relative to AAR (M) were calculated; representative colored slices (blue is the non-infarcted area, white is dead cells, red is viable cells) ($n = 8$ –11) (N), data are presented as individual plots and expressed as mean $\pm$ standard deviation; $^{*}P < 0.05$ IH vs. N, $^{§§}P < 0.05$ Met vs. Vh, Two-way ANOVA, Šidák *post-hoc* tests. (O–P) Metformin fails to improve systemic insulin sensitivity in IH mice. Insulin tolerance test was performed with intraperitoneal injection of insulin (0.5 mUI $\cdot$ kg $^{-1}$) (A); results are expressed in delta of glycemia from baseline before insulin administration and presented as mean $\pm$ standard deviation ($n = 6$) (B), $^{****}P < 0.0001$ IH vs. N, $^{§§§§}P < 0.0001$ Met vs. Vh, Two-way ANOVA, Šidák *post-hoc* tests. Schemes were made with [Biorender.com](https://www.biorender.com).

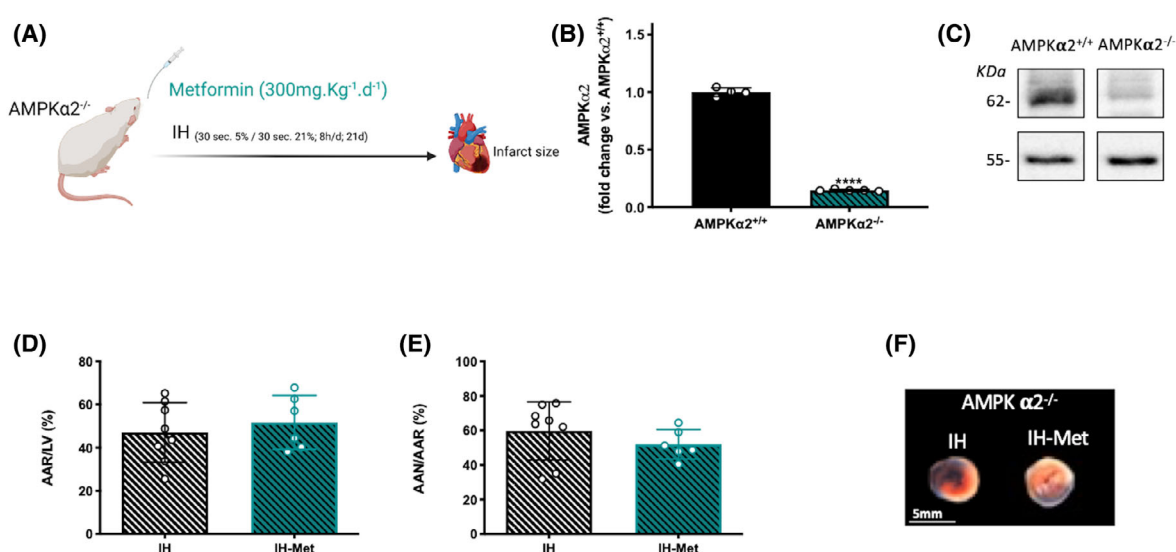


Fig. 6. Metformin fails to protect the heart in AMPK $\alpha 2^{-/-}$ exposed to intermittent hypoxia. AMPK $\alpha 2^{-/-}$ were exposed to 21 days of intermittent hypoxia (IH) and were submitted to an ischemia–reperfusion protocol (A), AMPK $\alpha 2$ expression in AMPK $\alpha 2^{-/-}$ mice (B) and representative image of western blot ($n = 4$) (C); area at risk (AAR) relative to Left ventricle (LV) (D) and area at necrosis (AN) relative to AAR (M)/AAR/LV (E) were calculated and representative colored slices (blue is the non-infarcted area, white is dead cells, red is viable cells) ($n = 6$ –8) (F), all data are presented as individual plots and expressed as mean $\pm$ standard deviation; $^{****}P < 0.0001$; t -test. Scheme was made with [Biorender.com](https://www.biorender.com).

glycolysis were upregulated. Additionally, oxidative and metabolic stress were both contributors to cardiac stress and to mitochondrial remodeling, two biological processes highlighted in our non-biased approach.

We demonstrate that IH induces a mitochondrial stress as it activates autophagy and mitophagy, revealed by changes in auto/mitophagic markers, such as BNIP3 and Parkin. This is consistent with previous studies showing that chronic IH alters mitochondrial function and/or modifies fuel usage in the heart [13,14]. Furthermore, we showed that chronic IH-induced autophagy and mitophagy is not observed in HIF-1 heterozygous mice, demonstrating that

IH-induced mitochondrial remodeling depends on HIF-1 upon IH. This aligns with the role of HIF-1 in inducing autophagy by activating the transcription of genes, including BNIP3 [28,29], one of the main autophagosome receptor activated by hypoxia [31]. HIF-1 has also been shown to positively regulate parkin-mediated mitophagy in acute kidney injury [32] and in diabetes [33]. In this study, we applied a chronic stress characterized by 21 days of 8 h a day of IH. This stimulus is recognized to mimic severe OSA and induce pathologies associated with OSA [34,35]. Chronic IH is now well recognized to sustainably activate HIF-1 [10]. In apneic patients at high cardiovascular risk, HIF-1

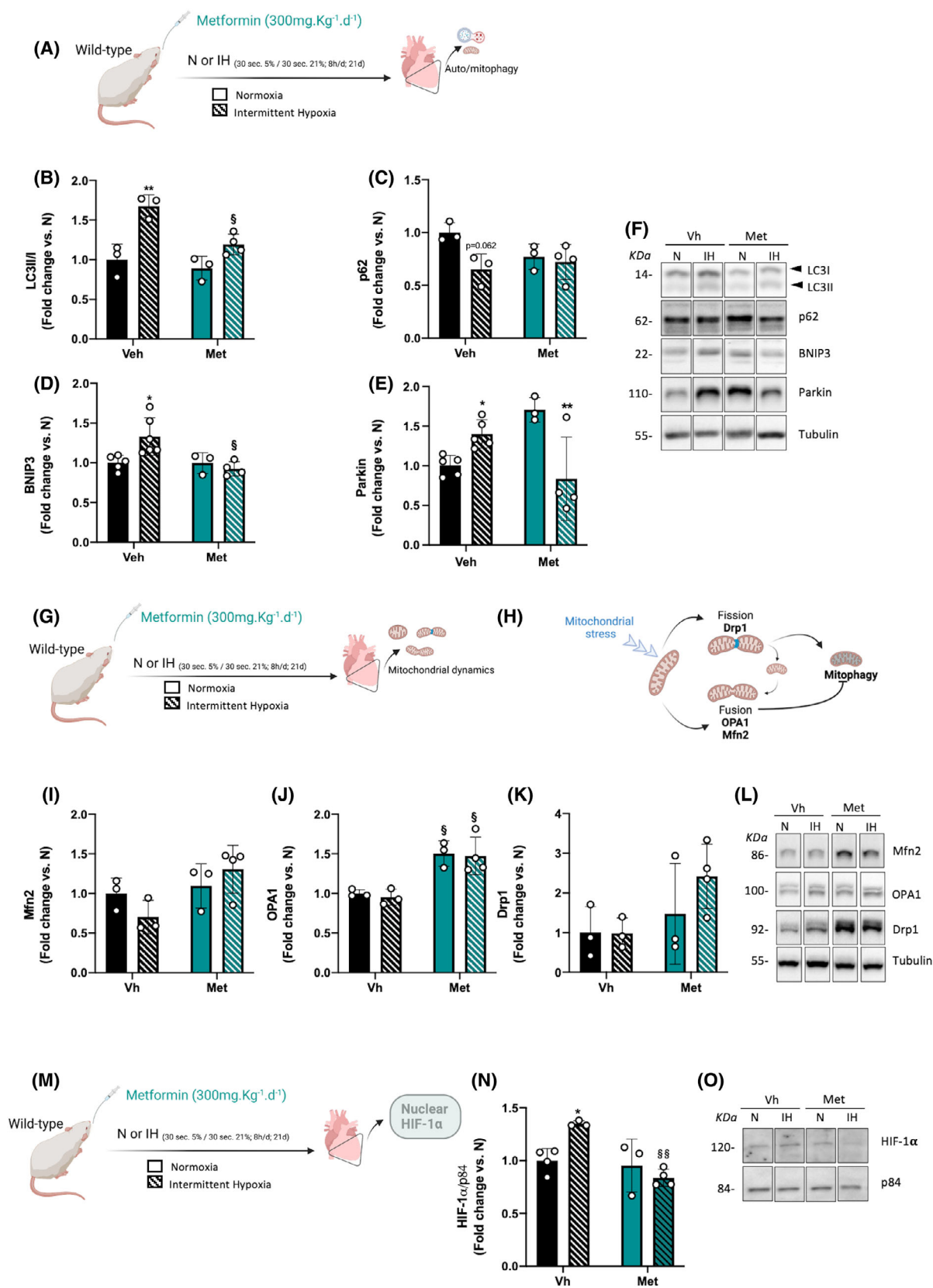


Fig. 7. Metformin decreases intermittent hypoxia-induced mitophagy and HIF-1 nuclear localization. (A–L) Metformin decreases IH-induced mitophagy. Wild-type mice were exposed to 21 days of Normoxia (N) or Intermittent Hypoxia (IH) (1-min cycle of FiO_2 5–21%) and treated with vehicle (Vh, CmCNa 0.01%, 0.1 mL·10 g⁻¹) or metformin (Met, 300 mg·kg⁻¹·day⁻¹) (A); *Autophagy, mitophagy*: LC3 II relative to LC3 I ratio (B), p62 (C), BNIP3 (D), Parkin (E) expressions and representative images of western blot ($n = 3–5$) (F); *Mitochondrial dynamics*: Wild-type mice exposed to 21 days of N or IH and treated with vehicle or metformin (G), scheme of protein explored and involved in mitochondrial dynamics (H), Mfn2 (I), OPA (J), Drp1 (K), representative images of western blot ($n = 3–4$) (L); (M–O) Metformin decreases IH-induced increase in HIF-1 nuclear localization. Nuclear HIF-1 expression was measured in 21 days of N and IH mice treated with vehicle or metformin (M), nuclear HIF-1 expression (N) and representative images ($n = 3–4$) (O), all data are presented as individual plots and expressed as mean $\pm$ standard deviation; * $P < 0.05$, ** $P < 0.01$ N vs. IH, $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ Met vs. Vh, two-way ANOVA, Sidák *post-hoc* tests. Scheme were made with [Biorender.com](https://biorender.com).

expression is positively correlated with the severity of OSA defined by the number of hypoxic cycles *per hour* [15]. Consistently, in pre-clinical models, HIF-1 has been demonstrated to be responsible for an increase in vascular remodeling [36], mean arterial blood pressure, infarct size [6,7,15], cardiomyocyte apoptosis [15], perivascular fibrosis, and cardiac function alteration [37,38]. Indeed, HIF-1 has been shown to induce calcium homeostasis alterations [7,13], ER stress [7,39] and more recently, to impair mitochondrial function [13,14]. All of these alterations, which are linked to HIF-1 upon IH, are potent stimulators of mitophagy, a dynamic process aiming to clear the mitochondrial pool [20]. This is consistent with the data provided by the transcriptomic analysis and the experiments performed in HIF-1^{+/-} mice, demonstrating that HIF-1 is responsible for IH-induced myocardial mitochondrial stress that generates mitophagy.

Our current work reveals a decrease in AMPK signaling in the hearts of hypoxic mice. This result was unexpected considering that AMPK is a critical energy sensor activated by mitochondrial dysfunction, a decrease in ATP concentration, and an increase in ADP-AMP/ATP ratio. Consistent with our result, a decrease in AMPK activation has been similarly reported in human adult cardiomyocytes exposed to chronic IH [40]. Inasmuch as the lack of explanation for such inhibition of AMPK by IH, we decided to investigate the effect of metformin, an AMPK activator widely used for efficiently treating type 2 diabetes [41], that is also a pathology with a high incidence in apneic patients [42]. The cardiovascular benefits of metformin are a subject of controversy, depending on its efficiency in lowering glycemia [43]. Interestingly, we demonstrated that metformin significantly reduced infarct size in mice exposed to chronic IH without affecting systemic metabolism, as metformin treatment failed to improve systemic insulin resistance in IH mice. Importantly, under IH conditions, the cardioprotective effect of metformin is dependent on AMPK2, as it failed to decrease infarct size in AMPK2^{-/-} mice.

These findings are of particular significance, suggesting that metformin could be used exclusively as a cardioprotective agent in apneic patients. This highlights a potential repositioning of metformin for these patients, requiring a better understanding of the mechanisms involved in metformin cardioprotective effects under IH.

Maintaining energy homeostasis requires the coordination of a large array of compensatory mechanisms, some of which are triggered by AMPK activation. In rodents exposed to 6 to 10 weeks of IH, studies have demonstrated that AMPK activation by melatonin or adiponectin alleviates cardiomyocyte apoptosis and improves cardiac function by inhibiting oxidative stress [24], inducing ER-phagy [27] and activating autophagy and mitophagy [25,26]. However, these studies did not demonstrate any direct impact of AMPK activation on mitophagy. In those studies, AMPK activation indirectly induced mitophagy to protect against prolonged IH (6–10 weeks). These findings differ from our study in which mice were exposed to only 3 weeks of IH. In agreement with previous studies demonstrating a mitochondrial dysfunction in hearts from mice exposed to 3 weeks of IH [13], we propose that mitochondria have already been submitted to a stress and have undergone remodeling characterized by an increase in mitophagy markers (without any modification in mitochondrial dynamics at this time). Interestingly, after 3 weeks, AMPK activation by metformin prevents the mitochondrial stress induced by IH, suggesting that earlier in the exposure (3 vs. 6–10 weeks), AMPK targets a key pathway that is involved in the myocardial deleterious response to chronic IH. Thus, we hypothesized that AMPK activation could impact HIF-1 activity, involved in IH-induced mitochondrial remodeling after 3 weeks of IH. Furthermore, HIF-1 is the most important transcription factor activated by chronic IH [10,44,45], recognized to play a major role in myocardial susceptibility to ischemia–reperfusion [6,9,13] and mitochondrial dysfunction [13].

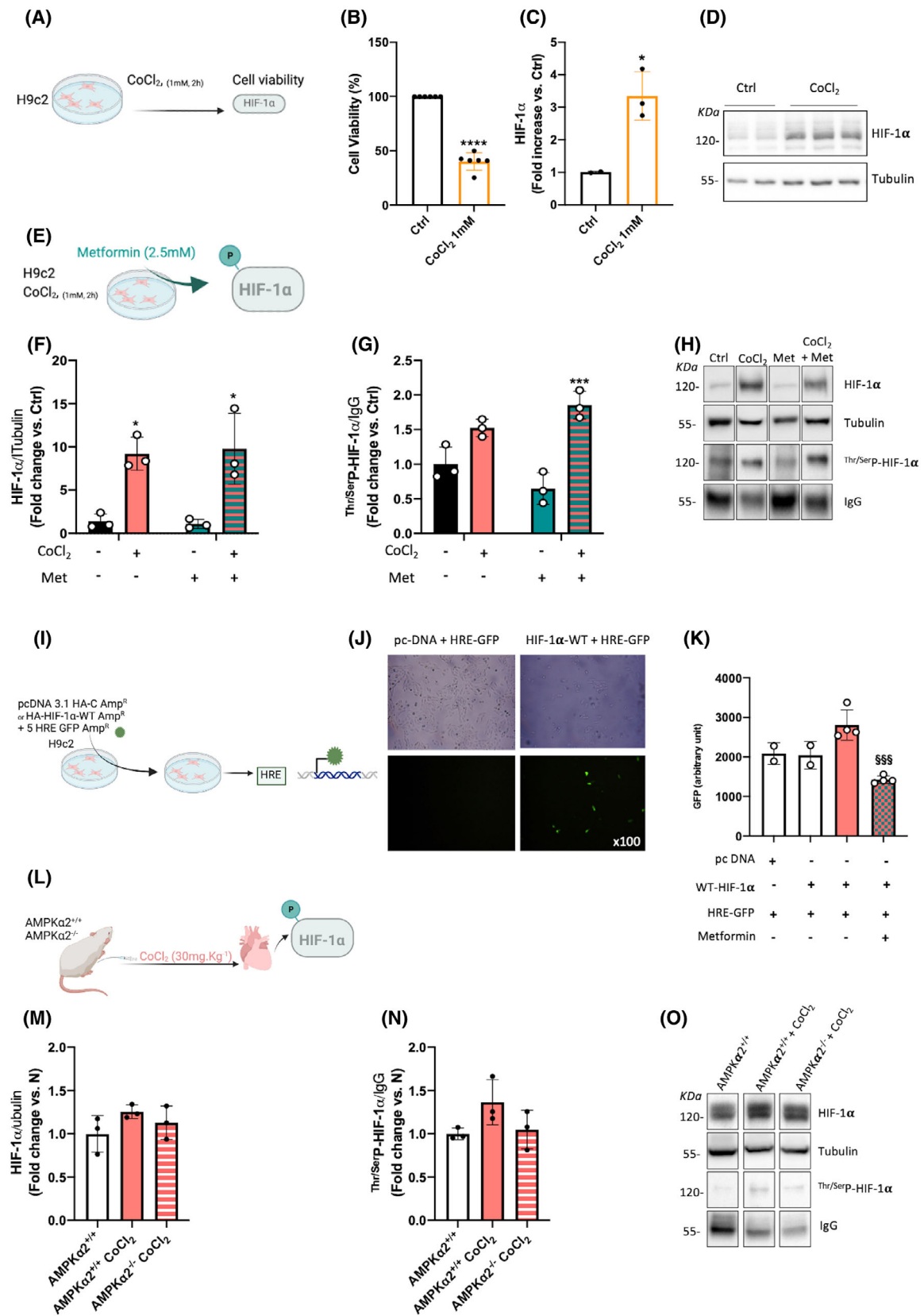


Fig. 8. Metformin phosphorylates HIF-1 and decreases HIF-1 activity. (A–D) CoCl₂ model validation. H9C2 were treated or not (Ctrl) with CoCl₂ (1 mM, 2 h) (A); cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) ($n = 6$) (B); quantification of HIF-1 expression relative to tubulin (C) and representative image of western blot ($n = 2–3$) (D); $*P < 0.05$, $****P < 0.05$ CoCl₂, t -test. (E–H) Metformin induces HIF-1 phosphorylation. H9c2 cells were treated with CoCl₂ (1 mM, 2 h) and Vehicle (Vh, CmCNa, 0.1%) or metformin (Met, 2.5 mM) (E), HIF-1 (F), ^{Thr/Ser}-P-HIF-1 (G) expression assessed after immunoprecipitation and western blot; representative images ($n = 3$) (H). (I–K) Metformin decreases HIF-1 activity. H9c2 cells were co-transfected with control (pcDNA 3.1 HA-C Amp^R) or WT-HIF-1 (HA-HIF-1 α -WT Addgene Amp^R) and HRE-GFP (5 HRE-GFP Amp^R) plasmids and treated or not with metformin (2.5 mM) (I), representative image of GFP fluorescence; magnification $\times 100$ (J), fluorescence was measured using Ex at 475 nm and Em spectrum 504–560 nm ($n = 2–4$) (K), data are presented as individual plots and expressed as mean $\pm$ standard deviation; $*P < 0.05$, $***P < 0.001$ CoCl₂ vs. Ctrl, $***P < 0.001$ Met vs. Vh, two-way ANOVA, Šidák *post-hoc* tests. (L–O) AMPK2 plays a role in HIF-1 phosphorylation. AMPK2^{+/+} and AMPK2^{-/-} were treated with CoCl₂ (30 mg·kg⁻¹) (L), HIF-1 (M), ^{Thr/Ser}-P-HIF-1 (N) expression assessed after immunoprecipitation and western blot; representative image ($n = 3$) (O), data are presented as individual plots and expressed as mean $\pm$ standard deviation; one-way ANOVA, Tukey's *post-hoc* tests. Scheme were made with [Biorender.com](https://biorender.com).

Thus, we sought to determine if metformin can directly impact sustained HIF-1 activation induced by chronic IH. Here, we demonstrated that metformin abolished the IH-induced increase in HIF-1 α nuclear localization, strongly suggesting that it can reduce its transcriptional activity [10,44]. HIF-1 α primary sequence contains 15 threonine/serine phosphorylation sites playing a role in HIF-1 O₂-dependent degradation, HIF-1 α cytosolic retention [46], HIF-1 dimerization and HIF-1 transcriptional activity [47]. We demonstrated *in vitro* that metformin significantly increases HIF-1 phosphorylation and decreases its activity when HIF-1 is sustainably stabilized by CoCl₂ and overexpressed by transfection, respectively. Interestingly, HIF-1 α phosphorylation failed to be increased by CoCl₂ in AMPK α 2 deficient mice, suggesting that among the proteins able to phosphorylate HIF-1 α , AMPK α 2 could participate. Some studies reported that AMPK and HIF-1 exert reciprocal regulation in order to maintain cellular homeostasis [48]. However, very few studies indicate that HIF-1 activity can be regulated by AMPK-dependent activation [48]. AMPK activation correlates with either an increase or a decrease in expression of HIF-1 α expression. In addition, data were mainly provided in *Caenorhabditis elegans* or in cancer cells [49]. Indeed, in *C. elegans*, Hwang *et al.* [50] demonstrated that HIF-1 α is phosphorylated by AMPK to reduce HIF-1 activation. In lymphoma and colon cancer cells, AMPK α 1 loss has been associated with an increase in HIF-1 α expression and glycolysis, thereby promoting cells growth and proliferation [51]. In our study, we highlight that metformin blunts HIF-1 activity by increasing its phosphorylation, partially through AMPK α 2 activation. This highlights the mechanism by which metformin could emerge as a drug, shielding the heart from the stress provoked by chronic IH.

In conclusion, our findings demonstrate that metformin efficiently counteracts the detrimental effects of chronic IH, providing a protection against cardiomyocyte death induced by ischemia–reperfusion. Beyond its established use for diabetes management, our data suggest that metformin may hold promise for patients with OSA and cardiovascular risks. Mechanistically, metformin triggers AMPK-dependent HIF-1 phosphorylation, resulting in reduced HIF-1 activity and alleviation of mitochondrial stress (see Graphical Abstract). This opens new avenues for exploring the potential effects of metformin as a modulator of HIF-1 activity in diverse pathologies including cancer and heart failure, where both AMPK and HIF-1 are involved.

Materials and methods

Materials

In vitro model, cell culture, and design

H9C2 cardiomyoblasts were cultivated in classic conditions (DMEM high glucose, 10% SVF, 1% penicillin/streptomycin, 37 °C, 5% CO₂) in the presence or absence of CoCl₂ (1 mM, 1 h), used as a HIF-1 α stabilizer. H9C2 were exposed to CoCl₂ with or without chloroquine (50 μ M) in order to induce and assess autophagic flux. H9C2 were also exposed to CoCl₂ in order to evaluate the effect of metformin treatment (2.5 mM) on HIF-1 α phosphorylation. Finally, the activity of HIF-1 was determined using an HRE-GFP plasmid (5 HRE-GFP Amp^R) transfected 24 h before metformin treatment. This test was performed in control condition (pcDNA 3.1 HA-C Amp^R) and in H9C2 simultaneously transfected with a WT-HIF-1 α (HA-HIF-1 α -WT Amp^R) plasmid overexpressing HIF-1 α . Plasmids were provided by Addgene (Watertown, MA, USA). GFP fluorescence intensity after metformin treatment was measured using Clariostar[®] fluorimeter (Ex at 475 nm and Em spectrum 504–560 nm).

Rodent model of intermittent hypoxia

Animals

The protocol was approved by the French minister (APAFIS#23725-2020012111137561.v2 approved in 2020, February 20th). All experiments were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (decree 2013-118 and orders of 1st February 2013); ARRIVE guidelines were followed. Male Swiss/SV129 mice with a heterozygous deletion of the gene encoding for the O₂-sensitive HIF-1 α subunit of HIF-1 (HIF-1 α ^{+/-}) and their wild-type (HIF-1 α ^{+/+}), aged 8–12 weeks were furnished by 'Plateforme de Haute Technologie Animale', Grenoble Alpes University. Male AMPK α 2^{+/-} mice (C57/Bl6 background) were furnished by 'Service Commun des Animaleries de Rockefeller' (Université Claude Bernard Lyon1). All animals were housed in the animal care facility of the HP2 laboratory (number: A3851610006). They were maintained on a 12/12 h light–dark cycle and had *ad-libitum* access to food and water.

Chronic intermittent hypoxia

Exposure to IH or N has been described previously [9]. Briefly, after an acclimatation period, mice were exposed in their housing cages to 21 days of normoxia (N) or intermittent hypoxia (IH), 8 h per day during their sleeping period. Intermittent hypoxia consisted of 60 s cycles alternating 30 s of hypoxia (hypoxic plateau at 5% FiO₂) and 30 s of N (normoxic plateau at 21% FiO₂). Normoxic mice were exposed to similar air–air cycles in order to avoid bias from noise and turbulence related to gas flow.

Experimental design

One week after arrival, mice were randomly assigned to be exposed to N or IH, and six experimental sets were constituted. In experimental set 1, two groups were constituted ($N = 6$ mice): HIF-1 α ^{+/+}-N, HIF-1 α ^{+/+}-IH ($n = 3$ per group). Set 1 was used for RNA-seq analysis. In experimental set 2, four groups were constituted ($N = 24$ mice): HIF-1 α ^{+/+}-N, HIF-1 α ^{+/+}-IH, HIF-1 α ^{+/-}-N, and HIF-1 α ^{+/-}-IH. Set 2 was used for western blotting ($n = 3$ –6 per group) in order to assess mitochondrial stress and AMPK activation. In experimental set 3, four groups were constituted ($N = 48$ mice): Vh-N, Vh-IH, Met-N, and Met-IH; mice were treated daily with vehicle (Vh, CmCNA 0.1 mL·10 g⁻¹·day⁻¹) or metformin (Met, 300 mg·kg⁻¹·day⁻¹) in order to perform *in vivo* ischemia–reperfusion (I/R) and to measure infarct size ($n = 12$ per group). In experimental set 5, the same groups as in experimental set 4 were constituted ($N = 24$ mice) in order to perform an insulin tolerance test after 14 days of exposure and western blot after 21 days ($n = 3$ –6 per group).

In experimental set 6, two groups were constituted ($N = 16$ mice): IH and IH-Met; AMPK α 2^{-/-} mice were exposed to IH and treated daily with Vh (CmCNA 0.1 mL·10 g⁻¹·day⁻¹) or Met (300 mg·kg⁻¹·day⁻¹) in order to measure infarct size after I/R ($n = 8$ per group). In experimental set 7, three groups were constituted ($N = 9$ mice): AMPK α 2^{+/+} and AMPK α 2^{-/-} mice treated with CoCl₂ (30 mg·kg⁻¹) 1 h before heart sampling in order to perform biochemical analyses ($n = 3$ per group).

Design and exclusion criteria: concerning the biochemical experiments, depending on the experiment and based on our experience and on our practical feasibility, some designs were built with $n = 3$ –6. No exclusion was performed after the immunoblotting. Concerning the I/R protocol, animals dead before the end of the reperfusion were excluded.

Methods

RNA sequencing, data processing, and differential expression analysis

Heart tissues (> 100 mg) were sent to Active Motif and total RNA was extracted and qualified. For each sample, 1000 ng of total RNA was then used in Illumina's TruSeq Stranded mRNA Library kit (Cat# 20020594). Libraries were sequenced on Illumina NextSeq 550 as paired-end 42-nt reads. Sequence reads were analyzed with the STAR alignment – DESEQ2 software pipeline described in the Data Explanation document of the manufacturer was performed. Finally, enrichment analyses were conducted using Gene Ontology and Panther databases.

Western blot

Frozen heart biopsies were homogenized (Precellys® 24, Bertin Technologies, Montigny le Bretonneux, France) in lysis buffer as previously described and depending on the subfractionment [15]. Protein concentration was determined using the Bradford assay. Then, proteins (30–100 μ g) were separated by SDS/PAGE (8–12% acrylamide) and transferred to polyvinylidene fluoride membranes. Membranes were blocked (5% non-fat milk or BSA), then incubated overnight at 4 °C with the following primary antibodies: HIF-1 α (mab50046 Covalab, Bron, France), LC3II, LC3I(4108, Cell signaling, Boston, MA, USA), p62(p067, Merk, St Quentin Fallavier, France), BNIP3(B7931, Merk), Parkin(4211, Cell signaling), Mfn2(AV42419, Merk), OPA1(612 606, BD Transduction Lab., Franklin Lakes, NJ, USA), Drp1(611738, BD Transduction Lab.), 172ThrP-AMPK(2535, Cell signaling), AMPK α 2(2532, Cell signaling), 79serP-ACC(3661, Cell signaling), ACC1/2(3662, Cell signaling) (1 : 1000) and α tubulin(sc-5286, Santa Cruz, Dallas, TX, USA) or p84(sc-346, Santa Cruz) (1 : 2000). After that, appropriate horseradish peroxidase-conjugated anti-IgG(Santa-Cruz) (1 : 2500) was incubated for 1 h at room temperature. Enhanced chemiluminescence was performed with the ECL

western blot detection kit (Bio-Rad, Hercules, CA, USA) and video acquisition (ChemiDoc™ XRS+ System, Bio-Rad). The density of protein bands was analyzed (IMAGE J, NIH, Bethesda, MD, USA) for each sample and expressed relative to corresponding tubulin expression (loading control) and normalized to the appropriate control group. We performed western blot at least two times for each sample from mice.

Immunoprecipitation and western blot

H9c2 were lysed as previously described [13] and 500 µg of proteins were incubated with 2 µg of ^{Ser/Thr}-phosphoprotein(9631, Cell signaling) antibody overnight at 4 °C with rotation. The next day, samples were incubated for 2 h with Pure Proteome protein G magnetic beads at 4 °C and elution was performed using Laemmli buffer. Then, western blot was performed to detect HIF-1α.

Real-time quantitative PCR and ELISA test on HIF-1

As previously described [13], HIF-1α mRNA content was assessed by quantitative reverse-transcriptase PCR and normalized with hypoxanthine phosphoribosyltransferase (HPRT). Primers are the following: 5'ACGATGAACATC AAGTCAGCAACG3'/3'TCACAAATCAGCACCAAGCA CG5' for HIF-1 α and 5'TCTTTGCTGACCTGCTG GATTACAT3'/3'CCAGGGAAAGCAAAGTTTGCATT5' for HPRT. HIF-1 activity was assessed using an ELISA kit (Abcam, Cambridge, UK). Briefly, nuclear extracts were incubated in wells coated with oligonucleotides containing a hypoxia response element and anti-HIF-1α and horseradish peroxidase coupled-IgG were incubated; luminescence was measured by densitometry.

In vivo ischemia–reperfusion

In vivo ischemia–reperfusion procedure has been described previously [7]. Briefly, after N or IH exposure, mice were anesthetized intraperitoneally with a pentobarbital injection (60 mg·kg^{−1}). Animals were intubated with a tidal volume of 0.2 mL and a breathing rate of 160 min^{−1}. Body temperature was controlled and maintained at 37 °C. Then, pericardectomy was performed and a 7-0 silk suture was passed around the left interventricular artery to proceed to the coronary occlusion. After 45 min of ischemia, the ligature was removed and the myocardium was reperfused for 90 min. According to a previous study, 90 min is enough to induce stable necrosis areas [52]. At the end of reperfusion, the coronary artery was briefly re-occluded, and 1 mL Unisperse blue pigment was injected intravenously to delineate the area at risk (AAR). Then, hearts were excised and cut into five transverse slices. Each slice was incubated for 20 min in a 1% triphenyltetrazolium chloride solution at 37 °C to differentiate infarcted from viable myocardial

areas [7]. Extent of AAR and area of necrosis (AN) were blindly quantified by planimetric analysis (IMAGE J software) and corrected by the weight of each slice [53].

Insulin tolerance test

Whole-body insulin sensitivity was assessed by intraperitoneal insulin tolerance test. Injections of insulin (0.5 units·kg^{−1} total body weight) (NovoRapid; Novo Nordisk, Bagsvaerd, Denmark) were performed in conscious mice fasted for 6 h. Glycemia was measured by tail bleeding at different times (15, 30, 45, 60, and 90 min after insulin injection) using a glucometer (One Touch verio®).

Statistical analysis

All analyses were done with GRAPHPAD PRISM Software® (GraphPad, Boston, MA, USA). Two-way ANOVA followed by Šidák *post-hoc* tests was performed. One-way ANOVA followed by Tukey's *post-hoc* test was performed when it was appropriate. *T*-test was performed when two groups had to be compared. Tests were performed after normality was accepted (Shapiro–Wilkinson test). Statistical significance was set for *P* values < 0.05.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

EB conceived and supervised the study; EB, RM, LBe, AT, LBe designed experiments; SM, BB-M, AK, EB, SP performed experiments; AK, RM, LBe, LBU provided new tools (i.e., AMPKα2 knock-out mice, AMPK signaling pathway exploration); EB, SM, BB-M analyzed data; SM, BB-M, and EB wrote the manuscript; EB, RM, LBe, CA, J-LP, SP made manuscript revisions.

Data availability statement

The data that support the findings of this study are openly available in Dryad at http://datadryad.org/share/wPXj_4koQz7w-4UFARRrSoecXM7trTts4Fqev_iQFqk.

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