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Compound C inhibits hypoxic activation of HIF-1 independent of AMPK

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Abstract

The key transcription factor that regulates the cellular responses to hypoxia is hypoxia inducible factor-1 (HIF-1). The signaling mechanisms that regulate the hypoxic activation of HIF-1 are not fully understood. Our objective here was to test whether AMP-activated kinase (AMPK) was an upstream regulator of HIF-1. Our results show that AMPK is not required for the hypoxic activation of HIF-1. Interestingly, the AMPK inhibitor, Compound C, inhibits the hypoxic activation of HIF-1 independent of AMPK. Furthermore, we demonstrate that Compound C functions as a repressor of HIF-1 by inhibiting respiration and suppressing mitochondrial generated ROS.

Keywords

Hypoxia inducible factor 1; AMP-activated kinase; Compound C; hypoxia; mitochondria; reactive oxygen species

1. Introduction

Hypoxia is the reduction in normal oxygen tension and occurs during acute and chronic vascular disease, pulmonary disease and cancer. HIF-1 is the master transcription factor that regulates the cellular responses to hypoxia [1]. HIF-1 is composed of two subunits, an oxygen-sensitive HIF-1 α subunit, and a constitutively expressed HIF-1 β subunit. HIF-1 activity is dependent on the availability of the HIF-1 α subunit. Under normal oxygen conditions (21% O₂), HIF-1 α is targeted for ubiquitin-mediated degradation by an E3 ubiquitin ligase complex [2]. This process is dependent on the hydroxylation of two proline residues by a family of prolyl hydroxylase (PHD) enzymes [3–5]. Under hypoxic conditions, hydroxylation is inhibited and HIF-1 α is stabilized [6]. To date the upstream regulators of HIF-1 are poorly understood. The reactive oxygen species (ROS) generated within complex III of mitochondria during hypoxia has been shown by multiple groups to be required and sufficient to activate HIF-1 [6–10]. However, the direct targets of the mitochondrial ROS or the upstream regulators of HIF-1 remain unresolved.

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Alternatively, it has been proposed that the mitochondria transduce hypoxic signals by regulating the availability of oxygen for the PHDs to hydroxylate HIF-1 α [11,12].

AMPK is a heterotrimeric serine/threonine kinase consisting of a catalytic α subunit and two regulatory β and γ subunits [13–15]. AMPK is ubiquitously expressed and functions as an intracellular fuel sensor by maintaining energy balance. Hypoxia, ischemia, and ROS are potent activators of AMPK [16–18]. Previous work has genetically shown that AMPK is not necessary for HIF-1 α protein stability [18]. Yet, other reports have suggested that Compound C prevents HIF-1 activation [19,20]. To resolve this discrepancy and to investigate whether AMPK is an upstream regulator of HIF-1 we used both Compound C and AMPK α null cells. Here we show that Compound C prevents the hypoxic activation of HIF-1 by suppressing mitochondrial generated ROS independent of AMPK.

2. Materials and methods

2.1 Cell culture and reagents

Ampka WT, *Ampka1*^{-/-}, *Ampka2*^{-/-}, and *Ampka1*^{-/-}*2*^{-/-} mouse embryonic fibroblasts (MEFs), obtained from B. Viollet, were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate, supplemented with 10% heat inactivated fetal bovine serum (Gibco), 100U/ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g/ml amphotericin B, and 20mM HEPES. The following reagents were used: Dimethylxaloylglycine (DMOG) (Frontier Scientific, Inc.), Compound C (Calbiochem), and AICA-Riboside (AICAR) (Calbiochem).

2.2 Oxygen conditions

Hypoxic conditions (1.5% O₂ and 5% CO₂ balanced with N₂) were achieved in a humidified variable aerobic workstation (INVIVO O₂, BioTrace), which contains an oxygen sensor that continuously monitors the chamber oxygen tension.

2.3 Immunoblot analysis

HIF-1 α protein was analyzed in nuclear extracts as previously described [21]. The anti-HIF-1 α antibody (Cayman Chemical) was used and an anti-POL II antibody (Santa Cruz Biotechnology, Inc.) as the loading control. For total cell lysates, the following antibodies were used for immunoblotting: AMPK α , Acetyl CoA Carboxylase (ACC), phospho- Acetyl CoA Carboxylase (Ser79) (all Cell Signaling Technology, Inc.), and an anti- α -tubulin antibody (Sigma-Aldrich, Inc.) to control for loading. A representative blot is shown of three independent experiments.

2.4 Transfections and reporter assays

Transfections were done using the *TransIT* Transfection reagent (Mirus) according to manufacturer's protocol. HIF-1 mediated transcriptional activity was measured using the HRE-Luciferase reporter gene construct, a pGL2 vector containing three hypoxia response elements from the *Pgk-1* gene upstream of firefly luciferase. Cells were subjected to conditions for 16 hours before lysates were collected. Luciferase values were determined using a dual-luciferase reporter assay kit (Promega). Values for firefly luciferase were normalized to *Renilla* luciferase under the control of the thymidine kinase promoter in the pRLTK vector. The data presented are the result of triplicate analyses and the error bars indicate standard error of the mean (SEM).

2.5 Measurement of cell death

Cell death was measured using a cytotoxicity detection kit (Roche Applied Science) according to manufacturer's protocol. This kit is based on the measurement of lactate dehydrogenase

(LDH) that is released into the medium by damaged cells. Cell death is presented as amount of LDH measured in the medium divided by the total LDH released after treatment with 1% Triton X-100. All cell death results are from four independent experiments and are represented as the mean value \pm standard error of the mean (SEM).

2.6 Measurement of ROS

Intracellular ROS generation was assessed using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes). ROS in the cells cause oxidation of DCFH, yielding the fluorescent product 2',7'-dichlorofluorescein (DCF). Cells were plated on Petri dishes and incubated with DCFH-DA (10 μ M) under various conditions. The media was then removed, cells were lysed, centrifuged to remove debris, and the fluorescence in the supernatant was measured using a spectrofluorometer (excitation, 500 nm; emission, 530 nm). Data were normalized to values obtained from normoxic, untreated controls.

2.7 Oxygen consumption

Cellular O₂ consumption rates were measured in aliquots of 1–2 \times 10⁶ subconfluent cells removed from flasks and studied in a magnetically stirred, water-jacketed (37°C) anaerobic respirometer fitted with a polarographic O₂ electrode (Oxytherm system; Hansatech Instruments). Oxygraph Plus software was used to determine oxygen consumption rate. The data presented is the result of triplicate analyses and the error bars indicate standard error of the mean (SEM).

3. Results

3.1 Compound C inhibits hypoxic activation of AMPK and HIF-1

Compound C is known to function as an ATP-competitive inhibitor of AMPK. To first demonstrate that Compound C inhibits the hypoxic activation of AMPK, cells were exposed to 21% O₂ (N), 1.5% O₂ (H), DMOG (D), and AICAR (A) \pm Compound C. To assess AMPK activation, lysates were immunoblotted using an antibody that recognizes the phosphorylation site (Ser79) on acetyl-CoA carboxylase (ACC), a direct target of AMPK [14,22] (Figure 1A). As expected, Compound C inhibits both the hypoxic and AICAR induced activation of AMPK, whereas, the hypoxic mimetic DMOG does not activate AMPK (Figure 1A). DMOG is a PHD inhibitor which serves as a hypoxic mimetic under normal oxygen conditions, whereas, AICA-Riboside (AICAR) mimics AMP, thereby acting as an activator of AMPK. To test the requirement of AMPK for the hypoxic activation of HIF-1, cells were exposed to hypoxia (1.5% O₂) or normoxia (21% DMOG \pm Compound C. Treatment with Compound C O₂) in the presence or absence of completely suppressed the stabilization of the HIF-1 α protein under hypoxia, whereas HIF-1 α protein level was unaffected with DMOG treatment (Figure 1B). These results were further confirmed by examining HIF-1 activity using the HRE-Luciferase assay (Figure 1C). Hypoxia and DMOG increased HRE-dependent luciferase induction in the untreated cells (Figure 1C). In contrast, cells treated with Compound C displayed a marked attenuation of luciferase induction under hypoxia but not in the presence of DMOG during normoxia (Figure 1C).

3.2 AMPK is not required for hypoxic activation of HIF-1

To genetically examine the requirement of AMPK for the hypoxic activation of HIF-1, the double knockout cells for AMPK α 1 and AMPK α 2, as well as, the individual null cells for AMPK α 1 and AMPK α 2 were used. First, *Ampka* WT and the *Ampka1*^{-/-}*2*^{-/-} cells were exposed to hypoxia (1.5% O₂) or normoxia (21% O₂) in the presence or absence of DMOG. Interestingly, the loss of both AMPK α 1 and AMPK α 2 did not affect HIF-1 α protein levels under hypoxia (Figure 2A). Moreover, *Ampka1*^{-/-} and *Ampka2*^{-/-} cells also displayed no

differences in HIF-1 α protein levels when subjected to hypoxia (Figure 2B). Lastly, the HRE-Luciferase assay displayed no difference between the *Ampka* WT and the *Ampka1^{-/-}2^{-/-}* cells under hypoxia (Figure 2C), providing genetic evidence that the hypoxic activation of HIF-1 is not dependent on AMPK, coinciding with data published by Laderoute *et al.* [18].

3.3 Compound C inhibits hypoxic activation of HIF-1 independent of AMPK

To resolve the discrepancy above, a HRE-Luciferase assay was used to examine HIF-1 activity in the *Ampka1^{-/-}2^{-/-}* in the presence or absence of Compound C. As shown by immunoblotting, these cells are null for AMPK α (Figure 3A). Similar to Figure 2A, hypoxic and DMOG treated *Ampka1^{-/-}2^{-/-}* cells stabilized the HIF-1 α protein (Figure 3B). Whereas, treatment with Compound C in the hypoxic *Ampka1^{-/-}2^{-/-}* cells inhibited HIF-1 α protein stability (Figure 3B). Furthermore, Compound C had no effect on DMOG treated *Ampka1^{-/-}2^{-/-}* cells (Figure 3B). Confirming the results shown in Figure 1C, WT cells treated with Compound C displayed a severe reduction in HRE-Luciferase expression when exposed to hypoxia in comparison to untreated WT cells (Figure 3C). Interestingly, hypoxic untreated *Ampka1^{-/-}2^{-/-}* cells showed an increase in HRE-Luciferase expression, whereas the hypoxic *Ampka1^{-/-}2^{-/-}* cells treated with Compound C displayed no induction of HRE-Luciferase under hypoxia (Figure 3C).

3.4 Compound C inhibits mitochondrial function

A functional electron transport chain is required for hypoxic activation of HIF-1. To examine if Compound C prevented HIF-1 activity by inhibiting oxidative phosphorylation, cells adapted to either glucose or galactose were treated with Compound C for 24, 48, and 72 hours and cell death was measured by LDH release (Figure 4A). Glucose adapted cells treated with Compound C displayed less than 20% cell death at all time points (Figure 4A). Untreated galactose adapted cells, which rely entirely on oxidative phosphorylation for survival, showed similar percentages of cell death. Galactose adapted cells display higher oxygen consumption than glucose adapted cells (14.4 nmol/min/million cells vs 6.34 nmol/min/million cells). Interestingly, galactose adapted cells that were treated with Compound C exhibited near 40% cell death at 48 hours and greater than 90% cell death at 72 hours (Figure 4A). Therefore, Compound C induces cell death in the presence of galactose. To further examine Compound C's effect on respiration, oxygen consumption was measured in WT cells in the presence or absence of Compound C (Figures 4B and 4C). In fact, Compound C inhibits respiration. The likely mechanism is electron transport inhibition since compound C inhibits respiration in the presence of the uncoupler FCCP (10 μ M). Previous studies have demonstrated that hypoxia increases the generation of oxidants during the ubiquinone cycle within complex III of the mitochondria and these oxidants are required and sufficient for HIF-1 activation [6–10]. Indeed, ROS is generated by hypoxia, as detected by the oxidation of the 2',7'-dichlorodihydrofluorescein (DCFH) dye and Compound C suppresses mitochondrial generated oxidants in response to hypoxia (Figure 4D). Treatment with DMOG had no effect on ROS generation (Figure 4D).

4. Discussion

Compound C is well established and a potent inhibitor of AMPK. Hypoxia is known to activate AMPK. Consistent with previous reports Compound C prevents hypoxia activation of AMPK. Compound C also inhibits the hypoxic activation of HIF-1, correlating with other reports that have suggested that AMPK is critical for HIF-1 activity by using Compound C under hypoxic conditions or exposure to the carcinogenic metal vanadate [19,20]. In contrast to the Compound C data, cells deficient in AMPK α 1 and α 2 are able to still activate HIF-1 during hypoxia. The novel finding in the present study is that Compound C still prevents the hypoxic activation of HIF-1 in cells deficient in both AMPK α 1 and α 2. This indicates that Compound C can

effectively suppress the hypoxic activation of HIF-1 independently of AMPK. Therefore, Compound C can be added to the list of known AMPK modulators, including AICAR and Metformin, which have off target effects. In addition, DN-AMPK adenoviruses have been shown to inhibit the activation of HIF-1, although these approaches also have off target effects [19,20]. Here we used AMPK α null cells, providing genetics in order to study the requirement of AMPK for the hypoxic activation of HIF-1.

Previous studies have demonstrated that a functional electron transport chain is necessary to produce an increase in ROS that subsequently stabilize HIF-1 α in response to hypoxia [6,7,9, 10]. Indeed we found that Compound C is also a mitochondrial inhibitor. The actual mitochondrial origin of ROS suppressed by Compound C remains unclear. Bell and colleagues have recently published that the ROS generated by the Q₀ site of complex III is required for hypoxic signaling [6]. Therefore, Compound C may be acting directly or indirectly at this site in order to suppress the hypoxic activation of HIF-1. Additionally, Compound C is part of a pyrazolo-pyrimidine class of protein kinase inhibitors, therefore it is possible that this compound is also inhibiting other kinase signaling pathways that regulate HIF-1 [23]. For example, p38 α MAPK is required for the hypoxic activation of HIF-1 [21]. Compound C may influence the p38 MAPK pathway, as well as, inhibit mitochondrial respiration in order to suppress hypoxic signaling. Cells treated with Compound C failed to survive in media containing galactose instead of glucose. Cells adapted to galactose rely on oxidative phosphorylation for survival. Compound C inhibited respiration as measured by oxygen consumption. Furthermore, Compound C suppresses the hypoxia generated increase in ROS, thus providing a mechanistic explanation for why Compound C is effective in suppressing the hypoxic activation of HIF-1. In conclusion, this study demonstrates that Compound C is not only an inhibitor of AMPK, but it inhibits the hypoxic activation of HIF-1 by suppressing mitochondrial generated ROS. These results further highlight the importance of mitochondria as a major regulator of the hypoxic activation of HIF-1.

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Abbreviations

HIF-1	Hypoxia inducible factor 1
AMPK	AMP-activated kinase
DMOG	Dimethyloxaloylglycine

AICAR

AICA-Riboside

ROS

Reactive Oxygen Species

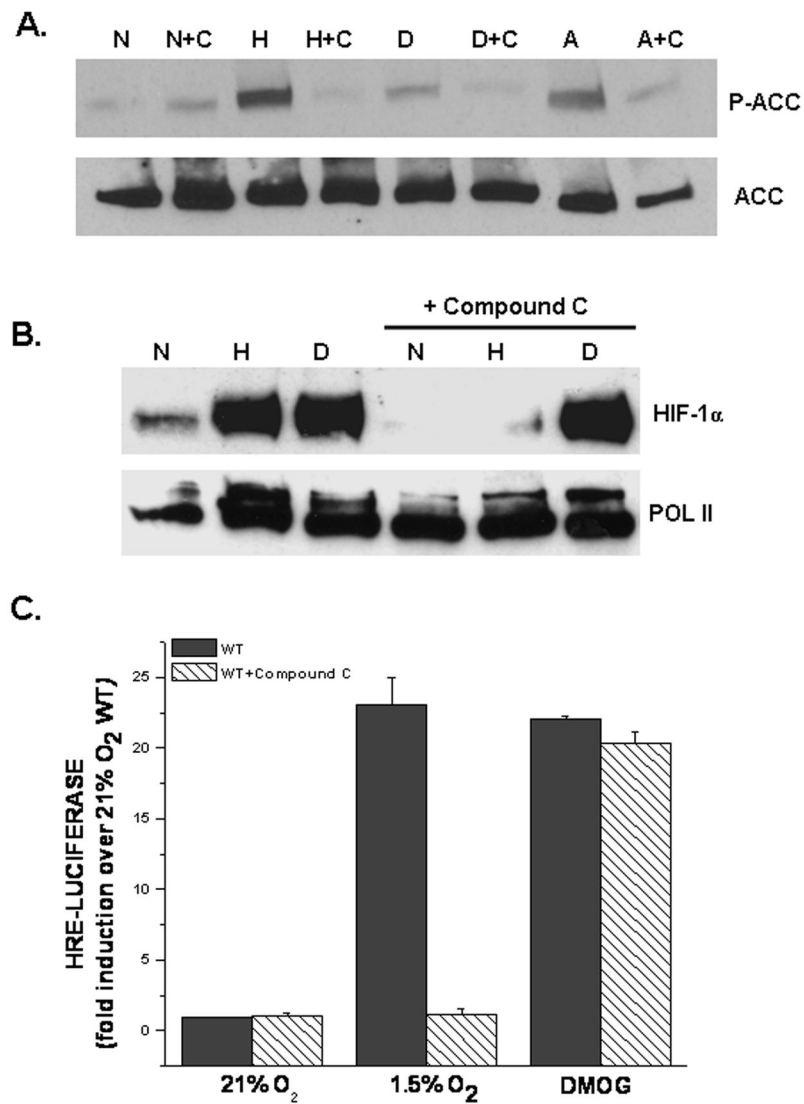


Figure 1. Compound C inhibits hypoxic activation of HIF-1

A. AMPK activation assessed by phospho-ACC protein in WT cells exposed to 21% O₂ (N), 1.5% O₂ (H), 1mM DMOG (D), or 0.5 mM AICAR (A) \pm 20 μ M Compound C (C) for 30 mins. Anti-ACC antibody used as loading control.

B. HIF-1 α protein levels in WT cells exposed to 21% O₂ (N) \pm 1mM DMOG (D) or to 1.5% O₂ (H) \pm Compound C for 2 hours. Anti-POL II antibody used as loading control.

C. WT cells transfected with a HRE-Luciferase reporter gene construct and exposed to 21% O₂ \pm 1mM DMOG or to 1.5% O₂ \pm Compound C for 16 hours before being harvested. Firefly luciferase activity was normalized to renilla luciferase activity and data reported as fold induction over 21% O₂.

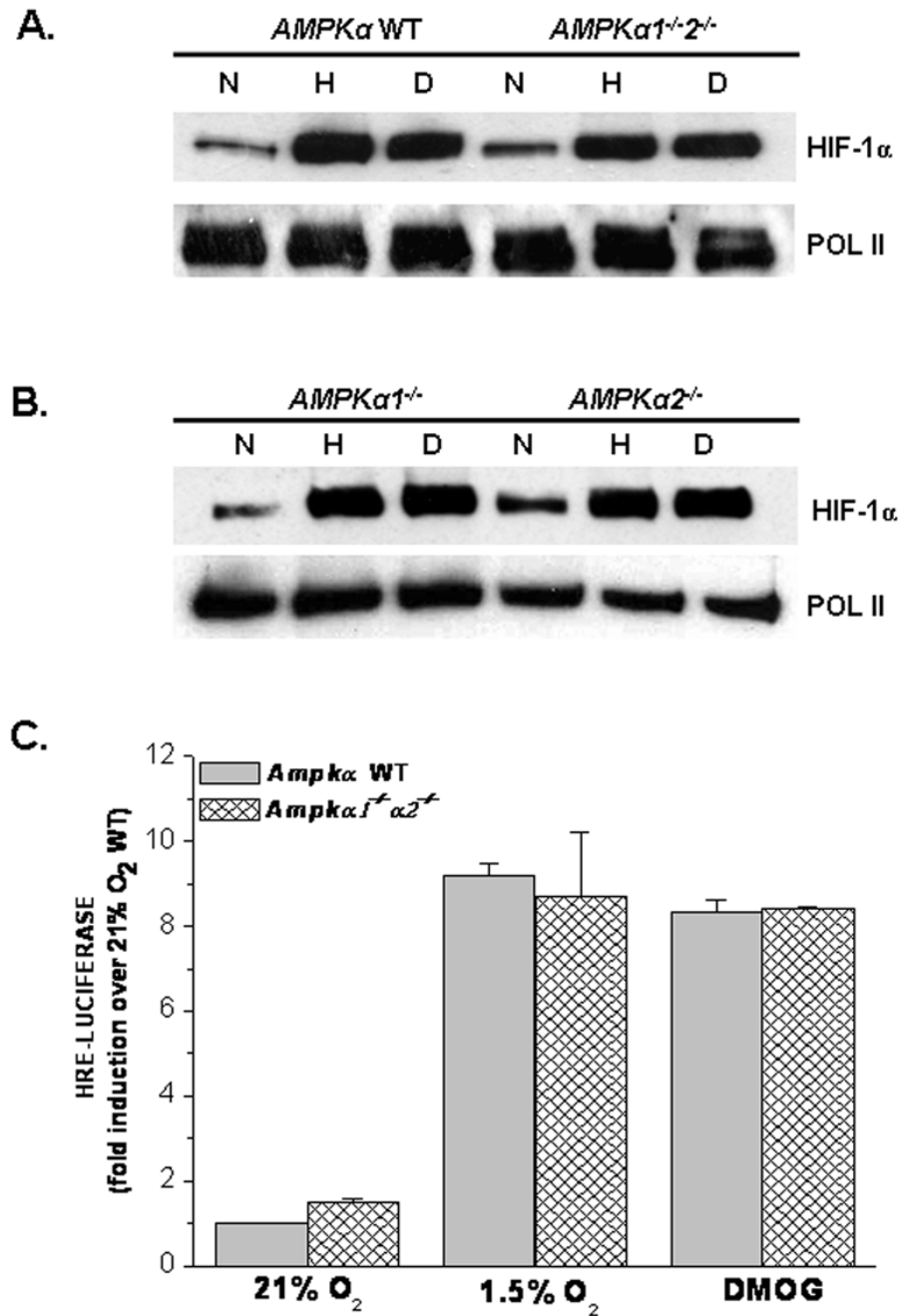


Figure 2. AMPK is not required for hypoxic activation of HIF-1

A. HIF-1 α protein levels in *AMPK α WT* and *AMPK $\alpha 1^{-/-} \alpha 2^{-/-}$* cells exposed to 21% O₂ (N) \pm 1mM DMOG (D) or to 1.5% O₂ (H) for 2 hours. Anti-POL II antibody used as loading control.

B. HIF-1 α protein levels in *AMPK $\alpha 1^{-/-}$* and *AMPK $\alpha 2^{-/-}$* cells exposed to 21% O₂ (N) \pm 1mM DMOG (D) or to 1.5% O₂ (H) for 2 hours. Anti-POL II antibody used as loading control.

C. *AMPK α WT* and *AMPK $\alpha 1^{-/-} \alpha 2^{-/-}$* cells transfected with a HRE-Luciferase reporter gene construct and exposed to 21% O₂ \pm 1mM DMOG or to 1.5% O₂ for 16 hours before being harvested. Firefly luciferase activity was normalized to renilla luciferase activity and data reported as fold induction over 21% O₂ *AMPK α WT*.

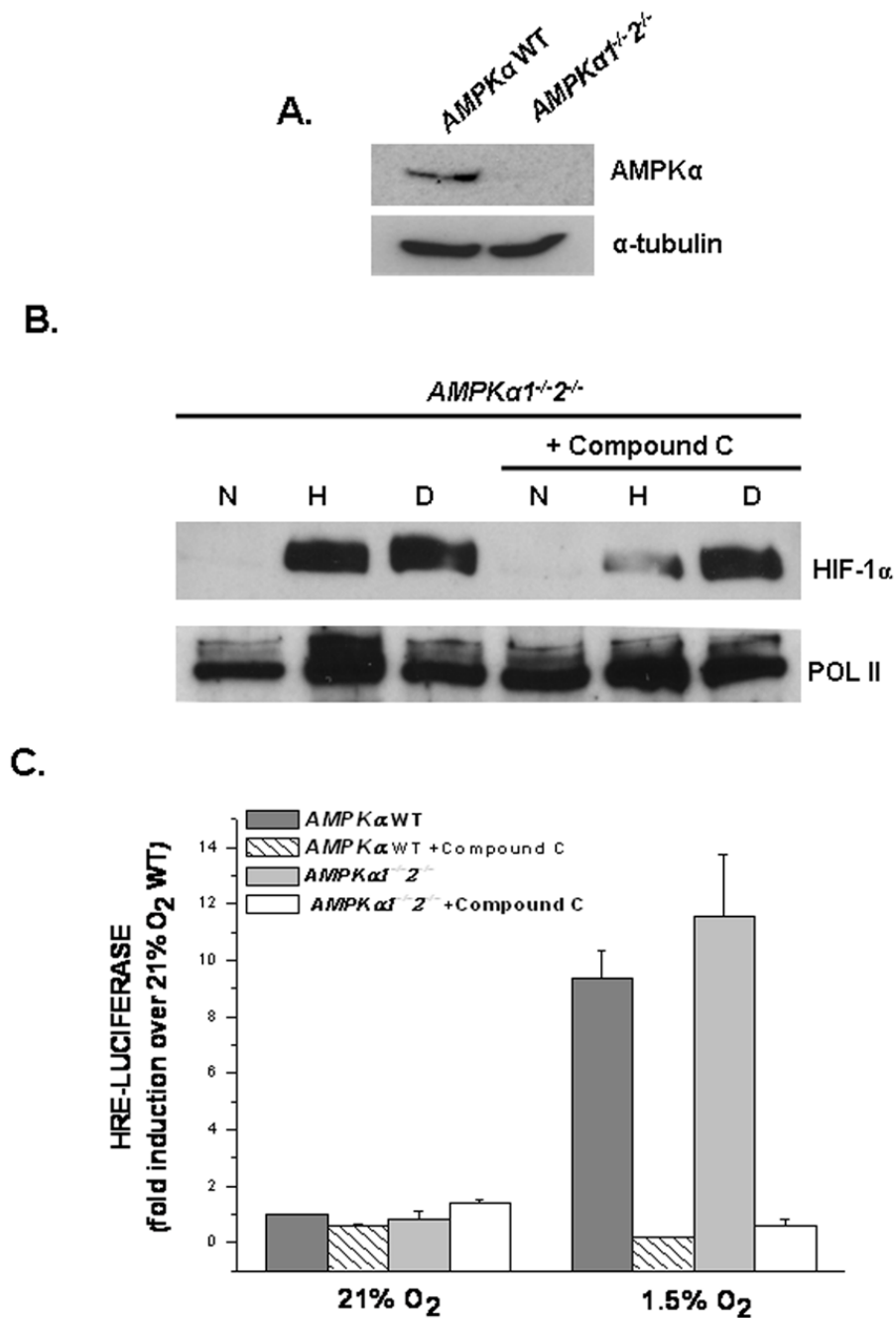
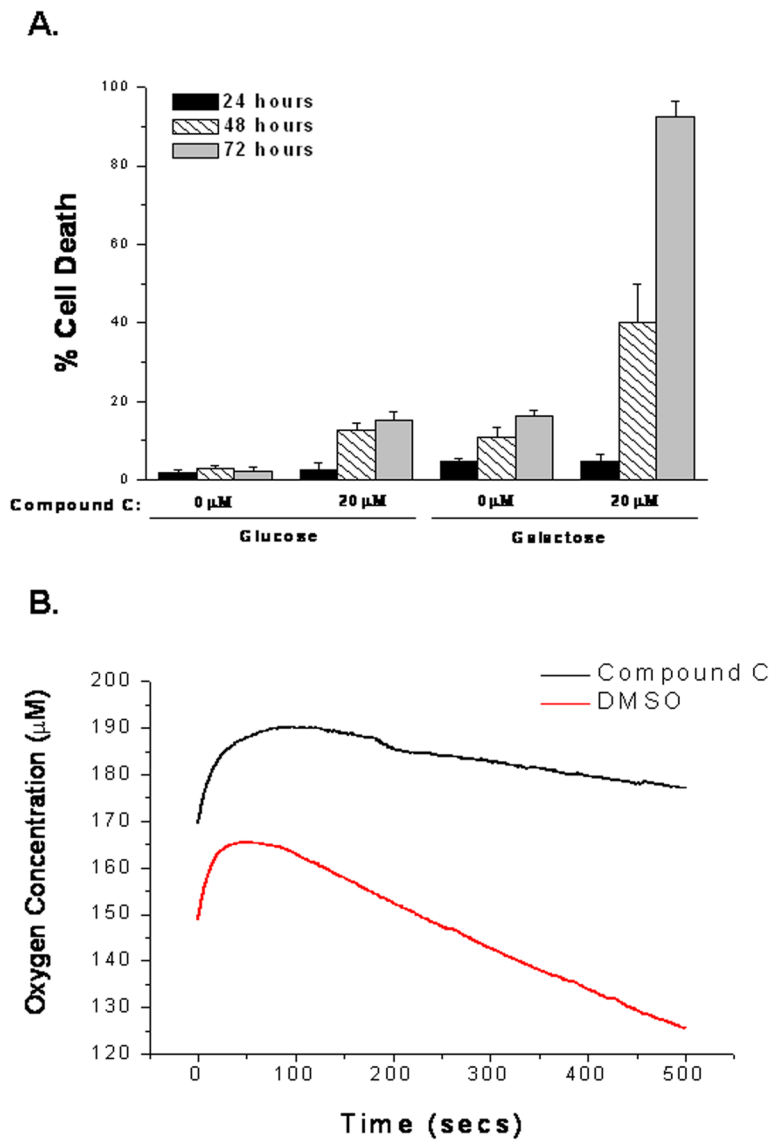


Figure 3. Compound C inhibits hypoxic activation of HIF-1 in AMPK α null cells

A. AMPK α protein levels in AMPK α WT and AMPK $\alpha 1^{-/-}\alpha 2^{-/-}$ cells. Anti-tubulin antibody used as loading control.

B. HIF-1 α protein levels in AMPK α WT and AMPK $\alpha 1^{-/-}\alpha 2^{-/-}$ cells exposed to 21% O₂ (N) \pm 1mM DMOG (D) or to 1.5% O₂ (H) \pm Compound C for 2 hours. Anti-POL II antibody used as loading control.

C. AMPK α WT and AMPK $\alpha 1^{-/-}\alpha 2^{-/-}$ cells transfected with a HRE-Luciferase reporter gene construct and exposed to 21% O₂ \pm 1mM DMOG or to 1.5% O₂ \pm 20 μ M Compound C for 16 hours before being harvested. Firefly luciferase activity was normalized to renilla luciferase activity and data reported as fold induction over 21% O₂ AMPK α WT.



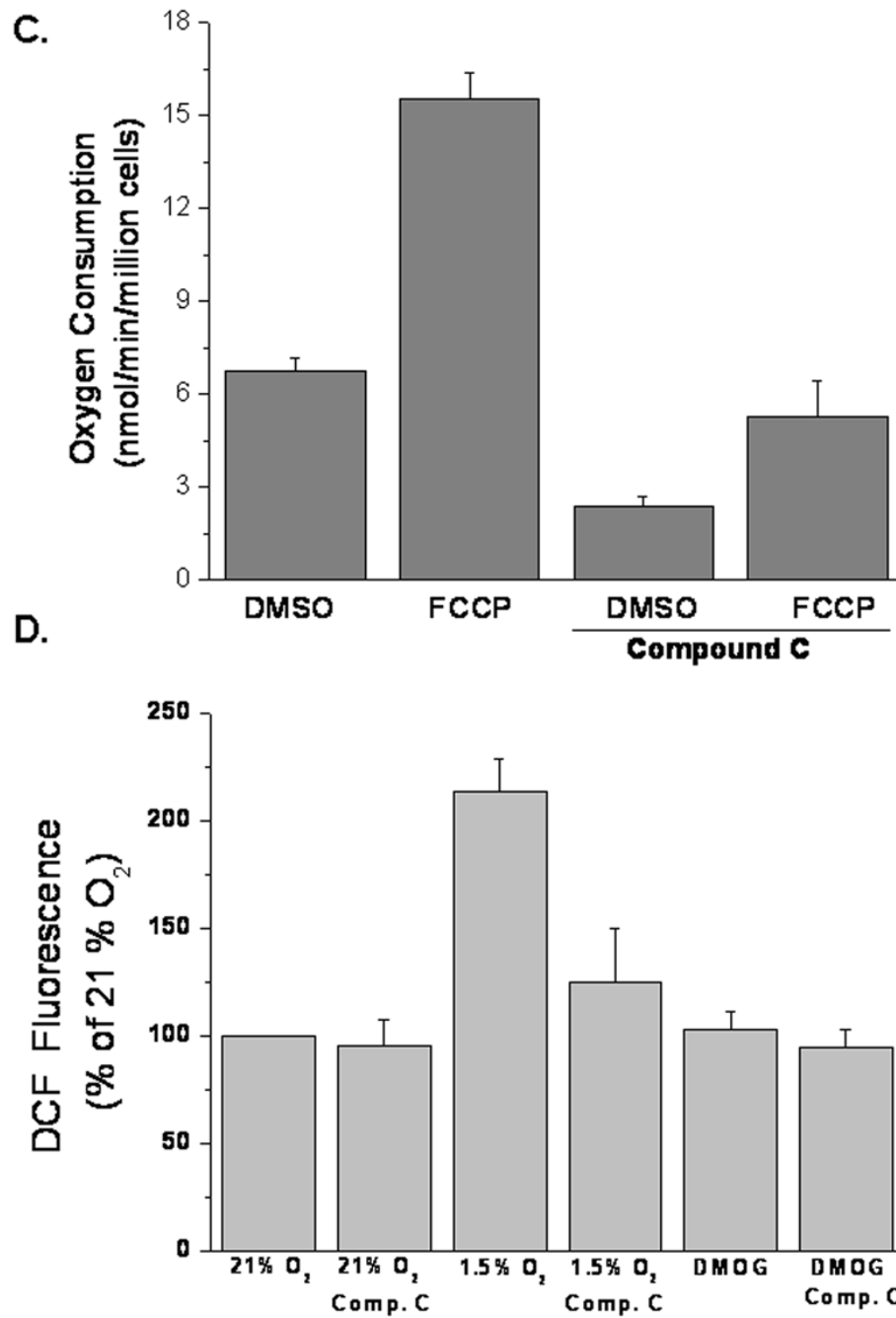


Figure 4. Compound C inhibits oxygen consumption and ROS generation

A. WT cells adapted to glucose or galactose \pm 20 μ M Compound C for 24, 48, and 72 hours and cell death assessed by LDH release.

B and C. Oxygen consumption of WT cells with 20 μ M Compound C or DMSO control in the presence of FCCP. N=3 mean \pm S.D.

D. ROS determined by incubating WT cells with DCFH-DA (10 μ M) exposed to 21% O₂, 1.5% O₂ \pm 20 μ M Compound C, or to 1mM DMOG \pm 20 μ M Compound C for 30 minutes. N=4 mean \pm S.D.