

Article

Cys340Ser Mutation Abolishing S-Nitrosylation Drives GRK2 Mitochondrial Localization and Dysfunction

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Highlights

What are the main findings?

- Blocking S-nitrosylation at Cys340 increases mitochondrial localization of GRK2, particularly under hypoxia/reoxygenation stress.
- Loss of S-nitrosylation impairs mitochondrial respiration, disrupts mitochondrial dynamics, and alters mitophagy, leading to mitochondrial dysfunction.

What are the implications of the main findings?

- S-nitrosylation functions as an endogenous regulatory mechanism limiting GRK2 mitochondrial toxicity in stressed cardiomyocytes.
- Targeting GRK2 activity or its post-translational modification may represent a therapeutic strategy in cardiac pathologies associated with mitochondrial dysfunction.

Abstract

In cardiac pathologies, levels of G protein-coupled receptor kinase 2 (GRK2)—which is involved in receptor desensitization and internalization—are elevated. Beyond these receptor-mediated effects, GRK2 also localizes to mitochondria, where it contributes to pathology. GRK2's activity can be inhibited via S-nitrosylation at Cysteine 340, a post-translational modification mediated by both endogenous and exogenous nitric oxide. Thus, S-nitrosylation is considered as an endogenous brake on GRK2's catalytic activity, counteracting its hyperactivity observed in disease states. However, it remains unclear whether S-nitrosylation also regulates GRK2's influence on mitochondrial function. This study aims to investigate how S-nitrosylation regulates mitochondrial localization and function of GRK2 under hypoxia/reoxygenation stress. To prevent S-nitrosylation at Cys340, we infected AC16 cardiac cells with adenoviruses carrying a GRK2 C340S (Ser) mutation. Our results indicate that inhibiting S-nitrosylation enhances mitochondrial localization of GRK2, especially in response to pathological stimuli. Additionally, mitochondrial function was impaired, as measured by oxygen consumption rates at ATP production. Furthermore, alterations in mitochondrial dynamics and mitophagy led to adverse outcomes when GRK2 was not subject to S-nitrosylation, presumably due to increased catalytic activity. Our findings underscore the importance of GRK2 regulation in cardiac pathologies and suggest that targeting GRK2 or its post-translational modifications may provide therapeutic benefits.



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1. Introduction

Cardiovascular diseases are among the major causes of morbidity and mortality worldwide, with impaired β -adrenoceptor (AR)-mediated inotropic and chronotropic responses representing a hallmark of their pathogenesis. This dysfunction is primarily driven by elevated levels of G protein-coupled receptor kinase 2 (GRK2), which promotes the desensitization and internalization of β 1- and β 2ARs. GRK2 functions as a negative feedback regulator of excessive β -AR stimulation; while its activation initially serves as a compensatory mechanism, sustained GRK2 activity ultimately leads to receptor inactivation, reduced cardiac performance, and pathological remodeling [1]. Elevated GRK2 levels have been reported in several cardiac disease states, and inhibition of GRK2 has been shown to improve cardiac function and enhance survival. In addition to its well-characterized canonical effects, increasing attention has been directed toward the non-canonical roles of GRK2. It has been shown to interact with a variety of intracellular non-receptor targets. Among these expanding GRK interactomes, heat shock protein 90 (hsp90) interaction facilitates mitochondrial location of GRK2 [2]. Mitochondrial localization of GRK2 is particularly enhanced under pathological conditions associated with oxidative stress, such as ischemia. GRK2 is phosphorylated at serine 670 (Ser670), enabling its binding to Hsp90 and subsequent translocation to mitochondria [3]. This localization impairs fatty acid-driven mitochondrial energy production and cardiomyocyte survival [3,4]. Importantly, inhibition of GRK2 phosphorylation at Ser670—thus preventing its mitochondrial translocation—has been shown to mitigate cardiac dysfunction following ischemia/reperfusion (I/R) injury [5]. GRK2 is also subject to various post-translational modifications, among which S-nitrosylation is particularly significant. S-nitrosylated GRK2 (SNO-GRK2) has been reported to exert cardioprotective effects [6], suggesting that S-nitrosylation serves as a regulatory brake against overactive GRK2. Both endogenous and exogenous S-nitrosothiol (SNOs) have been shown to inhibit GRK2 via S-nitrosylation at the Cys340 residue, thereby reducing its desensitizing activity on receptors and reactivating GPCR signaling [7]. Indeed, knock-in (KI) mice carrying a mutation at the GRK2 Cys340 site, rendering it resistant to S-nitrosylation via replacing the Cys with Ser (C340S), exhibit worsened cardiac function following I/R injury [6]. Moreover, loss of S-nitrosylation-mediated inhibition leads to increased GRK2 activity, contributing to cardiac dysfunction and remodeling with aging [8]. S-nitrosylation plays a key role in regulating mitochondrial function by modifying various mitochondrial proteins [9,10]. While mitochondrial NO bioactivity and SNO-proteins are generally considered protective, they may contribute to mitochondrial dysfunction under certain conditions [10,11]. Within this context, we aimed to investigate whether S-nitrosylation regulates the mitochondrial localization of GRK2 and how S-nitrosylated GRK2 influences mitochondrial function following ischemic injury.

2. Materials and Methods

2.1. Cell Culture and Hypoxia/Reoxygenation Model

The AC16 human cardiomyocyte cell line was purchased from Sigma Aldrich (USA) (SCC109; St. Louis, MO, USA) and cultured as indicated by the vendor. Briefly, AC16 cells were maintained in DMEM containing 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin solution. Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air. Cells were passaged at 80–90% confluency using 0.05% Trypsin-EDTA. Cells were transduced with adenoviruses encoding either GFP or

GRK2-C340S and incubated for 48 h [12]. GFP was used as a transfection control to allow verification of transfection efficiency and to ensure comparable expression conditions across experimental groups. The Hypoxia/Reoxygenation (H/R) model was conducted using a hypoxia incubator chamber (Billups Rothenberg, Inc., Del Mar, CA, USA). Prior to hypoxia, the culture medium was replaced with serum-free HBSS, and the cells were placed into the chamber, which was flushed with a gas mixture containing 95% N₂, 5% CO₂, and 1% O₂. After hypoxia, the cells were removed from the chamber, the medium was replaced with complete DMEM, and the cells were reoxygenated for 24 h at 37 °C under normoxic conditions (95% air and 5% CO₂).

2.2. Cell Viability

Cell viability was assessed using the MTT assay. MTT powder was dissolved in 1 mL of PBS and vortexed one day prior to the experiment. On the day of the assay, the MTT solution was pre-warmed to 37 °C. Following the completion of hypoxia and reoxygenation treatments, MTT solution was added to the cells at a final concentration of 10%. After a 2-h incubation, crystal formation was observed at the bottom of the wells, indicating the reduction of MTT by metabolically active cells. The supernatant was then discarded, and the formazan crystals were dissolved in 200–400 µL of DMSO. The resulting solutions were transferred to 96-well plates, and absorbance was measured spectrophotometrically at 590 nm using a MultiSkan plate reader (Thermo Fisher Scientific, Waltham, MA, USA). The mean optical density (OD) values, calculated from duplicate wells, were used to determine cell viability as a percentage.

2.3. MitoTracker Imaging

AC16 cardiomyocytes were labeled with MitoTracker Red CMXRos (579/599 nm; Invitrogen M7512; Waltham, MA, USA) following the manufacturer's instructions. A 1 mM stock solution was prepared by dissolving 50 µg of MitoTRACKER in 92 µL of DMSO. Cells were seeded and transduced as described, then incubated with 300 nM MitoTRACKER solution containing Hoechst 33342 nuclear stain (347/483 nm; Invitrogen H3570) in serum-free DMEM for 30 min at 37 °C. After staining, cells were washed twice with PBS and fixed in 4% paraformaldehyde (ThermoFisher J61899) for 15 min at room temperature followed by three PBS washes. Cells were imaged at 20× magnification using an EVOS M7000 Imaging System (Thermo Fisher Scientific, Waltham, MA, USA) under identical exposure settings across conditions. Quantification was performed by measuring total cellular fluorescence intensity (integrated density normalized to cell area) in ImageJ (<https://imagej.net>) from 3 images per condition.

2.4. Measurement of Intracellular Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) levels were assessed using the fluorescent probe DCFDA (10 µM) and imaged by confocal microscopy. Fluorescence was detected at excitation and emission wavelengths of 490 nm and 530 nm, respectively. Baseline fluorescence was recorded prior to stimulation, after which 100 µM H₂O₂ was applied to confirm dye responsiveness. Changes in ROS levels were quantified as fold increases in fluorescence intensity relative to baseline for each cell, and comparisons were performed between experimental groups.

2.5. Measurement of Mitochondrial Superoxide

Mitochondrial superoxide levels in AC16 cardiomyocytes were assessed using the fluorogenic dye CellROX Deep Red (644/665 nm; Invitrogen C10422) following the manufacturer's protocol. CellROX becomes fluorescent upon oxidation by reactive oxygen species, enabling detection of mitochondrial oxidative stress. A 5 µM CellROX working

solution containing Hoechst 33342 nuclear stain (347/483 nm; Invitrogen H3570) was prepared in PBS. Cells were seeded and transduced as described, washed twice with warm PBS and incubated with the CellROX/Hoechst solution at 37 °C for 30 min in the dark. Following incubation, cells were washed twice with warm PBS and fixed with 4% paraformaldehyde (ThermoFisher J61899) for 15 min at room temperature. Cells were then washed three times with cold PBS. Cells were imaged at 20× magnification using an EVOS M7000 Imaging System under identical exposure settings across conditions. Quantification was performed by measuring total cellular fluorescence intensity (integrated density normalized to cell area) in ImageJ from 3 images per condition.

2.6. Seahorse XF Cellular Bioenergetics

One day prior to the experiment, AC16 cells were seeded at 2×10^4 cells per well in XF96 Seahorse® (Agilent Technologies, Santa Clara, CA, USA) plates, and all measurements were normalized to cell number. On the day of the assay, the XF assay medium (XF DMEM pH 7.4) was supplemented with 10 mM glucose, 1 mM pyruvate, and 2 mM L-glutamine. Stock solutions of oligomycin (100 µM), FCCP (100 µM), and rotenone/antimycin A (50 µM) were prepared in XF assay medium and diluted to working concentrations according to the manufacturer's instructions. The inhibitors were loaded into ports A (oligomycin, final 1.5 µM), B (FCCP, final 1.0 µM), and C (rotenone/antimycin A, final 0.5 µM) of the hydrated sensor cartridge (done according to the manufacturer's instructions). Cell culture medium was replaced with 180 µL/well of pre-warmed assay medium, and the plate was incubated for 60 min in a non-CO₂ incubator (HERAcell vios 160i LK CO₂ incubator; Thermo Fisher Scientific, Waltham, MA, USA) set to 37 °C and 0% CO₂. Following sensor calibration, the cell plate was loaded into the Seahorse XF Analyzer (Agilent Technologies, Santa Clara, CA, USA), and the Mito Stress Test was performed according to the standard protocol.

The XF96 uses optical microensors to measure the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in the media of cells. OCR is a measure of O₂ utilization in oxidative phosphorylation by mitochondria, while ECAR is a measure of glycolysis. The mitochondrial electron transport chain inhibitor oligomycin (1.5 µM), the uncoupling agent carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP; 1 µM), and a mixture of the mitochondrial electron transport chain inhibitors rotenone and antimycin A (0.5 µM) were sequentially injected according to the manufacture's protocol, and OCR and ECAR were measured. OCR readings were normalized to the number of cardiomyocytes plated per well (2×10^4) for all conditions. Normalizing the OCR data to cell number ensured consistent comparison between the conditions.

Mitochondrial respiration was assessed by quantifying the oxygen consumption rate (OCR) as an indicator of oxidative phosphorylation by mitochondria. During the test, inhibitors were delivered sequentially to interrogate specific components of the electron transport chain. Oligomycin (1.5 µM), inhibits ATP synthase, allowing calculation of mitochondrial ATP-linked respiration from the drop in OCR after its addition. Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP; 1 µM) is an uncoupling agent that dissipates the proton gradient, driving the electron transport chain to operate at maximum capacity. The resulting increase in OCR defines maximal respiration. Rotenone and antimycin A (0.5 µM each) inhibit Complex I and Complex III respectively, shutting down mitochondrial electron flow. The remaining OCR represents non-mitochondrial respiration. From these inhibitor-driven changes in OCR, we calculated standard mitochondrial parameters: basal respiration (initial OCR reflecting ATP production and proton leak), ATP-linked respiration (OCR reduction after oligomycin), proton leak (basal OCR not coupled to ATP synthesis) and maximal respiration (peak OCR following FCCP addition), spare respiratory capacity (maximal minus basal respiration) and non-mitochondrial respiration (OCR

after rotenone/antimycin A). These calculations enable quantification of mitochondrial function by linking OCR shifts to inhibition or stimulation of specific electron transport chain complexes.

2.7. Subcellular Fractionation and Western Blot

Mitochondria were prepared from cells as previously described [3,4]. Briefly, after cells were washed with Dulbecco's phosphate-buffered saline, they were scraped into MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.5) supplemented with 1 mM EDTA and homogenized using a glass–glass Dounce homogenizer. Homogenate was then centrifuged at $600\times g$ for 10 min at 4 °C. The supernatant was collected, centrifuged again at $600\times g$ for 10 min, and the resulting supernatant was subjected to centrifugation at $5500\times g$ for 20 min at 4 °C to pellet mitochondria. The mitochondrial pellet was washed in fresh MSH buffer (without EDTA) and centrifuged once more at $5500\times g$ for 20 min. The resulting pellet was referred to as the mitochondrial fraction. Cell or mitochondria pellets were resuspended in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with $1\times$ protease inhibitor and $1\times$ phosphatase inhibitor cocktails 2 and 3 (Millipore Sigma, Burlington, MA, USA). Homogenates were allowed to equilibrate for 1 h at 4 °C, followed by centrifugation ($13,000\times g$ for 20 min) at 4 °C. Supernatant was then collected followed by the BCA protein assay. For immunoblotting, samples were prepared in Laemmli sample buffer and loaded on precast gels and transferred onto Trans-Blot Turbo Transfer membranes (Bio-Rad, Hercules, CA, USA). The membrane was blocked in LI-COR block buffer for 1 h at room temperature, followed by exposure to primary antibody at 4 °C. The primary antibodies used were as follows: GRK2 (13990-1-AP; ProteinTech, Rosemont, IL, USA), GRK2 (05-465; Sigma Aldrich, St. Louis, MO, USA), Drp1 (8570; Cell Signaling Technology, Danvers, MA, USA), Fis1 (32525; Cell Signaling Technology, Danvers, MA, USA), Mfn1 (14739; Cell Signaling Technology, Danvers, MA, USA), Mfn2 (11925; Cell Signaling Technology, Danvers, MA, USA), Opa1 (80471; Cell Signaling Technology), Pink (6946; Cell Signaling Technology, Danvers, MA, USA), Parkin (4211; Cell Signaling Technology, Danvers, MA, USA), LC3 (3868; Cell Signaling Technology, Danvers, MA, USA), Tom20 (42406; Cell Signaling Technology, Danvers, MA, USA), VDAC1 (sc-390996; Santa Cruz Biotechnology, Dallas, TX, USA), and GAPDH (sc-32233; Santa Cruz Santa Cruz Biotechnology, Dallas, TX, USA). Membranes were washed three times with PBS containing 0.1% Tween-20 and exposed to fluorescent conjugated secondary antibodies: rabbit 680 (A21109; Invitrogen, Waltham, MA, USA), mouse 680 (A28183; Invitrogen, Waltham, MA, USA), rabbit 800 (926-32211; LI-COR Biosciences, Lincoln, NE, USA), and/or mouse 800 (926-32210; LI-COR Biosciences, Lincoln, NE, USA). Imaging was done using LI-COR Odyssey software Image Studio Version 5.5 and quantification was done using Image Studio Lite Version 5.2.

2.8. Statistical Analysis

Data are presented as mean $\pm$ SEM. Differences between groups were evaluated for significance using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons (GraphPad Prism Version 10, GraphPad Prism Inc., San Diego, CA, USA). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$; p -value less than 0.05 was considered statistically significant.

3. Results

Cell viability decreased following hypoxia, as expected (Figure 1A). In cells infected with Ad.GRK2.C340S, where GRK2 cannot undergo S-nitrosylation, viability was further reduced compared to Ad.GFP-infected hypoxic cells. Hypoxia increased MitoTracker fluorescence intensity, reflecting increased mitochondrial content and/or membrane potential, with a more pronounced effect in GRK2.C340S-expressing cells (Figure 1B). Intracellular

ROS levels measured by DCFDA fluorescence were significantly increased under hypoxic conditions in both Ad.GFP- and Ad.GRK2-C340S-infected cells. Notably, the greatest elevation was observed in hypoxic Ad.GRK2-C340S cells (Figure 1C). In contrast, mitochondrial superoxide levels assessed by CellROX Deep Red fluorescence did not show statistically significant differences among groups (Figure 1D), although a modest upward trend was observed in hypoxic Ad.GRK2-C340S-infected cells.

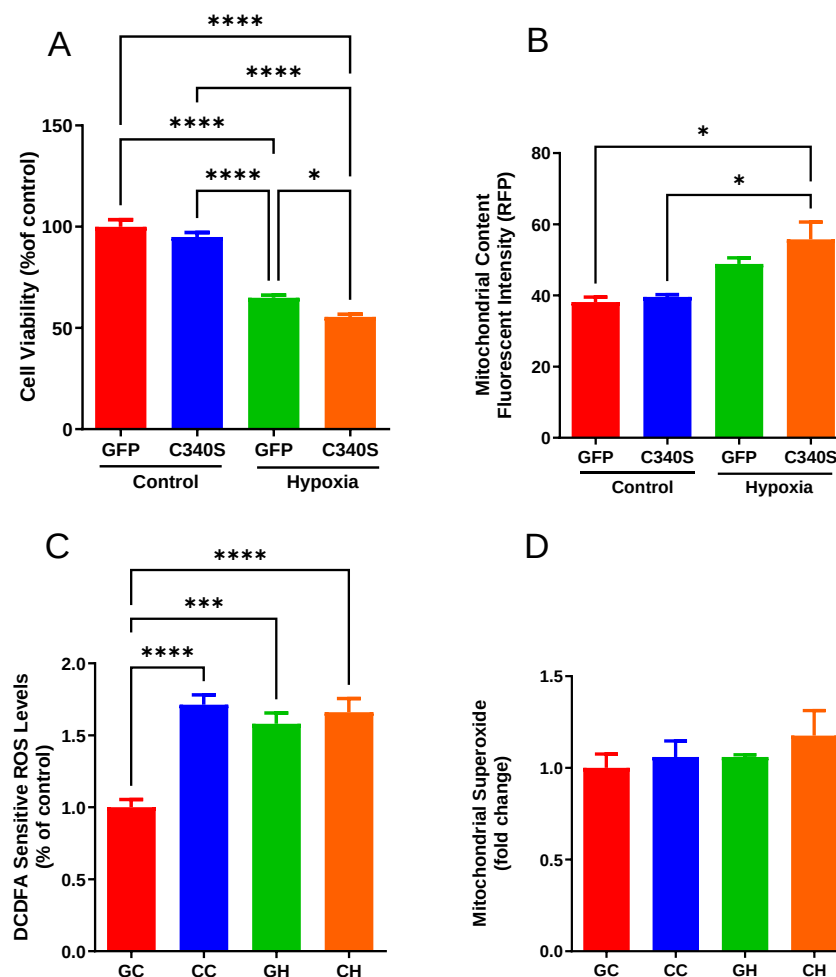


Figure 1. Cell viability measured by MTT staining (A); quantitative analysis of MitoTracker fluorescence intensity (B); H_2O_2 -induced ROS production measured by DCFDA fluorescence (C); quantitative analysis of CellROX Deep Red fluorescence intensity (D). Statistical significance was determined by ANOVA followed by Bonferroni's post hoc test. Data are shown as mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. Representative images are provided in Supplementary Data S1.

Mitochondrial function was evaluated using the Seahorse XF Mito Stress Test (Figure 2). As expected, measurements in control cells confirmed that hypoxia impairs cellular bioenergetics and mitochondrial function (Figure 2 blue vs. green line). Furthermore, under normoxic conditions, mitochondrial function was compromised in cells infected with Ad.GRK2.C340S compared to Ad.GFP controls (Figure 2 red vs. green line). This impairment was further exacerbated by hypoxia, with Ad.GRK2.C340S-infected cells showing the most severe decline in mitochondrial respiration and function (Figure 2 orange vs. red line). Impairments in cellular OCR in Ad.GRK2.C340S-infected cells under hypoxia resulted in decreased mitochondrial respiration parameters (Figure 2B–G).

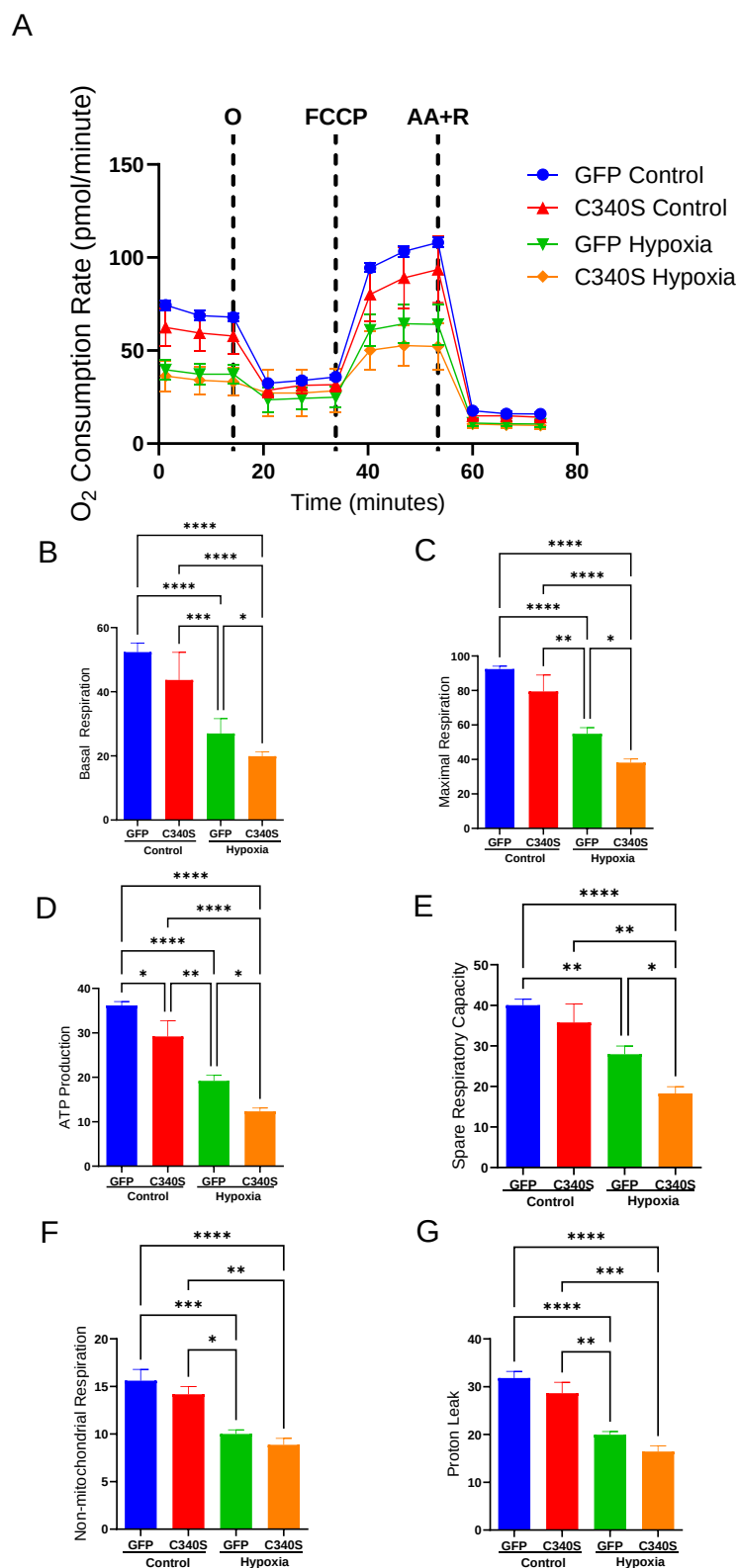


Figure 2. OCR trace over time with injection of inhibitors indicated (O: oligomycin, FCCP, AA + R: Antimycin A and Rotenone) (A), basal respiration (B), maximal respiration (C), ATP production (D), spare respiratory capacity (E), non-mitochondrial respiration (F), proton leak (G). Statistical significance was determined by ANOVA followed by Bonferroni's post hoc test. Data are shown as mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

Following confirmation of fraction purity, mitochondrial GRK2 levels were measured (Figure 3). GRK2 expression was markedly elevated in cells transduced with

Ad.GRK2.C340S, consistent with successful overexpression. Upon exposure to hypoxia, GRK2 levels increased further in these cells. However, because adenoviral transduction already induced high baseline GRK2 expression, direct comparison with GFP-infected controls was not appropriate. The absence of a shared baseline precluded a meaningful assessment of absolute GRK2 upregulation due to hypoxia. To overcome this, we calculated the fold change in mitochondrial GRK2 expression after hypoxia separately for both GFP- and GRK2.C340S-infected cells. Comparison of these fold changes revealed that loss of S-nitrosylation enhances the translocation of GRK2 to mitochondria in response to hypoxic stress.

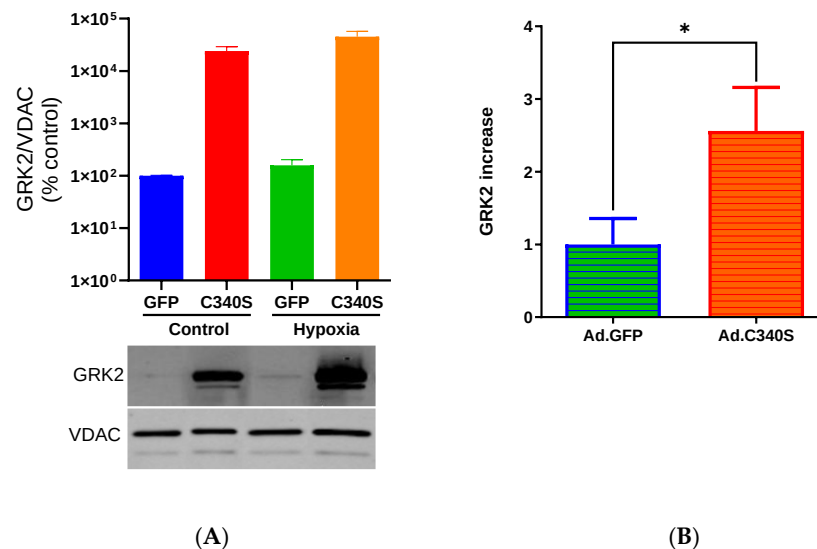


Figure 3. Group comparison of mitochondrial GRK2 expression (A); comparison of fold increase in GRK2 levels between Ad.GFP- and Ad.GRK2.C340S-infected cells (B). Statistical significance was determined by ANOVA followed by Bonferroni's post hoc test for GRK2/VDAC ($p = 0.0003$) and by t -test for GRK2 fold increase ($p = 0.0436$). Data are shown as mean $\pm$ SEM; * $p < 0.05$ ($n = 5$ per group). Representative immunoblots demonstrating GRK2 protein levels in mitochondrial fractions of AC16 cells infected with Ad.GFP or Ad.GRK2-C340S under normoxic or hypoxia/reoxygenation (H/R) conditions. VDAC served as a mitochondrial loading control. Densitometric analysis was performed using Li-Cor Image Studio software and normalized to the respective loading controls. All samples included in each comparative analysis were run on the same gel. Cropped blots are presented for clarity; uncropped blots with molecular weight markers are provided in Supplementary Data S2.

Subsequently, the levels of key mitochondrial proteins involved in mitochondrial dynamics were assessed. In Ad.GFP-infected cells, the mitochondrial fission protein Drp1 showed a trend toward reduced levels; however, this change did not reach statistical significance. In contrast, levels of the fission protein Fis1 were significantly reduced after hypoxia in both groups compared with normoxic Ad.GFP controls (Figure 4).

Protein levels of the mitochondrial fusion proteins Mfn1 (A), Mfn2 (B), and Opa1 (C) were analyzed under normoxic and hypoxic conditions. Hypoxia was associated with a general reduction in fusion protein levels in both Ad.GFP- and Ad.GRK2.C340S-infected cells, with no significant differences observed between the two groups (Figure 5).

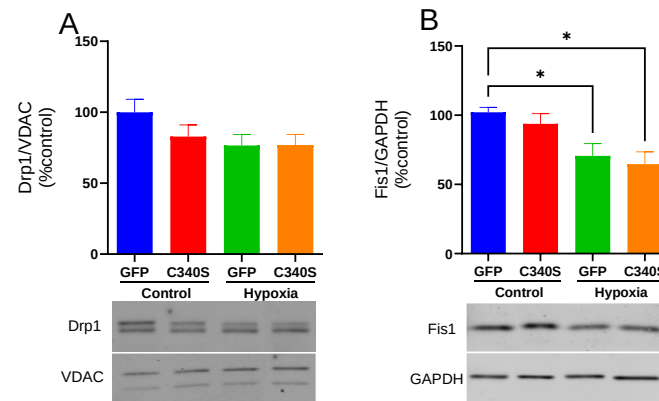


Figure 4. Group comparison of mitochondrial Drp1 (A) and Fis1 (B) expressions. Statistical significance was determined by ANOVA followed by Bonferroni's post hoc test. Data are shown as mean $\pm$ SEM; ns, $p = 0.2021$ for Drp1; *, $p < 0.05$ ($p = 0.0041$) for Fis1 ($n = 5-7$ per group). Representative immunoblots demonstrating Drp1 and Fis1 protein levels in mitochondrial fractions and total cell lysates of AC16 cells infected with Ad.GFP or Ad.GRK2-C340S under normoxic or hypoxia/reoxygenation (H/R) conditions. VDAC served as a mitochondrial loading control and GAPDH served as a total protein marker. Densitometric analysis was performed using Li-Cor Image Studio software and normalized to the respective loading controls. All samples included in each comparative analysis were run on the same gel. Cropped blots are presented for clarity; uncropped blots with molecular weight markers are provided in Supplementary Data S2.

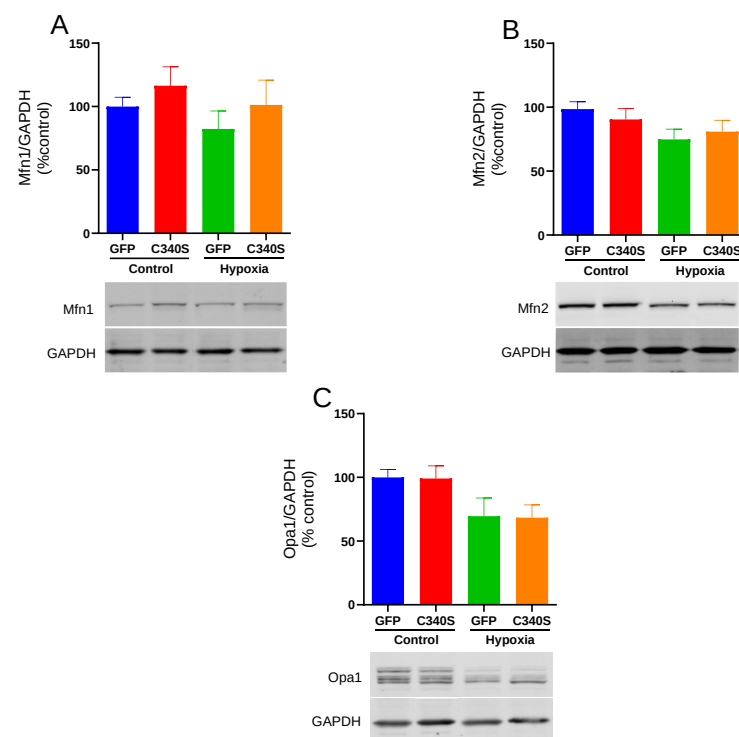


Figure 5. Group comparison of Mfn1 (A), Mfn2 (B) and Opa1 (C) expressions. Statistical significance was determined by ANOVA followed by Bonferroni's post hoc test. Data are shown as mean $\pm$ SEM; ns, $p = 0.4591$ for Mfn1; $p = 0.1796$ for Mfn2; $p = 0.0552$ for Opa1 ($n = 7-9$ per group). Representative immunoblots demonstrating Mfn1, Mfn2 and Opa1 protein levels in total cell lysates of AC16 cells infected with Ad.GFP or Ad.GRK2-C340S under normoxic or hypoxia/reoxygenation (H/R) conditions. GAPDH served as a total protein marker. Densitometric analysis was performed using Li-Cor Image Studio software and normalized to the respective loading controls. All samples included in each comparative analysis were run on the same gel. Cropped blots are presented for clarity; uncropped blots with molecular weight markers are provided in Supplementary Data S2.

Protein levels of mitophagy-related markers were analyzed under normoxic and hypoxic conditions. Hypoxia significantly reduced the expression of the mitophagy regulators Parkin (A) and Pink1 (B) in both Ad.GFP- and Ad.GRK2.C340S-infected cells. In parallel, hypoxia significantly increased LC3-II/VDAC levels (C), indicating enhanced autophagosome formation, while expression of the mitochondrial outer membrane protein Tomm20 (D) remained unchanged across all conditions (Figure 6).

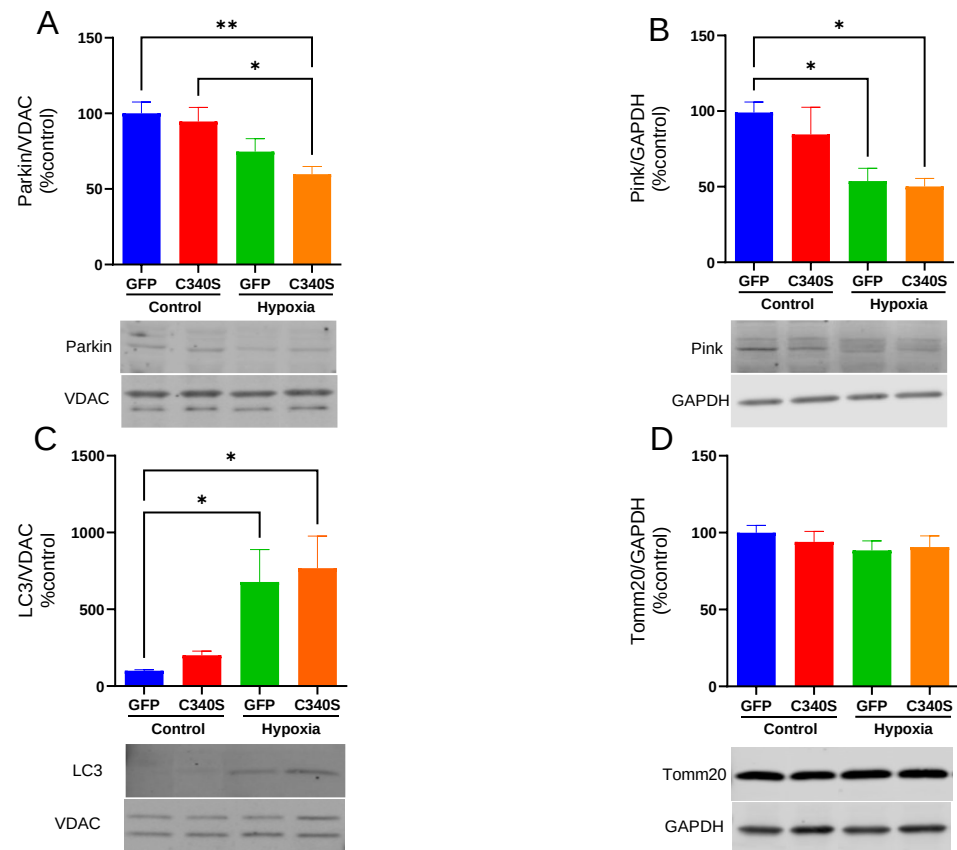


Figure 6. Group comparison of Parkin (A), Pink (B), LC3 (C) and Tomm20 (D) expressions. Statistical significance was determined by ANOVA followed by Bonferroni's post hoc test. Data are shown as mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$ $p = 0.0027$ for Parkin; $p = 0.0093$ for Pink (both bands); $p = 0.0043$ for LC3; ns, $p = 0.6020$ for Tomm20 ($n = 8$ – 10 per group). Representative immunoblots demonstrating Parkin, Pink, LC3 and Tomm20 in mitochondrial fractions and total cell lysates of AC16 cells infected with Ad.GFP or Ad.GRK2-C340S under normoxic or hypoxia/reoxygenation (H/R) conditions. VDAC served as a mitochondrial loading control and GAPDH served as a total protein marker. Densitometric analysis was performed using Li-Cor Image Studio software and normalized to the respective loading controls. All samples included in each comparative analysis were run on the same gel. Cropped blots are presented for clarity; uncropped blots with molecular weight markers are provided in Supplementary Data S2.

4. Discussion

Our study demonstrates that S-nitrosylation-mediated inhibition of GRK2 is critically important in its regulation of mitochondrial regulation of function. The C340S mutation disrupts S-nitrosylation at Cys340, thereby eliminating this regulatory modification of GRK2. This altered regulation is associated with the mitochondrial dysfunction observed, even in the absence of hypoxic stress. Loss of S-nitrosylation of GRK2 resulted in reduced mitochondrial ATP production, with a more pronounced effect after hypoxia/reoxygenation stress. Mitochondrial parameters such as maximal respiration and ATP production were markedly lower in GRK2-C340S-overexpressing cells compared to Ad.GFP controls. These functional

impairments were accompanied by changes in protein expression, and mitochondrial GRK2 content was elevated when S-nitrosylation was disrupted. The increase in mitochondrial signal observed in control GFP-expressing cells following hypoxia is consistent with an adaptive response to metabolic stress. Hypoxia activates compensatory pathways that promote mitochondrial remodeling and biogenesis to preserve cellular energy homeostasis, largely through hypoxia-inducible signaling and downstream regulators of mitochondrial turnover. Importantly, although hypoxia alone increased mitochondrial content in control cells, this effect was significantly more pronounced in GRK2-C340S-overexpressing cells. This suggests that GRK2 modification potentiates the normal hypoxia-induced mitochondrial response rather than initiating it independently.

Our findings highlight a critical interplay between mitochondrial GRK2 accumulation and both respiration and dynamics. Specifically, hypoxia-induced mitochondrial GRK2 accumulation was associated with diminished ATP production and oxygen consumption, effects that were exacerbated by impaired S-nitrosylation. This bioenergetic dysfunction likely arises from disruption of electron transport chain activity and reduced ATP synthesis.

Mitochondria, essential for energy production, calcium homeostasis, and redox balance [13], are highly dynamic organelles regulated by coordinated processes including fission, fusion, and mitophagy [14]. Drp1-mediated mitochondrial fission facilitates mitochondrial division, while fusion—regulated by MFN1, MFN2 (outer membrane) and OPA1 (inner membrane)—allows exchange of mitochondrial content [15]. Mitophagy, a selective form of autophagy, ensures removal of damaged mitochondria through pathways such as PINK1/Parkin [16]. In our study, hypoxic conditions were associated with reduced levels of both fission and fusion proteins, suggesting impaired mitochondrial dynamics (Figure 4). This imbalance could impair mitochondrial quality control and energy metabolism. Reduced Drp1 expression may hinder fission, resulting in fewer, enlarged mitochondria with diminished bioenergetic efficiency [17]. Although DRP1 inhibition protects the heart from acute I/R injury [18], prolonged or genetic ablation of DRP1 can impair mitophagy and promote mitochondrial dysfunction, exacerbating cardiac injury [19]. Similarly, downregulation of fusion proteins like MFN1/2 has been associated with cardiac dysfunction due to impaired clearance of damaged mitochondria (Figure 5) [20,21]. Enlarged, interconnected mitochondria are less responsive to stress and may contribute to inefficient respiration and ATP production.

We also observed decreased levels of mitophagy markers Parkin and PINK1 with elevated GRK2 activity due to loss of S-nitrosylation at Cys340, accompanied by elevated LC3 and unchanged TOMM20 levels after hypoxia (Figure 6). This profile suggests impaired mitophagy despite possible activation of general autophagy. Although elevated LC3 is typically interpreted as a marker of enhanced autophagy [22], the concurrent reduction in Parkin and PINK1 indicates selective inhibition of mitochondrial quality control. Hypoxia may downregulate mitophagy-specific proteins, leading to the accumulation of dysfunctional mitochondria and increased cellular stress [23]. This discrepancy between increased autophagy (LC3) and reduced mitophagy (Parkin, PINK1) reflects a failure to effectively clear damaged mitochondria. Our model shares similarities with aged mitochondria, which also show impaired fission/fusion dynamics and reduced mitophagy, ultimately contributing to neurodegeneration in conditions like Alzheimer's disease [24]. Although protein expression profiles between Ad.GFP- and Ad.C340S-infected hypoxic cells were comparable, Seahorse analysis revealed marked mitochondrial dysfunction in the mutant group (Figure 2). This suggests that the C340S mutation impairs mitochondrial function via post-translational mechanisms rather than altered protein abundance. These effects could involve disrupted ETC assembly, altered enzymatic activity, or compromised substrate use.

Our results are consistent with previous studies identifying GRK2 as a pro-death kinase, with its pathological effects partly mediated by mitochondrial localization and its contribution to mitochondrial dysfunction [3–5]. GRK2 mitochondrial accumulation has been observed in ischemic brain injury and is proposed as an early marker of Alzheimer's disease [25]. GRK2's mitochondrial translocation, facilitated by phosphorylation at Ser670 and binding to HSP90, is linked to ischemia-induced cell death and permeability transition pore opening [3]. In GRK2-overexpressing mice, mitochondrial localization occurs even under basal conditions, correlating with reduced fatty acid oxidation and increased ROS [4]. Conversely, GRK2.S670A mutant mice, which resist mitochondrial translocation, show improved cardiac function and metabolic profiles [5]. Additionally, elevated cardiac GRK2 suppresses fatty acid oxidation and impairs mitochondrial responses to β -adrenergic stimulation [26], while post-MI GRK2 inhibition enhances mitochondrial integrity and function [27]. GRK2 deficiency also improves mitochondrial function after IR injury in skin tissue by downregulating Drp1 expression [28].

However, GRK2's role in mitochondrial regulation appears to be context dependent. In fibroblasts and skeletal muscle, GRK2 has been reported to enhance ATP production and mitochondrial biogenesis [29,30]. Overexpression of the GRK2 carboxy-terminal domain (β ARK-ct) displaces GRK2 from the plasma membrane and promotes its mitochondrial translocation during LPS stimulation, enhancing mitochondrial DNA transcription and reducing ROS and cytokine production [31]. Endothelial-specific GRK2 deletion leads to vascular abnormalities, inflammation, and lipid accumulation [32]. Moreover, GRK2 protects against acute mitochondrial injury from ionizing radiation by modulating mitochondrial dynamics [33] and supports vascular function and mitochondrial stability under hypoxia [34]. These discrepancies underscore the tissue- and stimulus-specific functions of GRK2, with cardiac models predominantly implicating GRK2 in mitochondrial dysfunction during acute stress [27].

One limitation of this study is that adenoviral-mediated overexpression of GRK2-C340S obscured direct comparisons with control cells under hypoxia, restricting interpretation of fold changes in endogenous GRK2 regulation. Additionally, since our model involved 24 h of hypoxia followed by 24 h of reoxygenation, we were unable to assess immediate post-hypoxic changes or capture dynamic responses at different stages of reoxygenation. It is possible that compensatory mechanisms were activated during this period to promote cellular protection. Notably, mitophagy-related proteins have been reported to peak during the early phase of reoxygenation [14], suggesting that our observations likely reflect cumulative changes occurring by 24 h post-reoxygenation rather than transient early responses.

In conclusion, our study reveals a novel role for S-nitrosylation in regulating GRK2 mitochondrial localization. Loss of this post-translational modification promotes GRK2 accumulation in mitochondria, leading to impaired mitochondrial respiration and altered dynamics. Restoring S-nitrosylation may therefore mitigate these detrimental effects due to its endogenous inhibition of GRK2's catalytic activity. These findings highlight the importance of GRK2 regulation under pathological conditions and suggest that therapeutic strategies aimed at restoring or mimicking S-nitrosylation may offer protection in diseases characterized by impaired NO signaling and mitochondrial dysfunction.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells15050458/s1>, Supplementary Data S1: Figure S1. Representative images of MitoTracker staining in GFP Control Cells. Figure S2. Representative images of MitoTracker staining in GFP Hypoxic Cells. Figure S3. Representative images of MitoTracker staining in C340S Control Cells. Figure S4. Representative images of MitoTracker staining in C340S Hypoxia Cells. Figure S5. Representative images of MitoSox staining in GFP Control Cells. Figure S6. Rep-

representative images of MitoSox staining in GFP Hypoxic Cells. Figure S7. Representative images of MitoSox staining in C340S Control Cells. Figure S8. Representative images of MitoSox staining in C340S Hypoxia Cells. Supplementary Data S2: Original Blots.

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Abbreviations

The following abbreviations are used in this manuscript:

GRK2	G protein-coupled receptor kinase 2
β-AR	Beta Adrenoceptor
H/R	Hypoxia/Reoxygenation
NO	Nitric Oxide

References

1. Cannavo, A.; Liccardo, D.; Koch, W.J. Targeting cardiac β-adrenergic signaling via GRK2 inhibition for heart failure therapy. *Front. Physiol.* **2013**, *4*, 264. [\[CrossRef\]](#)
2. Pflieger, J.; Gresham, K.; Koch, W.J. G protein-coupled receptor kinases as therapeutic targets in the heart. *Nat. Rev. Cardiol.* **2019**, *16*, 612–622. [\[CrossRef\]](#)
3. Chen, M.; Sato, P.Y.; Chuprun, J.K.; Peroutka, R.J.; Otis, N.J.; Ibbett, J.; Pan, S.; Sheu, S.S.; Gao, E.; Koch, W.J. Prodeath signaling of G protein-coupled receptor kinase 2 in cardiac myocytes after ischemic stress occurs via extracellular signal-regulated kinase-dependent heat shock protein 90-mediated mitochondrial targeting. *Circ. Res.* **2013**, *112*, 1121–1134. [\[CrossRef\]](#)
4. Sato, P.Y.; Chuprun, J.K.; Ibbett, J.; Cannavo, A.; Drosatos, K.; Elrod, J.W.; Koch, W.J. GRK2 compromises cardiomyocyte mitochondrial function by diminishing fatty acid-mediated oxygen consumption and increasing superoxide levels. *J. Mol. Cell Cardiol.* **2015**, *89*, 360–364. [\[CrossRef\]](#)
5. Sato, P.Y.; Chuprun, J.K.; Grisanti, L.A.; Woodall, M.C.; Brown, B.R.; Roy, R.; Traynham, C.J.; Ibbett, J.; Lucchese, A.M.; Yuan, A.; et al. Restricting mitochondrial GRK2 post-ischemia confers cardioprotection by reducing myocyte death and maintaining glucose oxidation. *Sci. Signal.* **2018**, *11*, eaau0144. [\[CrossRef\]](#) [\[PubMed\]](#)
6. Huang, Z.M.; Gao, E.; Fonseca, F.V.; Hayashi, H.; Shang, X.; Hoffman, N.E.; Chuprun, J.K.; Tian, X.; Tilley, D.G.; Madesh, M.; et al. Convergence of G protein-coupled receptor and S-nitrosylation signaling determines the outcome to cardiac ischemic injury. *Sci. Signal.* **2013**, *6*, ra95. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Whalen, E.J.; Foster, M.W.; Matsumoto, A.; Ozawa, K.; Violin, J.D.; Que, L.G.; Nelson, C.D.; Benhar, M.; Keys, J.R.; Rockman, H.A.; et al. Regulation of beta-adrenergic receptor signaling by S-nitrosylation of G-protein-coupled receptor kinase 2. *Cell* **2007**, *129*, 511–522. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Lieu, M.; Traynham, C.J.; de Lucia, C.; Pflieger, J.; Piedepalumbo, M.; Roy, R.; Petovic, J.; Landesberg, G.; Forrester, S.J.; Hoffman, M.; et al. Loss of dynamic regulation of G protein-coupled receptor kinase 2 by nitric oxide leads to cardiovascular dysfunction with aging. *Am. J. Physiol. Heart Circ. Physiol.* **2020**, *318*, H1162–H1175. [\[CrossRef\]](#)
9. Ozawa, K.; Komatsubara, A.T.; Nishimura, Y.; Sawada, T.; Kawafune, H.; Tsumoto, H.; Tsuji, Y.; Zhao, J.; Kyotani, Y.; Tanaka, T.; et al. S-nitrosylation regulates mitochondrial quality control via activation of parkin. *Sci. Rep.* **2013**, *3*, 2202. [\[CrossRef\]](#)
10. Montagna, C.; Cirotti, C.; Rizza, S.; Filomeni, G. When S-Nitrosylation Gets to Mitochondria: From Signaling to Age-Related Diseases. *Antioxid. Redox Signal.* **2020**, *32*, 884–905. [\[CrossRef\]](#)

11. Piantadosi, C.A. Regulation of mitochondrial processes by protein S-nitrosylation. *Biochim. Biophys. Acta* **2012**, *1820*, 712–721. [[CrossRef](#)]
12. Brinks, H.; Boucher, M.; Gao, E.; Chuprun, J.K.; Pesant, S.; Raake, P.W.; Huang, Z.M.; Wang, X.; Qiu, G.; Gumpert, A.; et al. Level of G protein-coupled receptor kinase-2 determines myocardial ischemia/reperfusion injury via pro- and anti-apoptotic mechanisms. *Circ. Res.* **2010**, *107*, 1140–1149. [[CrossRef](#)]
13. Nguyen, B.Y.; Ruiz-Velasco, A.; Bui, T.; Collins, L.; Wang, X.; Liu, W. Mitochondrial function in the heart: The insight into mechanisms and therapeutic potentials. *Br. J. Pharmacol.* **2019**, *176*, 4302–4318. [[CrossRef](#)]
14. Anzell, A.R.; Fogo, G.M.; Gurm, Z.; Raghunayakula, S.; Wider, J.M.; Maheras, K.J.; Emaus, K.J.; Bryson, T.D.; Wang, M.; Neumar, R.W.; et al. Mitochondrial fission and mitophagy are independent mechanisms regulating ischemia/reperfusion injury in primary neurons. *Cell Death Dis.* **2021**, *12*, 475. [[CrossRef](#)]
15. Zanfardino, P.; Amati, A.; Perrone, M.; Petruzzella, V. The Balance of MFN2 and OPA1 in Mitochondrial Dynamics, Cellular Homeostasis, and Disease. *Biomolecules* **2025**, *15*, 433. [[CrossRef](#)]
16. Zhao, X.; Wang, Z.; Wang, L.; Jiang, T.; Dong, D.; Sun, M. The PINK1/Parkin signaling pathway-mediated mitophagy: A forgotten protagonist in myocardial ischemia/reperfusion injury. *Pharmacol. Res.* **2024**, *209*, 107466. [[CrossRef](#)]
17. Favaro, G.; Romanello, V.; Varanita, T.; Andrea Desbats, M.; Morbidoni, V.; Tezze, C.; Albiero, M.; Canato, M.; Gherardi, G.; De Stefani, D.; et al. DRP1-mediated mitochondrial shape controls calcium homeostasis and muscle mass. *Nat. Commun.* **2019**, *10*, 2576. [[CrossRef](#)]
18. Ong, S.B.; Subrayan, S.; Lim, S.Y.; Yellon, D.M.; Davidson, S.M.; Hausenloy, D.J. Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. *Circulation* **2010**, *121*, 2012–2022. [[CrossRef](#)]
19. Ikeda, Y.; Shirakabe, A.; Maejima, Y.; Zhai, P.; Sciarretta, S.; Toli, J.; Nomura, M.; Mihara, K.; Egashira, K.; Ohishi, M.; et al. Endogenous Drp1 mediates mitochondrial autophagy and protects the heart against energy stress. *Circ. Res.* **2015**, *116*, 264–278. [[CrossRef](#)]
20. Chen, Y.; Liu, Y.; Dorn, G.W., 2nd. Mitochondrial fusion is essential for organelle function and cardiac homeostasis. *Circ. Res.* **2011**, *109*, 1327–1331. [[CrossRef](#)]
21. Papanicolaou, K.N.; Kikuchi, R.; Ngoh, G.A.; Coughlan, K.A.; Dominguez, I.; Stanley, W.C.; Walsh, K. Mitofusins 1 and 2 are essential for postnatal metabolic remodeling in heart. *Circ. Res.* **2012**, *111*, 1012–1026. [[CrossRef](#)]
22. Tanida, I.; Ueno, T.; Kominami, E. LC3 and Autophagy. *Methods Mol. Biol.* **2008**, *445*, 77–88. [[CrossRef](#)]
23. Guo, Z.; Tian, Y.; Liu, N.; Chen, Y.; Chen, X.; Yuan, G.; Chang, A.; Chang, X.; Wu, J.; Zhou, H. Mitochondrial Stress as a Central Player in the Pathogenesis of Hypoxia-Related Myocardial Dysfunction: New Insights. *Int. J. Med. Sci.* **2024**, *21*, 2502–2509. [[CrossRef](#)]
24. Martín-Maestro, P.; Gargini, R.; García, E.; Perry, G.; Avila, J.; García-Escudero, V. Slower Dynamics and Aged Mitochondria in Sporadic Alzheimer's Disease. *Oxidative Med. Cell. Longev.* **2017**, *2017*, 9302761. [[CrossRef](#)]
25. Obrenovich, M.E.; Smith, M.A.; Siedlak, S.L.; Chen, S.G.; de la Torre, J.C.; Perry, G.; Aliev, G. Overexpression of GRK2 in Alzheimer disease and in a chronic hypoperfusion rat model is an early marker of brain mitochondrial lesions. *Neurotox. Res.* **2006**, *10*, 43–56. [[CrossRef](#)]
26. Zhai, R.; Varner, E.L.; Rao, A.; Karhadkar, S.; Di Carlo, A.; Snyder, N.W.; Sato, P.Y. Myocardial GRK2 Reduces Fatty Acid Metabolism and β -Adrenergic Receptor-Mediated Mitochondrial Responses. *Int. J. Mol. Sci.* **2022**, *23*, 2777. [[CrossRef](#)]
27. Ciccarelli, M.; Sorriento, D.; Fiordelisi, A.; Gambardella, J.; Franco, A.; Del Giudice, C.; Sala, M.; Monti, M.G.; Bertamino, A.; Campiglia, P.; et al. Pharmacological inhibition of GRK2 improves cardiac metabolism and function in experimental heart failure. *ESC Heart Fail.* **2020**, *7*, 1571–1584. [[CrossRef](#)]
28. Wang, Y.; Wu, Y.; Zhou, M.; Wang, P.; Luo, J.; Rui, Y. GRK2 deletion improves the function of skin flap following ischemia-reperfusion injury by regulating Drp1. *Am. J. Transl. Res.* **2021**, *13*, 223–233.
29. Fusco, A.; Santulli, G.; Sorriento, D.; Cipolletta, E.; Garbi, C.; Dorn, G.W., 2nd; Trimarco, B.; Feliciello, A.; Iaccarino, G. Mitochondrial localization unveils a novel role for GRK2 in organelle biogenesis. *Cell. Signal.* **2012**, *24*, 468–475. [[CrossRef](#)]
30. Manfredi, L.H.; Ang, J.; Peker, N.; Dagda, R.K.; McFarlane, C. G protein-coupled receptor kinase 2 regulates mitochondrial bioenergetics and impairs myostatin-mediated autophagy in muscle cells. *Am. J. Physiol. Cell Physiol.* **2019**, *317*, C674–C686. [[CrossRef](#)]
31. Sorriento, D.; Fusco, A.; Ciccarelli, M.; Rungi, A.; Anastasio, A.; Carillo, A.; Dorn, G.W., 2nd; Trimarco, B.; Iaccarino, G. Mitochondrial G protein coupled receptor kinase 2 regulates proinflammatory responses in macrophages. *FEBS Lett.* **2013**, *587*, 3487–3494. [[CrossRef](#)]
32. Ciccarelli, M.; Sorriento, D.; Franco, A.; Fusco, A.; Del Giudice, C.; Annunziata, R.; Cipolletta, E.; Monti, M.G.; Dorn, G.W., 2nd; Trimarco, B.; et al. Endothelial G protein-coupled receptor kinase 2 regulates vascular homeostasis through the control of free radical oxygen species. *Arterioscler. Thromb. Vasc. Biol.* **2013**, *33*, 2415–2424. [[CrossRef](#)]

33. Franco, A.; Sorriento, D.; Gambardella, J.; Pacelli, R.; Prevete, N.; Procaccini, C.; Matarese, G.; Trimarco, B.; Iaccarino, G.; Ciccarelli, M. GRK2 moderates the acute mitochondrial damage to ionizing radiation exposure by promoting mitochondrial fission/fusion. *Cell Death Discov.* **2018**, *4*, 25. [[CrossRef](#)] [[PubMed](#)]
34. Gatto, C.; Rusciano, M.R.; Visco, V.; Ciccarelli, M. GRK2 and Mitochondrial Dynamics in Cardiovascular Health and Disease. *Int. J. Mol. Sci.* **2025**, *26*, 2299. [[CrossRef](#)]

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