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PKA activity exacerbates hypoxia-induced ROS formation and hypoxic injury in PC-12 cells

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Abstract

Hypoxia is a primary factor in many pathological conditions. Hypoxic cell death is commonly attributed to metabolic failure and oxidative injury. cAMP-dependent protein kinase A (PKA) is activated in hypoxia and regulates multiple enzymes of the mitochondrial electron transport chain, thus may be implicated in cellular energy depletion and hypoxia-induced cell death.

Wild type (WT) PC-12 cells and PKA activity-deficient 123.7 PC-12 cells were exposed to 3, 6, 12 and 24h hypoxia (0.1% or 5% O₂). Hypoxia, at 24 h 0.1% O₂, induced cell death and increased reactive oxygen species (ROS) in WT PC-12 cells. Despite lower ATP levels in normoxic 123.7 cells than in WT cells, hypoxia only decreased ATP levels in WT cells. However, menadione-induced oxidative stress similarly affected both cell types. While mitochondrial COX IV expression remained consistently higher in 123.7 cells, hypoxia decreased COX IV expression in both cell types.

N-acetyl cysteine antioxidant treatment blocked hypoxia-induced WT cell death without preventing ATP depletion. Transient PKA cat. expression in 123.7 cells partially restored hypoxia-induced ROS but did not alter ATP levels or COX IV expression.

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We conclude that PKA signaling contributes to hypoxic injury, by regulating oxidative stress rather than by depleting ATP levels. Therapeutic strategies targeting PKA signaling may improve cellular adaptation and recovery in hypoxic pathologies.

Keywords

PC-12 cells; Protein Kinase A (PKA); Reactive oxygen species (ROS); ATP; Cytochrome oxidase

1. Introduction

Hypoxia, characterized by restricted oxygen supply disrupting cell metabolism, has previously been reported to induce apoptosis to an extent critically dependent on the severity and the duration of the hypoxic injury [1- 4]. Traumatic CNS injury, stroke and cardiopulmonary disease are associated with ischemic events and are among the most prevalent causes of death in the U.S. [5]. Hypoxia has been shown to produce reactive oxygen species (ROS), disrupting brain metabolism [6, 7]. During hypoxia, oxygen-dependent oxidative phosphorylation processes decline and cells switch to anaerobic metabolism, potentially triggering significant energy imbalance, since the breakdown of one mole glucose by glycolysis produces only 2 ATP compared to 36-38 ATP produced in aerobic conditions by combined oxidative phosphorylation and glycolytic processes. Therefore, identifying the signaling events that may contribute to hypoxic survival and alleviate oxidative damage may provide new therapeutic options.

Hypoxia has been shown to decrease the activity of mitochondrial enzymes involved in oxidative phosphorylation, including Complexes I, II, and IV of the electron transport chain in PC-12 cells [8-10]. Mitochondrial redox carriers, primarily complexes I and III, can leak electrons and produce superoxide ($O_2^{\cdot-}$), rapidly dismutated by mitochondrial SOD to form hydrogen peroxide (H_2O_2) in resting cells [4, 6, 7, 10, 11]. Both $O_2^{\cdot-}$ and H_2O_2 are required for cellular signaling, however their overproduction is associated with multiple pathologies. Thus, a drop in mitochondrial enzymatic activity, increasing $O_2^{\cdot-}$ production may potentially disrupt mitochondrial $O_2^{\cdot-}/H_2O_2$ homeostasis, leading to oxidative injury and cell death.

The cAMP-dependent, Protein Kinase A (PKA) signaling pathway has been reported to induce both pro- and anti-apoptotic pathways, and its role in hypoxic cell survival is controversial [12-14]. Increased PKA activity during hypoxia has been reported in bone, mouse macrophage, cardiomyocytes and endothelial cells and is enhanced by ROS [15-20]. A recent study showed that under hypoxia, catalytically active PKA is sequestered in the mitochondria in a process involving ROS and causing cellular injury [17]. Furthermore, PKA inhibitors were shown to protect against ischemia-reperfusion injury in the mouse heart [17, 19] and prevent oxidative stress and memory impairment in a rat model of Alzheimer's disease [21]. However, activation of PKA has also been shown to be neuroprotective in a variety of neuronal injury models [22]. PKA phosphorylates and regulates mitochondrial chain enzymes [4, 17-20, 23-25] and could potentially increase leakage of electrons and superoxide formation, and decrease ATP production. Consequently, the PKA pathway may

play an important role in the regulation of metabolic pathways and energy production, critical to hypoxic survival.

To elucidate the role of PKA signaling in hypoxia, we used rat PC-12 cells, a model of oxygen sensitive neuronal-like cells that closely resemble carotid body type I cells [26-28]. 123.7 cells are genetically altered PC-12 cells carrying a mutated PKA I regulatory subunit (RI) that prevents cAMP binding and the release of the active PKA catalytic subunit. While PKA expression is comparable in both cell types, this mutation results in minimal PKA activity in the 123.7 PC-12 cells [29]. 123.7 cells stimulation with 5 μ M of cAMP induces only 12% PKA I activity and 16% PKA II activity [29]. Therefore, using these cells may allow us to investigate the role of PKA enzymatic activity while preserving its expression and potentially also protein-protein interactions. We hypothesize that PKA activity alters cell metabolism, enhances oxidative stress and energy depletion during hypoxia, inducing PC-12 hypoxic cell death. Understanding the role of PKA activity in hypoxic regulation of cellular metabolic responses, oxidative stress, and cell survival may provide novel therapeutic opportunities for the treatment of pathologies involving hypoxia.

2. Materials and Methods

2.1 Cell culture and hypoxic exposures

WT and PKA-deficient (123.7) PC-12 cells were kindly provided by Dr. JA Wagner from Cornell University [29] and grown at 37°C on collagen coated plates in RPMI1640 medium (Gibco/Invitrogen, Carlsbad, CA), supplemented with 10% horse serum and 5% fetal bovine serum. Cells were exposed to hypoxia (0.1% or 5% O₂, 5% CO₂, balanced N₂) or to normoxia (21% O₂, 5% CO₂, balanced N₂) for up to 24 h, using a custom-designed, computer controlled incubator chamber attached to an external O₂/CO₂ computer-driven controller (Biospherix, Redfield, NY) as previously described [3, 30]. Normoxic cells served as controls and were compared to hypoxic cells.

2.2 Cell viability

Cell viability was measured as previously described [29, 30-33] by assessment of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction (MTT; Sigma-Aldrich, St. Louis, MO). 100 μ l MTT solution (5mg/ml) per mL media was added to each well during the last hour of hypoxic exposure. Cells were collected by centrifugation, washed, and resuspended in DMSO for 24h to solubilize the formazan produced from MTT reduction. Absorption of reduced MTT was measured at 540 nm using a Multiskan EX plate reader (Thermo electron corporation, Milford, MA) and values for DMSO blanks were subtracted. Cell survival was also analyzed using Trypan Blue (J.T. Baker, Philipsburg, NJ) exclusion as a marker of live cells with intact membranes. Cells were collected, resuspended in PBS/trypan, counted using a hemacytometer, and total cell number was calculated and averaged.

2.3 cAMP determination

Intracellular cAMP levels were determined using a chemiluminescent immunoassay kit according to the manufacturer's instructions (Upstate, Temecula, CA). Briefly, standards and

sample mixed with an anti-cAMP antibody and an alkaline phosphatase-labeled cAMP conjugate were added to a 96-well plate precoated with a capture antibody. The conjugate competes with native cAMP in the standards and samples for binding sites on the antibody. Thus, chemiluminescence levels are inversely related to the amount of cAMP in the standards and samples. Luminescence was detected using a SpectraFluor Plus (Tecan, San Jose, CA).

2.4 ATP determination

Intracellular ATP levels were determined using a bioluminescence ATP determination kit according to the manufacturer's instructions (Alexis Biochemicals, San Diego, CA).

Luminescence was detected using a SpectraFluor Plus (Tecan, San Jose, CA).

2.5 Reactive oxygen species

Formation of intracellular oxidant species was detected by fluorescence, using the cell permeable, non-fluorescent 2'-7' dichlorodihydrofluorescein diacetate (DCFH₂-DA; Molecular Probes, Eugene, OR), which is de-esterified in cells by endogenous esterases and trapped. 2'-7' dichlorofluorescein is oxidized by intracellular oxidant species to fluorescent 2'-7' dichlorofluorescein. Cells were loaded for 1h with 5 μ M DCFH₂-DA, washed, and fluorescence was measured at 485 nm excitation and 535 nm emission using a SpectraFluor Plus (Tecan, San Jose, CA). For antioxidant treatments, cells were pretreated with 2.5 mM N-acetyl cysteine (Sigma-Aldrich, St. Louis, MO) for 1 hour prior to hypoxic exposure. For oxidant treatments, normoxic cells were exposed to 20 μ M menadione (Sigma-Aldrich, St. Louis, MO).

2.6 Preparation of cell lysates and western blotting

Cells were collected by centrifugation and pellets were resuspended in RIPA buffer (PBS, 1% NP-40, 0.5% Deoxycholate, 1% SDS, 1 mM Sodium orthovanadate, 0.5 mM PMSF, 10 mg/ml Aprotinin, 20 mg/ml Leupeptin), incubated on ice for 30 min., briefly sonicated, and centrifuged at 4°C at 15,000 rpm for 10 min. Total protein concentration in the supernatant was determined using a DC-Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Immunoblotting with COX IV antibody (Cell Signaling Technology, Danvers, MA) or PKA α catalytic subunit (Santa Cruz Biotechnology, Dallas, TX) was performed as previously described [30, 32, 33]. Membranes were stained with Ponceau-S to verify proper transfer, and immunoblotted for β -actin (Sigma-Aldrich, St. Louis, MO) to verify equal protein loading.

2.7 Cell transfection

Transfections were performed using the FuGENE HD transfection reagent (Roche Applied Science, Indianapolis, IN). Plasmids encoding active PKA cat- α were kindly provided by Dr. G. Stanley McKnight, and used as previously described [37]. pcDNA 3.0 (Invitrogen, Carlsbad, CA) was used as a vector control with FuGene reagent.

2.8 PKA activity

PKA activity was analyzed as previously described [34]. Cells were lysed and total protein concentration in the supernatant was determined as described above. Duplicate samples (50 µg protein) were diluted in Kinase Buffer (25 mM Hepes, pH 7.4; 20 mM MgCl₂; 20 mM β-glycerophosphate; 0.1 mM Sodium orthovanadate; 2 mM DTT, 1 mL dd H₂O), in the presence or absence of a PKA inhibitory peptide, PKI 6-22 amide (26.7 µM, Calbiochem, La Jolla, CA) to account for PKA-independent phosphorylation. Reaction Buffer (200 µM PKA substrate peptide (Kemptide); 55.6 nM ³²P-ATP; Kinase Buffer) was added and samples were incubated for 30 min at 30°C in a water bath. Aliquots of each sample were removed from the tube and spotted on Whatman filter paper p81 (Whatman International Ltd, Maidstone, England). Filter papers were washed for 5 minutes with 1% phosphoric acid and the waste was drained. Washes were repeated for 15 min with 1% phosphoric acid, and 15 minutes with ddH₂O to remove non-specifically bound radioactivity. Radioactivity, representative of ³²P incorporation into the substrate, was counted using a Packard Tri-carb Liquid Scintillation Analyzer 2100TR (Perkin Elmer Life & Analytical Sciences, Shelton, CT). PKA-mediated ³²P incorporation was calculated by subtracting ³²P incorporation in presence of the PKI 6-22 amide inhibitory peptide.

2.9 Statistical analysis

Statistical analyses were performed using SigmaStat software version 3.5.1 (Systat Software, Inc., San Jose, CA). Data were presented as the means ± SD of individual experiments. Significant differences were determined by one-way or two-way Analysis of variance (ANOVA) followed by Tukey HSD post-hoc t-tests, unless indicated otherwise in the figure legends. Significance was defined as p<0.05.

3. Results

3.1 Effect of PKA activity on cell survival to hypoxia

To determine the hypoxic conditions that would show differential vulnerability to hypoxic injury between the two cell types, cells were exposed to mild (5 % O₂) and severe hypoxia (0.1% O₂) for various times up to 24 h. In order to compensate for variability that may stem from differences in cell confluence, MTT values at 3 and 6h hypoxia were compared to normoxic values at 6h while 12 and 24h hypoxic values were compared to 24h normoxic values. Severe hypoxia (SH; 0.1% O₂) induced significant cell death by 24h in WT cells (Figure 1A; p<.01 RA24 h vs. SH 24h), while a milder hypoxic challenge (5% O₂) did not affect cell survival (Figure 1A). In contrast, 123.7 cell survival was not affected by 24h at 0.1% O₂ (Figure 1B), and MTT values increased at both levels of hypoxia at 12 and 24h (Figure 1B; p<.01 RA vs. SH). Showing significant differences in WT cells but not in 123.7 cells survival, 0.1% O₂ SH was selected for all subsequent experiments aimed at determining the mechanisms underlying 123.7 cells relative tolerance to hypoxia. While normoxic 123.7 cells MTT values tended to be consistently lower when compared to WT cells at 6h, this trend did not reach significance and disappeared at 24h (Figure 1A vs. 1B). MTT values represent mitochondrial reduction of 3-(4,5-dimethyldiazol-2yl)-2,5,-diphenyltetrazolium bromide and depend on mitochondrial function, and thus may be affected by the number of cells. To determine whether lower baseline MTT values stemmed

from differences in mitochondrial reducing ability or from lower starting cell counts in 123.7 cells, viable cells were counted using Trypan Blue assays. Results obtained from trypan blue positive cell counts indicated that cell exposures to SH were started at similar confluence and that lower MTT values in 123.7 cells may indeed stem from differences in cell metabolism rather than from lower initial cell numbers (Figure 1C). Increased MTT values found in hypoxic 123.7 cells were not reflected by Trypan Blue cell counts, suggesting that this increase was not due to increased 123.7 cells proliferation but rather to increased mitochondrial activity during hypoxia. Additionally, decreasing WT cells Trypan Blue count but not in 123.7 cells after 24h SH confirmed that WT cells were more susceptible to hypoxia (Figure 1C).

These findings suggest that decreased PKA activity increases PC-12 cells tolerance to hypoxia. The consistent difference in baseline cellular reductive capacity at 6h, as indicated by lower MTT reduction, and the increase at 12 and 24h SH in 123.7 cells, suggest that the metabolic function of the two cell lines may differ and further implicates PKA signaling in the regulation of cellular metabolism.

3.2 Effect of PKA activity on ROS production and hypoxic injury

exposure to 24h SH significantly increased ROS (Figure 2A) and decreased cell viability (Figure 2B) in WT cells. In contrast, 123.7 cells showed no significant alterations in DCF fluorescence or cell viability following SH exposure, suggesting that PKA activity contributes to hypoxia-induced ROS response (Figure 2A). Pre-treatment of WT cells with the antioxidant N-acetyl cysteine (NAC) reduced ROS levels (Figure 2A) and improved cell survival assessed by Trypan blue exclusion (Figure 2B), indicating that oxidative injury contributes to hypoxia-induced PC-12 cell death.

3.3 Effect of menadione-induced ROS on cell survival

The absence of hypoxia-induced oxidative injury in 123.7 cells could also indicate their ability to remove toxic oxidative species very efficiently, rather than a lower ROS production in hypoxia. To determine whether 123.7 cells were more tolerant to oxidative injury than WT cells, cells were treated with the quinone compound menadione (20 μ M) for 6 hours. Menadione induces oxidative stress and decreases glutathione, depleting antioxidant defenses. Susceptibility to menadione-induced injury was similar in WT and 123.7 cells (Figure 3). Therefore, 123.7 cells' relative tolerance to hypoxia most likely results from lower ROS production in response to hypoxia rather than from increased tolerance to oxidative stress.

3.4 Effect of PKA activity on cellular energetic status

To determine whether the lack of PKA activity in 123.7 cells alters their cellular energy requirements, underlying their relative tolerance to SH, ATP and cAMP levels were measured. ATP levels significantly decreased in hypoxic WT cells by 24h, while they remained unchanged in 123.7 cells (Figure 4A). Furthermore, 123.7 cells had lower basal ATP levels, suggesting that the lack of PKA activity may either decrease 123.7 cells energy requirements or improve their ability to efficiently use reserve energy stores (Figure 4A). The hypoxia-induced ATP decrease in WT cells at 24 h SH, when significant cell death

occurred. However, constitutively lower baseline ATP levels in 123.7 cells did not appear to be detrimental to cell survival (Figure 2B). NAC pretreatment prevented WT cell death (Figure 2B) but failed to restore ATP levels (Figure 4A), suggesting that in WT cells, ROS may be implicated in hypoxia-induced cell death independently of energy depletion. Failure to restore ATP levels while cell numbers did not decrease suggest that SH-induced ATP decrease is not caused by a decrease in WT viable cells.

Mutation of the PKA regulatory subunit in 123.7 cells prevents cAMP binding and could potentially alter cAMP levels within the cells. However, cAMP levels did not differ between WT and 123.7 cells, or upon exposure to 24h of severe hypoxia (Figure 4B) and were not significantly affected by NAC treatment in either of the cell types (data not shown), suggesting that cAMP levels are not altered by hypoxia, oxidative stress, or PKA activity.

3.5 Effect of PKA and ROS on COX IV expression

COX IV is a key oxygen sensitive regulatory subunit of cytochrome c oxidase, the terminal enzyme of the electron transport chain. COX IV expression decreased after 24h SH in both cell lines (Figure 4C). However, COX IV baseline expression was higher in 123.7 cells and remained higher than in WT cells after 24h SH (Figure 4C), suggesting that PKA activity may play a role in the regulation of COX IV expression in these cells. Pretreatment with NAC did not prevent the SH-induced decrease in COX IV expression (Figure 4C), suggesting again that hypoxia-induced metabolic changes do not entirely result from oxidative stress.

3.6 Transfection of 123.7 cells with active PKA catalytic α subunit

To restore PKA activity in 123.7 cells, cells were transfected with a plasmid encoding for active PKA catalytic α subunit (PKA cat- α). Over-expression of PKA cat- α in both RA and SH was verified by activity assay (Figure 5A), and by immunoblotting (Figure 5B), confirming transfection efficiency. While PKA cat- α transfection similarly increased its expression in RA and SH, PKA activity significantly increased in hypoxic transfected 123.7 cells.

3.7 Effect of PKA cat- α transfection on hypoxia-induced ROS production and cell death

Transfection of PKA cat- α in 123.7 cells significantly increased hypoxia-induced ROS production at 24h relative to their RA controls (Figure 6A). However, hypoxia-induced DCF increase in 123.7 cells remained lower than in hypoxic WT cells. Furthermore, increased ROS formation in transfected 123.7 cells only resulted in a slight but not significant decrease in cell viability after hypoxic challenge, as shown by Trypan Blue exclusion assays (Figure 6B). While slightly decreasing cell viability, hypoxia-induced oxidative stress in transfected 123.7 cells may not have been sufficient to significantly affect viability, suggesting that ROS may not be cytotoxic below a certain threshold. Thus, the transfection may have only partially restored PKA cat- α activity in 123.7 cells or PKA may not have localized properly in the cell to affect ROS production and cell viability similarly to WT cells. Moreover, PC-12 cells do not transfect easily [35, 36] and there may be a significant number of cells with low or no PKA expression. Nevertheless the increase in ROS and slightly higher cell death in hypoxic transfected cells suggest that PKA plays a role in hypoxia-induced toxicity.

3.8 Effect of PKA cat- α transfection on COX IV expression

Transfection of 123.7 cells with PKA cat- α did not significantly alter COX IV expression (Figure 6C) relative to empty vector controls or untransfected cells. However, we cannot rule out that cells did not uniformly transfect or that sustained rather than transient PKA activation may be necessary for COX IV downregulation to the levels observed in WT cells.

3.9 Effect of PKA cat- α transfection on energetic status

PKA cat- α transfection did not alter ATP levels in 123.7 relative to empty vector transfected cells (Figure 6D). ATP levels in transfected 123.7 cells remained significantly lower than in WT cells and were not affected by hypoxia (Figure 6D). Again, we cannot exclude partial transfection or low mitochondrial localization, or that a longer or stronger PKA activation could restore ATP level to that of WT cells. However 123.7 cells may also have developed an alternative, less energy consuming adaptation strategy.

4. Discussion

While hypoxia-induced signaling events have been extensively studied, the role of PKA has yet to be clarified. Activation of PKA during hypoxia has been reported in multiple cell types and is enhanced by ROS [15-20]. To examine the role of PKA in hypoxia-induced signaling, PC-12 WT cells and PKA activity deficient 123.7 cells, were exposed to hypoxia. Based on the current literature, we hypothesized that PKA activity may regulate PC-12 hypoxic cell death via oxidative stress and cellular energy depletion. Induction of cell death in WT PC-12 cells following 24 hours hypoxia correlated with increased ROS production and energy depletion, while 123.7 cells did not show significant cell death or ROS formation. WT cells treatment with an antioxidant prevented cell death but not energy depletion and 123.7 cells were more resistant to hypoxia despite lower ATP levels. These data support our hypothesis that PKA activity contributes to hypoxia-induced oxidative injury in PC-12 cells but also suggest that hypoxia-induced PC-12 cell death does not occur because of energy depletion. These findings agree with earlier reports of PKA-induced cardiac ROS and ROS-mediated activation of PKA during hypoxia [16, 17, 19, 37], and of PKA inhibitors protective effects against ischemia-reperfusion injury in the rabbit heart [17, 19].

Cellular metabolism is highly dependent on the ability of the mitochondrial electron transport chain to generate ATP. Hypoxia decreases the $V_{\max}$ of the mitochondrial electron transport chain oxygen-dependent cytochrome oxidase, disrupting the flow of electrons and inducing ROS leakage at Complexes I and III [10, 38, 39]. PKA phosphorylation of complexes I, IV and V of the mitochondrial electron transport chain may contribute to its deleterious effect [4, 17-20, 23-25]. In mouse fibroblasts, PKA phosphorylation of the 18 kD subunit of complex I increases its activity [18]. COX IV has been identified as a key regulatory subunit of the mammalian cytochrome c enzymatic complex, the rate limiting enzyme of the respiratory chain that uses O_2 and cytochrome c as substrates. COX IV activity is regulated by hypoxia and by PKA-dependent pathways [4, 18-20, 23-25]. Phosphorylation of COX IV decreased its activity by 40-70% in bovine heart and in hypoxic rat macrophages, resulting in increased ROS formation [19]. Additionally, the RI α

regulatory subunit of PKA directly binds to the Vb subunit of COX IV and inhibits its activity [20]. PKA phosphorylation turns on COX IV allosteric inhibition at high ATP/ADP ratio while dephosphorylation restores its activity [4, 23-25]. Therefore, the PKA-dependent increased activity of Complex I and decreased activity of Complex IV may result in disruption of the electron flow and increased ROS leakage [4, 18-20, 23-25]. In addition, inhibition or knock down of PKA in HeLa cells has been shown to inhibit mitochondrial ATP synthesis, directly affecting mitochondrial ATP synthase, without significant effect on membrane potential or decrease in expression levels [40]. In the current study, we examined whether PKA inactivation could also affect COX IV expression. COX IV expression was significantly higher in 123.7 cells relative to WT cells during both RA and SH, suggesting either an increase in mitochondrial enzyme or in mitochondria number in absence of PKA activity. Srinivastan et al. reported that PKA inhibition during hypoxia has a significant protective effect on mitochondrial respiratory capacity, preventing PKA-mediated activity decrease and COX IV degradation [17]. The absence of PKA activity preventing COXIV phosphorylation-induced downregulation [19, 20] may also result in low basal ATP levels by inhibiting the mitochondrial ATP synthase [40] in 123.7 cells, therefore preventing allosteric ATP inhibition and contributing to the maintenance of mitochondrial energy production in 123.7 cells even under drastically reduced oxygen conditions. While hypoxia decreased COX IV expression in both cell types, expression remained higher in 123.7 cells and was not affected by PKA cat- α transient expression, suggesting that hypoxia-induced decreased COX IV activity and expression in low oxygen conditions may also involve additional PKA-independent mechanisms. Alternatively, transfected PKA cat- α may not adequately localize in the mitochondria to affect COX IV expression [17]. Antioxidant treatment did not affect COX IV expression in either cell type, suggesting that decreased COX IV expression in hypoxia was not induced by increased ROS.

We examined whether 123.7 cells could maintain lower ROS levels in hypoxia by efficiently removing ROS rather than by failing to generate ROS. However, both WT and 123.7 cells were similarly susceptible to menadione-induced injury. Menadione increases oxidant species, depletes cellular glutathione and conjugates with thiol dependent proteins. Therefore, superior antioxidant and increased ROS removal capacities should have protected 123.7 cells from menadione-induced injury. Our data suggest that both cell types are vulnerable to oxidant injury and that differences in ROS induction by hypoxia rather than in antioxidant capacity may account for these cells'; differential susceptibility to hypoxia.

Hypoxia-induced cell death has been widely attributed to cellular energy failure [41]. To determine whether PKA activity contributes to hypoxia-induced energy depletion, we examined ATP and cAMP levels in both cell types exposed to 24h SH. In agreement with a previous study, cAMP levels were similar in normoxic or hypoxic WT and 123.7 cells [29]. As expected, severe hypoxia decreased ATP in WT cells. In contrast, while 123.7 cells had lower ATP basal levels, no further decrease was observed after 24 hours of hypoxic exposure. These lower baseline ATP levels in 123.7 cells were unexpected as these cells very efficiently performed ATP-consuming cell functions such as proliferation and adaptation to hypoxia, increasing their reducing capabilities as shown by elevated MTT reduction values during hypoxia, which may suggest a superior ability to increase their use of reserve capabilities. Additionally, decreased mitochondrial ATP synthase in absence of PKA could

play a role [40]. To assess the role of ROS in PKA-dependent hypoxic cell death, cells were treated with NAC, prior to exposure to 24h SH. Although the antioxidant function of NAC was previously attributed solely to its role in glutathione biosynthesis, several studies suggest that NAC can also directly detoxify ROS [42-44]. Treatment of WT cells with NAC reduced ROS levels in hypoxia and improved cell survival, confirming that oxidative stress contributes to hypoxia-induced PC-12 cell death. While preventing WT cell death, NAC pre-treatment failed to restore ATP levels, suggesting that energy depletion during hypoxia may occur independently from oxidative stress.

To determine whether restoring PKA activity in 123.7 cells would abrogate differences in hypoxic susceptibility and energy homeostasis between WT and 123.7 cells, 123.7 cells were transfected with plasmids encoding for active PKA catalytic α subunits. Transfection resulted in significantly higher PKA activity and increased hypoxia-induced ROS levels albeit to a lesser extent than in WT cells, confirming that PKA activity contributes to hypoxia-induced oxidative injury in our experimental model. However, intracellular ATP levels were not significantly altered relative to empty vector controls and while a slight decrease in viability occurred after 24h SH in transfected 123.7 cells, it did not reach statistical significance. These discrepancies may result from the well-established and previously reported low transfectability of PC-12 cells [35, 36]. While we could express active PKA in 123.7 cells to a certain extent, all the cells may not have PKA activity. Alternatively, these data suggest that additional signaling events could contribute to hypoxia-induced ROS formation and cell death in WT cells.

These findings may also indicate that the transfected PKA cat- α did not localize to the mitochondria or that metabolic adaptation may have occurred in 123.7 cells following prolonged PKA inactivation that could confer these cells higher tolerance to hypoxia by decreasing their energy requirements, and may prevent efficient upregulation of metabolic processes following re-activation of the pathway.

5. Conclusions

5. 1 While we have uncovered a critical role for PKA-dependent disruption of cellular metabolism in hypoxia, resulting in ROS-induced cytotoxicity, the process is complex and hypoxic adaptation is multifactorial.
5. 2 Failure of antioxidant treatment to restore ATP levels while preventing hypoxic cell death suggests that energy failure is not a consequence of ROS increase and that PKA-dependent ROS formation, rather than energy depletion, mediates hypoxic cell death in hypoxia.
5. 3 This observation, together with the increased hypoxic tolerance of 123.7 cells despite their consistently low ATP levels suggest that PC-12 cells can adapt to decreased energy levels. Therefore hypoxia-induced energy depletion in itself doesn't appear to be lethal to PC-12 cells.
5. 4 Modulating PKA pathways may decrease hypoxia-induced oxidative injury. Thus, this work mandates further examination into the role of PKA in the hypoxic response.

5. 5 Additional cellular targets of PKA need to be identified that could be modulated to improve cellular adaptation and recovery in the many pathologies characterized by periods of hypoxia.

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References

- Dematteis M, Godin-Ribuot D, Arnaud C, Ribuot C, Stanke-Labesque F, Pepin JL, Levy P. Cardiovascular consequences of sleep-disordered breathing: contribution of animal models to understanding of the human disease. *ILAR J.* 2009; 50:262–281. [PubMed: 19506313]
- Banasiak KJ, Haddad GG. Hypoxia-induced apoptosis: effect of hypoxic severity and role of p53 in neuronal cell death. *Brain Res.* 1998; 797:295–304. [PubMed: 9666152]
- Gozal E, Sachleben LR Jr, Rane MJ, Vega C, Gozal D. Mild Sustained and Intermittent Hypoxia Induce Apoptosis in PC-12 Cells via Different Mechanisms. *Am J Physiol Cell Physiol.* 2005; 288:C535–C542. [PubMed: 15537711]
- Solaini G, Baracca A, Lenaz G, Sgarbi G. Hypoxia and mitochondrial oxidative metabolism. *Biochim Biophys Acta.* 2010; 1797:1171–1177. [PubMed: 20153717]
- Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, et al. American Heart Association Statistics Subcommittee. Heart disease and stroke statistics— 2015 update: a report from the American Heart Association. *Circulation.* 2015; 131:e29–322. [PubMed: 25520374]
- Guzy RD, Shumacker PT. Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. *Exp Physiol.* 2006; 91:807–819. [PubMed: 16857720]
- Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J.* 2009; 417:1–13. [PubMed: 19061483]
- Vijayasarathy C, Damle S, Prabu SK, Otto CM, Avandahi NG. Adaptive changes in the expression of nuclear and mitochondrial encoded subunits of cytochrome c oxidase and the catalytic activity during hypoxia. *Eur J Biochem.* 2003; 270:871–879. [PubMed: 12603320]
- Khan SA, Nanduri J, Kinsman B, Kumar GK, Joseph J, Kalyanaraman B, Prabhakar NR. NADPH oxidase 2 mediates intermittent hypoxia-induced mitochondrial complex I inhibition: relevance to blood pressure changes in rats. *Antioxid Redox Signl.* 2011; 14(4):533–542.
- Beyer RE. An Analysis of the Role of Coenzyme Q in Free Radical Generation and as an Antioxidant. *Biochem Cell Biol.* 1992; 70:392.
- Mailloux RJ. Teaching the fundamentals of electron transfer reactions in mitochondria and the production and detection of reactive oxygen species. *Redox Biol.* 2015; 4:381–398. [PubMed: 25744690]
- Saito A, Hayashi T, Okuno S, Ferrand-Dranke M, Chan PH. Overexpression of copper/zinc superoxide dismutase in transgenic mice protects against neuronal cell death after transient focal ischemia by blocking activation of the bad cell death signaling pathway. *J Neurosci.* 2003; 23:1710–1718. [PubMed: 12629175]
- Insel PA, Zhang L, Murray L, Yokushi H, Zambon AC. Cyclic AMP is both a pro-apoptotic and anti-apoptotic second messenger. *Acta Physiol.* 2012; 204:277–287.
- Torii S, Okamura N, Suzuki Y, Ishizawa T, Yasumoto K, Sogawa K. Cyclic AMP represses the hypoxic induction of hypoxia-inducible factors in PC 12 cells. *J Biochem.* 2009; 146(6):838–844.
- Zhang YL, Tavakoli H, Chachisvilis M. Apparent PKA activity responds to intermittent hypoxia in bone cells: a redox pathway? *Am J Physiol Heart Circ Physiol.* 2010; 299:H225–H235. [PubMed: 20453101]

16. Shaikh D, Zhou Q, Chen T, Ibe JCF, Raj JU, Zhou G. cAMP-dependent protein kinase is essential for hypoxia-mediated epithelial-mesenchymal transition, migration and invasion in lung cancer. *Cell Signal*. 2012; 24:2396–2406. [PubMed: 22954688]
17. Srinivasan S, Spear J, Chandran K, Joseph J, Kalyanaraman B, Avadhani NG. Oxidative stress induced mitochondrial protein kinase A mediates cytochrome C oxidase dysfunction. *PLOS One*. 2013; 8:1–15.
18. Scacco S, Vergari R, Scarpulla RC, Technikova-Dobrova Z, Sardanelli A, Lambo R, Lorusso V, Papa S. cAMP-dependent Phosphorylation of the Nuclear Encoded 18-kDA Subunit of Respiratory Complex I and Activation of the Complex in Serum-starved Mouse Fibroblast Cultures. *J Biol Chem*. 2000; 275:17578–17582. [PubMed: 10747996]
19. Prabu SK, Anandatheerthavarada HK, Raza H, Srinivasan S, Spear JF, Avadhani NG. Protein Kinase A-mediated Phosphorylation Modulates Cytochrome c Oxidase Function and Augments Hypoxia and Myocardial Ischemia-related Injury. *J Biol Chem*. 2006; 281:2061–2070. [PubMed: 16303765]
20. Yang WL, Iacono L, Tang WM, Chin KV. Novel function of the regulatory subunit of Protein Kinase A: Regulation of cytochrome c oxidase activity and cytochrome c release. *Biochemistry*. 1998; 37:14175–14180. [PubMed: 9760254]
21. Eftekharzadeh B, Ramin M, Khodaghali F, Moradi S, Tabrizian K, Sharif R, Azami K, Beyer C, Sharifzadeh M. Inhibition of PKA attenuates memory deficits induced by p-amyloid (1-42), and decreases oxidative stress and NF- κ B transcription factors. *Behav Brain Res*. 2012; 226:301–308. [PubMed: 21856333]
22. Tanaka K. Alteration of Second Messengers during Acute Cerebral Ischemia –Adenylate Cyclase, cAMP Dependent Protein Kinase, and cAMP Response Element Binding Protein. *Prog Neurobiol*. 2001; 65:173–207. [PubMed: 11403878]
23. Beauvois B, Rigoulet M. Regulation of cytochrome c oxidase by adenylic nucleotides. Is oxidative phosphorylation feedback regulated by its end products? *IUBMB Life*. 2001; 52(3-5):143–152. [PubMed: 11798026]
24. Kadenbach B, Bender E, Vogt S. High efficiency versus maximal performance – the cause of oxidative stress in eukaryotes: a hypothesis. *Mitochondrion*. 2013; 13:1–6. [PubMed: 23178790]
25. Helling S, Huttemann M, Ramzan R, Kin SH, Lee I, Muller T, Langenfeld E, Meyer HE, Kadenbach B, Vogt S, Marcus K. Multiple phosphorylations of cytochrome c oxidase and their functions. *Proteomics*. 2012; 12:950–959. [PubMed: 22522801]
26. Greene LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci USA*. 1976; 73:2424–2428. [PubMed: 1065897]
27. Greene LA, Tischler AS. PC-12 pheochromocytoma cultures in neurobiological research. *Adv Cell Neurobiol*. 1982; 3:373–414.
28. Seta K, Kim HW, Ferguson T, Kim R, Pathrose P, Yuan Y, Lu G, Spicer Z, Millhorn DR. Genomic and physiological analysis of oxygen sensitivity and hypoxia tolerance in PC12 cells. *Ann N Y Acad Sci*. 2002; 971:379–388. [PubMed: 12438156]
29. Ginty DD, Glowacka D, DeFranco C, Wagner JA. Nerve growth factor-induced neuronal differentiation after dominant repression of both type I and type II cAMP-dependent protein kinase activities. *J Biol Chem*. 1991; 266:15325–15333. [PubMed: 1651325]
30. Zhang S, Gozal D, Sachleben LR Jr, Rane MJ, Klein J, Gozal E. Hypoxia induces an autocrine-paracrine survival pathway via platelet-derived growth factor (PDGF)-B/PDGF- β receptor/phosphatidylinositol 3-kinase/Akt signaling in RN46A neuronal cells. *FASEB J*. 2003; 17:1709–1711. [PubMed: 12958184]
31. Mosmann T. Rapid colorimetric assay for cellular growth and survival. *J Immunol Methods*. 1983; 65:55–63. [PubMed: 6606682]
32. Gozal E, Simakajornboon N, Dausman JD, Xue YD, Corti M, El-Dahr S, Gozal D. Hypoxia induces selective SAPK/JNK-2-AP-1 pathway activation in the nucleus tractus solitarius of the conscious rat. *J Neurochem*. 1999; 73:665–674. [PubMed: 10428063]

33. Rane MJ, Pan Y, Singh S, Powell DW, Wu R, Cummins T, Chen Q, McLeish KR, Klein JB. Heat shock protein 27 controls apoptosis by regulating Akt activation. *J Biol Chem*. 2003; 278:27828–27835. [PubMed: 12740362]
34. Uhler MD, Carmichael DF, Lee DC, Chrivia JC, Krebs EG, McKnight GS. Isolation of cDNA clones coding for the catalytic subunit of mouse cAMP-dependent protein kinase. *Proc Natl Acad Sci USA*. 1986; 83(5):1300–1304. [PubMed: 3456589]
35. Willis D, Parameshwaran B, Shen W, Molloy GR. Conditions providing enhanced transfection efficiency in rat pheochromocytoma PC12 cells permit analysis of the activity of the far-upstream and proximal promoter of the brain creatinine kinase gene. *J Neurosci Methods*. 1999; 92(1-2):3–13. [PubMed: 10595698]
36. Covello G, Siva K, Adfami V, Denti MA. An electroporation protocol for efficient DNA transfection in PC12 cells. *Cytotechnology*. 2014; 66:543–553. [PubMed: 23846478]
37. Nagasaka S, Katoh H, Niu CF, Matsui S, Urushida T, Satoh H, Watanabe Y, Hayashi H. Protein Kinase A Catalytic Subunit Alters Cardiac Mitochondrial Redox State and Membrane Potential Via the Formation of Reactive Oxygen Species. *Circ J*. 2007; 71:429–436. [PubMed: 17322647]
38. Budinger GR, Chandel N, Shao ZH, Li CQ, Melmed A, Becker LB, Schumacker PT. Cellular energy utilization and supply during hypoxia in embryonic cardiac myocytes. *A J Physiol*. 1996; 270:L44–L53.
39. Chandel NS, Budinger SGR, Choe SH, Schumaker PT. Cellular respiration during hypoxia. *J Biol Chem*. 1997; 272:18808–18816. [PubMed: 9228055]
40. Sugawara K, Fujikawa M, Yoshida M. Screening of protein kinase inhibitors and knockdown experiments identified four kinases that affect mitochondrial ATP synthesis activity. *FEBS Lett*. 2013; 587:3843–3847. [PubMed: 24157360]
41. Skulachev VP. Bioenergetic aspects of apoptosis, necrosis, and mitoptosis. *Apoptosis*. 2006; 11:473–485. [PubMed: 16532373]
42. DeVries N, DeFlora S. N-Acetyl-L-Cysteine. *J Cell Biochem*. 1993; 17F:S270–S277.
43. Issels RD, Nagele A, Eckert KG, Wilmanns W. Promotion of cystine uptake and its utilization for glutathione biosynthesis induced by cysteamine and N-acetylcysteine. *Biochem Pharmacol*. 1988; 37:881–888. [PubMed: 3345201]
44. Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetyl cysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic Biol Med*. 1989; 6:593–597. [PubMed: 2546864]

Figure 1. Cellular Tolerance Hypoxia

MTT Assay assessment of WT (A) and 123.7 (B) cell viability exposed to hypoxia (SH) at 0.1% O₂ (open bars), or 5% O₂ (grey bars) or to normoxia (RA). Data are expressed as O.D.₅₄₀ means $\pm$ SD (n=5), * p<.01 SH vs. RA control at one time point, + p<.01 SH 0.1% vs. SH 5% at one time point. **Trypan Blue Exclusion Assay.** (C) Assessment of WT (open bars) and 123.7 cells (black bars) live cells count after 24 h normoxia (RA) or 24h hypoxia at 0.1% O₂ (SH). Data are expressed as means of live cell counts $\pm$ SD (n=10), *p<.001 RA vs. SH 0.1% O₂.

Figure 2.

A. Reactive Oxygen Species Production assessed by DCF Fluorescence of WT cells (open bars) and 123.7 cells (black bars) exposed to 24h normoxia (RA) or hypoxia (SH), with or without N-acetyl cysteine (NAC). Data are expressed as relative fluorescence unit means $\pm$ SD (n=5), *p<.05 RA vs. SH; #p<.05 SH vs. SH + NAC. **B. Effect of Antioxidant treatment on Trypan Blue Exclusion Assay.** Assessment of WT (open bars) and 123.7 cells (black bars) live cell count in presence or absence of N-acetyl cysteine (NAC). Data are expressed as means live cell counts $\pm$ SD (n=5), * p<.05 RA vs. SH; #p<.05 SH vs. SH + NAC.

Figure 3. Effect of Exogenous ROS on Cell Survival

Trypan Blue Exclusion Assay assessment of cell viability of WT (open bars) and 123.7 cells (black bars) after menadione (MEN) treatment. Data are expressed as mean live cell count $\pm$ SD (n=5), *p<05 MEN vs. vehicle treated cells in each cell line.

Figure 4. Cellular Energetic Status

A. ATP levels. ATP levels in WT cells (open bars) and 123.7 cells (black bars) exposed to normoxia (RA) or hypoxia (SH) with or without N-acetyl cysteine (NAC). Data are expressed as means of relative luminescence units (RLU) $\pm$ SD (n=4-7), ** p<005 WT-RA vs 123.7-RA, * p<01 WT-RA vs. WT-SH. **B. cAMP levels.** cAMP levels in WT cells (open bars) and 123.7 cells (black bars) exposed to normoxia (RA) or hypoxia (SH) Data are expressed as means $\pm$ SD (n=5-6), p= NS. **C. COX IV Expression.** Western blot of COX IV immunoreactivity in WT cells and 123.7 cells exposed to normoxia (RA) or hypoxia (SH) with or without N-acetyl cysteine (NAC). Data are representative of 3 individual experiments (n=3).

Figure 5. Effect of PKA cat- α Transfection

A. PKA activity assay 123.7 cells transfected with the active catalytic subunit of Protein Kinase A (PKA cat- α) or control vector (Vect) and exposed to hypoxia (SH) or to normoxia (RA). Data are expressed as counts per minute (CPM) means $\pm$ SD (n=5), * p<.005 PKA cat α transfected cells vs. vector controls of the same oxygen condition. **B. PKA cat α expression.** Western blot of active catalytic subunit of Protein Kinase A (PKA cat- α) immunoreactivity in PKA cat- α -transfected 123.7 cells compared to vector transfected cells. (Vect) exposed to normoxia (RA) and hypoxia (SH). Data are representative of 7 individual experiments (n=7).

Figure 6. Effect of PKA cat- α Transfection

A. Hypoxia-induced ROS Levels. DCF fluorescence in WT cells (open bars) and 123.7 cells transfected with PKA catalytic subunit (cat α ; black bars) or with empty vector control ((Vect; grey bars) and exposed to normoxia (RA) or to hypoxia (SH). Data are expressed as relative fluorescence units (RFU) $\pm$ SD (n=5), * p<005 as indicated or PKA cat α transfected cells vs. vector controls of the same oxygen condition, **p<001 RA 24h vs. SH 0.1% 24h. **B. Hypoxia-induced Cell Death.** Trypan Blue exclusion cell count representing live WT cells (open bars) and 123.7 cells transfected with PKA catalytic subunit (cat α ; black bars) or with empty vector control ((Vect; grey bars) and exposed to normoxia (RA) or to hypoxia (SH) for 24h. Data are expressed as means live cell counts $\pm$ SD (n=10), * p<05 RA 24h vs. SH 0.1% 24h. **C. COX IV expression** Expression of cytochrome oxidase IV (COX IV) in 123.7 cells exposed to normoxia (RA) or to 24 h hypoxia (SH) and transfected with the active catalytic subunit of Protein Kinase A (PKA cat- α) or empty vector control (Vector). Data are representative of 5 individual experiments (n=5). **D. ATP Levels** in WT cells (open bars) and 123.7 cells transfected with PKA catalytic subunit (cat α ; black bars) or with empty vector control ((Vect; grey bars) and exposed to normoxia (RA) or to hypoxia (SH) for 24h. Data are expressed as mean relative luminescence units (RLU) $\pm$ SD (n=5-7), * p<005 RA 24h vs. SH 0.1% 24h for each condition.