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Hypoxia and hypoxia mimetic cooperate to counteract tumor cell resistance to glucose starvation preferentially in tumor cells with mutant p53

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ABSTRACT

We demonstrated that exogenous pyruvate promotes survival under glucose depletion in aerobic mutant p53 (R175H) human melanoma cells. Others subsequently indicated that mutant p53 tumor cells undergo p53 degradation and cell death under aerobic glucose-free conditions. Since glucose starvation occurs in hypoxic gradients of poorly vascularized tumors, we investigated the role of p53 siRNA under hypoxia in wt p53 C8161 melanoma using glucose starvation or 5 mM physiological glucose. p53 Silencing decreased survival of glucose-starved C8161 melanoma with pyruvate supplementation under hypoxia (⩽1% oxygen), but increased resistance to glycolytic inhibitors oxamate and 2-deoxyglucose in 5 mM glucose, preferentially under normoxia. Aiming to counteract hypoxic tumor cell survival irrespective of p53 status, genetically-matched human C8161 melanoma harboring wt p53 or mutant p53 (R175H) were used combining true hypoxia (⩽1% oxygen) and hypoxia mimetic $CoCl_2$. No significant decrease in metabolic activity was evidenced in C8161 melanoma irrespective of p53 status in 2.5 mM glucose after 48 h of physical hypoxia. However, combining the latter with 100 μM $CoCl_2$ was preferentially toxic for mutant p53 C8161 melanoma, and was enhanced by catalase in wt p53 C8161 cells. Down-regulation of MnSOD and LDHA accompanied the toxicity induced by hypoxia and $CoCl_2$ in 5 mM glucose, and these changes were enhanced by oxamate or 2-deoxyglucose. Our results show for the first time that survival of malignant cells in a hypoxic microenvironment can be counteracted by hypoxia mimetic co-treatment in a p53 dependent manner.

1. Introduction

Evolving solid tumors become progressively distant from vascularized regions, being exposed to a restrictive microenvironment characterized by sub-optimal levels of oxygen and glucose. This requires induction of the hypoxia inducible factor (HIF-1α) to facilitate adaptative survival in response to these metabolic stresses [1,2]. Transient activation of HIF-1α may be protective under physiological conditions in response to stress. However, prolonged and severe hypoxia can lead to oxidative stress and cytotoxicity [3]. To counteract the scarce glucose in poorly vascularized tumors, the hypoxic environment induces a high rate of glycolysis promoting addiction to glucose [4–8]. During the shift from mitochondrial oxidative phosphorylation towards glycolysis, HIF-1α favours induction of lactate dehydrogenase A (LDHA) which regulates the conversion of pyruvate to lactate, a hallmark of glycolysis [9,10]. A consequence of tumor cell adaptation to low oxygen and sub-physiological glucose is lower efficacy of cancer therapy, since survival under severe hypoxia may select for mutant cells with decreased response to some cancer treatments [3,11,12]. Tolerance to oxygen restriction also contributes to tumor cell survival by helping to down-regulate expression of key pro-apoptotic genes like Bid and Bax [13]. Although cells with wild type p53 are more likely to undergo apoptosis, cell cycle arrest or senescence in response to DNA damage, their response may be hampered in hypoxia [14–16]. Using genetically-matched human C8161 melanoma with wt p53 or mutant p53 (R175H), our laboratory showed that overnight glucose depletion caused cell death irrespective of p53 status counteracted by N-acetylcysteine [17]. However, glucose depletion preferentially permitted survival of mutant p53 cells with pyruvate supplementation [17]. Others reported more recently that mutant p53 breast cancer cell lines MDA-231,

Abbreviations: mutant p53 R175H, mutant p53 Arg 175His; wt, wild type; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species.

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MDA-468 exposed for 16 h to glucose depletion undergo deacetylation and autophagic degradation of mutant p53 protein and cell death, effect not seen in unmatched wt p53 cells [18]. Both of the latter reports used cells under aerobic conditions [17,18] underscoring that glucose depletion and high metabolic demand leads to tumor cell death, because of unfulfilled glucose addiction [7]. Cancer cells are frequently maintained *in vitro* in complete medium containing 10% serum and 20 mM glucose, which is about 4-fold higher than the physiological glucose levels [17,18]. However, the response to glucose restriction is more likely to occur under hypoxia, which is experimentally induced using either hypoxia mimetics like $CoCl_2$ or by true hypoxia ($\leqslant$1% oxygen) [19,20]. To learn about the role of p53 under glucose starvation or 5 mM glucose, we first used p53siRNA and scrambled siRNA sequences in parental wt p53 C8161 melanoma. Since the consequences of gene silencing are not identical to those involving p53 mutation, we also investigated the glucose dependent responses in genetically-matched human C8161 melanoma harboring wt p53 or mutant p53 (R175H) [17], attempting to learn whether hypoxia and $CoCl_2$ cooperate to enhance tumor cell death in physiological 5 mM glucose or lower glucose.

2. Materials and methods

2.1. Cell lines

(a) Parental human C8161 melanoma harboring wt p53 [21]. (b) Human C8161 melanoma harboring wt or mutant p53 status – isogenic C8161 melanoma were obtained by retroviral transduction of parental wt p53 C8161 cells. We used calcium chloride transfection of Phoenix cells with pWZL-Hygro plasmid harboring a human p53 arginine-175 histidine mut gene, which is a dominant-negative mutant p53 plasmid [17]. Control cells were retrovirally transduced with the empty pWZL-Hygro plasmid. Both plasmids were kindly provided by Dr. Scott Lowe, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. Verification of p53 status was achieved by immune precipitation of mutant p53 with monoclonal antibody Pab 240 (SC-99) which is mutant p53-specific under non denaturing conditions; wt p53 was identified by its lack of immune precipitation with Pab 240 (SC-99) under non denaturing immune precipitation and reactivity with monoclonal antibody (Pab DO-1-SC-126) under comparable conditions. Immune precipitated proteins were subjected to SDS–PAGE, bi directionally blotted onto nitrocellulose membranes and p53 identified by the DO-1 (SC126) monoclonal antibody, which recognizes both wt and mutant p53 in denatured form. Both antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA).

2.2. Glucose and serum starvation

Experiments involving glucose depletion were carried out by seeding the cells overnight in a complete medium consisting of Dulbecco's Modified Medium (DME) Sigma Cat # D1152 containing 4.5 g/lL glucose ($\sim$23 mM) supplemented with 4 mM glutamine and 10% fetal calf serum. After allowing 20 h for cell attachment, adherent cells were extensively washed 3 times in isotonic phosphate-buffered saline pH 7.3, followed by addition of Dulbecco's Modified Eagle's Medium Sigma Cat # D5030, supplemented with 4 mM glutamine and bicarbonate without glucose or sodium pyruvate. Glucose and fetal calf serum concentrations were added to the glucose-free D5030 medium, as indicated in each experiment and physiological glucose was estimated around 5 mM levels [22].

2.3. Hypoxia

Cells were incubated for different times (24 or 48 h) in low oxygen ($\leqslant$1% O_2) in hypoxic C-474 chamber equipped with Pro-Ox 110 oxygen controlling regulators (Biospherix) which provided ($\leqslant$1%). Whenever indicated, $CoCl_2$ was added during cell seeding prior to subjecting the cells to low oxygen or normoxia.

2.4. Transient p53 siRNA silencing

This was carried out using the Invitrogen Neon electroporation system (Cat No. MPK5000) containing 5×10^6 cells resuspended in 100 µl buffer R (Cat No. MPK10025R) and 4 µl containing 10 nM of p53 siRNA (H) duplex standardized to silence p53 (Cat # SC-29435). Cells were treated in parallel with a comparable concentration of negative control siRNA-A (Cat # SC-36868), a non-targeting duplex of the same length as the specific p53 siRNA. Both siRNA types were obtained from Santa Cruz Biotechnology. Cells were subjected to electroporation using 2 pulses (width 30 and 1150 voltage) and subsequently seeded to 35% confluence in medium devoid of antibiotics in 96 well plates. After 12 h, cultures were washed and subjected to the treatments in glucose-free medium with glucose supplementation as indicated, for further assays within 48 h.

2.5. Relative toxicity/metabolic activity and live–dead assays

(a) Toxicity/metabolic activity was estimated with Alamar Blue (resazurin) which measures intracellular redox mitochondrial activity by quantitating the cell-catalyzed conversion of non-fluorescent resazurin to fluorescent resorufin [23]. Alamar Blue was added to a 10% final concentration to each one of 96 well plates after the appropriate treatment, the dye is non-toxic, allows fluorescent quantitation, permits re-use for further investigation such as morphological, biochemical and clonogenic analyses. As such, this assay is valuable as an endpoint of proliferation or relative metabolic activity, rather than a kinetic measure for monitoring cell growth. For these experiments, cells (10,000) were allowed to adhere overnight in 96 well TC microtiter dishes. After the corresponding treatments, Alamar Blue (BioSource, Camarillo, CA, USA) was added without removing medium containing dead cells, and fluorescence was measured 4 h later in a Fluoroskan Ascent microplate reader with an excitation of 544 nm and an emission of 590 nm.

(b) Live–dead ratio was determined by adding Calcein AM and propidium iodide directly to the cell cultures. Subsequently, cytofluorometry was used to determine the ratio of live cells with (Calcein – green fluorescence) and dead (propidium iodide – red fluorescence) in an Isocyte laser spectrofluorometer [22].

2.6. *A*poptosis-associated PARP cleavage

This was investigated in cell lysates following SDS–PAGE and immune blotting using an antibody PARP (SC-7150 from Santa Cruz Biotechnology), followed by reaction with protein A conjugated to peroxidase and chemiluminescence. Subsequently, images were digitalized and quantitated to define the ratio of cleaved to intact PARP [17,19].

2.7. Statistical studies

One-Way Analysis of Variance (ANOVA) test was conducted with SigmaPlot 11.0 (Systat Software, Inc, San Jose, CA, USA), to determine whether there was a statistically significant differences evaluated versus the control groups. In each Figure, $^*p \leqslant 0.005$, showed a statistically significant difference whenever indicated [17].

3. Results

3.1. p53 siRNA decreases survival of pyruvate supplemented glucose-depleted cells under hypoxia but promotes resistance to oxamate and 2-DG in 5 mM glucose

In parental wt p53 C8161 melanoma cells, p53 siRNA was introduced at high efficiency by electroporation to silence wt p53 expression compared to that seen in the same cells in which scrambled negative-control siRNA was similarly electroporated. Within 3 days of electroporation, cells were assayed by plating them in medium supplemented with the glucose concentrations indicated in each case. Immune blotting confirmed that p53 siRNA decreased p53 protein expression when normalized to reference actin levels (Fig. 1A). In hypoxic cells depleted of glucose and supplemented with 5 mM pyruvate, p53 siRNA decreased the number of viable cells, indicated by greater uptake of the cell impermeable DNA-binding propidium iodide. Moreover, within the live cell population, those electroporated with p53 siRNA showed decreased green fluorescence following addition of Calcein AM (Fig. 1B). This may be linked to lower live cell associated esterase activity induced by p53 siRNA, an early event that precedes decreased cell viability, as shown by others [22]. However, with 5 mM glucose supplementation, p53 siRNA increased resistance to oxamate and 2-deoxyglucose preferentially under normoxia, although this effect was decreased under hypoxia (Fig. 1C).

3.2. Metabolic activity under physical hypoxia and PARP cleavage by exposure to 2.5 mM glucose and 100 μM $CoCl_2$ are preferentially decreased in mut p53 C8161 cells

Genetically-matched C8161 melanoma harboring a wt p53 or mutant p53 R175H cell were used. A mutation within one allele of the p53 tumor suppressor gene can inactivate the remaining wild-type allele in a dominant-negative manner and in some cases can exert additional activities, known as mutant p53 'gain of function' [24]. Diminished function in cells transduced with mutant p53 (R175H) was seen by an over-expressed dysfunctional p53 protein, since there was negligible expression of the p53-activated p21WAF1 protein, compared to that in the corresponding wt p53 cells. [17]. When these matched cells were cultured for 48 h under normoxia in either 2.5 or 10 mM glucose, those harboring mutant p53 showed greater metabolic activity, even in the presence of 100 μM $CoCl_2$ (Fig. 2, upper left). However, when these cells were assayed in parallel following 48 h in ⩽1% O_2, metabolic activity of cells with mutant p53 in 2.5 mM glucose was preferentially decreased in the presence of 100 μM $CoCl_2$ (Fig. 2, lower left). We also confirmed that $CoCl_2$ increases PARP cleavage counteracted by NAC (N-acetylcysteine) in mutant p53 cells after 18 h in 1.25 mM glucose and 2% dialyzed serum. $CoCl_2$ treatment in low glucose increased AMPK phosphorylation (pAMPK) presumably via oxidative stress since this was counteracted by NAC (Fig. 2, right) [25].

3.3. Catalase and 100 μM $CoCl_2$ potentiate hypoxia-mediated cell death in C8161 cells in a p53-regulated manner

Since wt p53 C8161 melanoma cells showed relatively greater resistance to glucose starvation, cells were exposed to hypoxia and/or $CoCl_2$, in 5 mM glucose including glycolytic inhibitors like oxamate or 2-deoxyglucose, and exogenous superoxide dismutase or catalase whenever indicated. This revealed a lower survival of wt p53 cells jointly treated with catalase, hypoxia and $CoCl_2$. In contrast, in 5 mM glucose, control and superoxide dismutase-treated mutant p53 C8161 melanoma retained viability, but this was decreased in the presence of the glycolytic inhibitors when these cells were treated with hypoxia and $CoCl_2$, (Fig. 3).

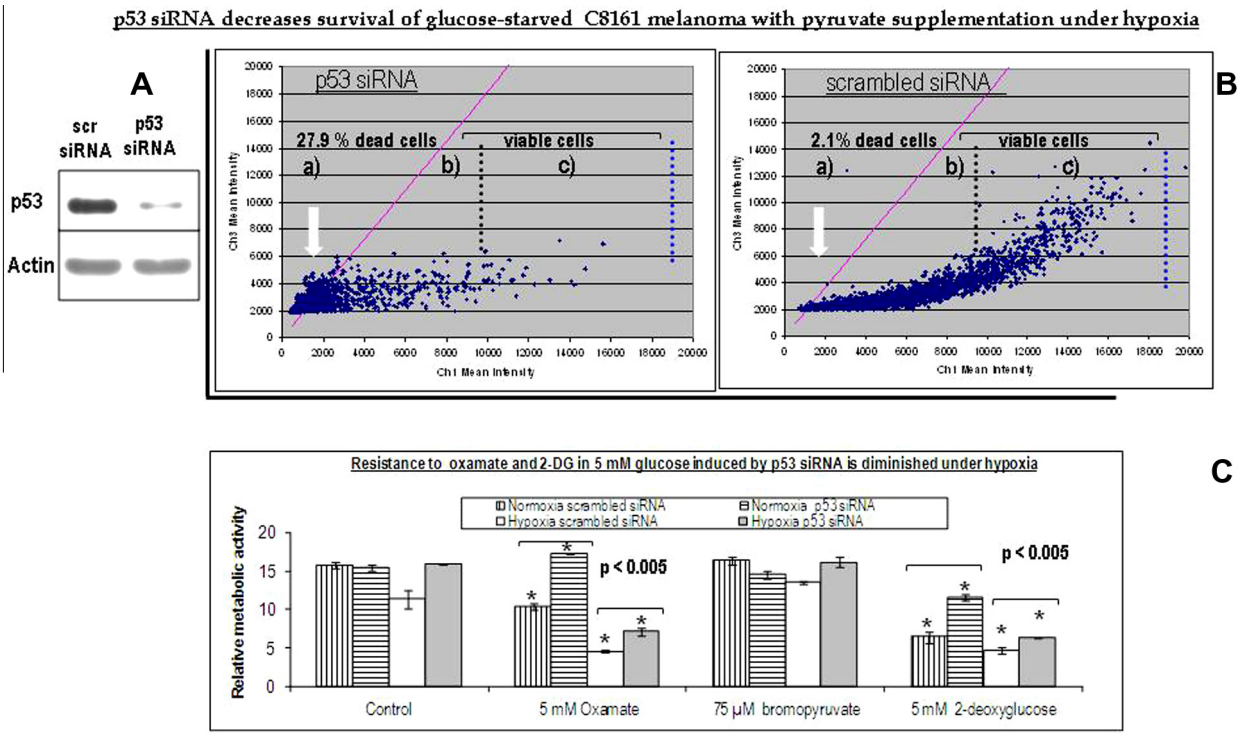

Fig. 1. C8161 melanoma cells electroporated with p53 siRNA or negative control sequences were assayed for: (A) changes in p53 expression normalized to actin by immune blotting; (B) % of live/dead cells following glucose depletion and pyruvate supplementation under hypoxia measured by laser-based 2 channel fluorescence; (C) resistance to oxamate, 3-bromopyruvate or 2-deoxyglucose under normoxia or hypoxia, measured by Alamar Blue fluorescence. The bars shown in (B) and (C) represent the median value of relative percentage of metabolic activity for each treatment group ± standard deviation (SD); $n = 4$.*$P < 0.005$ there was a statistically significant difference [17].

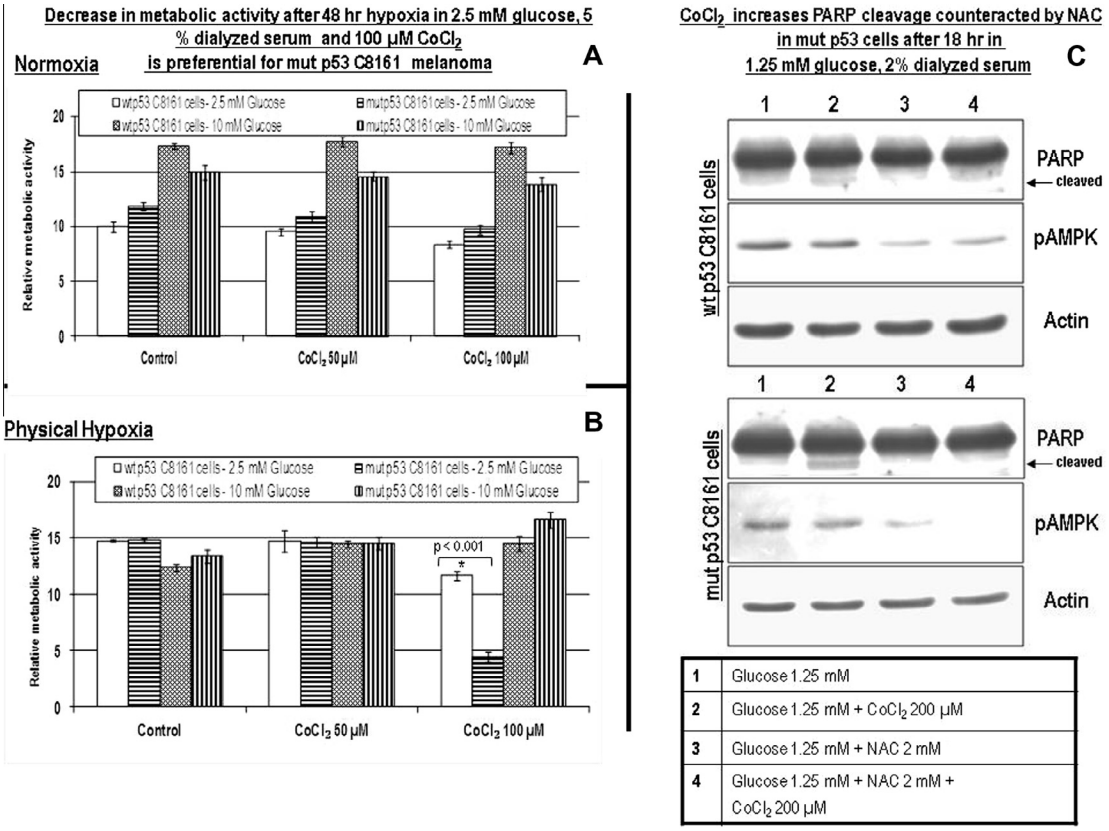

Fig. 2. Cells seeded in complete medium overnight were washed to remove glucose. Subsequently glucose-free medium supplemented with 2.5 or 10 mM glucose and 5% dialyzed serum including $CoCl_2$ at the concentrations indicated was added to the cells which were maintained for 48 h in (A) normoxia or (B) hypoxia (⩽1% oxygen). (C) $CoCl_2$ increases PARP cleavage counteracted by NAC in mutant p53 C8161 cells (lower panel) after 18 h in 1.25 mM glucose, 2% dialyzed serum.

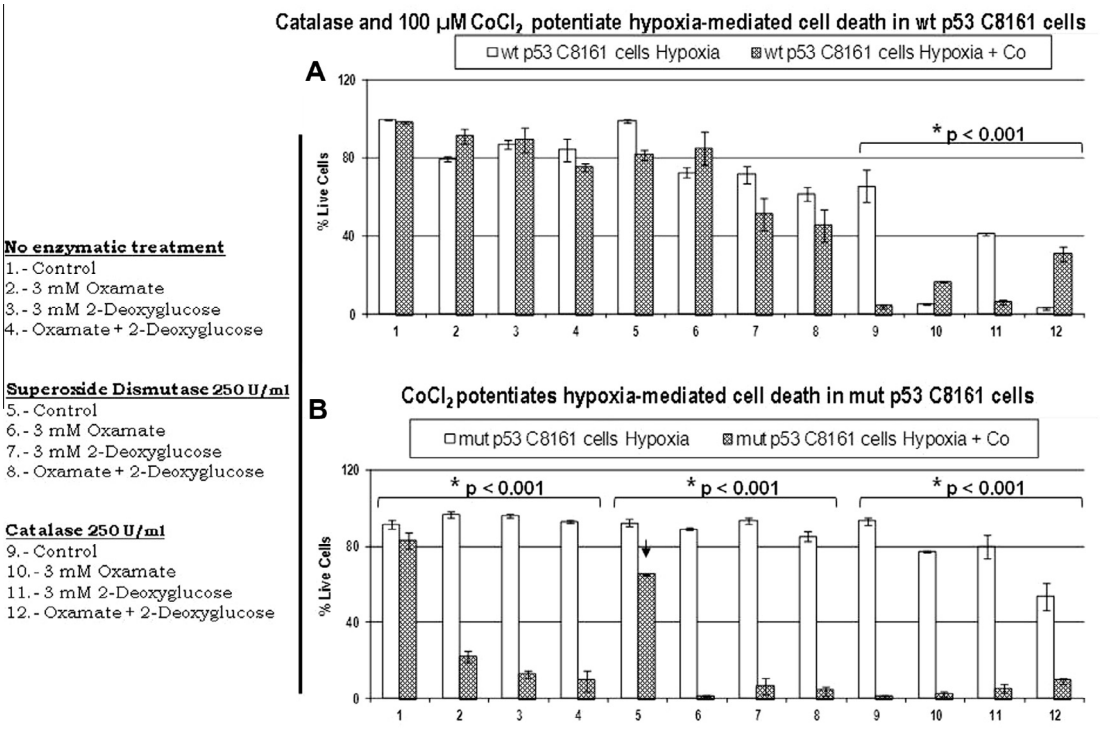

Fig. 3. Sparse cells were seeded in complete medium with $CoCl_2$ for pre-conditioning whenever indicated for 48 h prior to changing to medium with 5 mM glucose, 5% dialyzed serum with the additions of enzymes and glycolytic inhibitors and $CoCl_2$ indicated in each case, followed by 48 h under hypoxia. (A) wt p53 C8161 cells; (B) mut p53 C8161 cells.

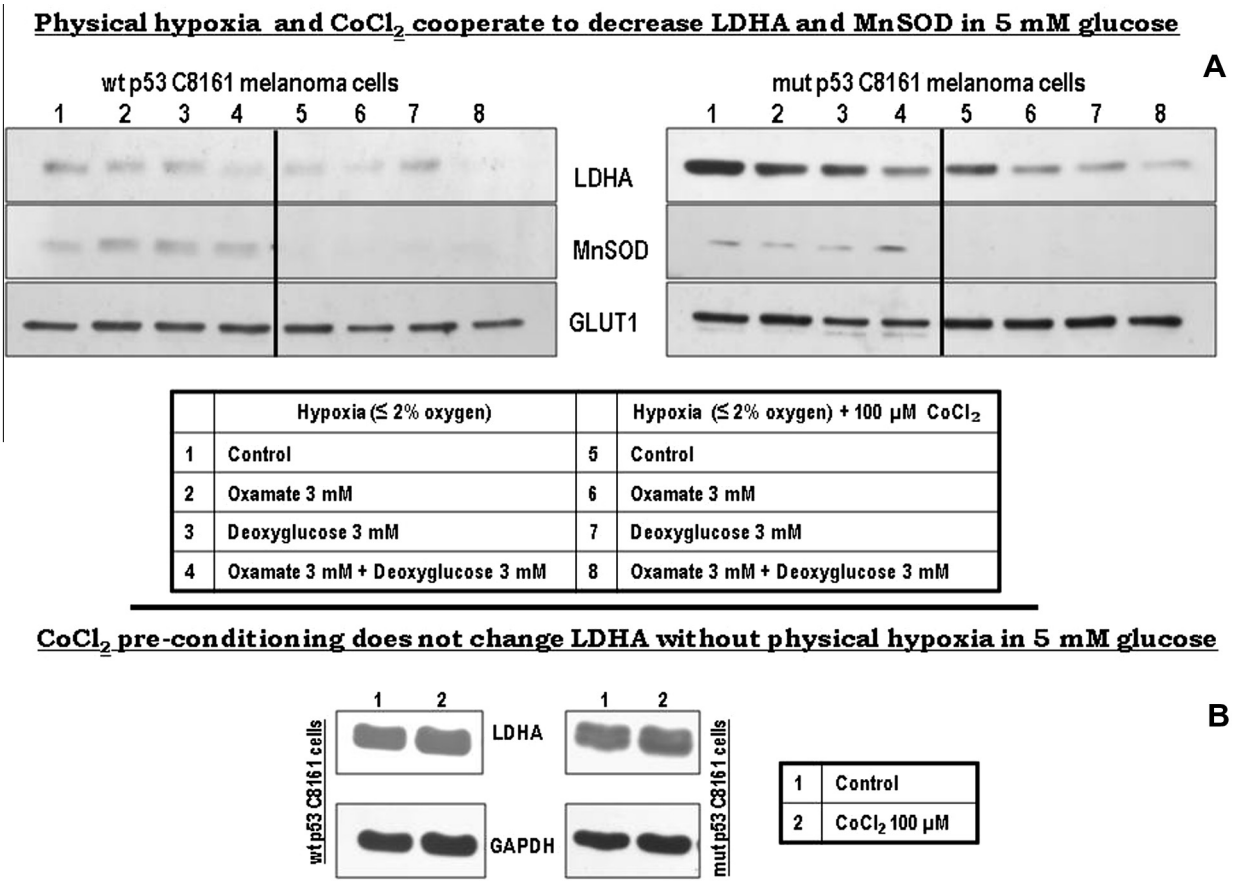

Fig. 4. (A) wt p53 or mutant p53 C8161 cells were seeded in medium with 10% serum and 20 mM glucose including 100 µM $CoCl_2$ for 12 h. All cultures were transferred to hypoxia in medium with 5 mM glucose and 5% dialyzed serum by 12 h, followed by 6 h of re-oxygenation +/− $CoCl_2$ and the additions indicated in each case for 12 h. (B) cells were treated as above indicated +/− $CoCl_2$ under normoxic conditions. In both cases, cells were harvested and analyzed by immune blotting [17].

3.4. Hypoxia and $CoCl_2$ cooperate to decreases LDHA and MnSOD in 5 mM glucose

Since superoxide anion is induced by glucose starvation and may enhance mitochondrial oxidative stress [26], we investigated whether MnSOD, was unequally affected by hypoxia (⩽1% oxygen) in the absence or presence of $CoCl_2$ in genetically-matched C8161 cells with differing p53 status exposed to 5 mM glucose. No significant difference in MnSOD expression related to p53 status was seen under hypoxia, but joint treatment with hypoxia and $CoCl_2$ essentially depleted MnSOD from both cell types. Since glycolytic LDHA is also induced by hypoxia [9,10], its p53-associated modulation by hypoxia ± $CoCl_2$ was also studied. This revealed that LDHA is increased in hypoxic mutant p53 cells compared to their wt p53 counterparts. However, adding $CoCl_2$ down-regulated LDHA preferentially in mutant p53 cells, compared to its expression in the absence of $CoCl_2$ (upper Fig. 4A). In contrast to the greater LDHA expression under hypoxia in mutant p53 cells, no p53-associated difference in LDHA expression was seen in C8161 melanoma when assayed under normoxia, irrespective of $CoCl_2$ addition (lower Fig. 4A).

4. Discussion

Previous reports have indicated that glucose depletion of melanoma [17] and carcinoma cells [18] promotes tumor cell death. However, we showed that glucose depletion with pyruvate supplementation preferentially permitted survival of mutant p53 cells [17]. Both of these reports used cells under aerobic conditions [17,18]. Since glucose starvation occurs in hypoxic gradients of poorly vascularized tumors, we now introduced p53 siRNA or mutant p53R175H into human melanoma cells, to investigate how they responded to glucose starvation or to physiological 5 mM glucose under hypoxia or normoxia in the presence or absence of glycolytic inhibitors. This revealed that p53 siRNA decreases survival of glucose-starved C8161 melanoma with pyruvate supplementation under hypoxia. In 5 mM glucose, p53 siRNA rather increased resistance to oxamate or 2-deoxyglucose particularly under normoxia. These results suggested that diminishing wt p53 by silencing decreases the ability of pyruvate to counteract glucose starvation. Reciprocally, p53 siRNA silencing seems to contribute to survival in 5 mM glucose (Fig. 1). In experimental systems, hypoxia is alternatively induced in a low oxygen chamber or aerobically by hypoxia mimetics like $CoCl_2$. When matched C8161 melanoma with wt p53 or mutant p53 R175H are compared, the latter become more susceptible in 2.5 mM glucose under hypoxia (⩽1% O_2), particularly when the low oxygen treatment is accompanied by the hypoxia mimetic $CoCl_2$. This report is the first to show that hypoxia and $CoCl_2$ cooperate to preferentially enhance loss of metabolic activity in mutant p53 C8161 melanoma exposed to sub-physiological glucose, when compared to their isogenic wt p53 counterpart subjected to the same conditions (Fig. 2). The cooperation between hypoxia and $CoCl_2$ was also seen in 5 mM glucose when C8161 melanoma cells were treated with glycolytic inhibitors like oxamate or 2-deoxyglucose or when exogenous catalase was added (Fig. 3). The catalase-mediated potentiation of toxicity in cells jointly treated with hypoxia and $CoCl_2$ may be related to results showing that epidermal growth factor up-regulates

HIF-1α expression via H_2O_2 production, with PEG-catalase diminishing this up-regulation in ovarian carcinoma cells [28]. C8161 melanoma cells responded to joint hypoxia and $CoCl_2$ by down-regulating significantly MnSOD (SOD2) [29] and glycolytic LDHA (Fig. 4). We hypothesize that lower MnSOD and LDHA jointly induced by hypoxia and $CoCl_2$ may augment the oxidative stress produced by each treatment individually, leading to accumulation of mitochondrial superoxide anion [26,27] and to lower glycolysis [9,10]. It will be interesting to examine in other tumor cells whether dual treatment with hypoxia and CoCl2 also down-regulate LDHA and SOD2 proteins, since these 2 proteins increase in aggressive glycolytic pheochromocytomas and paragangliomas [29]. Taken together, our results suggest that p53 inactivation by silencing or mutation confers susceptibility to glucose starvation, particularly under hypoxia. Our results also show that hypoxia and hypoxia mimetics are not redundant, and may cooperate together to counteract survival of aggressive cancer cell variants capable of resisting the stress of hypoxic tumor microenvironments.

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