Glycogen Synthase Kinase 3 Regulates Cell Death and Survival Signaling in Tumor Cells under Redox Stress

Abstract
Targeting tumor-specific metabolic adaptations is a promising anticancer strategy when tumor defense mechanisms are restrained. Here, we show that redox-modulating drugs including the retinoid N-(4-hydroxyphenyl)retinamide (4HPR), the synthetic triterpenoid bardoxolone (2-cyano-3,12-dioxoooleana-1,9(11)-dien-28-oic acid methyl ester), arsenic trioxide (As2O3), and phenylethyl isothiocyanate (PEITC), while affecting tumor cell viability, induce sustained Ser9 phosphorylation of the multifunctional kinase glycogen synthase kinase 3β (GSK3β). The antioxidant N-acetylcysteine decreased GSK3β phosphorylation and poly(ADP-ribose) polymerase cleavage induced by 4HPR, As2O3, and PEITC, implicating oxidative stress in these effects. GSK3β phosphorylation was associated with up-regulation of antioxidant enzymes, in particular heme oxygenase-1 (HO-1), and transient elevation of intracellular glutathione (GSH) in cells surviving acute stress, before occurrence of irreversible damage and death. Genetic inactivation of GSK3β or transfection with the non-phosphorylatable GSK3β-S9A mutant inhibited HO-1 induction under redox stress, while tumor cells resistant to 4HPR exhibited increased GSK3β phosphorylation, HO-1 expression, and GSH levels. The above-listed findings are consistent with a role for sustained GSK3β phosphorylation in a signaling network activating antioxidant effector mechanisms during oxidoreductive stress. These data underlie the importance of combination regimens of antitumor redox drugs with inhibitors of survival signaling to improve control of tumor development and progression and overcome chemoresistance.

Introduction
Recent innovative approaches to cancer chemoprevention and treatment focus on tumor-specific metabolic adaptations as drug targets. In particular, some redox drugs appear to deeply compromise tumor bioenergetics [1]. Antitumor agents belonging to this class of chemicals, including the chemopreventive agent N-(4-hydroxyphenyl)retinamide (4HPR) [2], the synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid methyl ester (CDDO-Me; RTA402 or bardoxolone methyl) [3], and phytochemicals such as phenylethyl isothiocyanate (PEITC) [4], are under scrutiny in clinical studies. Accordingly, patterns of the antioxidant machinery are currently used as biomarkers in clinical trials for metabolic disorders and cancer.
The main cytoprotective pathways against cell death originate from growth factor–activated phosphatidylinositol 3-kinase and AKT and by transient activation of the mitogen-activated protein kinase p42/p44 MAPK (ERK1/2) through ras-mediated pathways. These signaling molecules, and related transcription factors, are part of a stress-sensing network that can modulate the activity and expression of effectors involved in redox homeostasis, in concert with modulation of glutathione (GSH) metabolism and the activation of a battery of antioxidant enzymes [5].

The multifunctional glycogen synthase kinase 3b (GSK3β) is a key convergence point of survival pathways mediated by Ser7Thr protein kinases most related to PAK, PKG, PKC (AGC) kinases (AKT, protein kinase C (PKC), and cAMP-dependent protein kinase (PKA)), ERK1/2, and Wnt. Accordingly, GSK3β inhibitors are under clinical scrutiny in both neurodegenerative disorders and in cardioprotection. GSK3β activity depends on the balance between the activating Tyr216 phosphorylation and the inactivating phosphorylation at Ser9. Recent evidence indicates that active GSK3β in tumors can be proapoptotic or antiapoptotic depending on the cell type or subcellular localization [6]. Recent findings highlight a role for active GSK3β in cancer stem cell maintenance [7], reinforcing the interest for GSK3β inhibitors in cancer treatment.

We have previously shown that 4HPR and CDDO-Me induce tumor cell death by ATP depletion and mitochondrial bioenergetic failure leading to necrosis-like death, associated with inhibition of basal or insulin-like growth factor-1 (IGF-1)–induced AKT phosphorylation and disruption of IGF-1 survival function [8–10]. Here, we investigated modulation of GSK3β phosphorylation and antioxidant enzyme expression, in particular heme oxygenase-1 (HO-1), and poly(ADP-ribose) polymerase (PARP) cleavage as a sign of apoptosis, in the same responsive cellular models under pharmacologic redox stress. Our results show that the redox imbalance induced by 4HPR and other redox drugs in retinoblastoma Y79 cells and by CDDO-Me and 4HPR in human prostate cancer cells is associated with increased Ser9 phosphorylation of GSK3β requiring glucose metabolism and results in activation of a defense response involving the induction of HO-1, GSH, and nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) [11] levels. Remarkably, GSK3 gene silencing, or expression of the GSK3β-S9A mutant, decreased HO-1 induction in CDDO-Me–treated cells. These findings highlight a correlation between GSK3β Ser9 phosphorylation and the antioxidant response, suggesting that redox stress induced by either oxido-reductive or metabolic perturbation uses a redox code that targets sensitive signaling pathways. The regulatory network described here suggests that curtailing the energy demanding antioxidant response could increase the efficacy of redox anticancer drugs.

Materials and Methods

Cell Cultures and Reagents

Human retinoblastoma Y79 cells (ATCC HTB-18), androgen-independent PC3 (ATCC CRL-1435), and DU145 (ATCC HTB-81) human prostate adenocarcinoma cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and certified by the Interlab Cell Line Collection of the Biological Bank and Cell Factory core facility (member of the European BioBanking and Molecular Resources Research Infrastructure), IRRCS AOU S. Martino-IST, (Genoa, Italy). The cells were propagated in RPMI 1640 supplemented with either 15% (retinoblastoma cells) or 10% (prostate cancer cells) heat-inactivated FBS and 2 mM glutamine. For adherent cultures, retinoblastoma cells were seeded on poly-D-lysine–coated dishes for 24 hours and then transferred to a chemically defined N1 medium (Invitrogen, Life Technologies, Monza, Italy). 4HPR was kindly provided by Dr James A. Crowell, Division of Cancer Prevention, National Cancer Institute (Bethesda, MD) and Dr Gregg Bullard, McKessonBio (Rockville, MD). Cells were treated with 4HPR dissolved in ethanol in a 10 mM stock solution. Human recombinant IGF-1 (PeproTech EC, London, United Kingdom) was diluted in 0.1 N acetic acid and used at the final concentration of 10 to 100 ng/ml. In the experiments with IGF-1, the cells were maintained for 24 hours in N1 defined medium without insulin. The synthetic triterpenoid CDDO-Me was kindly provided by Dr Michael B. Sporn (Dartmouth Medical School, Hanover, NH). The following reagents used were purchased from Sigma-Aldrich (Milano, Italy): the antioxidants N-acetylcysteine (NAC), desferoxamine mesylate (DFX), and GSH ethyl ester (GSH-EE), the oxidants As2O3, PEITC, and DL-buthionine-[S,R]-sulfoximine, and the mitogen-activated protein kinase kinase kinase (MEK) inhibitor bis[amino(2-aminophenyl)thio)methylene]butanedinitrile (U0126) from BIO-MOL (Plymouth Meeting, PA); the p90 ribosomal S6 kinase (p90RSK) inhibitor BI-D178 was provided by Dario R. Alessi (MRC Protein Phosphorylation Unit, University of Dundee, Scotland, United Kingdom). All pretreatments with antioxidants (NAC, DFX, and GSH-EE) or other reagents were carried out for 2 hours before drug administration.

Cell Viability and Cytotoxicity Assays

Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, by lactate dehydrogenase release and ATP levels as previously described [8–10]. The absorbance was determined with a microtiter plate reader (Molecular Devices Corp, Sunnyvale, CA). Detection of cell membrane integrity was also performed by propidium iodide staining (1 μg/ml) and flow cytometry as previously described [9]. Analysis was performed on 10,000 gated cells to exclude cell debris using a CyAn ADP flow cytometer (Beckman Coulter, Milano, Italy).

Total and Reduced GSH Determination

Total GSH (reduced GSH plus glutathione disulfide (GSSG)) was determined by the enzymatic recycling method with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (Ellman’s reagent) (BioxyTech GSH-400 colorimetric assay; OxisResearch, Portland, OR). Total protein content was determined before metaphosphoric acid precipitation by the DC Protein Assay (Bio-Rad, Milano, Italy). Total GSH content was calculated as nanomoles per milligram of protein and expressed as percent relative to controls. Determination of GSH with monochlorobimane (mBCl; Molecular Probes, Life Technologies; 200 nM, 37°C for 1 hour) was carried out in cells suspended in cold phosphate-buffered saline using a microplate spectrophotometer (SpectraMax Gemini XPS; Molecular Devices) or by flow cytometry [12].

Reactive Oxygen Species Detection

The cells were incubated with 5 μM carboxymethyl dichlorofluorescin diacetate (CM-H2DCFDA) (Molecular Probes) 20 minutes before the end of treatments and analyzed by flow cytometry as described [8,9].

Determination of Glucose Uptake

Glucose uptake was determined with the hydrolyzable fluorescent probe (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxy-D-glucose (2-NBDG) and the nonhydrolyzable 6-NBDG (Molecular
**Results**

**GSK3β Ser9 Phosphorylation Correlates with Cell Death Activation**

The anticancer drugs 4HPR and CDDO-Me induce tumor cell death by bioenergetic failure due to loss of mitochondrial transmembrane potential ($\Delta \psi_m$), ATP depletion, cell membrane damage, and down-regulation of the AKT survival pathway [9,10]. In this study, we explored redox stress and survival signaling in the cell death pathways induced by CDDO-Me in prostate adenocarcinoma PC3 and DU145 cells and by 4HPR in Y79 retinoblastoma cells, focusing on the role of GSK3β in these processes.

Treatment with 4HPR induced time-dependent GSK3β phosphorylation in Y79 cells that persisted at 24 hours and was coincident with extensive PARP cleavage (Figure 1), in line with activation of caspase-3, DNA fragmentation, and nuclear apoptosis in dying cells [9]. Similar to the effects of CDDO-Me in prostate cancer cells [10], 4HPR in Y79 cells induced GSK3β phosphorylation that parallels decreased AKT phosphorylation [9]. While AKT stimulation by IGF-1 was inhibited by 4HPR, GSK3β Ser9 phosphorylation was sustained (Figure 1). Perturbation of IGF-1 signaling by 4HPR correlated with the lack of effect of IGF-1 on PARP cleavage. Reactive oxygen species (ROS) elevation by 4HPR impairs cellular bioenergetics in Y79 cells, as indicated by ATP depletion and disruption of $\Delta \psi_m$ [9]. The master energy sensor AMPK can be activated during redox stress concomitant with autophagy, hypoxia, and nutrient deprivation [16]. Increased phosphorylation at Thr172 of AMPK and its substrate acyl CoA carboxylase pACC at Ser79 confirmed that GSK3β hyperphosphorylation plays a role in 4HPR-induced cell death in critical energy conditions under redox stress.

**GSK3β Ser9 Phosphorylation Is Associated with an Antioxidant Response**

GSK3β phosphorylation has been associated with cytoprotective mechanisms to preserve homeostasis during metabolic stress [6]. Since 4HPR and CDDO-Me can kill tumor cells by redox stress

![Figure 1](image-url)
[3,9], we asked whether GSK3β phosphorylation correlates with an increased expression of antioxidant enzymes. HO-1 catalyzes the rate-limiting step in heme catabolism as an adaptive response to oxidative injury. Cytosolic Cu/Zn SOD serves to detoxify ROS. G6PD, the limiting enzyme of the pentose phosphate pathway (PPP), provides reducing equivalents (NADPH). GSH peroxidase detoxifies peroxides through GSH oxidation. Expression of all four pro-survival molecules was upregulated in 4HPR-treated Y79 cells at 24 hours (Figure 2A). Dose-dependent HO-1 and Cu/Zn SOD expression were induced in a limited concentration range, declining with the severity of cell damage (Figure 2B) [9]. GSK3β can regulate the activity of the transcription factor Nrf2, which in turn controls the expression of G6PD, HO-1, and enzymes of GSH metabolism [17]. Indeed, Nrf2 was increased within a defined range of concentrations and declined at higher doses of 4HPR in Y79 cells (Figure 2B). Due to the potential clinical application of HO-1 pharmacological targeting [18], we limited further analysis to this enzyme. Oxidative stress triggered by 4HPR in prostate tumor cells has been extensively studied [19]; however, the antioxidant response was not explored. In PC3 cells, 4HPR (10 μM) induced time-dependent HO-1 expression and GSK3β phosphorylation, along with PARP

Figure 2. (A) Induction of antioxidant enzymes is associated with PARP cleavage induced by 4HPR in Y79 cells. Densitometric analysis was carried out by normalizing protein expression versus GAPDH and calculation of fold increase over control samples. The dividing vertical line indicates the junction between control and lanes spliced from the blot. (B) Dose-dependent increase of GSK3β phosphorylation and HO-1 and Nrf2 expression in 4HPR-treated Y79 cells. (C) Time-dependent GSK3β phosphorylation, HO-1 expression, and PARP processing in PC3 cells treated with 4HPR. (D) Dose-dependent GSK3β phosphorylation, HO-1 and Nrf2 expression, PARP and caspase-8 processing in PC3 and DU145 cells treated with CDDO-Me (24 hours). pAMPK induction indicates ATP decline, in line with previous results [10]. Pretreatment for 2 hours with the antioxidant NAC (10 mM) (E) or DFX (200 μM) (F) decreases GSK3β phosphorylation, PARP cleavage, and HO-1 expression induced by 4HPR (2.5 μM, 24 hours). (G) Similar modulation by NAC of the molecular pattern analyzed under the effect of PEITC. HO-1 overexpression is visible with the combination of As2O3 and NAC. In all panels, results from representative experiments are shown.
Involves ROS and GSH
levels of GSH in tumors and in cell lines has been reported[30]. The β-cleavage (Figure 2) [30] was associated with transient HO-1 expression and pAMPK increase, both declining in late apoptotic cells showing strong caspase-8 processing (Figure 2D). In Y79 cells, CDDO-Me at relatively low dose (250-500 nM, 24 hours) caused massive cell lysis and extensive PARP cleavage indicative of severe cell damage [20], while GSK3β Ser9 phosphorylation or HO-1 expression was not induced (Figure W1A).

To assess whether oxidative stress in general stimulates GSK3β phosphorylation, we investigated the effects of different redox drugs and antioxidants. Besides 4HPR, we used the clinically relevant prooxidants PEITC (2.5 μM) [4] and As2O3 (50 μM) [21]. Cell viability, as assessed by ATP levels, remarkably declined in Y79 cells treated with PEITC, As2O3, and CDDO-Me (Figure W1B). PEITC and As2O3, similarly to 4HPR [9,22], increased ROS in Y79 cells (Figure W1C). CDDO-Me can alter redox in different tumor types [23]; however, it did not increase ROS in Y79 or in PC3 cells (Figure W1, C and D). The antioxidant NAC (10 mM) that provides l-cysteine released by esterases for GSH synthesis and the iron chelator DFX (200 μM) [25], PEITC (2.5 μM) [4] and As2O3 (50 μM) [21] stimulated GSK3β Ser9 phosphorylation and PARP cleavage in Y79 cells at 24 hours (Figure 2G). This effect was prevented by NAC, which also abrogated HO-1 expression induced by PEITC in Y79 cells but further increased HO-1 expression induced by As2O3 (Figure 2G). This paradoxical effect could be due to the particular chemistry of reaction between GSH and trivalent arsenic, producing arsenic radicals and GSH-arsenic conjugates that inhibit GSH reductase (GR), blocking regeneration of oxidized GSH [26]. Enhanced HO-1 expression by As2O3 in combination with other antioxidants has been reported [27]. In contrast, PEITC and its bioactivation products form GSH conjugates that do not apparently inhibit GR [26,28]. Taken together, these data suggest that GSK3β Ser9 phosphorylation occurs during acute redox imbalance triggering cell death [9,10] and correlates with activation of a transient antioxidant defense.

Redox Imbalance Affecting GSK3β Ser9 Phosphorylation Involves ROS and GSH
Prevention of PARP cleavage induced by 4HPR, As2O3, and PEITC by NAC in Y79 cells (Figure 2) prompted us to investigate whether redox stress affecting GSK3β was associated with altered GSH metabolism. Determination of reduced GSH with the fluorescent probe mBCl [12] and flow cytometry analysis revealed discrete subpopulations in Y79 cells, differing in size and structural complexity (Figure 3, A and B). They were distinguished by different mBCl mean fluorescence intensity (MFI; bright and dim fluorescence population) represented neuronal precursors undergoing spontaneous death upon differentiation [29] (Figure 3, A, left, and B). DL-Buthionine-[S,R]-sulfoximine was used as control to test GSH depletion. The existence of heterogeneous populations with different levels of GSH in tumors and in cell lines has been reported [30]. The MFI of the bright fluorescence population at 24 hours after 4HPR administration was transiently elevated over controls (Figure 3C). Interestingly, GSH increase at 24 hours in the bright population was consistent with bell-shaped ROS kinetics that peaked at 6 hours and progressively declined at 24 hours after 4HPR administration [9]. These data are in line with a transient adaptive increase of GSH that can be associated with a biphasic response to lethal oxidative stress [31]. The fraction of bright fluorescent cells declined in a dose- and time-dependent manner, shifting in the dim and non-fluorescent dying population (Figure 3B; data at 16 hours of treatment are not shown). Thus, total GSH levels in the whole cell population treated with 5 μM 4HPR was decreased to approximately 50% of controls at 24 hours (average decrease of 50.2 ± 10.6%; Figure 3D).

A significant reduction of total GSH (reduced GSH plus GSSG), as determined with DTNB, was observed in Y79 cells after 24 hours of treatment with 2.5 μM 4HPR (40.8 ± 0.5% of control; mean GSH levels in controls were 10.16 ± 1.82 nmol/mg protein; Figure 3E), confirming the data obtained with mBCl (Figure 3D). In the cells pretreated with NAC (10 mM, 2 hours), intracellular total GSH was elevated over controls, both in the absence or presence of 4HPR (Figure 3E), as expected. Lack of effect of methionine to inhibit GSH efflux [32] excluded that GSH decline was due to extracellular release (data not shown). As2O3 and CDDO-Me substantially depleted GSH levels in Y79 cells, in contrast with PEITC that did not exert significant effects (Figure W2A). In PC3 and DU145 prostate tumor cells, GSH was significantly decreased by CDDO-Me at 24 hours of treatment (Figure W2B).

The Y79 bright and dim fluorescence populations were collected by cell sorting. The bright population showed increased basal as well as 4HPR-induced GSK3β phosphorylation relative to the total population and was able to cleave PARP in response to 4HPR (2.5 μM, 24 hours; Figure W2C). To assess whether intracellular GSH content could influence GSK3β phosphorylation, we treated the cells with a cell permeable GSH-EE (10 mM) for 16 and 24 hours. Cells treated with GSH-EE showed increased basal GSK3β phosphorylation, consistent with the previous result. In contrast, PARP cleavage, HO-1 expression (Figure W2D), and ROS induced by 2.5 μM 4HPR (Figure W2E) were decreased, indicating antioxidant effects similar to NAC. It is possible that NAC, also capable of influencing energy metabolism [33], exerts a more tunable effect on redox balance, while an abrupt uptake of GSH-EE could result in reductive stress in Y79 cells [34]. Taken together, these data suggest that GSK3β phosphorylation can signal redox perturbations, including variation in GSH levels, away from the equilibrium state.

4HPR-Resistant Cells Show Elevated GSH Levels and Enhanced GSK3β Phosphorylation
To clarify the function of GSK3β in redox stress, we analyzed GSK3β phosphorylation in established 4HPR-resistant cells. Y79, PC3, and DU145 cells surviving chronic administration of 4HPR starting from 1 μM and progressively reaching the 2.5 to 5 μM concentration range were isolated from long-term cultures and cloned by limiting dilution (Figures 4 and W3) [22]. Y79 cells resistant to 2.5 μM 4HPR (Y79-R2.5) showed significantly limited damage (Figure W2C). To assess whether intracellular GSH content could influence GSK3β phosphorylation, we treated the cells with a cell permeable GSH-EE (10 mM) for 16 and 24 hours. Cells treated with GSH-EE showed increased basal GSK3β phosphorylation, consistent with the previous result. In contrast, PARP cleavage, HO-1 expression (Figure W2D), and ROS induced by 2.5 μM 4HPR (Figure W2E) were decreased, indicating antioxidant effects similar to NAC. It is possible that NAC, also capable of influencing energy metabolism [33], exerts a more tunable effect on redox balance, while an abrupt uptake of GSH-EE could result in reductive stress in Y79 cells [34]. Taken together, these data suggest that GSK3β phosphorylation can signal redox perturbations, including variation in GSH levels, away from the equilibrium state.
significantly increased in 4HPR-resistant Y79 cells cultured without 4HPR for 1 week relative to the wt cells (Figure 4B). PC3 and DU145 cells resistant to 5 μM 4HPR (PC3-R5, DU145-R5) showed increased GSK3β phosphorylation, HO-1 and GCLC expression (Figure 4, C and E, respectively), and high basal GSH and NAD(P)H levels relative to the wt cells (Figure 4, D and F), a result in line with the stabilization of a pGSK3β-mediated redox-sensitive signaling cascade in stressed PC3 and DU145 cells[10]. These data suggest that increased GSK3β phosphorylation could be part of an adaptive mechanism to tolerate long-term exposure to redox stress. This observation was corroborated by immunohistochemical analysis of human prostate tumor specimens showing elevated pGSK3β and HO-1 levels (Figure W4), in line with the evidence that oxidative stress parallels prostate tumor progression[35,36].

**GSK3β Ser9 Phosphorylation Influences HO-1 Expression**

To further investigate the role of GSK3β in redox remodeling, we analyzed HO-1 expression in PC3 and DU145 cells transiently transfected with an siRNA specific for GSK3α/β. Reduction of GSK3β expression has been linked to increased cellular distress in CDDO-Me–treated and untreated prostate cancer cells [10]. Since GSK3α can compensate for lack of GSK3β in some functions [37] and the role of GSK3α is poorly defined in cell death, we silenced both GSK3α and GSK3β to better evaluate the role of GSK3 in this context. We then focused on GSK3β because of the expanding development of GSK3β pharmacologic inhibitors in clinical settings, even if isoform-specific GSK3 inhibitors are lacking. Treatment with CDDO-Me (1 μM) for 24 hours strongly induced HO-1 expression in wt and non-specific siRNA-transfected prostate cancer cells, while HO-1 was significantly decreased in GSK3β-depleted cells (Figure 5A). Similarly, transfection with the non-phosphorylatable GSK3β-S9A mutant caused a marked decline in HO-1 induction by 4HPR and CDDO-Me in the same cell lines (Figure 5, B and D), an indication that GSK3β Ser9 phosphorylation signaling is crucial to regulate HO-1 induction.

PC3 cells stably transfected with the GSK3β-S9A mutant grew slower relative to the mock-transfected cells and were more sensitive to CDDO-Me and 4HPR treatment (Figure W3D). Both PC3 and DU145 cells stably transfected with the GSK3β-S9A mutant showed elevated levels of GSH and NAD(P)H [11] relative to controls (Figure 5, C and E, respectively), suggestive of an adaptive mechanism to compensate a precarious redox balance in these cells.

**Glucose Metabolism Supports GSK3β Phosphorylation Induced by Prooxidants**

To resist redox stress, cells activate metabolic adaptations to feed antioxidant defenses. Since GSK3β has been shown to play a role in
glucose-dependent survival pathways [38], we focused on glucose metabolism. The glucose antagonist 2-deoxyglucose (2DG), a non-hydrolysable glucose analog, blocks glycolysis but undergoes further metabolism through the PPP. In Y79 cells, 2DG decreased GSK3β phosphorylation and HO-1 expression during acute (24 hours) exposure to 4HPR and As2O3 (Figure 6, A and B). Energy loss, in particular ATP [9], in these conditions was confirmed by increased Thr172 phosphorylation of AMPK, recently implicated in NADPH balance under metabolic stress [39]. Glucose metabolism through the oxidative PPP partly contributes to NADPH homeostasis in cells under oxidative stress. As shown in Figure 2A, G6PD expression was induced in Y79 cells treated with 4HPR. Indeed, G6PD activity, after a transient up-regulation at 16 hours, declined at 24 hours after 4HPR administration (Figure 6C). Accordingly, the G6PD inhibitor 6-aminonicotinamide (6AN, 1 mM) decreased GSK3β phosphorylation and HO-1 induction by 4HPR (Figure 6D). The 89-kDa PARP cleavage fragment was decreased by 2DG and 6AN (Figure 6, A, B, and D). Anomalous regulation of PARP cleavage in caspase-independent oxidative cell death, similar to that induced by 4HPR [9], has been described [40]. These represent signs of cellular distress; both 2DG (50 mM) and 6AN (1 mM) in glucose-containing medium (5 mM glucose), alone or in combination with 4HPR, remarkably decreased MTT reduction and ATP levels in Y79 cells (Figure 6E). In Y79 cells resistant to 4HPR, glucose metabolism, as assessed by the uptake of the glucose analogue 6-NBDG or 2-NBDG and flow cytometry, was higher relative to wt Y79 cells (Figure 6F), in line with the elevated NAD(P)H levels (Figure 4B) [11]. The lack of GSK3β phosphorylation in acutely stressed cells with impaired glucose metabolism, coupled with the enhanced requirement for glucose in 4HPR-resistant Y79 cells, suggests that GSK3β phosphorylation is glucose-dependent under stress conditions.

**ERK1/2 and RSK Are Implicated in GSK3β Phosphorylation Induced by 4HPR in Y79 Cells**

Phosphorylation of Ser9 on GSK3β and of Ser21 on GSK3α is mainly operated by AKT. In Y79 and prostate cancer cells, AKT phosphorylation was decreased by 4HPR and CDDO-Me, respectively, possibly due to energy depletion and redox stress [41,42] that we observed in both models [9,10] and ceramide production in the case of 4HPR [43]. We then considered other possible stress-activated pathways. GSK3 can fall under the control of ERK1/2 under oxidative stress [44–46]. The role of ERK1/2 phosphorylation in cell survival or death, depending on the duration of the signal, is well documented [45–47]. ERK1/2 phosphorylation was sustained at 24 hours after 4HPR (2.5 μM) administration in Y79 cells (Figure 7A). The MEK inhibitor UO126 decreased GSK3β...
phosphorylation, slightly lowered HO-1 expression, and inhibited PARP cleavage (Figure 7A), suggesting a role in cell death for ERK1/2 [45]. Remarkably, UO126 rescued loss of viability and cell membrane damage and prevented GSH decrease induced by 4HPR at 48 hours (Figure 7B and C, respectively). Accordingly, NAC abolished ERK1/2 phosphorylation in 4HPR-treated cells (Figure 7D).

Figure 5. (A) GSK3α/β gene silencing lowers HO-1 induction by CDDO-Me (1 μM, 24 hours) in PC3 and DU145 cells. The potentiation of CDDO-Me cytotoxicity in these conditions has been published previously [10]. Optical density of bands was normalized versus GAPDH and fold increase expression was calculated relative to control samples set as 1. (B) Transfection with the GSK3β-S9A mutant decreases HO-1 induced by 4HPR and CDDO-Me in PC3 cells. (C) PC3 cells transfected with the GSK3β-S9A mutant show elevated levels of GSH and NAD(P)H. (D) Lower induction of HO-1 in DU145 cells transfected with the GSK3β-S9A mutant in response to 4HPR and CDDO-Me treatment. (E) Elevated GSH and NAD(P)H levels in DU145-GSK3β-S9A cells relative to the mock-transfected cells. *P < .05 and ***P < .001, statistically significant difference between GSK3β-S9A mutants and mock-transfected cells.
Regulation of GSH metabolism by ERK1/2 is well documented; however, the negative or positive effects of ERK1/2 activity on GSH levels are still a matter of debate [45,46]. GSK3β can be phosphorylated at Ser9 by the stress-activated p90RSK. Phosphorylation of p90RSK can be controlled by ERK1/2 but also by other pathways including phosphatidylinositol 3-kinase and phosphoinositide-dependent kinase-1 (PDK-1) [48]. Phosphorylation at Thr356/Ser360 of RSK3, which is activated and can phosphorylate GSK3β under oxidative stress [44], was effectively elevated by 4HPR and decreased by UO126 (Figure 7A). The specific p90RSK inhibitor BI-D1780 [49] abolished 4HPR-stimulated GSK3β phosphorylation, induced PARP cleavage alone and in combination with 4HPR, abolished HO-1 induction more effectively than UO126, and increased loss of viability caused by 4HPR (Figure 7, E and F), suggesting a partially ERK1/2 independent, pro-survival role for RSK3 in Y79 cells. The data obtained indicate that acute oxidative stress induced by 4HPR activates a signaling pathway with an ERK1/2-dependent branch mainly involved in cell death and an RSK-dependent cytoprotective branch, both converging on GSK3β phosphorylation and influencing cell fate in Y79 cells.

**Discussion**

In the tumor cells analyzed here, we previously described a short-term model of cell death due to bioenergetic failure [9,10]. In particular, in prostate tumor cells we demonstrated that GSK3β genetic inactivation favors necrosis and ATP loss [10]. The data obtained in this study show a relationship between GSK3β expression or phosphorylation and the modulation of the antioxidant asset of the cell, in terms of HO-1 expression and intracellular GSH, during oxidative stress. The modulation of GSK3β phosphorylation, concomitant with
an energy demanding antioxidant response, thus appears as coordinated processes in line with a dual role of GSK3β in cytoprotection and cell death [50]. Several lines of evidence support this suggestion: 1) Short-term genetic depletion of GSK3β by siRNA or transfection with the GSK3β-S9A mutant, both converging on deficient GSK3β Ser9 signaling, decreased HO-1 induction in cells during acute oxidative stress. 2) 4HPR-resistant retinoblastoma and prostate cancer cells, representing models of adaptation to chronic stress, showed elevated GSK3β phosphorylation, intracellular GSH, and NADPH. 3) Antioxidants prevented GSK3β phosphorylation, paralleled by inhibition of PARP cleavage, a marker of nuclear apoptosis. 4) In bioenergetic stress models due to impaired glucose metabolism, GSK3β phosphorylation along with HO-1 expression was decreased, and 4HPR cytotoxicity was increased.

Our data indicate that GSK3β responds to an ample array of redox perturbations, however within a limited range of either oxidative or reductive equilibrium. GSK3β Ser9 phosphorylation was modulated by ROS-mediated signals in Y79 cells treated with 4HPR, PEITC, and As2O3, in keeping with previous observations [44,51]. In CDDO-Me–treated prostate tumor cells, where GSK3β Ser9 phosphorylation and HO-1 were induced, no ROS burst was observed; however, in these cells GSH declined, an event shared with...
Y79 cells exposed to PEITC and As$_2$O$_3$. Interestingly, GSK3β responded to GSH-EE treatment, possibly sensing a disproportionate reducing milieu [34]. GSK3β might be part of a mechanism that propagates signals of mitochondrial damage, independently of ROS overproduction, coupled with thiol imbalance.

We speculate that one possible function of GSK3β is to alert defenses against redox instability and that GSK3β phosphorylation and coordinated redox changes, including HO-1 induction, promoting apoptotic cell death and delaying necrosis in acute damage (Figure W5). In fact, transient GSK3β genetic inactivation, which exacerbates cell necrosis in apoptotic cells treated with CDDO-Me [10], lowered the ability to induce HO-1. Interestingly, HO-1 expression has been shown to modulate apoptotic and necrotic cell death pathways [52,53]. In this context, the antioxidant effects of HO-1 could favor a reducing milieu required for caspase activation [54]. Further, prostate tumor cells stably transfected with the GSK3β-S9A construct competing with the endogenous GSK3β showed lower levels of HO-1 under stress. Decreased HO-1 expression in cells depleted of GSK3β or the GSK3β-S9A mutant might indicate that GSK3β Ser9–mediated signaling is a crucial event to induce HO-1. In GSK3β-S9A cells, elevated basal GSH and NADPH were suggestive of an acquired tolerance to a precarious redox equilibrium due to a pool of unphosphorylatable GSK3β-S9A during selection.

Our data indicate that modulation of GSK3β activity could help preserve a balanced milieu and bioenergetic resources in severe oxidative injury by enhancing redox cycling or stimulation of GSH metabolism. This suggestion is supported by the data indicating that acute exposure to 4HPR in Y79 cells stimulates transient elevation of GSH and G6PD activity producing NADPH. NADPH, a cofactor of GR, is provided by enhanced glucose metabolism through the PPP, feeding the main antioxidant defense of the cell [55]. In line with this, glucose metabolism inhibitors repressed GSK3β phosphorylation and activation of the antioxidant response. Most importantly, glucose uptake was enhanced in 4HPR-resistant Y79 cells, suggesting that gradual stabilization of some of the antioxidant mechanisms activated upon acute exposure was functional to the selection leading to drug resistance. In 4HPR-resistant prostate clones, GSK3β phosphorylation, GSH, NAD(P)H, and HO-1 were elevated over the wt counterparts, implying that GSK3β Ser9 phosphorylation helps adapt to chronic redox and metabolic stress.

The function of GSK3β does not appear identical in retinoblastoma Y79 and in the prostate tumor cells analyzed here. One major difference might be the lack of caspase-8 in Y79 cells. In fact, GSK3β inhibits caspase-8 [6], whose activation was enhanced by LiCl or GSK3β gene silencing in CDDO-Me–induced prostate tumor cell death [10]. Opposing roles for GSK3 in cell-specific apoptotic programs, even in the same cell type, have been described [6,56]. In non-tumor cells, GSK3β phosphorylation propagates survival signals [6], and upon oxidative stress, the change in GSK3β phosphorylation varies depending on the cell type. For example, in kidney cells oxidative injury has been shown to mitigate GSK3β phosphorylation [57]. Accordingly, GSK3β inhibitors are used in clinical trials for neurodegenerative and cardiovascular disorders [6]. In several tumor models, GSK3β has been proposed as a tumor suppressor [58,59]; in contrast, GSK3β promotes tumorigenesis and development of ovarian, colon, liver, and pancreatic carcinomas. In prostate tumor cells, GSK3β inactivation is regarded as a potential therapeutic target [6,10].

The definition of the molecular events leading to persistent GSK3β phosphorylation and related redox remodeling requires further investigation. In Y79 cells, the result that the MEK inhibitor UO126 preserves cell viability and GSH in 4HPR-stressed cells indicates that pro-survival RSK signaling [44] is partly independent of ERK1/2 in this GSK3β-mediated regulatory network. Reciprocal regulation between GSK3β, ERK1/2, and RSK has been described [60,61] (Figure W5). In this context, hyperresponsive signaling and inadequate inactivation of ERK1/2 could contribute to the severity of cell damage; however, we cannot exclude that redox imbalance inactivates redox-sensitive protein phosphatases [62]. GSK3β has been shown to regulate Nrf2 activity, allowing up-regulation of antioxidant phase II genes including HO-1 and GSH regulators [17]. The transient induction of Nrf2 we observed is suggestive of a functional adjustment mediated by GSK3β phosphorylation, possibly by catalyzing the formation of a phosphodegron [17], to adapt GSH metabolism and HO-1 expression during acute oxidative stress.

Tumor-specific oxidative stress received attention as a promising therapeutic target [1,63]. Clinicopathologic traits of constitutive oxidative stress in retinoblastoma [64,65] have encouraged clinical studies of redox therapeutics, including As$_2$O$_3$. Chemopreventive derivatives of natural redox compounds like 4HPR, CDDO-Me, and PEITC have been shown to prompt homeostatic repair in early stages of carcinogenesis, while inducing tumor cell death [63,66]. The data presented here suggest that the energetic resources needed to counteract acute cell damage conflict with the high metabolic demand of frank tumor cells, eventually leading to tumor cell death. In contrast, adaptation to chronic stress in drug-resistant cells stably engages some of the same homeostatic antioxidant mechanisms (Figure W5). Cell stress evokes interweaved events, after which the severity of damage or successful channeling of cytoprotective signaling and effector mechanisms prevails. In this context, our novel observations on drug resistance assign a role for GSK3β in redox remodeling to determine cell fate.

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