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p38^{MAPK} and ERK1/2 dictate cell death/survival response to different pro-oxidant stimuli *via* p53 and Nrf2 in neuroblastoma cells SH-SY5Y

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ABSTRACT

Redox changes are often reported as causative of neoplastic transformation and chemoresistance, but are also exploited as clinical tools to selectively kill tumor cells. We previously demonstrated that gastrointestinal-derived tumor histotypes are resistant to ROS-based treatments by means of the redox activation of Nrf2, but highly sensitive to disulfide stressors triggering apoptosis *via* the redox induction of Trx1/p38^{MAPK}/p53 signaling pathway.

Here, we provide evidence that neuroblastoma SH-SY5Y has a complete opposite behavior, being sensitive to H_2O_2, but resistant to the glutathione (GSH)-oxidizing molecule diamide. Consistent with these observations, the apoptotic pathway activated upon H_2O_2 treatment relies upon Trx1 oxidation, and is mediated by the p38^{MAPK}/p53 signaling axis. Pre-treatment with different antioxidants, pharmacological inhibitor of p38^{MAPK}, or small interfering RNA against p53 rescue cell viability. On the contrary, cell survival to diamide relies upon redox activation of Nrf2, in a way independent on Keap1 oxidation, but responsive to ERK1/2 activation. Chemical inhibition of GSH neo-synthesis or ERK1/2 phosphorylation, as well as overexpression of the dominant-negative form of Nrf2 sensitizes cells to diamide toxicity. In the searching for the molecular determinant(s) unifying these phenomena, we found that SH-SY5Y cells show high GSH levels, but exhibit very low GPx activity. This feature allows to efficiently buffer disulfide stress, but leaves them being vulnerable to H_2O_2-mediated insult. The increase of GPx activity by means of selenium supplementation or GPx1 ectopic expression completely reverses death phenotype, indicating that the response of tumor cells to diverse oxidative stimuli deeply involves the entire GSH redox system.

1. Introduction

Since its first identification in 1985 by Cerutti [1], the intimate relationship between pro-oxidant conditions and cancer development has been widely investigated. Today, non-physiological alterations of the intracellular redox state are well established hallmarks of tumor biology, due to their implication in several transforming events, such as self-sufficiency in growth signals [2], apoptosis evading [3], sustained angiogenesis [4,5], autophagy [6,7] and tissue invasion [8,9]. This has been exploited by chemotherapeutic strategies based on the use of ROS-generating molecules (*e.g.*, 2-metoxyestradiol and anthracyclins) to selectively kill transformed cells [10–12]. In order to optimize therapeutic design and reduce possible development of resistance phenomena, research is attempting to deeply investigate the mechanisms underlying such an intrinsic alteration of redox homeostasis in cancer cells. An intense metabolism and multiple mutations in proteins controlling redox homeostasis are important causative determinants of the high levels of ROS [13], which, in turn, induce a selective pressure to survive under these extreme conditions. In this scenario, the modulation of glutathione (GSH), which represents one of the major indicators of the intracellular redox environment, as well as the control on transcriptional activity of redox sensitive factors is emerging as possible strategies [12]. Among the transcription factors able to sense oxidative stress, the nuclear erythroid factor 2 (NE-F2)-related factor 2 (Nrf2) and p53 are well-characterized examples [14]. Besides the canonical

Abbreviations: AMS, 4-acetamido-4-maleimidylstilbene-2,2-disulfonic acid; ARE, antioxidant response elements; BSO, buthionine sulfoximine; DMSO, dimethyl sulfoxide; DMTU, dimethylthiourea; DN Nrf2, dominant negative Nrf2 protein; DNP, 2,4-dinitrophenylhydrazine; ERK1/2, extracellular signal related kinase 1 and 2; GI-GPx, gastrointestinal isoform of glutathione peroxidase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSHest, reduced glutathione ethyl ester; GSSG, glutathione disulfide; HO-1, heme oxygenase-1; JNK, c-Jun-NH_2-terminal kinase; Keap1, kelch-like ECH-associated protein 1; MAPK, mitogen activated protein kinase; NAC, N-cetylcysteine; Nrf2, nuclear erythroid factor 2 (NE-F2)-related factor 2; p62/SQSTM1, protein of 62 kDa sequestrosoma1; PARP, polyADP-ribose polymerase; ROS, reactive oxygen species; Trx1, thioredoxin 1.

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regulation of Nrf2 mediated by Kelch-like ECH-Associated Protein 1 (Keap1), recent pieces of evidence indicate that Nrf2 and p53 are functional competitors for the same DNA promoter regions [15], thereby identifying a new sophisticated level of control of Nrf2 trans-activation. In particular, it has been indicated that p53 binds to the antioxidant response elements (ARE), inhibiting, in such a way, Nrf2 transcriptional activity of the battery of genes involved in the antioxidant defense. The high incidence of p53 mutations in human tumors together with its pivotal role in protecting from DNA damage [16,17] and in the induction of apoptosis [18] makes the crosstalk between these transcription factors a very intriguing subject of tumor biology that deserves to be investigated in depth. Indeed, the involvement of Nrf2 in resistance development during cancer treatment has been extensively reported [19,20] and a role for some dysfunctions in the Nrf2/Keap1 interaction has been also suggested to allow cancer progression [21]. In this regard, it is also worth to mention that a novel redox-independent activation of Nrf2 has been shown to proceed through the interaction of Keap1 with p62/SQSTM1 [22,23]. The formation of Keap1/p62 heterodimer does not involve any redox alteration of Keap1 cysteines. However, it allows Nrf2 to be released and to accumulate into the nucleus, by means of a direct competition between Keap1/p62 and Keap1/Nrf2 complexes for Keap1 binding. The tumorigenic role of Nrf2 has been also very recently reported by DeNicola et al. [24], who demonstrated that the overexpression of the oncogenic mutants of Ras, Raf and Myc results in the up-regulation of Nrf2 and in the activation of the antioxidant/detoxifying systems downstream of it. The great impact that this finding might have in cancer research relies upon the evidence that Nrf2 can represent the converging point of different oncogenic pathways, in a way completely independent on the mutagenic events causing cell transformation.

In this context, we have previously demonstrated that oxidative stress of different nature (ROS *versus* disulfide stress) can evocate opposite responses (cell death or survival), in the gastric adenocarcinoma cell line AGS, by means of the redox activation of specific signaling pathways [14]. In particular, we identified in the redox-dependent dissociation of Keap1/Nrf2 complex the event responsible for the resistance to ROS-mediated insults, and in the redox-activation of trx1/p38^{MAPK}/p53 signaling axis the pathway governing the induction of the apoptosis upon treatment with disulfide stressors (*e.g.*, diamide). Here, we try to extend the knowledge of cell response to oxidative stress and provide evidence that GSH concentration and glutathione peroxidase 1 (GPx1) activity are the main determinants of tumor cell response to disulfide molecules and H_2O_2, respectively. In particular, we demonstrate that, in contrast to AGS cells, neuroblastoma SH-SY5Y have high intracellular levels of GSH, which allow them to directly dampen disulfide stressors-mediated insult *via* ERK/Nrf2 signaling pathway. Nevertheless, the lack of a significant GPx activity renders them sensitive to H_2O_2 treatment and induces apoptosis in a p53-dependent manner.

2. Materials and methods

2.1. Materials

Diamide, dimethyl sulfoxide (DMSO), dimethylthiourea (DMTU), H_2O_2, sodium selenite (Na_2SeO_3), propidium iodide, goat anti-mouse and anti-rabbit IgG (H + L)-horseradish peroxidase conjugate, buthionine sulfoximine (BSO) and N-acetylcysteine (NAC) were from Sigma (St. Louis, MO); GSH and GSSG were from Roche Applied Science (Monza, Italy); Oxyblot detection kit was from Intergen (Purchase, NY); nitrocellulose membrane was from Bio-Rad Laboratories (Hercules, CA); Alexa Fluor488-conjugated secondary antibody was from Molecular Probes (Eugene, OR); ChemiGlow chemoluminescence substrate was from Alpha Innotech Corporation (San Leandro, CA). All other chemicals were from Merck (Darmstadt, Germany).

2.2. Cell cultures and transfection

Human neuroblastoma cells SH-SY5Y and gastric adenocarcinoma AGS were purchased from the European Collection of Cell Culture and grown at 37 °C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's-F12 medium (DMEM-F12) medium or F12, respectively, supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 0.1% penicillin/streptomycin (Lonza, Milan, Italy). 24 h after plating, SH-SY5Y cells were transfected by electroporation using a GenePulser xcell system (Bio-Rad). Downregulation of p53 was obtained by means of transfection with a small interference RNA (siRNA) against p53 [25]. Protein overexpression was obtained by means of transfection with: (i) pmaxFP^{TM}-Green-C vector containing the dominant negative Nrf2 protein (DN-Nrf2) [25]; (ii) pcDNA3 vector containing the wild-type form of mouse glutathione peroxidase 1 (GPx1) kindly provided by Prof. Regina Brigelius-Flohé, Department of Biochemistry of Micronutrients, German Institute of Human Nutrition Potsdam-Rehbruecke, Nuthetal, Germany. Cells transfected with siRNA duplex not sharing homology with any other human mRNAs (siScr), or empty pmaxFP^{TM}-Green-C and pcDNA3 vectors were used as control.

2.3. Treatments

Diamide solution (10 mM) was prepared in DMSO, whereas H_2O_2 was dissolved in water to reach the concentration of 100 μM. All solutions were prepared just before the experiments and treatments were done for 1 h in serum-containing medium. Then the medium was removed, cells were washed and let growing in fresh medium. As control, equal volumes of DMSO or water were added to untreated cells. BSO was used at the concentration of 1 mM, added 12 h before treatments and maintained throughout the experiments. DMTU, GSHest and NAC were supplemented to culture medium at the concentration of 20, 10 and 5 mM, respectively, added 1 h before treatments and maintained throughout the experiments. Na_2SeO_3 was used to induce GPx expression. In particular it was dissolved in water and added to cell medium at the final concentration of 25 or 50 nM and maintained for two weeks. Cell permeable ERK 1/2, JNK and p38^{MAPK} inhibitors, namely U0126, SP600125 and SB203580, (Calbiochem-Novabiochem, La Jolla, CA) were added at concentration of 0.26, 10 and 15 μM, for 1 h before the addition of diamide or H_2O_2, maintained during treatment, and re-added during the recovery phase.

2.4. Analysis of cell viability and apoptosis

After 24 h of recovery, adherent and detached cells were combined and stained with 50 μg/ml propidium iodide prior to analysis by a FACScalibur instrument (BD Biosciences, San Josè, CA). Apoptotic cells were evaluated by calculating peak areas of hypodiploid nuclei (sub-G1) [26]. Alternatively, cells were counted after Trypan blue staining by optic microscope.

2.5. Western blot analyses

Total and nuclear extracts were obtained as previously reported [25], electrophoresed by SDS-PAGE and blotted onto nitrocellulose or PVDF membrane (Bio-Rad). Polyclonal anti phospho-p38^{MAPK} (Thr180/Tyr182), anti phospho-ERK1/2-p42/44 (Cell Signaling Technology, Beverly, MA), anti-Nrf2, anti-Bax, anti-JNK, anti-p38^{MAPK}, anti-histone H2B, anti ERK1/2, anti Trx1 (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal anti-p53 and anti-tubulin (Sigma), anti-heme oxygenase-1 (HO-1) (BD Biosciences),

anti polyADP-ribose polymerase (PARP), anti-phospho-JNK (Santa Cruz Biotechnology), anti-caspase9 and caspase3 (Cell Signaling Technology) were used as primary antibodies. The specific protein complex, formed upon incubation with specific secondary antibodies, was identified using a Fluorchem Imaging system (Alpha Innotech, M-Medical, Milano, Italy) after incubation with Chemi-Glow chemiluminescence substrate (Alpha Innotech).

2.6. Redox Western blot

Trx1 redox state was analyzed as previously described [14]. Briefly, cells were treated with cold TCA at final concentration of 10% for 30 min at 4 °C. Proteins were then precipitated at 12,000 × *g* for 10 min and pellets incubated for 30 min in cold acetone at 4 °C. Further, pellets were dissolved in 20 mM Tris/HCl, pH 8.0 containing 15 mM AMS (4-acetamido-4-maleimidylstilbene-2,2-disulfonic acid, Molecular Probes) and incubated at room temperature for 3 h to allow modifying free thiols. Trx1 redox forms were then separated on 4–12% bis–Tris MES gel in non-reducing loading buffer and blotted onto nitrocellulose or PVDF. Polyclonal anti-Trx1 (SantaCruz Biotechnology) was used as primary antibody.

2.7. Measurement of glutathione, protein carbonyls and GPx activity

Intracellular GSH and GSSG were assayed by HPLC as previously described [27]. Carbonylated proteins were detected using the Oxyblot detection kit (Intergen). Briefly, 20 μg of proteins were reacted with 2,4-dinitrophenylhydrazine (DNP) for 15 min at 25 °C. Samples were resolved on 12% SDS-PAGE and DNP-derivatized proteins were identified by immunoblot using an anti-DNP antibody. GPx activity was measured spectrophotometrically, using H_2O_2 as substrate, by means of a glutathione reductase-coupled reaction, in which the decrease of NADPH is considered proportional to GPx-mediated H_2O_2 reduction. GPx activity was therefore expressed as nmol of NADPH consumed min^{-1} mg $prot^{-1}$.

2.8. Fluorescence microscopy

Cells were cultured on coverslips, fixed with 4% paraformaldehyde and permeabilized. Monoclonal anti-ser-139-phosphorylated histone H2A.X (Upstate Biotechnology), and successively probed with an Alexa Fluor488-conjugated secondary antibody. To visualize nuclei with the cell permeable DNA-specific dye Hoechst 33342 (Calbiochem-Novabiochem). Images were digitized either with a Cool Snap video camera connected to a Nikon Eclipse TE200 fluorescence microscope.

2.9. Protein determination

Proteins were determined by the method of Lowry et al. [28].

2.10. Data presentation

All experiments were done at least three different times unless otherwise indicated. The results are presented as means ± SD. Statistical evaluation was conducted by ANOVA, followed by Bonferroni's test. Differences were considered to be significant at $p < 0.05$.

3. Results and discussion

3.1. SH-SY5Y cells show different sensitivity to different pro-oxidant stimuli

We previously identified that the gastric adenocarcinoma cells AGS were highly resistant to ROS-producing drugs, but sensitive to treatment with thiol-oxidizing chemicals. In order to generalize this observation, we selected another tumor histotype, the neuroblastoma SH-SY5Y, and treated the cells with H_2O_2, or with diamide, which acts by oxidizing cellular sulfhydryls, without producing ROS directly. Cells were treated with 100 and 200 μM H_2O_2 or diamide: concentrations that are in the range of those usually employed in studies of toxicology, and that, also, overlap those we previously used on AGS cells [14]. After 1 h-incubation, H_2O_2 and diamide were removed, cells washed and let growing in fresh medium, and the extent of apoptosis analyzed cytofluorometrically after 24 h-recovery. It is worth noting that, owing to the different cellular metabolism of H_2O_2 and diamide, the strength of oxidative stress produced upon the administration of

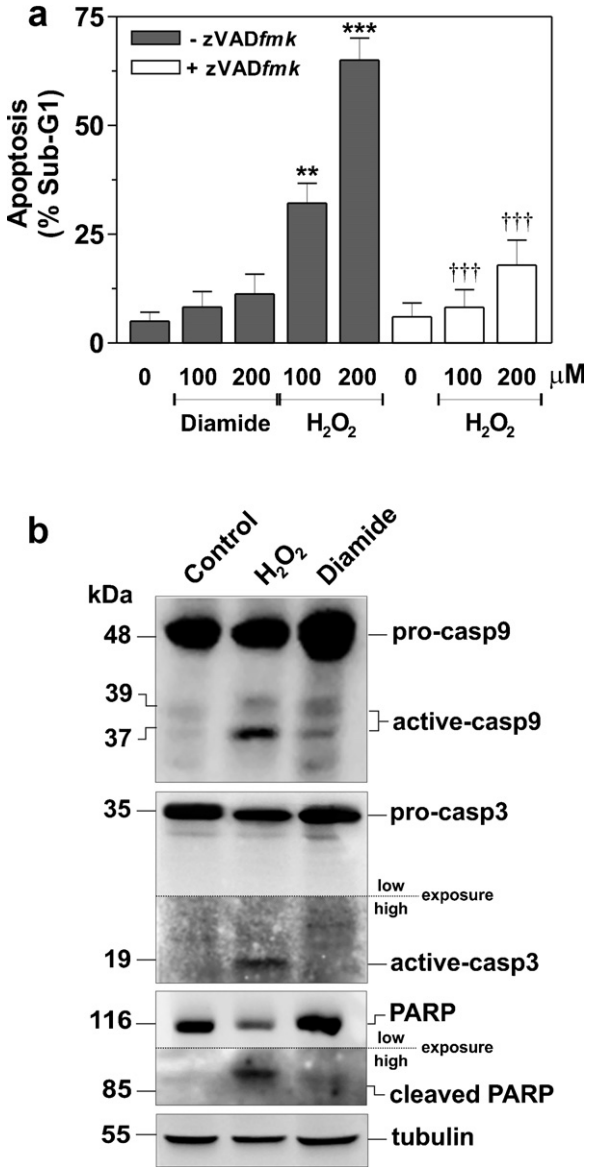

Fig. 1. Hydrogen peroxide, but not diamide, triggers caspase-dependent apoptosis in SH-SY5Y cells. (a) SH-SY5Y cells were treated with 100 or 200 μM H_2O_2 or diamide, in the presence or absence of the pan-caspase inhibitor zVAD*fmk* after 24 h. Apoptosis (*SubG1*) was cytofluorometrically analyzed upon propidium iodide staining, with cells incubated with DMSO (*time* 0) used as control. Data are expressed as % of SubG1 (apoptotic) cells and represent the mean ± SD of $n = 4$ independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$ (H_2O_2 *versus* control). $^{\dagger\dagger\dagger}p < 0.001$ (−zVAD*fmk versus* +zVAD*fmk*). (b) After 18 h-treatment, 30 μg of total protein extract was loaded onto each lane for the detection of the full length and cleaved caspase9, caspase3 and PARP. Tubulin was used as loading control. Due to the different specificity of the anti-caspase3 and anti-PARP antibodies to recognize the cleaved and full-length form of the proteins, two different exposures (1 min, *low*; 10 min, *high*) are shown.

the two drugs is not comparable in terms of absolute concentrations. However, data reported in Fig. 1a indicated that SH-SY5Y underwent caspase-dependent apoptosis (subG1) in a dose-dependent manner when incubated with H_2O_2, with the percentage of apoptosis ranging from 35% to 70%. By contrast, they were much less sensitive to diamide treatment. This dichotomy in cell response was also confirmed by Western blot analyses. In particular, SH-SY5Y cells treated with H_2O_2 showed the proteolysed form of caspase9, caspase3 and PARP after 18 h of recovery (Fig. 1b). On the contrary, no significant increase in the immunoreactivity of these proteins was observed upon recovery from diamide treatment, indicating that H_2O_2, but not diamide, induced the intrinsic pathway of apoptosis. Such difference in cell fate was also confirmed by the occurrence of oxidative damage to proteins and DNA, with a marked increase of protein carbonyls and phospho-H2A.X-positive nuclear foci evident only upon treatment with H_2O_2 (Supplementary Figure 1).

Supplementary material related to this article found, in the online version, at doi:10.1016/j.bcp.2012.02.003.

3.2. H_2O_2 and diamide specifically modulate Nrf2 and p53 transcription factor

Consistent with our previous results, we investigated whether the different cell response to H_2O_2 and diamide relied upon the activation of p53 or Nrf2 by Western blot analyses of their nuclear levels. Fig. 2a shows that the nuclear fraction of p53 increased time-dependently after 3 h-recovery from H_2O_2 administration, whereas no significant change occurred at the same time of recovery from diamide incubation. On the contrary, Nrf2 increased rapidly after diamide treatment but transiently accumulated also in response to H_2O_2, confirming data from the literature [29] and our previous observations of a direct sensitivity of Nrf2 to H_2O_2 [14]. We then verified the acquisition of Nrf2 or p53 transcriptional

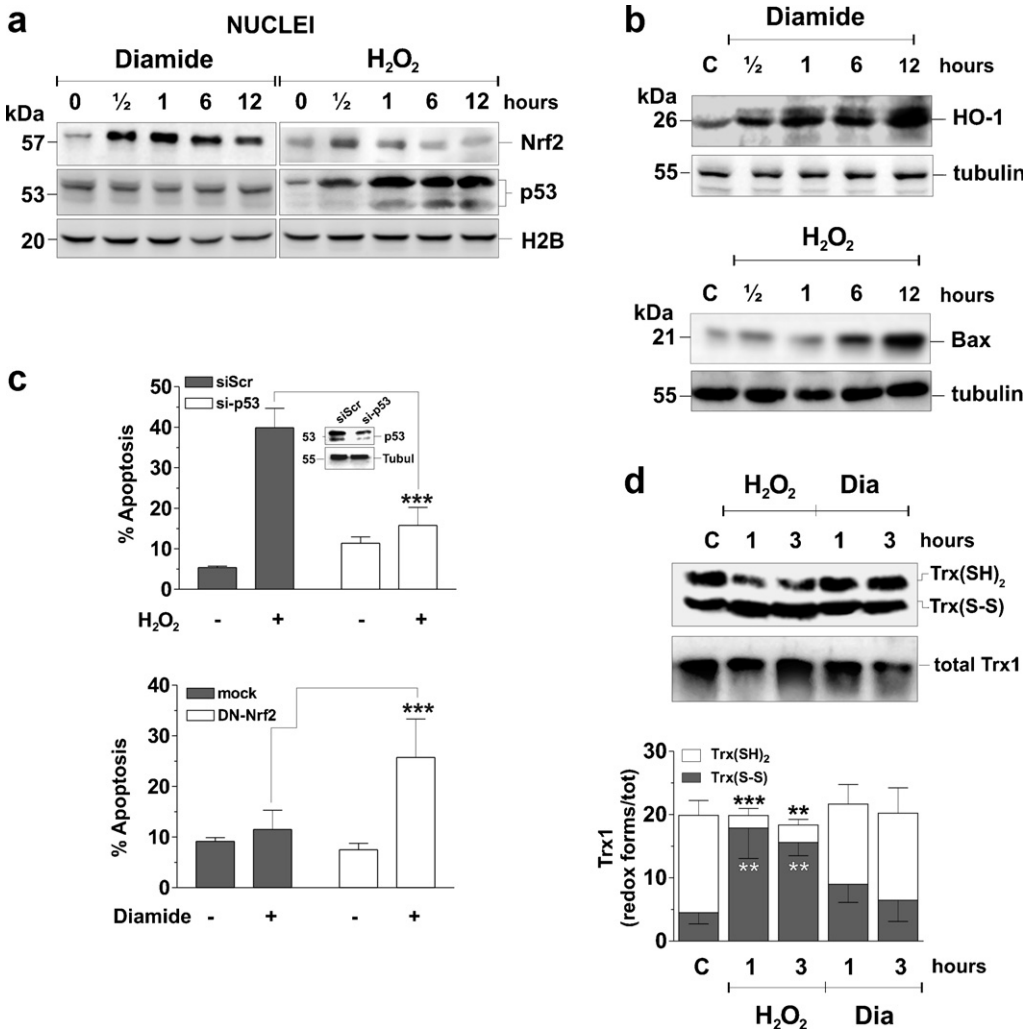

Fig. 2. p53 mediates apoptosis upon H_2O_2 treatment, whereas Nrf2 is the pro-survival factor involved in response to diamide. (a) SH-SY5Y cells were treated with 100 μM H_2O_2 or diamide. At the indicated time points, 20 μg of nuclear enriched extracts was loaded onto each lane for the detection of Nrf2 and p53. Histone 2B (H2B) was used as loading control. (b) Alternatively, 30 μg of total protein extract was loaded onto each lane for the detection of HO-1 and Bax. Tubulin was used as loading control. (c) SH-SY5Y cells were transfected with siRNA against p53 (*si-p53*) or with a scrambled sequence (*siScr*). The efficiency of transfection was evaluated by Western blot analyses of p53 and is shown in the inset. After 12 h from transfection cells were treated with 100 μM H_2O_2. Similarly, cells were transfected with the dominant negative mutant of Nrf2 (*DN-Nrf2*) or with an empty vector (*mock*). After 48 h from transfection, cells were treated with 100 μM diamide. After 24 h, apoptosis (*SubG1*) was cytofluorometrically analyzed. Data are expressed as % of apoptosis and represent the mean ± SD of $n = 4$ independent experiments. $^{***}p < 0.001$. (d) SH-SY5Y cells were treated with 100 μM H_2O_2 or diamide. At the indicated time points, cells were precipitated in TCA and dissolved in 20 mM Tris/HCl, pH 8.0 containing AMS to allow modifying free thiols. Trx1 redox forms were then separated on 4–12% SDS-PAGE in non-reducing conditions. Reduced (AMS-bound) Trx1 [*Trx(SH)$_2$*] and oxidized Trx1 [*Trx(S-S)*] are shown. Total Trx1 was used as control. Density of reduced and oxidized Trx1 (reported below the immunoblots) was calculated using the software Quantity one (Bio-Rad) and reported as arbitrary units. Data are expressed as means ± SD of $n = 3$ independent experiments. $^{***}p < 0.001$; $^{**}p < 0.01$.

activity by Western blot analyses of the expression levels of their target genes, heme-oxygenase 1 (HO-1) and Bax, respectively. Fig. 2b shows that HO-1 increased rapidly during the recovery from diamide incubation, whereas Bax accumulated only after 6–12 h of recovery from H_2O_2 administration, suggesting that survival response to diamide was mediated by Nrf2, whereas H_2O_2-induced apoptosis relied on p53 activation. To corroborate these hypotheses, we transfected SH-SY5Y cells with an siRNA against p53 or, alternatively, with the dominant negative form of Nrf2 (DN-Nrf2) and analyzed cytofluorometrically the extent of apoptosis. Fig. 2c shows that p53 knocking down significantly decreased the percentage of sub-G1 (apoptotic) cells induced by H_2O_2, while DN-Nrf2 transfected cells became sensitive to diamide.

Given the redox-dependent activation of p53 and Nrf2 *via* Trx1 and Keap1 oxidation [14], we evaluated the redox state of Trx1 and Keap1 by redox Western blot. Fig. 2d indicates that a marked decrease in reduced/oxidized ratio of Trx1 occurred only during the recovery from H_2O_2 incubation, whereas diamide did not induce any visible change. Conversely, neither H_2O_2 nor diamide alters Keap1 redox state (data not shown), suggesting that other Keap1-independent mechanism(s) should be responsible for the activation of Nrf2.

3.3. $p38^{MAPK}$ and ERK1/2 are the mediators of cell response to H_2O_2 and diamide

p53 activation has been reported to be responsive to $p38^{MAPK}$ downstream of Trx1 oxidation. Similarly, it has been described that phosphorylation of Nrf2 by a series of kinases (*e.g.*, ERK 1/2) affects its fate and distribution [30–32]. In particular, phosphorylation of Nrf2 on Ser^{40} is required for Nrf2 to be released from Keap1, whereas mutant Nrf2 at Ser^{40} remained bound to Keap1 [32]. However, it is worthwhile mentioning that H_2O_2 treatment has been reported to broadly activate ERK, JNK, and $p38^{MAPK}$ in human colon cancer SW620 [33] and in SH-SY5Y cells [34], and a previous study also demonstrated that both diamide and H_2O_2 activate ERK and $p38^{MAPK}$ *in vitro* [35]. In the light of these observations, we analyzed the activation state of MAPK by Western blot analyses of the basal and phospho-active forms of ERK1/2, JNK and $p38^{MAPK}$ upon recovery from incubation with H_2O_2 and diamide. Fig. 3a and b show that ERK1/2 was the only member always activated upon treatment with 100 μM diamide, whereas H_2O_2 induced the activation of all the enzymes analyzed, although with different modalities. In particular, ERK1/2 appeared phospho-activated only at 3 h of treatment, and rapidly returned to the control levels, thus

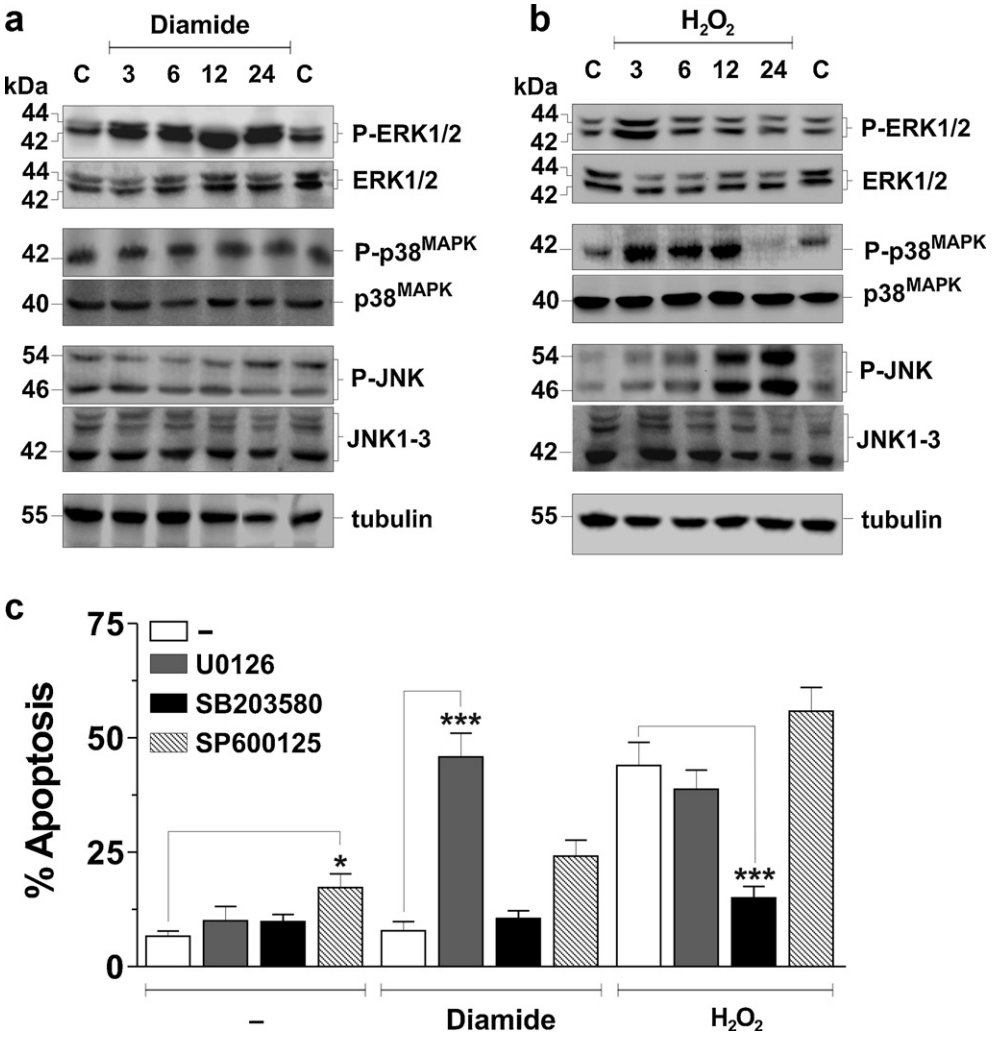

Fig. 3. ERK and $p38^{MAPK}$ are the upstream kinases involved in the modulation of cell viability. SH-SY5Y cells were treated with diamide (a) or H_2O_2 (b). At the indicated time points, 20 μg of total protein extract was loaded onto each lane for the detection of basal and phospho-active forms of ERK1/2, JNK and $p38^{MAPK}$. Tubulin was used as loading control. (c) SH-SY5Y cells were treated with diamide or H_2O_2 in the presence or absence of the ERK1/2 inhibitor U0126, JNK inhibitor SP600125, or $p38^{MAPK}$ inhibitor SB203580. After 24 h, apoptosis (*SubG1*) was cytofluorometrically analyzed. As control (−) cells were incubated with DMSO. Data are expressed as % of apoptosis and represent the mean ± SD of $n = 4$ independent experiments. $^{***}p < 0.001$, $^{*}p < 0.05$.

resembling the kinetics of Nrf2 nuclear translocation in response to H_2O_2 exposure. $p38^{MAPK}$ became rapidly and completely phosphorylated already at 3 h to be restored only after 24 h-treatment. Conversely, JNK phosphorylation seemed to be delayed with respect to $p38^{MAPK}$, as it reached the maximum values only at 12 and 24 h-treatment. To verify the exact role of each MAPK in the cell response to the different pro-oxidant insults, we treated SH-SY5Y cells with H_2O_2 or diamide, in the presence of the pharmacological inhibitors of ERK1/2, JNK and $p38^{MAPK}$, namely U0126, SP600125 and SB203580, respectively. Cytofluorometric analyses performed after 24 h-recovery show that ERK1/2 inhibition resulted in a significant increase of

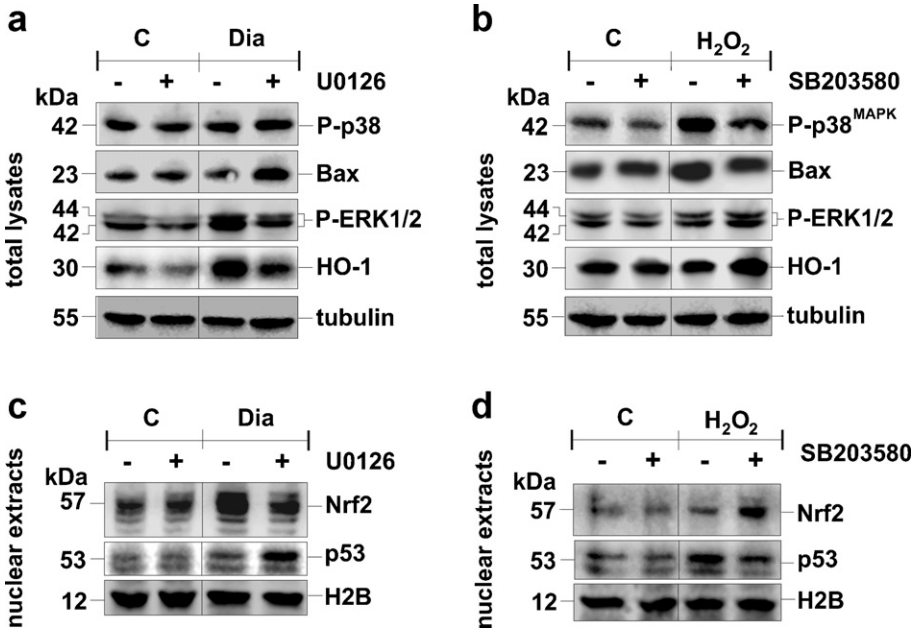

Fig. 4. ERK and $p38^{MAPK}$ are the activating kinases of Nrf2 and p53, and their induction is mutually exclusive.
SH-SY5Y cells were treated for 3 h with 100 μM diamide or H_2O_2. Twenty μg of total protein extract (a and b) was loaded onto each lane for the detection of phospho-$p38^{MAPK}$ and phospho-ERK1/2, Bax, HO-1, whereas 30 μg of nuclear enriched extract (c and d) was loaded onto each lane for the detection of Nrf2 and p53. Tubulin and H2B were used as loading control of total and nuclear extract, respectively.

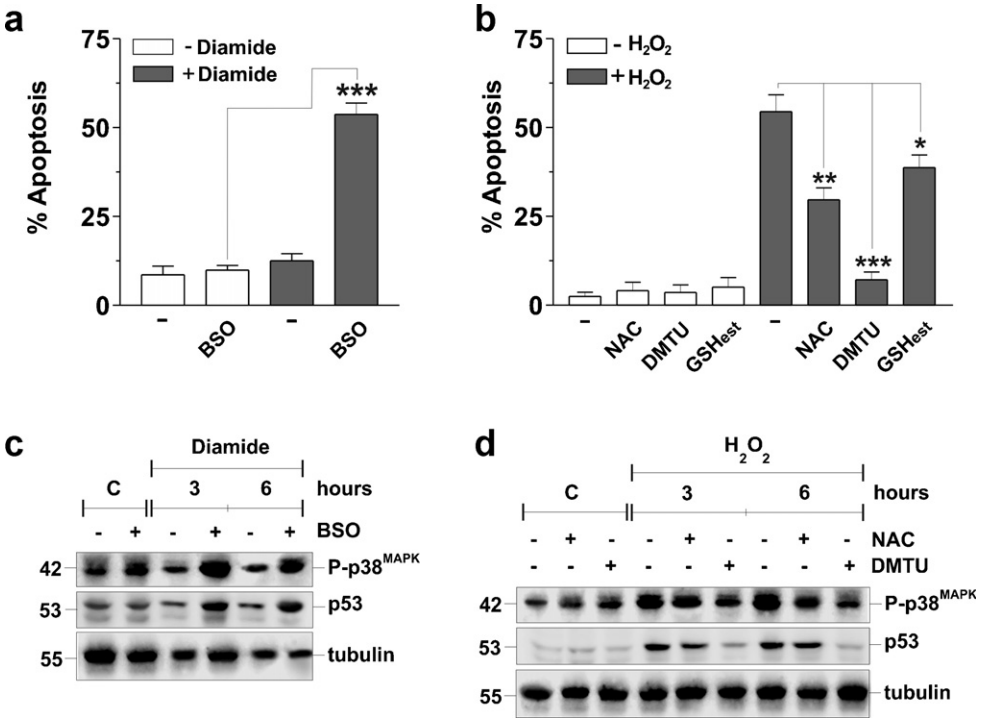

Fig. 5. Glutathione availability affects diamide-mediated effects, but not H_2O_2 toxicity. SH-SY5Y cells were treated with (a) 100 μM diamide in the presence or absence of BSO or, alternatively (b) with 100 μM H_2O_2 in the presence or absence of the antioxidants NAC and DMTU, or with GSH-ethyl ester (GSHest). After 24 h, apoptosis (*SubG1*) was cytofluorometrically analyzed. Data are expressed as % of apoptosis and represent the mean ± SD of $n = 4$ independent experiments. $^{*}p < 0.01$; $^{**}p < 0.01$; $^{***}p < 0.001$. SH-SY5Y cells were treated with 100 μM diamide, in the presence or absence of BSO (c), or H_2O_2, in the presence or absence of NAC and DMTU (d). At the indicated time points, 20 μg of total protein extract was loaded onto each lane for the detection of phospho-$p38^{MAPK}$ and p53. Tubulin was used as loading control.

apoptosis when the cells were treated with diamide, whereas $p38^{MAPK}$ inhibition, carried out upon H_2O_2 incubation, yielded a decrease of subG1 percentages (Fig. 3c), indicating that they were the master regulators of cell survival and cell death observed in our experimental conditions. Interestingly, JNK inhibitor did not result in any significant modulation of cell response to both H_2O_2 and diamide, but altered *per se* the viability of SH-SY5Y (Fig. 3c) and cell cycle progression by blocking the cells in G2/M phase (data not shown). This evidence perfectly correlates with our previous observations performed in the same cell line, and argue for an active role of JNK in the replicative capability of neuroblastoma cells [36]. On the basis of these results, we focused on ERK1/2 and p38MAPK and analyzed whether their inhibition could impact on signal transduction leading to cell death or survival. Western blot analyses shown in Fig. 4a indicate that ERK1/2 inhibition induced a decrease in the expression level of HO-1 and, concomitantly, in the nuclear content of Nrf2 in cells treated with diamide (Fig. 4c). On the contrary, $p38^{MAPK}$ inhibition resulted in a marked reduction of both Bax level (Fig. 4b) and nuclear accumulation of p53 (Fig. 4d). It is worth noting that, the unresponsiveness of Nrf2 to diamide caused by ERK1/2 inhibition, was associated with a visible accumulation of p53 into the nuclear compartment (Fig. 4c), whereas a marked increase of nuclear Nrf2, upon H_2O_2 treatment, was clearly evident upon the inhibition of $p38^{MAPK}$ (Fig. 4d).

3.4. GSH and GPx are the redox modulators of cell response to H_2O_2 and diamide

Since we previously found that changes of GSH redox state were tightly associated with the pattern of cell response (death *versus* resistance) [14], we investigated this issue also in SH-SY5Y cells upon 12 h-incubation with 1 mM BSO, the specific inhibitor of GSH neo-synthesis, before adding diamide. Apoptotic extent was then evaluated cytofluorometrically after 24 h of recovery. Data reported in Fig. 5a show that GSH depletion rendered the cells sensitive to diamide with apoptosis values reaching 50%. By contrast, we enriched the intracellular antioxidant pool by means of 1 h-incubation with: 10 mM of the ethyl ester form of GSH (GSHest), 5 mM NAC, or 20 mM DMTU. Under these conditions, SH-SY5Y cells were protected against H_2O_2-mediated apoptosis, although at different extents (Fig. 5b). Indeed, DMTU, which acts as direct H_2O_2 scavenger, completely rescued cells from death, whereas NAC and GSHest, which are canonical thiol antioxidants, partially contributed to the pro-survival response, with a decrease in the percentage of apoptosis of only 45% and 29%, with respect to H_2O_2-treated cells. We then selected DMTU and NAC as the most effective anti-apoptotic compounds and performed Western blot analyses of the pro-apoptotic axis $p38^{MAPK}$/p53. Fig. 5c shows that phospho-active $p38^{MAPK}$ and p53 increased in GSH-depleted cells when incubated with diamide, whereas they decreased in

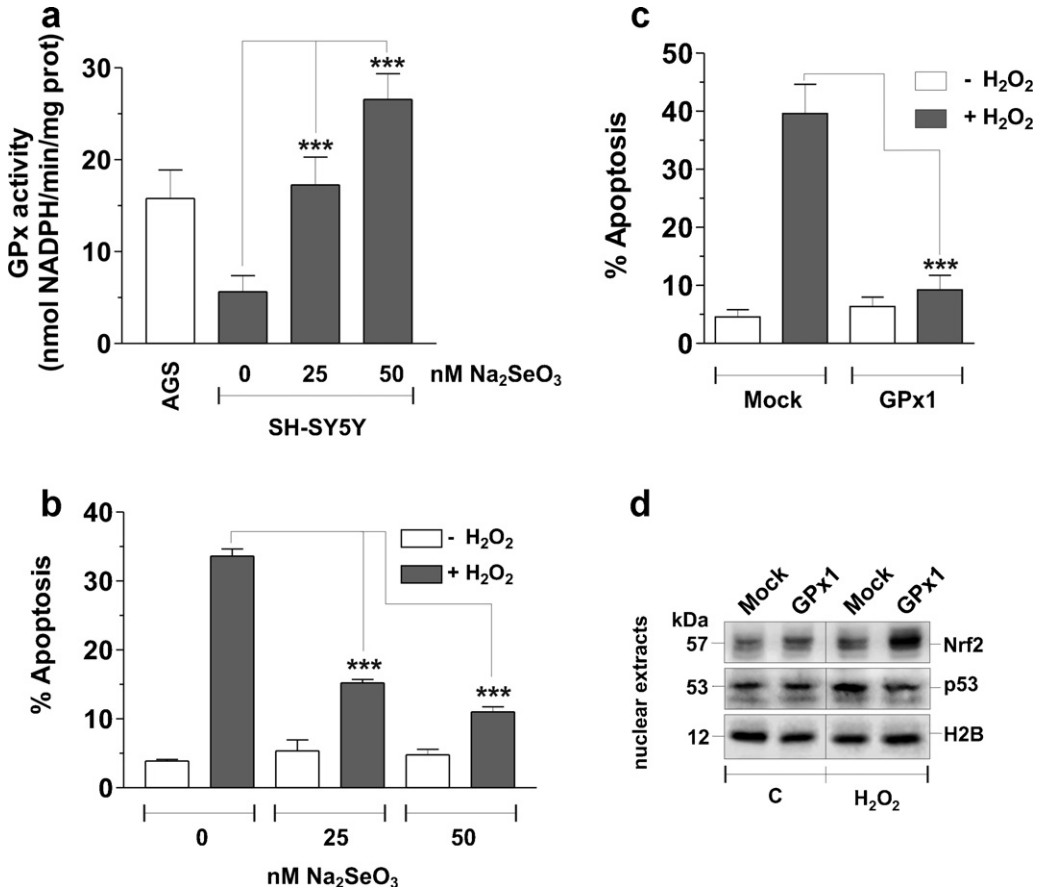

Fig. 6. GPx activity buffers H_2O_2 toxicity independently on GSH concentration. (a) SH-SY5Y cells were let growing for two weeks with different concentrations of Na_2SeO_3, and GPx activity was measured spectrophotometrically. AGS were used as positive control. Data are expressed as nmol of NADPH consumed min^{-1} mg $prot^{-1}$ and represent the mean ± SD of $n = 6$ independent experiments. $^{***}p < 0.001$. (b) After two week-administration of 25 or 50 nM Na_2SeO_3, SH-SY5Y cells were treated with 100 μM H_2O_2. After 24 h, apoptosis (*SubG1*) was cytofluorometrically analyzed. Data are expressed as % of apoptosis and represent the mean ± SD of $n = 4$ independent experiments. $^{***}p < 0.001$. (c) SH-SY5Y cells were transfected with a pCDNA3 vector containing the WT form of GPx or with an empty vector (*mock*). After 48 h from transfection cells were treated with 100 μM H_2O_2. After 24 h, apoptosis (*SubG1*) was cytofluorometrically analyzed. Data are expressed as % of apoptosis and represent the mean ± SD of $n = 4$ independent experiments. $^{***}p < 0.001$. (d) SH-SY5Y cells were treated for 3 h with 100 μM H_2O_2. Thirty micrograms of nuclear enriched extract was loaded onto each lane for the detection of Nrf2 and p53. H2B was used as loading control.

H_2O_2-treated cells when pre-incubated with DMTU and NAC, in a manner resembling their anti-apoptotic effect (Fig. 5d).

To comprehend the redox bases underlying the different sensitivity to H_2O_2 and diamide in SH-SY5Y cells with respect to what previously reported for AGS, we measured GSH content in both cells lines. Taking into account a cell volume of ~10 μl/mg protein [37], we found that the molar concentration of GSH calculated in SH-SY5Y cells was significantly higher than in AGS (7.65 ± 0.78 mM *versus* 1.65 ± 0.35 mM, $n = 10$, $p < 0.001$). This result nicely correlated with the resistance of the neuroblastoma cell line to diamide toxicity; nevertheless, it did not explain SH-SY5Y sensitivity to H_2O_2. Since AGS cells have been previously reported to be equipped with the additional gastrointestinal isoform of GPx (GPx2 or GI-GPx) [38], we wondered to evaluate differences in GPx activity between AGS and SH-SY5Y. Fig. 6a shows that the basal GPx activity measured in AGS cells was three-fold higher with respect to that evaluated in SH-SY5Y. However, two week-incubation with 25 or 50 nM Na_2SeO_3, which is usually employed to induce the expression of GPx, increased peroxidatic activity in a dose-dependent manner, up to values comparable, or even higher, to those measured in AGS (Fig. 6a). We then treated SH-SY5Y cells with H_2O_2 and analyzed cytofluorometrically the extent of apoptosis. As shown in Fig. 6b, SH-SY5Y grown in the presence of Na_2SeO_3 were resistant to H_2O_2-mediated apoptosis, and the rescue of cell viability directly correlated with the concentration of Na_2SeO_3 used to up-regulate GPx activity. To confirm these data, we also overexpressed GPx1, the most abundant form of GPx, which preferentially catalyzes the reduction of H_2O_2. After two day-incubation with Na_2SeO_3, necessary to provide selenium to the new-synthesized proteins, GPx1 activity was measured to be 40.75 ± 2.15 nmol NADPH × min × mg of total protein, therefore we treated SH-SY5Y cells with H_2O_2 and analyzed the extent of apoptosis cytofluorometrically. Fig. 6c shows that GPx1 overexpressing cells are totally protected from H_2O_2 toxicity. In line with this results, Western blot analyses performed on nuclear extracts indicated that Nrf2 accumulated in GPx1-overexpressing cells during the recovery from H_2O_2 treatment, whereas p53 immuno-reactive levels remained unchanged (Fig. 6d), confirming that survival/death response to H_2O_2-mediated oxidative stress depended on the amount of H_2O_2 lasting upon GPx-mediated scavenging.

4. Conclusions

The capability of several tumor cell lines to face pro-oxidant conditions and overcome oxidative damages is among the principal alterations causing tumor transformation and resistance to chemotherapeutics. A large number of molecules commonly employed today in cancer therapy induces selective tumor killing by interfering with the maintenance of DNA integrity or by impairing the efficiency of the DNA replication machinery. Moreover, many of these drugs induce oxidative stress as side effect of their mechanism of action. Therefore, the overexpression of detoxifying/antioxidant systems, such as those up-regulated by Nrf2 is consistent with the generation of a neoplastic phenotype, whose aggressiveness directly correlates with the capability to evade apoptosis induced by chemotherapeutic approaches. To corroborate this hypothesis, it has been very recently demonstrated that Nrf2 is induced by diverse oncogenes, such as K-Ras, B-Raf and Myc, in order to decrease ROS concentration and to mediate oncogenesis [24].

We previously demonstrated that H_2O_2 treatment induces Keap1 oxidation and its detachment from Nrf2 in tumor histotypes efficiently equipped with GPx (*e.g.*, gastric or intestinal adenocarcinoma cells). This molecular event does not take place upon treatment with disulfide stressors that, conversely, triggers apoptotic cell death *via* the redox activation of the Trx1/$p38^{MAPK}$/p53-dependent signaling cascade. These pieces of evidence argue for two important issues: (i) disulfide stress is not able to induce oxidation of Keap1 cysteines and, in turn, Nrf2 activation; (ii) alteration of thiol redox homeostasis could be a valuable tool to circumvent Nrf2-mediated resistance to ROS-based chemotherapy.

In the searching for possible clinical implications of these results, in this work we have highlighted two important considerations. The first one deals with the fact that tumor histotypes resistant to disulfide stressors obviously exist. The second, and more important, is that, also in this case, resistance response is governed by Nrf2. However, as we previously reported [14], no Keap1 oxidation is observed upon disulfide stress. This could reasonably depend on at least three distinct issues: (i) the redox potential of Keap1 reactive cysteines; (ii) the different chemical structure and steric hindrance of H_2O_2 and diamide; (iii) GSH concentration and GPx activity of the cell. While the first two points are of general application and could affect all redox reactions involving Keap1, the last one is cell specific. In particular, in this work, we have provided evidence that high GSH concentration can dampen disulfide stress, but are inefficient to counteract H_2O_2 toxicity, unless complemented with high levels of GPx activity. This depends on the fact that GSH can react directly with diamide (rate constant = $3 \times 10^2\ M^{-1}\ s^{-1}$) [39], whereas GSH-mediated reduction of H_2O_2 can occur almost exclusively if catalyzed by GPx. SH-SY5Y cells perfectly fit this condition, since they are very rich in GSH, but show low levels of GPx. In agreement with the biochemical features above mentioned, pre-treatment with the ROS scavenger DMTU, overexpression of GPx1, or addition of selenite to the culture media, as tool to induce GPx1 expression, completely abolish H_2O_2-induced toxicity; but further increase of GSH, or thiol enrichment only partially prevents this phenomenon. On the contrary, depletion of GSH by BSO sensitizes SH-SY5Y cells to diamide and activates apoptosis *via* the $p38^{MAPK}$/p53-mediated signaling pathway. It has been exhaustively reported that BSO enhances apoptotic response of neuroblastoma to different stimuli by increasing the steady-state concentration of ROS [40,41]. Therefore, although not directly investigated in this work, it is reasonable to hypothesize that also in our conditions, BSO-dependent GSH depletion results in ROS production, which ultimately activates $p38^{MAPK}$/p53 signaling pathway.

The second important concern arising from this work is that, although Keap1 oxidation does not occur upon treatment with disulfide stressors, nevertheless, the anti-apoptotic response involves Nrf2 as principal mediator. In the searching for alternative pathways underlying this event, able to circumvent the redox-dependent detachment of Keap1 from Nrf2, we identified ERK1/2 phosphorylation as the pivotal process responsible for Nrf2 activation. Several reports indicate that also this pathway is redox sensitive, but independent on the direct oxidation of Keap1 [42–44]. Indeed, it has been reported that members of MAPK family, and in particular ERK1/2, are able to phosphorylate Nrf2 on Ser^{40} in a way allowing its detachment from Keap1 [32,45]. Interestingly, this signaling pathway has been indicated: (i) to increase antioxidant defense, mainly GSH neo-synthesis and HO-1 expression [44,46]; (ii) to be induced upon stressful conditions, such as chemotherapeutic treatments [44,46,47]; (iii) to be mutually exclusive of the $p38^{MAPK}$-mediated apoptotic pathways [14,46,48]. Interestingly, these observations are all in accordance with the results obtained in this work. In particular, we have also observed that diamide administration results in a significant increase of GSH levels starting from 6 h of treatment (data not shown). These data further confirm Nrf2 activation and ultimately underline the importance of GSH redox buffer against disulfide stress in neuroblastoma cells.

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