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Original Contribution

A study of the relative importance of the peroxiredoxin-, catalase-, and glutathione-dependent systems in neural peroxide metabolism

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ABSTRACT

Cells are endowed with several overlapping peroxide-degrading systems whose relative importance is a matter of debate. In this study, three different sources of neural cells (rat hippocampal slices, rat C6 glioma cells, and mouse N2a neuroblastoma cells) were used as models to understand the relative contributions of individual peroxide-degrading systems. After a pretreatment (30 min) with specific inhibitors, each system was challenged with either H_2O_2 or cumene hydroperoxide (CuOOH), both at 100 μM. Hippocampal slices, C6 cells, and N2a cells showed a decrease in the H_2O_2 decomposition rate (23–28%) by a pretreatment with the catalase inhibitor aminotriazole. The inhibition of glutathione reductase (GR) by BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) significantly decreased H_2O_2 and CuOOH decomposition rates (31–77%). Inhibition of catalase was not as effective as BCNU at decreasing cell viability (MTT assay) and cell permeability or at increasing DNA damage (comet test). Impairing the thioredoxin (Trx)-dependent peroxiredoxin (Prx) recycling by thioredoxin reductase (TrxR) inhibition with auranofin neither potentiated peroxide toxicity nor decreased the peroxide-decomposition rate. The results indicate that neural peroxidatic systems depending on Trx/TrxR for recycling are not as important as those depending on GSH/GR. Dimer formation, which leads to Prx2 inactivation, was observed in hippocampal slices and N2a cells treated with H_2O_2, but not in C6 cells. However, Prx-SO_3 formation, another form of Prx inactivation, was observed in all neural cell types tested, indicating that redox-mediated signaling pathways can be modulated in neural cells. These differences in Prx2 dimerization suggest specific redox regulation mechanisms in glia-derived (C6) compared to neuron-derived (N2a) cells and hippocampal slices.

The main pathways to decompose hydrogen peroxide involve catalase (CAT), cytoplasmic glutathione peroxidase (GPx1), and peroxiredoxin (Prx) [1]. Decomposition of H_2O_2 by CAT is greatly dependent on H_2O_2 concentration [2]; this enzyme presents lower affinity for H_2O_2 than GPx and Prx. It is generally accepted that GPx and Prx are the main routes for H_2O_2 metabolism when it is present at relatively low levels, whereas at higher H_2O_2 levels CAT would have a prominent role [2,3]. The most used CAT inhibitor, aminotriazole (AT), has demonstrated the importance of CAT in protecting cells against oxidative stress [2,3].

The catalytic action of GPx requires a regenerating system composed of glutathione/glutathione reductase (GSH/GR) [4]. GPx activity generates glutathione disulfide (GSSG) that is reduced back to GSH by GR, using NADPH as electron donor. Interference with the NADPH supply can impair GR function and, consequently, GPx turnover [5,6]. Respiratory metabolites, such as glutamate, glutamine, and β-hydroxybutyrate can also modulate GSSG recycling to GSH [7]. Another way to interfere with GSSG recycling, and hence with the GPx turnover, is by GR inhibition. BCNU, the best known irreversible inhibitor of human GR [8], is largely used in preclinical studies [9], sensitizes glioblastoma cells, and is currently used in chemotherapy for brain tumors [10].

Prx's are a family of thiol-dependent peroxidases identified under various names and functions [11]. The ability to protect glutamine

Abbreviations: AF, auranofin; AT, aminotriazole; CuOOH, cumene hydroperoxide; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CAT, catalase; Chaps, 3-((3-cholamidopropyl)dimethylammonio)-1-propane sulfonate hydrate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NPSH, nonprotein thiol group; PI, propidium iodide; Prx, peroxiredoxin; GPx, glutathione peroxidase; Tris, tris(hydroxymethyl)aminomethane; Trx, thioredoxin; TrxR, thioredoxin reductase.

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synthase from inactivation was associated with one of their initial designations, thiol-specific antioxidant [12]; however, a range of functions unrelated to antioxidant activity has been attributed to several Prx family members. Later on, the enzymes were renamed to thioredoxin peroxidases or peroxiredoxins, the most used terms to identify Prx's [11]. The study of Prx's gained special attention when their catalytic activity was proven to compete with GPx in H_2O_2 decomposition [1,13] and their rate constants were recognized to be in the range of 10^7 M^{-1} s^{-1} [14–16], comparable to those of CAT (10^7 M^{-1} s^{-1}) and GPx1 (~10^8 M^{-1} s^{-1}) [2,17].

The six known mammalian Prx isoforms (Prx1–6) are classified as typical 2-Cys, atypical 2-Cys, or 1-Cys Prx's according to the number of cysteines and to the mechanism of catalysis. Hydrogen peroxide and alkyl peroxides are catalytically reduced by a Prx conserved cysteine. The 1-Cys Prx members, such as Prx6, have a conserved peroxidatic cysteine that forms the sulfenic acid during catalysis, but, unlike 2-Cys Prx's, the sulfenic acid cannot be reduced by Trx/TrxR. Other thiols, such as GSH, and ascorbic acid are regarded as reductants [11,22,23]. In 2-Cys Prx's, such as Prx2, the catalytic cycle of Prx involves some odd characteristics [18]: at low levels of H_2O_2, Prx forms sulfenic acid on its peroxidatic Cys. This Cys is attacked by a resolving Cys from a second subunit, generating a dimer linked by disulfide bridges. This dimer can be readily reduced back to its thiol form by the Trx/TrxR system. However, at higher oxidative status, the catalytic Cys residues can be converted to sulfinic or sulfonic acid, known as overoxidation states of Prx. These sulfinic (SO_2)- or sulfonic (SO_3)-modified Cys's can be reduced again to Cys (thiol state) by sulfiredoxin in a slow process. It is believed that impairment of the 2-Cys Prx activity due to dimerization or overoxidation regulates signaling cascades [19,20], and inhibition of the Trx/TrxR backup system has proven to be efficient in disrupting the Prx catalytic cycle [20,21].

The debate to ascertain the most prominent enzymatic peroxide-degrading system is not new. In this regard, erythrocytes have been used as an important cell model to investigate relevant aspects of peroxide metabolism. For instance, the search to identify the most important enzyme in H_2O_2 decomposition started with Cohen and Hochstein [24], attributing major importance to GPx. Since then, GPx, CAT, or both [2,25–28] were considered major routes for H_2O_2 decomposition. In studies using mice lacking GPx1, erythrocytes were almost normal, displaying a lack of increased sensitivity to H_2O_2 [13], whereas tissues of CAT-null mice presented minor sensitivity to oxidative stress [29]. CAT-null erythrocytes are highly susceptible to oxidation by exogenous H_2O_2 [30], whereas GPx-deficient erythrocytes are more susceptible to oxidation by organic peroxides [31]. Even though the contribution of CAT in endogenous H_2O_2 metabolism has been shown to be negligible [2,13,25–28], a major role for CAT in clearing exogenous H_2O_2 *in vivo* and *in vitro*, a function also attributed to GPx [30], received experimental support [2,32]. More recently, the importance of Prx2 has been added to this scenario. Mice lacking Prx2 developed hemolytic anemia, presenting oxidative-damaged erythrocytes [33] and elevated levels of endogenous H_2O_2 [1,21,30]; therefore Prx2 has been implicated in endogenous H_2O_2 removal in blood, in agreement with data suggesting that Prx2 is able to provide antioxidant protection in other cells [1,21,30]. These results contrast with the absence of spontaneous hemoglobin oxidation or hemolysis in erythrocytes of CAT- or GPx-deficient mice [1,21,30].

In neural cells, the debate around the roles of CAT and GPx (or both) as major mechanisms for H_2O_2 clearance remains open, but it is clear that the simultaneous inhibition of both enzymes decreases the H_2O_2 decomposition rate and renders cells more vulnerable to oxidative damage [34,35]. Literature about neural cells gives more attention to CAT and GPx [36,37], and little is known about the relative contribution of Prx to neural peroxide metabolism. Ascertaining that Prx2 has high turnover rate [14–16] comparable to GPx, along with the finding that the Prx2-deficient mouse has hematological abnormalities [33], has brought more attention to Prx as a physiologically relevant peroxide-degrading enzyme.

Similar to the erythrocyte enzyme, neuronal/glial GPx is important for H_2O_2 and organic peroxide metabolism [35,38,39]. The importance of GPx and CAT in the H_2O_2 metabolism of neural cells has been demonstrated using inhibitors, such as mercaptosuccinate and AT, respectively [35,40]. In primary glial cell culture, a decrease in the activity of one enzyme, either GPx or CAT, was not able to decrease exogenous H_2O_2 decomposition; however, lower H_2O_2 clearance was observed when both inhibitors were present, confirming the involvement of both enzymes in H_2O_2 metabolism. It was also shown that GPx activity depends on NADPH recycling, because impairment in the pentose phosphate pathway compromises H_2O_2 clearance [38,41,42]. In neural cells, GPx is dependent on GSH metabolism, because inhibition of GSH synthesis limits the importance of GPx [36]. At first glance, the CAT contribution was less relevant; however, inhibition by AT rendered astrocytes more sensitive to oxidative stress caused by iron overload, as demonstrated in GPx-null mutants exposed to organic peroxides [38,39]. Treatment of synaptosomes with AT leads to a more pronounced decrease in H_2O_2 consumption compared to BCNU treatment [7]. During the maturation of the central nervous system, GPx — but not CAT — expression was a critical factor in protecting oligodendrocytes, whereas mature cells were equally dependent on GPx and CAT [34,43].

Most of the work on peroxide metabolism in neural cells disregards the participation of Prx's [36,44–46]; however, the importance of Prx's in the central nervous system has begun to be recognized [47]. Changes in the expression of Prx's or in the backup system (Trx/TrxR) are related to neuroprotection/neurotoxicity, as demonstrated in overexpression models, ischemia–reperfusion studies, and neurodegenerative models, providing evidence of the importance of Prx in neural cells [47–49]. However, in the central nervous system, little is known about the relative contributions of CAT, GPx, and especially Prx's to peroxide decomposition.

Aiming to contribute to the understanding of the mechanisms of peroxide removal by neural cells, we took advantage of three largely used models: hippocampal slices, representing a model tissue, and C6 glioma and N2a neuroblastoma cells, as representatives of glial and neuronal cells. The relative importance of CAT, GPx, and Prx's was tested by impairing their peroxide decomposition capacity. For this purpose, an indirect approach was used to decrease GPx or Prx turnover, i.e., impairing recycling of GSSG and the disulfide form of Trx by inhibitors of GR (BCNU) and TrxR (auranofin). AT was used to inhibit CAT. This approach was successful in decreasing the exogenous H_2O_2 or cumene hydroperoxide (CuOOH) decomposition in the three models studied (hippocampal slices, C6 and N2a cells). After a pretreatment with inhibitors, a subsequent challenge with H_2O_2 or CuOOH (100 μM) was used to detect short-term effects on cell viability and permeability and DNA damage. The dimerization and overoxidation states of Prx2 were also evaluated.

Material and methods

Chemicals

Trypsin, fetal bovine serum, low-melting-point agarose (Gibco), and DMEM (Dulbecco's modified Eagle's medium) were purchased from Invitrogen (São Paulo, SP, Brazil). Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), NADPH, 5,5′-dithiobis(2-nitrobenzoic acid), CuOOH, Triton X-100, xylenol orange, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), silver nitrate, (3-(3-cholamidopropyl)dimethylammonio)-1-propane sulfonate hydrate (Chaps), GSSG, CAT, SDS–PAGE reagents, mouse monoclonal anti-β-actin peroxidase antibody, propidium iodide (PI), GR, NEM (*N*-ethylmaleimide), and auranofin (AF) were purchased from Sigma–Aldrich (São Paulo, SP,

Brazil) and nitrocellulose membranes from Bio-Rad (São Paulo, Brazil). AT was purchased from ChemService, Inc. (West Chester, PA, USA) and BCNU from Bristol–Myers Squibb (São Paulo, SP, Brazil). Goat anti-rabbit IgG, anti-peroxiredoxin 2, and anti-peroxiredoxin-SO_3 were from Abcam (Cambridge, MA, USA) and molecular-weight standards from GE Healthcare (São Paulo, SP, Brazil). Dimethyl sulfoxide (DMSO), H_2O_2, and perchloric acid were from Merck (São Paulo, SP, Brazil). All other reagents used were of analytical grade.

Animals

Adult male Wistar rats (60–90 days) from our own breeding colony were used. They were maintained in a ventilated room at constant temperature (23–25 °C) on a 12-h light/dark cycle with water and food available ad libitum. All protocols involving animal use were approved by the local ethics committee (CEUA).

Preparation of hippocampal slices

Rats were killed by decapitation and the hippocampus was dissected on ice. Transverse slices (0.4 mm) were cut with a McIlwain chopper and trimmed to identical sizes in a basic Hepes medium (124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Hepes, pH 7.4, 12 mM glucose, and 0.1 mM $CaCl_2$), which was gassed with O_2 before use [50].

Cell culture

Mouse N2a neuroblastoma cells (clone C1300) and rat C6 glioma cells (clone CCL107) were purchased from the American Type Culture Collection (Rockville, MD, USA). Both cell lines were kept in DMEM supplemented with 10% (w/v) fetal bovine serum and 2 mM L-glutamine with penicillin (100 U/ml) and streptomycin (100 μg/ml) in a 5% (v/v) CO_2 humidified atmosphere at 37 °C. The cells were seeded on a 24-well plate at a density of 2×10^5 cells/well and incubated for 24 h in plastic flasks at a density of 10^6 cells/flask. Before the treatment, the culture medium, DMEM, was replaced by Hepes medium.

Inhibition studies

The effects of AT [51] and BCNU [52] (inhibitors of CAT and GR, respectively) were analyzed in rat hippocampal slices and C6 and N2a cells. The effects of TrxR inhibition were investigated by treating cells and tissue with AF [53,54]. Tissue or cells were preincubated with Hepes and the indicated concentrations of AT, BCNU, or AF for 30 min at 37 °C. After the preincubation, slices and cells were exposed to H_2O_2 or CuOOH for a further 60–90 min, as indicated in the figure legends.

Antioxidant enzyme assays and peroxide determination

For antioxidant enzyme assays, the hippocampal slices were treated as described previously, homogenized in 20 mM Hepes, pH 7.0, and centrifuged at 15,000*g* for 30 min at 4 °C. Enzyme activities were then determined in the supernatant according to standard methods [55–57] for CAT, GR, or TrxR activity, respectively. Enzyme activities were expressed as a percentage of untreated sample, and protein determination was carried out by the method of Bradford [58] using bovine serum albumin as standard. Nonprotein thiol groups (NPSH) were determined colorimetrically [59].

Peroxide decomposition was determined by PCA–FOX assay as described previously [60]. Aliquots of incubation medium were collected at various time points to determine peroxide decomposition, as indicated.

Cell damage parameters

The conversion of the dye MTT to a formazan [61] is simply referred to as cell viability.

Standard procedures were used to assess membrane integrity by using PI fluorescence. After treatments, hippocampal slices or cultured cells were incubated for 30 min in the presence of PI, and the fluorescence was recorded using a fluorescence plate reader at λ_{ex} 535 nm and λ_{em} 617 nm, as detailed elsewhere [62].

Comet assay was performed according to the procedure described by Singh and co-authors [63] with some modifications. After treatments as described above, hippocampal slices were manually homogenized in 100 μl PBS, 20 mM EDTA, and 10% (v/v) DMSO (pH 7.4), and slides were prepared by mixing the cell suspension with 0.75% (w/v) low-melting-point agarose. For cultured cells, they were detached by incubating in the presence of 0.05% (w/v) trypsin and DMEM, followed by centrifugation for 10 min at 1000*g* at 37 °C. Excess supernatant was discarded, and 0.75% (w/v) low-melting-point agarose was added to the pellet. The mixture of cells/agarose was transferred to a microscope slide, covered with a coverglass, and allowed to solidify for 15 min at 10 °C. After solidification, the slides without their coverglasses were placed in lysing buffer solution (2.5 M NaCl, 100 mM disodium EDTA, 10 mM Tris, 1% (v/v) Triton X-100, and 10% (v/v) DMSO, pH 10.0) for 2 h at 4 °C. Subsequently, the slides were incubated in alkaline buffer (0.3 M NaOH and 1 mM EDTA, pH 13) for 20 min and were electrophoresed at 25 V, 300 mA for 20 min. After electrophoresis, slides were immersed in neutralizing buffer (0.4 M Tris–HCl, pH 7.5); fixed for 10 min in buffer containing trichloroacetic acid, zinc sulfate, and glycerol; and left to dry overnight. Finally, the DNA was stained with silver nitrate [64]. Images of 100 randomly selected nuclei (50 nuclei from each replicated slide) were analyzed for each treatment. Nuclei were scored visually for comet tail size based on an arbitrary scale of 0–4 (from undamaged, 0, to maximally damaged, 4). Thus, a damage index could range from 0 (all nuclei without tail) to 400 (all nuclei with maximally long tails), obtained by multiplying the number of cells in a given category by their corresponding class number. Slides were viewed on an Olympus BX41 microscope, and images were transferred to a computer with a digital camera. Cell counts were made by a researcher unaware of experimental groups.

Immunodetection

The Prx2 and Prx-SO_3 immunodetection was performed by Western blot. After treatment, hippocampal slices and C6 and N2a cells were homogenized in NEM buffer (final concentrations 100 mM NEM, 40 mM Hepes, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, with protease inhibitors, pH 7.4, and supplemented with 10 μg/ml catalase). To allow thiol blockade by NEM, the cells were incubated at room temperature for 15 min and then Chaps was added to a final concentration of 1% (w/v). This procedure has been reported to efficiently trap the oxidation states of Prx's, avoiding artificial dimer formation during sample processing [65]. As shown under Results, a low dimer amount could be detected in the untreated samples, proving the efficiency of this protocol.

Electrophoresis buffer (final concentration 2.1% (w/v) SDS, 10.5% (w/v) glycerol, 65.8 mM Tris, pH 6.8, 0.053% (w/v) bromophenol blue) was added to samples before SDS–PAGE. Proteins (30 μg of total protein/track) were separated by SDS–PAGE and, after electrotransfer to nitrocellulose membranes, were probed overnight with Prx2 and Prx-SO_3 primary antibodies. The immunoblots were developed using ECL Plus detection reagents, and β-actin immunocontent was used as the protein loading control.

Statistical analysis

Significant differences among groups were detected using one-way analyses of variance, followed by Duncan's post hoc test when necessary. For comet assay, the nonparametric Mann–Whitney *U* test

was used. Significance was accepted at $p<0.05$. Data are expressed as a percentage of control (means ± SE), unless otherwise stated.

Results

Inhibition of CAT, GR, and TrxR impairs peroxide consumption

Pretreatment of hippocampal slices for 30 min with inhibitors of CAT (AT), GR (BCNU), and TrxR (AF) decreased the corresponding activity by 30–55%, depending on the enzyme and concentration of the inhibitor (Figs. 1A–C). The use of BCNU decreased the levels of NPSH around 40% (Fig. 1D). Either peroxide (H_2O_2 or CuOOH at 100 μM), whether alone or in combination with inhibitors, was not able to change the NPSH levels beyond those shown in Fig. 1D (data not shown). Hippocampal slices were exposed for 60 min to H_2O_2 to establish a nontoxic concentration. Fig. 1E shows that cell viability was decreased by acute H_2O_2 treatment only at a concentration of 1 mM or higher. As the treatment with 100 μM H_2O_2 did not change the viability of hippocampal cells, this concentration was used to investigate any potential synergistic effect with inhibitors. The basal H_2O_2 decomposition rate was about twice as high compared to CuOOH (Table 1).

Peroxide decomposition was evaluated to determine if pretreatment with AT, BCNU, or AF would decrease the decomposition of exogenously added peroxide in the three models of neural tissue: hippocampal slices, C6 (glial) cells, and N2a (neuronal) cells. Peroxide decomposition profiles over 60 min of incubation are presented in Supplementary Fig. 1, which shows that H_2O_2 and CuOOH were consumed at different rates in the different models (Table 1). The peroxide decomposition rate was estimated after 5 min of incubation, and the relative effects of inhibitors are presented in Table 2.

CAT inhibition decreased the rate of H_2O_2 decomposition by about 25% (23–27%) in all three models studied. BCNU was very effective at decreasing H_2O_2 and CuOOH decomposition rates (70–77%) in hippocampal slices. The ability of C6 cells to dispose of H_2O_2 was reduced by 40% by BCNU, but this effect was much more pronounced for CuOOH consumption (67% inhibition). The H_2O_2 decomposition rate was also reduced (31%) in N2a cells treated with BCNU, which represents a much lower effect compared to hippocampal slices and C6 cells (40–70%). In N2a cells, CuOOH decomposition rates were decreased by 38% after BCNU treatment.

Table 1
Peroxide consumption rate in three neural models.

Cell/tissue	H_2O_2	CuOOH	Ratio	*p*
Hippocampal slices	5.0 ± 0.4 (6)	2.2 ± 0.7 (5)	2.3	0.001
C6 cells	8.9 ± 1.7 (3)	4.0 ± 1.0 (3)	2.2	0.01
N2a cells	6.0 ± 1.1 (3)	3.3 ± 0.6 (3)	1.8	0.05

Values (μmol/ml/min) are presented as means ± SE (n). The ratio between the H_2O_2 and the CuOOH consumption rate is also presented and statistical differences are presented as p values.

A specific TrxR inhibitor, auranofin (0.1 μM), reduced TrxR activity by 38% (Fig. 1B). In contrast to the large decrease in peroxide consumption rates found for the GR inhibitor BCNU, the inhibition of TrxR by AF presented almost no effect on the peroxide consumption rate, except for in C6 cells, which showed 38 and 18% decreases in H_2O_2 and CuOOH decomposition rates, respectively (Table 2).

Cell viability

When evaluating cell viability by MTT assay, we observed that CAT inhibition did not affect this parameter in any neural model studied (Figs. 2A–C). N2a cells were more susceptible to H_2O_2, observed as a decrease in viability, which was worsened by the pretreatment with AT (Fig. 2C), indicating a synergistic toxic effect of H_2O_2 and CAT inhibition.

BCNU was unable to decrease viability in hippocampal slices or in C6 and N2a cells (Figs. 3A–C). When cells were pretreated with BCNU, the H_2O_2 and CuOOH toxicity was clearly increased in all models used

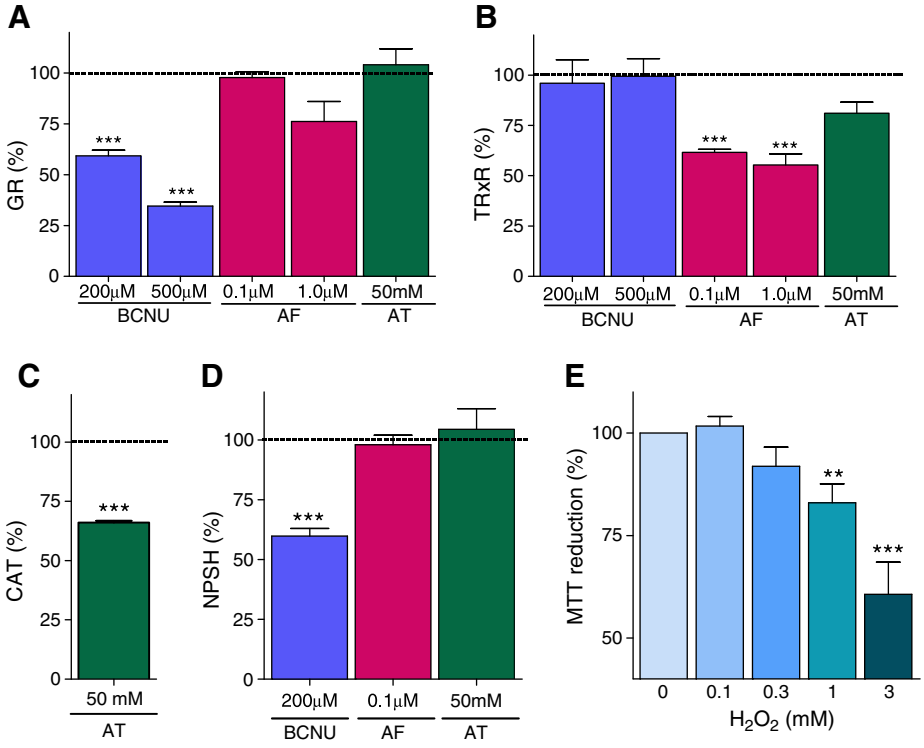

Fig. 1. Activity of the antioxidant enzymes, NPSH, and cell viability in hippocampal slices treated with BCNU, AT, or AF. The hippocampal slices were pretreated for 30 min with vehicle, BCNU, AT, or AF and the enzymatic activity was measured after 60 min of incubation: (A) GR, (B) TrxR, and (C) CAT activities. (D) NPSH levels. (E) MTT reduction after 60 min of exposure to H_2O_2. Results (means ± SE; $n=3$) are expressed as percentage of control. Statistical differences from untreated vehicle group (dashed line) are indicated (**$p<0.01$ or ***$p<0.001$).

Table 2
Reduction in peroxide consumption by enzymatic inhibitors.

Cells/tissue[a]	AT	BCNU	AF
H_2O_2			
Hippocampal slices[b]	23.3	69.9	0.0
C6 cells	27.7	40.1	38.1
N2a cells	27.1	31.1	0.0
CuOOH			
Hippocampal slices	n.d.[c]	76.9	10.2
C6 cells	n.d.	66.7	18.5
N2a cells	n.d.	37.6	2.5

[a] Cells and tissue were pretreated with inhibitors for 30 min: aminotriazole (AT), BCNU, or auranofin (AF), as described in the Materials and Methods section. Thereafter the medium was replaced and peroxide levels were measured after 5 min of incubation. The values refer to the mean decrease (%) in the rate of peroxide consumption of untreated controls.
[b] Slices refers to hippocampal slices of adult male rats.
[c] n.d., not determined.

(Figs. 3A–C), in agreement with the impairment in peroxide-degrading rates observed in all three models (Table 2).

Inhibition of TrxR by AF was ineffective at interfering with cell viability in all models tested (Figs. 3A–C).

Cell permeability

In addition to the MTT viability test, the cell permeability was evaluated by estimating PI incorporation. Treatment with H_2O_2, with or without AT pretreatment, did not modify cell permeability under any condition or in any cell model tested (Figs. 2D–F). Pretreatment with BCNU or AF also did not alter cell permeability (Figs. 3D–F), but the combination of BCNU and H_2O_2 significantly increased cell permeability in hippocampal slices and N2a cells (Figs. 3D and F). BCNU pretreatment also potentiated CuOOH toxicity, as the permeability of N2a cells was markedly increased. The permeability of C6 cells was affected neither by AT (Fig. 2E) BCNU, AF, or peroxides nor by the combination of inhibitors and peroxides (Fig. 3E).

DNA damage

The single-cell or comet assay was used as a way to determine if an impairment in the peroxide-degrading capacity leads to higher DNA damage. AT pretreatment was able to increase DNA damage in C6 cells. It also increased H_2O_2-induced DNA damage in these cells (Fig. 2H). Exposure to H_2O_2 increased DNA damage in N2a cells, but this effect was not potentiated by AT pretreatment (Fig. 2I), whereas hippocampal slices were unaffected (Fig. 2G).

When cells were pretreated with BCNU, DNA damage was increased in C6 and N2a cells (Figs. 3H and I). Both peroxides greatly increased the index of DNA damage in C6 and N2a cells (Figs. 3H and I), which may have prevented the appearance of synergistic effects because of a possible ceiling effect, but even so it was slightly apparent in C6 cells (Fig. 3H). In our experimental setup, tissue (hippocampal slices) was much more resistant to DNA damage induced by H_2O_2 or CuOOH (Fig. 3G) than were C6 and N2a cells (Fig. 3H and I). Only a

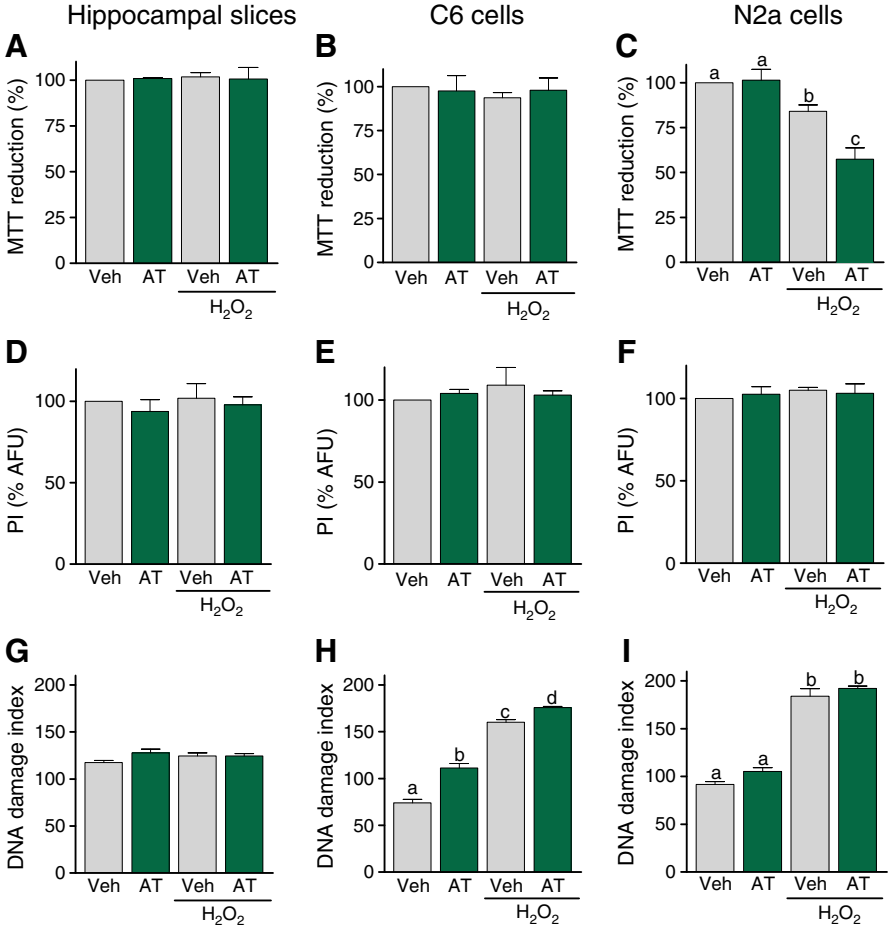

Fig. 2. Cell viability, membrane integrity, and DNA damage in hippocampal slices and C6 and N2a cells exposed to AT. After a 30-min preincubation with vehicle or AT, cells were exposed to H_2O_2 (100 μM, for 60–90 min) and (A–C) MTT reduction, as an index of cell viability; (D–F) PI fluorescence (AFU), as an index of cell permeability; and (G–I) comet assay, as an index of DNA damage, were measured as described under Material and methods. Results (means ± SE; $n = 3$) are expressed as a percentage of control. Columns not sharing the same letters are significantly different ($p < 0.05$).

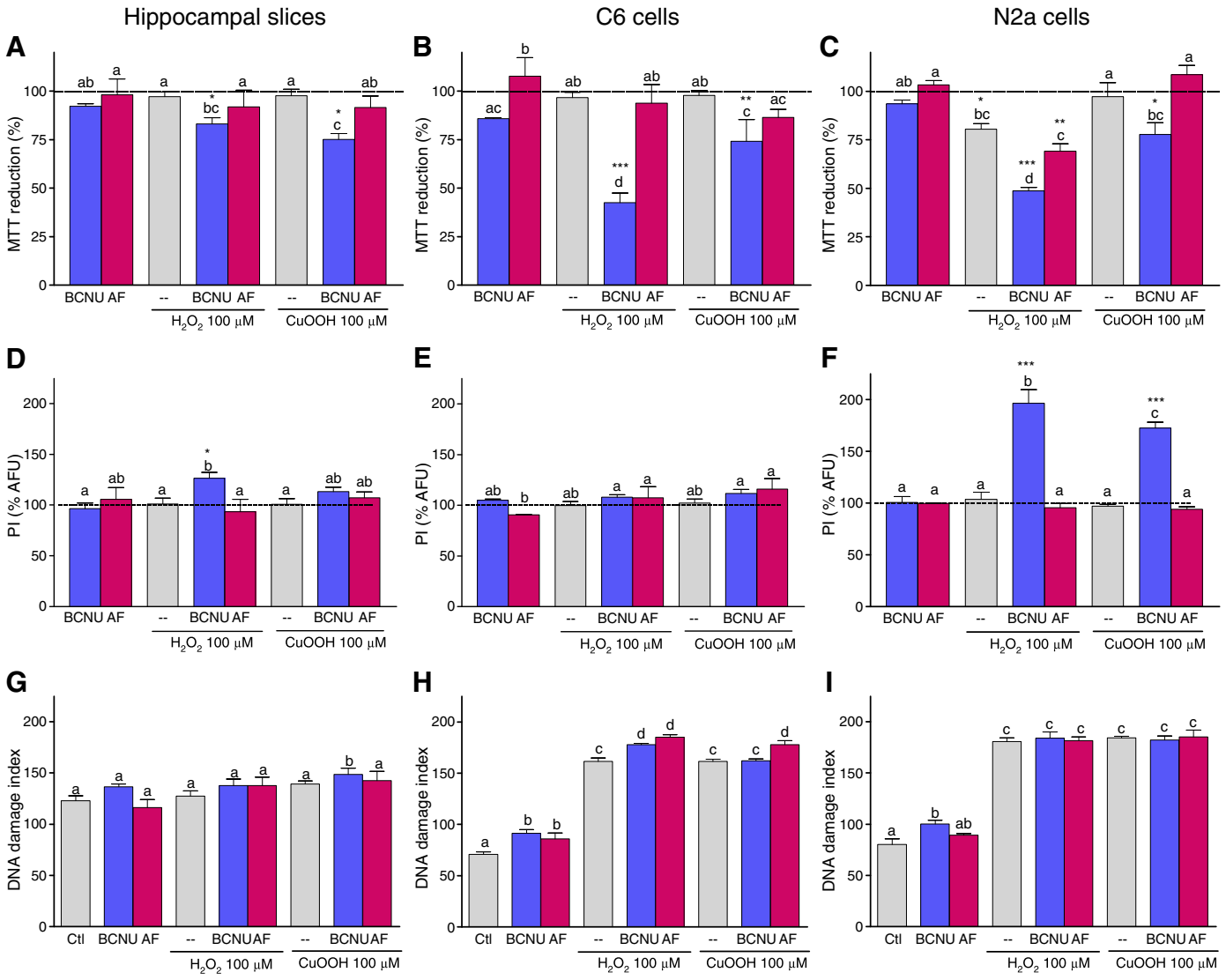

Fig. 3. Cell viability, membrane integrity, and DNA damage in hippocampal slices and C6 and N2a cells exposed to BCNU or AF. After a 30-min preincubation with vehicle, BCNU, or AF, cells were exposed to H_2O_2 or CuOOH (100 µM, for 60–90 min), and (A–C) MTT reduction, as an index of cell viability; (D–F) PI fluorescence (AFU), as an index of cell permeability; and (G–I) comet assay, as an index of DNA damage, were measured as described under Material and methods. Results (means ± SE; $n = 3$) are expressed as a percentage of control. Statistical differences from untreated vehicle group (dashed line) are indicated (*$p<0.05$, **$p<0.01$, or ***$p<0.001$). Columns not sharing the same letters are significantly different ($p<0.05$).

slight increase in DNA damage was observed in the CuOOH/BCNU group (Fig. 3G).

Inhibition of TrxR by AF was ineffective at interfering with DNA damage in hippocampal slices and N2a cells; however, AF or AF in combination with either peroxide increased DNA damage in C6 cells (Fig. 3H).

Peroxiredoxin 2 dimerization and overoxidation

At physiological conditions, Prx2 is a dimeric protein assembled by noncovalent bonds, which appears as a monomer in nonreducing SDS–PAGE, for hippocampal slices or C6 or N2a cells (Figs. 4A–C). Unlike C6 and N2a, untreated hippocampal slices presented an appreciable amount of Prx2 in the dimeric form (Fig. 4A), whereas C6 and N2a cells presented almost exclusively the monomeric form (Figs. 4B and C). In hippocampal slices and N2a cells, Prx2 dimerization was clearly increased by H_2O_2 treatment, but this treatment failed to induce dimerization in C6 cells (Fig. 4B).

Overoxidation of Prx2 (Prx-SO_3) was clearly increased in hippocampal slices and N2a and C6 cells treated with H_2O_2 (Figs. 4A–C).

Results overview

To give an overview of experiments comprising peroxide consumption, cellular viability and permeability, DNA damage, and Prx2 immunocontent, the main findings are summarized as follows: (a) BCNU pretreatment produced a more significant decrease in the rate of H_2O_2 decomposition in all three models tested, compared to AF. (b) Except for a moderate decrease in the peroxide decomposition rates in C6 cells (38 and 18% for H_2O_2 and CuOOH), inhibition of TrxR with 0.1 µM AF was ineffective at decreasing peroxide decomposition rates, which may explain the lack of toxicity in AF-treated hippocampal slices and cells. (c) Decreasing CAT activity by AT pretreatment was almost ineffective at decreasing cell viability or increasing cell permeability and DNA damage. (d) Upon addition of peroxides, the previous inhibition of GR (BCNU) sensitized hippocampal slices and C6 and N2a cells to 100 µM H_2O_2 and CuOOH, leading to an impairment in cell viability and

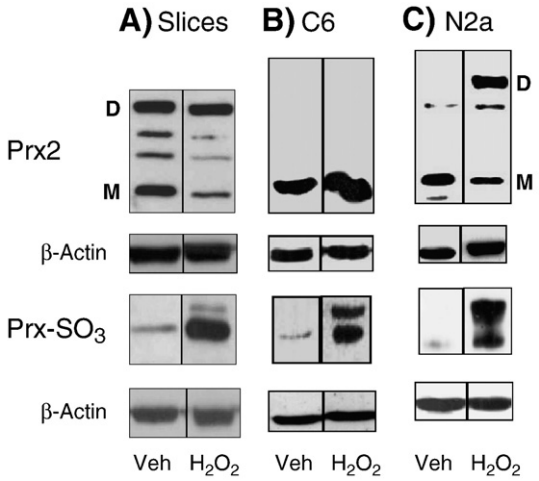

Fig. 4. Dimerization and overoxidation of Prx2 in hippocampal slices and C6 and N2a cells. The immunocontent of Prx2 from (A) hippocampal slices, (B) C6 cells, and (C) N2a cells is shown by representatives of three independent experiments. The monomeric (M) or dimeric (D) forms of Prx2 are indicated. Two nonspecific bands of unknown origin appeared between the dimer and the monomer of Prx2 in hippocampal slices. The results are from nonadjacent lanes from the same run. Samples were obtained after a 30-min preincubation with vehicle; after medium change, the cells were exposed to incubation buffer (Veh; control) or 100 μM H_2O_2 for 60 min, as described under Material and methods. Sample extraction was made in a NEM-containing buffer to prevent artificial oxidation of Prx2, and nonreducing SDS–PAGE was followed by immunodetection of Prx2 or Prx-SO_3 and β-actin, used as a loading control, as indicated.

permeability and increasing DNA damage. (e) AF was not toxic nor did it increase the toxicity of peroxides, except for a slight increase in DNA damage in C6 cells, which matched the lower decomposition rate induced by AF treatment in these cells. (f) CuOOH produced similar or lower toxic effects compared to H_2O_2 and it also potentiated BCNU toxicity. (g) When exogenous peroxide is added, Prx2 undergoes dimerization in hippocampal slices and N2a neuroblastoma cells, but not in C6 glioma cells. (h) Treatment of hippocampal slices or C6 or N2a cells with H_2O_2 was very effective at increasing the amount of overoxidized Prx2, suggesting the participation of Prx-SO_3 in cell signaling.

Discussion

Previous evidence [40] described that glial and neuronal cells are able to decompose H_2O_2 at appreciable rates, as corroborated by our findings (Table 1). In agreement with previously published data [35], this work shows that in our experimental setup a modest decrease (23–28%) in H_2O_2 decomposition was caused by AT pretreatment of hippocampal slices and N2a and C6 cells. Inhibition of TrxR by AF did not affect the ability of hippocampal slices or Na2 cells to decompose peroxides (Table 2), but there was a slight inhibition of C6 cells' ability to dispose of peroxides. This effect on C6 cells would be the underlying cause for a slight increase in DNA damage observed after AF pretreatment. GR inhibition by BCNU was very effective at decreasing H_2O_2 and CuOOH decomposition rates. This effect would be a consequence of lower glutathione levels in combination with a decreased GR activity, limiting the turnover rate of GPx or of other GSH-dependent enzymes, such as Prx6 [66].

BCNU was more effective than AT or AF at decreasing cell viability and permeability as well as at promoting DNA damage. Comparing the sensitivity of the different models, it can be observed that hippocampal slices were more resistant than glioma C6 or neuroblastoma N2a cells. N2a and C6 cells showed similar sensitivity to the pretreatment with BCNU, peroxides, or the combination of both, despite data reporting that neuronal cells are more vulnerable to oxidative challenge [36]. However, lower peroxide-degrading capacity would not always render cells vulnerable, unless subjected to a subsequent stress [9,38], as observed in the viability test in all models tested.

Furthermore, it is important to note that the three *in vitro* models are quite different in terms of full antioxidant capacity and metabolism. In fact, the hippocampal slices represent a whole tissue, in which neuroglial interactions are present. In this regard, the crucial role of astrocytes in offering glutathione precursors to neurons seems to be relevant [36,44]. In addition, hippocampal slices are 400 μm thick, limiting the access of substances to the inner parts, which may interfere with results. Moreover, it is important to point out that the cultured cells studied, from both glial (C6) and neuronal (N2a) sources, are tumor cell lines. This would be of interest considering that tumor cells can present a distinct antioxidant capacity compared to normal cells [67] and it should be taken as a limitation of such studies. Caution needs to be taken to avoid overinterpreting the data. Because our experimental framework encompasses a short period of time (60–90 min) and neural cell models, it is possible that the use of different cell models, compartmentalization of peroxide removal [68], or a different experimental framework would contribute to the detection of alternative mechanisms of peroxide metabolism.

Inhibition of catalase, glutathione reductase, or thioredoxin reductase

Regarding CAT inhibition, it was clearly less noxious in all models tested compared to BCNU treatment. These data leave no doubt that under the present experimental conditions CAT activity had a less important role than the other peroxide-degrading systems depending on GSSG recycling, even at the high exogenous H_2O_2 levels tested (100 μM). It is important to note that the potential interference of CAT inhibition with other enzymes involved in peroxide metabolism needs to be further investigated.

Inhibition of GR increases the oxidative burden to glial cells and causes a decrease in the capacity to decompose H_2O_2 [9]. This agrees with our data, because the inhibition of GR by BCNU impaired the cells' capacity to detoxify H_2O_2. However, the H_2O_2-degrading activity was not fully eliminated by inhibiting GR, indicating the activity of other H_2O_2-degrading systems. The sole inhibition of GR by BCNU was sufficient to produce increased cell damage, which was even more elevated after H_2O_2 or CuOOH treatment. Pretreatment with BCNU also displays a synergistic effect with copper toxicity in cortical cells [69]. Synergism of GR inhibition and cell toxicity was a phenomenon observed in all studied models, clearly underscoring the relevance of GSSG recycling to prevent cell vulnerability to H_2O_2 and organic peroxides. BCNU effects may have been potentiated by a decrease in glutathione levels; nevertheless it is clear that systems depending on glutathione would have an important role in peroxide disposal in neural cells, which includes, among others, GPx [30] and Prx6 [66].

Decreasing glutathione synthesis or CAT activity leads to an increase in lactate dehydrogenase release from astrocytes exposed to H_2O_2 (100 μM), which agrees with our findings, although additive or synergistic effects of a combination of GSH synthesis inhibition and AT treatment have not been observed [39]. Under H_2O_2 stress (100 μM, 15 min), GSSG comprised about 60% of total glutathione [9]. Interestingly, this treatment of astrocytes with BCNU prevented intracellular accumulation of GSSG, which was accompanied by an increase in total glutathione (the sum of intra- and extracellular GSH + 2 GSSG), demonstrating GSSG export [9]. GPx-null mutant astrocytes were very sensitive to H_2O_2 (100 μM, 24 h), but this sensitivity was not greatly increased by GSH synthesis inhibition and AT treatment; however, it was increased by iron overload [38]. The authors conclude that under unstressed conditions, GPx1 seems to be dispensable, but under sublethal peroxide (100 μM H_2O_2), it is evident that both GPx and CAT are required for rapid clearance of H_2O_2, with a prominent role for the GPx function. These results are in agreement with our findings showing the importance of GSSG recycling to support GPx activity at this level of H_2O_2. From data obtained after AF pretreatment, it was clear that a 38%

inhibition of TrxR neither greatly compromises peroxide decomposition nor significantly increases the toxic effect of both peroxides.

TrxR inhibition by AF increased the DNA damage and potentiated the effect of peroxides on C6 cells, but this sensitivity was not shared by hippocampal slices and N2a cells. These results point to a relative relevance of systems depending on Trx/TrxR activity in C6 cells. All three models (hippocampal slices, C6 and N2a cells) displayed lower cell viability, higher cell permeability (except for Na2 cells), or higher DNA damage when GSSG recycling was limited by pretreatment with BCNU.

A decrease in CAT and TrxR activities was less effective at inducing cell damage or at potentiating H_2O_2 toxicity, compared to inhibition of GR. Compared to CAT and systems depending on Trx/TrxR, our findings support the notion that peroxidatic enzymes depending on GSH, and hence on GSSG reduction by GR (e.g., GPx), are more important for cell protection and exogenous peroxide metabolism of neural cells.

Overoxidation and dimerization of peroxiredoxin 2

Considering that H_2O_2 induces a concentration-dependent inactivation of Prx, by dimerization or overoxidation [21], which is believed to be a regulatory signal [70], we investigated whether Prx was inactivated by dimerization or superoxidation. It was possible to observe that H_2O_2 induced an extensive dimerization of Prx2 in hippocampal slices and N2a cells, but not in C6 cells. Dimerization seems to occur in cells with low TrxR activity [21], indicating that hippocampal slices and N2a cells have distinct backgrounds for redox signaling compared to C6 cells. All cell models used showed significantly more Prx-SO_3 formation when treated with H_2O_2. These data support the notion that hippocampal slices and Na2 cells have diverse strategies/sensitivity to redox regulation compared to C6 cells, because Prx activity can be decreased by both dimerization and superoxidation.

Connecting peroxide-degrading systems

Studies on the interactions among different peroxide-metabolizing systems can shed light in this area. For instance, a recent work showed that GPx1 undergoes irreversible inactivation in erythrocyte aging or when exposed to increased levels of H_2O_2, because these events also increased Prx inactivation [71]. Related to these findings, a report demonstrated that GPx inhibition leads to an inhibition of CAT [72]. It is also known that CAT can be inactivated by high levels of H_2O_2 [34]; for instance 200 μM H_2O_2 could inactivate CAT in minutes in a study using primary astroglia-rich culture [72]. These data open the possibility that peroxide-degrading systems are somewhat linked to one another under oxidative conditions, because inactivation of one system can be related to the inactivation of a second system. The magnitude of the oxidative insult certainly influences the interplay between antioxidants and peroxide-degrading systems, which has been recently supported by data suggesting that glutaredoxin actively participates in the yeast mitochondrial 1-Cys Prx catalytic cycle [73].

The inhibition of GPx practically eliminates the ability of astrocytes to dispose of organic peroxides [38], which agrees with our data showing that the inhibition of only CAT (AT) or only TrxR (AF) did not produce higher sensitivity in all three models tested. The data obtained support the notion that CAT and TrxR-dependent Prx's are minor contributors to peroxide decomposition in neural cells and that the systems depending on GSH and GR activity would be more relevant. In addition to GPx [38], other systems depending on GSH would also be candidates for peroxide removal in neural cells, such as Prx6 [30,66].

It is known that Prx2 is inactivated, either by dimerization or by overoxidation, when oxidative stress exceeds certain levels, triggering signaling-dependent cell responses [1]. The data obtained clearly show that hippocampal slices and N2a cells resemble each other in presenting Prx2 dimer formation, an indication of a limited TrxR activity, whereas all neural models present a H_2O_2-dependent Prx-O_3 formation. Prx-O_3 formation in C6 cells, associated with an inability to retain dimeric Prx2, is a characteristic of high TrxR turnover rate in cells [21]. These differences would be related to specific signaling for each neural cell model, which needs further attention.

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