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Inflammatory Response and Glutathione Peroxidase in a Model of Stroke[1]

Nobuya Ishibashi,[2]* Olga Prokopenko,* Kenneth R. Reuhl,† and Oleg Mirochnitchenko[3]*

Stroke is one of the leading causes of death in major industrial countries. Many factors contribute to the cellular damage resulting from ischemia/reperfusion (I/R). Experimental data indicate an important role for oxidative stress and the inflammatory cascade during I/R. We are testing the hypothesis that the mechanism of protection against I/R damage observed in transgenic mice overexpressing human antioxidant enzymes (particularly intracellular glutathione peroxidase) involves the modulation of inflammatory response as well as reduced sensitivity of neurons to cytotoxic cytokines. Transgenic animals show significant reduction of expression of chemokines, IL-6, and cell death-inducing ligands as well as corresponding receptors in a focal cerebral I/R model. Reduction of DNA binding activity of consensus and potential AP-1 binding sites in mouse Fas ligand promoter sequence was observed in nuclear extracts from transgenic mice overexpressing intracellular glutathione peroxidase compared with normal animals following I/R. This effect was accompanied by modulation of the c-Jun N-terminal kinase/stress-activated protein kinase pathway. Cultured primary neurons from the transgenic mice demonstrated protection against hypoxia/reoxygenation injury as well as cytotoxicity after TNF-α and Fas ligand treatment. These results indicate that glutathione peroxidase-sensitive reactive oxygen species play an important role in regulation of cell death during cerebral I/R by modulating intrinsic neuronal sensitivity as well as brain inflammatory reactions. *The Journal of Immunology,* **2002, 168: 1926–1933.**

Both animal studies and clinical data indicate that brain ischemia/reperfusion (I/R)[4] injury is a complex, multistage process. It is initiated by the immediate damage caused by hypoxia as well as by the return of oxygenated blood, which injures the microvasculature (1). The so-called secondary damage involves neuronal injury from excitatory amino acids, intracellular Ca^{2+} accumulation, free radical generation, and apoptosis as well as microvascular processes. There is accumulating evidence that inflammatory-immunologic reactions are also involved in the pathogenesis of cerebral ischemia. Inflammatory cells such as neutrophils and macrophages infiltrate the ischemic brain in various models of ischemic stroke (2). In addition, cells resident in brain, such as astrocytes, microglia, and endothelia, have been found to be activated by cerebral I/R. These cells then become immunologically reactive and interact with each other by producing inflammatory mediators, including eicosanoids, reactive oxygen species (ROS), cytokines, and adhesion molecules. These molecules appear responsible for the accumulation of inflammatory cells in the injured brain, and the resulting inflammatory cascade may affect the survival of neurons subjected to ischemic injury.

ROS produced by the immune system are well-recognized toxic metabolites that can directly cause damage to brain cells. It has become apparent that ROS have a much broader role in the regulation of the immune response itself. For example, ROS might serve as endogenous signals, released by injured tissue to trigger activation of the immune system. ROS are important regulators of many intracellular signaling pathways, leading to the release of inflammatory mediators as well as those that represent reaction of different cell types to the mediators. The signaling pathways involve G proteins, protein tyrosine kinases, protein tyrosine phosphatases, Janus kinases, mitogen-activated kinases, caspases, a variety of transcription factors, etc. (3).

Many studies of the involvement of ROS in regulatory pathways have relied on the use of exogenous sources of ROS and therefore could not completely support their physiological role. To address the role of ROS, particularly those sensitive to intracellular glutathione peroxidase (GPx1) activity in brain I/R, we used transgenic mice overexpressing human GPx1 (4). Experiments with GPx1 transgenic mice subjected to intraluminal blockade of the middle cerebral artery (MCA) as a model system have shown that they are protected against brain I/R damage (5). Our GPx1 animals displayed a significant decrease in infarct volume (48%) compared with nontransgenic mice. Recently, in a model of kidney I/R we were able to show a significant inhibition of activation of neutrophil-attracting chemokines in those animals (6). The current report demonstrates that the increased level of GPx1 activity in transgenic mice modulates inflammatory response in focal brain I/R model of stroke and decreases the sensitivity of brain cells to induced cell death. The data obtained suggest that the development of therapeutic approaches aimed at inhibition of ROS production

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[4] Abbreviations used in this paper: I/R, ischemia/reperfusion; JNK, c-Jun N-terminal kinase; FADD, Fas-associated death domain protein; RIP, receptor interacting protein; TRADD, TNFR-associated death domain protein; SAPK, stress-activated protein kinase; FasL, Fas ligand; FAF, Fas-associated protein factor; SEK1/MKK4, SAPK/Erk kinase; GP, glutathione peroxidase; GPx1, intracellular GP; IP-10, inducing protein 10; LDH, lactate dehydrogenase; TRAIL, TNF-related apoptosis-inducing ligand; MCA, middle cerebral artery; MCP, monocyte chemoattractant protein; MIP, macrophage-inflammatory protein; MPO, myeloperoxidase; ROS, reactive oxygen species.

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may be beneficial not only for stroke but also for other inflammation-related neurodegenerative diseases.

Materials and Methods

Transgenic mice

The generation of transgenic mice with human GPx1 genes in a C57BL/6 × CBA/J background was previously reported (4, 7). The mouse line GPE23 (containing 200 copies of the human GPx1 gene) was used for these studies. To obtain nontransgenic and heterozygous transgenic animals for the experiments, transgenic founders were bred with (C57BL/6 × CBA/J)F_1 mice.

Focal cerebral ischemia

The experimental procedure was previously described (5, 8). These conditions provide reproducible I/R injury in brain of normal and transgenic mice. In brief, 6-mo-old normal or transgenic males, weighing 35 ± 5 g, were subjected to focal I/R using the intraluminal suture method. Mice were deeply anesthetized using 2–3% isoflurane in a 30% oxygen/70% nitrous oxide gas mixture. Body temperatures were monitored and maintained at 37 ± 0.5°C with a feedback-regulated heating pad. After exposing the left carotid artery, 12 ± 1 mm of 5-0 monofilament nylon suture was inserted into the internal carotid artery through the external carotid artery stump just beyond the MCA branch. The animals underwent MCA occlusion for 1 h and were subjected to reperfusion by removing the nylon suture. After recovering from the anesthesia, mice were maintained in an air-conditioned room at 24°C during reperfusion periods. Mice were sacrificed at different time points after surgery. Brains were immediately removed after perfusion with cold PBS and rapidly frozen in liquid nitrogen for obtaining extracts and RNA analysis.

MPO activity assay

Myeloperoxidase (MPO) activity was measured spectrophotometrically at 630 nm, as described previously (9). The 1-ml assay mixture contained 100 μl of heat-inactivated extract, 80 mM phosphate buffer (pH 5.4), and tetramethylbenzidine and H_2O_2 in final concentrations of 1.6 and 3.0 mM, respectively. MPO activity was expressed in units, where 1 U represents the amount of enzyme degrading 1 μmol H_2O_2/min, and was standardized with the protein content of the extract (units per milligram of protein).

Lipid peroxidation assay

Lipid peroxidation in the brain was assessed by measurement of malondialdehyde and 4-hydroxy-2(*E*)-nonenal, using a lipid peroxidation kit (Calbiochem, San Diego, CA) according to the manufacturer's protocol. Lipid peroxidation was normalized to the protein content of the supernatant.

Preparation of RNA and RNase protection assay

Total RNA was isolated from brain samples using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to manufacturer's protocol. The RNA concentration was measured by a spectrophotometer. RNase protection assay was performed using a Riboquant kit and an mCK-3, mCK-5, and mAPO-3 Multiprobe Template Set (BD PharMingen, San Diego, CA) according to the manufacturer's protocol. The mCK-3 Multiprobe Template Set contains RNA probes for the mouse chemokines lymphotactin, RANTES, eotaxin, macrophage-inflammatory protein (MIP)-1β, MIP-1α, MIP-2, inducing protein 10 (IP-10), monocyte chemoattractant protein (MCP)-1, and TCA-3; the mCK-5 Multiprobe mCK-5 Template Set contains RNA probes for mouse cytokines TNF-β, leukotriene-β, TNF-α, IFN-γ, IFN-β, TGF-β1, and TGF-β2, and the mAPO-3 Multiprobe Template Set contains RNA probes for mouse TNFR superfamily proteins Fas ligand (FasL), Fas, Fas-associated death domain protein (FADD), Fas-associated phosphatase, Fas-associated protein factor (FAF), TNF-related apoptosis-inducing ligand (TRAIL), TNFR p55, TNFR-associated death domain protein (TRADD), receptor interacting protein (RIP), and caspase-8. RNA probes and for positive controls (L32 and GAPDH) were generated in vitro using a T7 polymerase transcription kit and [α-^{32}P]UTP as a label. A standard curve was made using undigested probes as markers, and the identities of RNase-protected bands were established. To compare RNA amounts in the protected bands, the films were scanned with an imaging densitometer (GS-670; Bio-Rad, Hercules, CA), and final values were factored relative to GAPDH levels.

Western blotting

Proteins were analyzed by Western blot analysis as reported previously (6). In brief, tissue samples were homogenized in RIPA buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, aprotinin, pepstatin, and phosphatase inhibitors (10 mM NaF, 1 mM $NaVO_4$, 1.5 mM Na_2MoO_4, 1 mM benzamidine, 20 mM glycerophosphate, and 20 mM *p*-nitrophenyl phosphate)). SDS-PAGE (5×) sample buffer was added to the final concentrations of 50 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM DTT, 0.006% bromophenol blue, and 10% glycerol. Lysates were centrifuged at 13,000 × *g* for 20 min, and supernatants were used for 10% PAGE. After electrophoresis gels were electroblotted onto a polyvinylidene difluoride membrane and analyzed using anti-Fas (sc-1023) and anti-FasL (sc-6237) Abs (both from Santa Cruz Biotechnology, Santa Cruz, CA), and phospho-SAPK/Erk kinase (SEK1/MKK4) (Thr^{261}) and phospho-c-Jun (Ser^{63}) Abs (Cell Signaling Technology, Beverly, MA). Proteins were detected with the Phototope-HRP Western Blot Detection kit (New England Biolabs, Beverly, MA).

Cortical cell cultures, experimental treatments, and quantification of neuron survival

Primary neuronal cultures were prepared as described by Berezovska et al. (10). In brief, cells were isolated from neocortex of embryonic day 16–17 normal and transgenic mice and placed in Neurobasal medium (Life Technologies) with 10% FBS. The neurons were plated on 0.025% poly-D-lysine-coated plates. After 1 h the medium was replaced with serum-free Neurobasal medium containing 2% B27 supplement. The cultures were maintained at 37°C with 5% CO_2 in a tissue culture incubator. Experiments were performed in 10- to 12-day cultures; >95% of the cells in the cultures were neurons, as judged by cell morphology and immunostaining with Abs against neurofilaments. Neurons at this age become vulnerable to hypoxia and glucose deprivation (11, 12). Cells in serum-free medium without glucose were exposed to hypoxia in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) by flushing the chamber with humidified 95% N_2/5% CO_2 as described previously (13). A progressive drop in pO_2 was measured with a Clark-style probe (model DO-166; Lazar Research Laboratory, Los Angeles, CA) installed in the chamber and placed in the culture dish with medium. pO_2 usually dropped to approximately 5 torr by the end of 4-h hypoxia. At the end of hypoxia, medium was changed to normal growth medium with or without TNF-α/FasL (recombinant murine TNF-α and SUPERFAS ligand were obtained from R&D Systems (Minneapolis, MN) and Alexis (San Diego, CA), respectively), and cultures were returned to the incubator in air and 5% CO_2. Neuronal cell viability was assessed by the release of lactate dehydrogenase (LDH) into the culture medium. LDH activity was measured by using a commercial kit (Cyto Tox 96 Cytotoxicity Assay; Promega, Madison, WI). The percent cytotoxicity was expressed as (experimental LDH release/maximum LDH release) × 100.

Preparation of nuclear extracts and EMSA

Nuclear extracts were prepared from sham-operated normal and transgenic mice as well as animals after ischemia and 60 min of reperfusion, as previously described (6). Brains were dissected to isolate tissues from ipsilateral and contralateral cortexes. Aliquots of the extract were stored at −80°C. Protein content was assayed using Bio-Rad protein reagent. The following oligonucleotides were used in EMSAs: 5′-CGCTTGATGAGTCAGCCGGAA-3′ containing AP-1 consensus site; several oligonucleotides bearing the potential AP-1 site were deduced from the mouse FasL promoter sequence: −282 to −252 (5′-CGCTGACTTGCTGAGTTGGACTCAGGCAGGC-3′), −233 to −215 (5′-TCTCAGTTAGCACAGAGAC-3′), and −119 to −103 (5′-AAGTGAGTGGCTGTCTC-3′). For each oligonucleotide, the two complementary strands were synthesized using an Applied Biosystems automated DNA synthesizer (Foster City, CA). These oligonucleotides were end-labeled using polynucleotide kinase and [γ-^{32}P]ATP (NEN Life Science Products, Boston, MA), annealed, and purified by PAGE. For EMSA, 3 μg of extract was incubated in a reaction mixture containing 20 mM HEPES (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.2 mM EDTA, 0.5% Nonidet, 1 μg poly(dI-dC), and 8% glycerol in a final volume of 20 μl for 20 min at 4°C. After preincubation, 10^5 cpm radiolabeled DNA probe was added, and the incubation was continued for 20 min at room temperature. The DNA-protein complexes were separated on native 5% polyacrylamide gels in 0.25× Tris-borate-EDTA buffer. Supershift assay was conducted after incubation of the nuclear extracts with Abs (0.5 μg of anti-mouse c-Fos, c-Jun, ATF-2, p50, p65 Abs; sc-52X, sc-45X, sc-6233, sc-372X, sc-7178X, respectively, from Santa Cruz Biotechnology) for 20 min at 4°C followed by EMSA.

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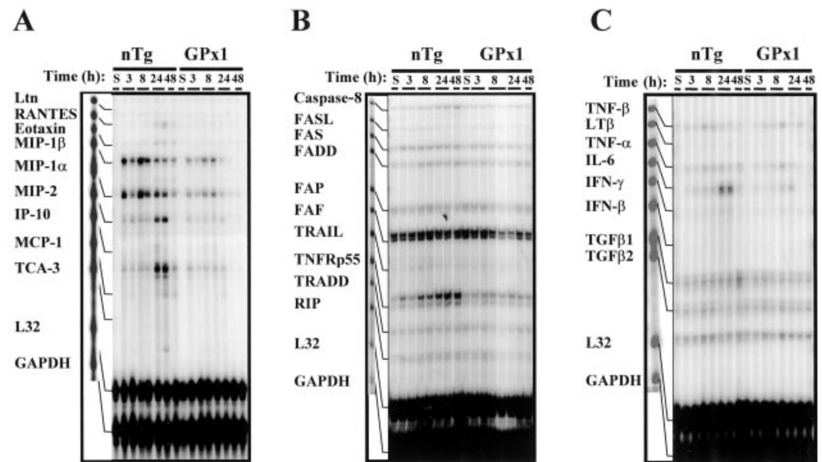

FIGURE 1. Effect of brain I/R on cytokine mRNA expression. RNase protection assay was performed using a Riboquant kit and mCK-3 (*A*), mAPO-3 (*B*), and mCK-5 (*C*) template sets (BD PharMingen). Five micrograms of total RNA from each sample was used for hybridization. The autoradiograms from this analysis show on the *left* the probe sets that were not treated with RNase. Also shown are the corresponding RNase-protected probes after hybridization and incubation in the presence of RNase. Two independent animals are shown for 3, 8, and 24 h points, and one animal for the 48 h point. S, Sham-operated animals, which were sacrificed at 8 h after operation. The gels are representative of three independent experiments. The results of the quantitative analysis are shown in Table I.

Results

Inhibition of neutrophil migration in brain infarcts by GP overexpression

To evaluate the presence of neutrophils in brain infarcts we measured MPO activity. It was reported by Matsuo et al. (14) that the increase in brain MPO activity after transient focal ischemia correlates closely with the neutrophil infiltration and that this process is implicated in postischemic brain injury. According to our measurements in normal mice, the level of MPO activity increases in infarct regions compared with noninfarcted contralateral cortex by 70% at 24 h after I/R (10.7 ± 1.7 vs 6.3 ± 0.8 U/g; $p < 0.05$; $n = 6$). In contrast, almost no changes in MPO activity were observed in GPx1 transgenic mice (6.2 ± 1.0 vs 6.5 ± 1.1 U/g, infarct and contralateral side, respectively; $n = 6$). Data from detailed histopathologic analysis and quantitative neutrophil counts in brain sections from normal and transgenic mice after I/R are well correlated with MPO measurements and are currently included in a manuscript submitted for publication.[5] Therefore, the results indicate that overexpression of glutathione peroxidase (GP) inhibits neutrophil transmigration after focal I/R.

Effect of GP overproduction on the level of inflammatory mediators

It is known that the inflammatory response is mediated by a series of regulatory proteins such as cytokines. To measure the expression of inflammatory genes we took advantage of Riboquant RNase protection kits and Multiprobe Template Sets mCK-3, mCK-5, and mAPO-3 (Fig. 1). To compare RNA amounts of the protected bands, exposed films were scanned by an imaging densitometer and final values were factored relative to GAPDH levels. Results are shown in Table I.

GP overexpression significantly inhibited the expression of several chemokines, especially MIP-1α, MIP-1β, MIP-2, IP-10, and MCP-1. Among other cytokines, TNF-α, IL-6, and FasL were most affected. Another death-inducing ligand, TRAIL, as well as a series of adapter proteins (FADD, TRADD, RIP, and FAF) were also inhibited. Interestingly, the expression of transmembrane receptors, Fas and TNFR p55, which are capable of triggering cell death in neurons and other brain cells, decreased >2.5 times.

Tissue extracts from two brain regions, the core of the infarct and the pyriform cortex, containing cells predominantly dying by necrosis and apoptosis, respectively, were analyzed by Western blotting to further characterize expression of FasL/Fas on a protein level. Fas and FasL proteins were dramatically lower in brains of GPx1 transgenic mice compared with normal animals at 8 and 24 h after I/R (Fig. 2). Importantly, the overall level of proteins in both regions was not very significantly different, indicating that mechanisms other than ligand/receptor levels are responsible for the character of the neuronal death pathway, necrotic or apoptotic. These mechanisms may involve levels of other mediators, differences in intrinsic cell sensitivity from those brain regions, or significantly deeper oxygen deprivation and ATP depletion in a core region of the infarct compared with the penumbral zone. It was recently reported that both TNF-α- and FasL-induced pathways could lead to apoptosis as well as necrosis (15, 16), and in at least one model system (17) it has been reported that cellular ATP concentration is a critical parameter in the decision between the two cell death pathways.

Induced cytotoxicity in primary neurons in vitro

Intracellular ROS produced during I/R as well as those triggered by ligand/receptor TNF superfamily proteins are active players in the execution of apoptosis and necrosis. Therefore, one of the primary mechanisms that might be involved in GPx1 protection is modulation of cell sensitivity to induction of cell death. According to our previously reported measurements, GP activity was more than two times higher in purified cortical neurons from GPx1 animals compared with that in normal mice.[5] The ability of antioxidants such as *N*-acetylcysteine to protect neuronal cells against TNF-α-induced apoptosis in cell culture has been reported (18). To address this issue we exposed cortical neurons from normal and transgenic mice to an in vitro model of I/R, anoxia/glucose depletion following reoxygenation/high glucose treatment, as well as different concentrations of exogenous TNF-α and FasL. Neuronal viability decreased significantly at 18 and 24 h of normoxia following hypoxic insult (Fig. 3*A*). Nevertheless, cortical neurons from transgenic mice revealed almost 2-fold greater resistance to anoxia/glucose depletion compared with cells from normal animals. Increased protection in response to TNF-α and FasL was also observed in GPx1 cell cultures (Fig. 3*B*). Moreover, cortical neurons overexpressing GPx1 were more resistant to combined treatment by anoxia/glucose depletion and TNF-α or FasL (Fig. 3*C*). A weak additive effect of both types of cell injury inducers was observed, probably due to the use of the same or similar death-executing mechanisms, which were saturated under experimental

[5] N. Ishibashi, O. Prokopenko, M. Weisbrot-Lefkowitz, K. R. Reuhl, and O. Mirochnitchenko. Glutathione peroxidase inhibits cell death and glial cell activation following experimental stroke. *Submitted for publication.*

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Table I. *Quantitative analysis of chemokine and cytokine expression by RNase protection assay*[a]

	Time (h)				
	0	3	8	24	48
RANTES					
nTg	1.00	1.28	1.28	2.23	1.42
GPx1	1.00	1.22	1.27	1.22[b]	1.23
Eotaxin					
nTg	1.00	1.53	1.61	2.57	2.45
GPx1	1.00	1.45	1.40	1.25[b]	1.25[b]
MIP-1β					
nTg	1.00	16.05	17.30	7.51	4.85
GPx1	1.00	2.74[b]	3.99[b]	1.42[b]	1.26[b]
MIP-1α					
nTg	1.00	14.3	13.74	8.52	2.97
GPx1	1.00	3.03[b]	3.84[b]	1.28[b]	1.16
MIP-2					
nTg	1.00	5.88	5.89	13.39	2.00
GPx1	1.00	1.77[b]	1.80[b]	1.90[b]	1.25
IP-10					
nTg	1.00	2.54	2.87	3.69	1.81
GPx1	1.00	1.42	1.46[b]	1.50[b]	1.30
MCP-1					
nTg	1.00	2.05	2.36	7.87	3.20
GPx1	1.00	1.65	1.79	1.35[b]	1.30[b]
TCA-3					
nTg	1.00	1.58	1.73	2.97	1.41
GPx1	1.00	1.45	1.34	1.22[b]	1.26
TNF-β					
nTg	1.00	2.79	3.24	2.54	1.85
GPx1	1.00	1.64[b]	2.12[b]	1.50	1.30
Leukotriene-β					
nTg	1.00	2.25	2.64	2.93	1.96
GPx1	1.00	1.90	2.21	2.43	1.71
TNF-α					
nTg	1.00	3.23	3.28	4.49	2.66
GPx1	1.00	1.57[b]	1.96[b]	2.03[b]	1.36[b]
IL-6					
nTg	1.00	3.39	3.74	9.73	2.08
GPx1	1.00	1.60[b]	2.04	2.35	1.47
IFN-γ					
nTg	1.00	2.10	2.30	2.81	1.57
GPx1	1.00	1.52	1.82	2.00[b]	1.47
IFN-β					
nTg	1.00	2.32	2.51	2.77	1.58
GPx1	1.00	1.64[b]	1.80[b]	2.03[b]	1.51
TGF-β1					
nTg	1.00	2.21	2.23	2.27	2.00
GPx1	1.00	1.44[b]	1.49[b]	1.70[b]	1.65
TGF-β2					
nTg	1.00	1.78	1.87	1.98	1.57
GPx1	1.00	1.39[b]	1.52[b]	1.57[b]	1.55
Caspase-8					
nTg	1.00	1.69	1.94	2.62	2.76
GPx1	1.00	1.13[b]	1.38[b]	1.25[b]	1.21[b]
FasL					
nTg	1.00	2.71	2.25	2.19	1.80
GPx1	1.00	1.20[b]	1.53[b]	1.32[b]	1.29
Fas					
nTg	1.00	4.56	3.87	2.89	3.14
GPx1	1.00	1.65[b]	1.85[b]	1.55[b]	1.50[b]
FADD					
nTg	1.00	1.75	3.78	3.72	3.24
GPx1	1.00	1.30[b]	1.53[b]	1.42[b]	1.42[b]
FAP[c]					
nTg	1.00	2.11	1.98	2.00	1.84
GPx1	1.00	1.44[b]	1.52[b]	1.51[b]	1.48
FAF					
nTg	1.00	2.87	3.66	3.23	3.08
GPx1	1.00	2.46	2.04[b]	1.70[b]	1.45[b]
TRAIL					
nTg	1.00	2.52	3.44	2.95	2.51
GPx1	1.00	1.62[b]	1.88[b]	1.66[b]	1.57[b]
RIP					
nTg	1.00	2.44	3.45	3.77	3.32
GPx1	1.00	1.68[b]	1.83[b]	1.62[b]	1.64[b]
TRADD					
nTg	1.00	1.90	3.34	3.62	3.22
GPx1	1.00	1.41	1.54[b]	1.95[b]	1.81[b]
TNFRp55					
nTg	1.00	2.87	3.86	5.50	4.89
GPx1	1.00	1.41[b]	1.78[b]	1.69[b]	1.57[b]

Table 1 *Continued*

[a] To compare RNA amounts in the protected bands, the films were scanned using an image densitometer (GS-670; Bio-Rad), and final values were factored relative to GAPDH levels. Only means are presented.

[b] Values of $p < 0.05$, GPx1 in comparison to nontransgenic mice (one-way ANOVA); $n = 5–6$ animals per group.

[c] FAP, Fas-associated phosphatase.

conditions. The data indicate that an increased level of GP in neurons interferes with the mechanisms of direct cellular toxicity involved in brain I/R injury.

Effect of GP on DNA binding activity, SEK1/MKK4, and c-Jun phosphorylation

Transcriptional regulation of FasL and Fas genes is mediated by several transcription factors, including AP-1, NF-κB, NF-AT, ATF-2, Egr3, etc. Most studies performed to date focus on mechanisms of activation involved in lymphocyte homeostasis. Two studies reported stress-induced activation of FasL expression via induction of the following sequences: c-Fos/c-Jun-specific AP-1 site or c-Jun/ATF-2-specific site (19, 20). Because there is significant evidence to indicate importance of AP-1 subunits in neural homeostasis and during focal I/R injury in particular (21, 22), we analyzed the DNA binding activity of AP-1 consensus oligonucleotides as well as published and potential AP-1 binding sites, identified using a TRANSFAC search in the mouse FasL promoter

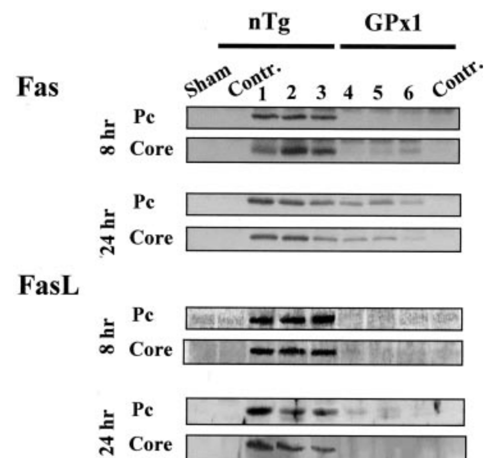

FIGURE 2. Fas and FasL expression in normal and GPx1 transgenic mice during I/R. Levels of Fas and FasL were evaluated by Western blot analysis using anti-Fas and FasL Abs. Samples were prepared from pyriform cortex (Pc) and core regions of the infarct at 8 and 24 h of reperfusion. Samples from sham-operated animals as well as contralateral regions of the brain were used as a control. Analysis of three independent animals per each group is shown.

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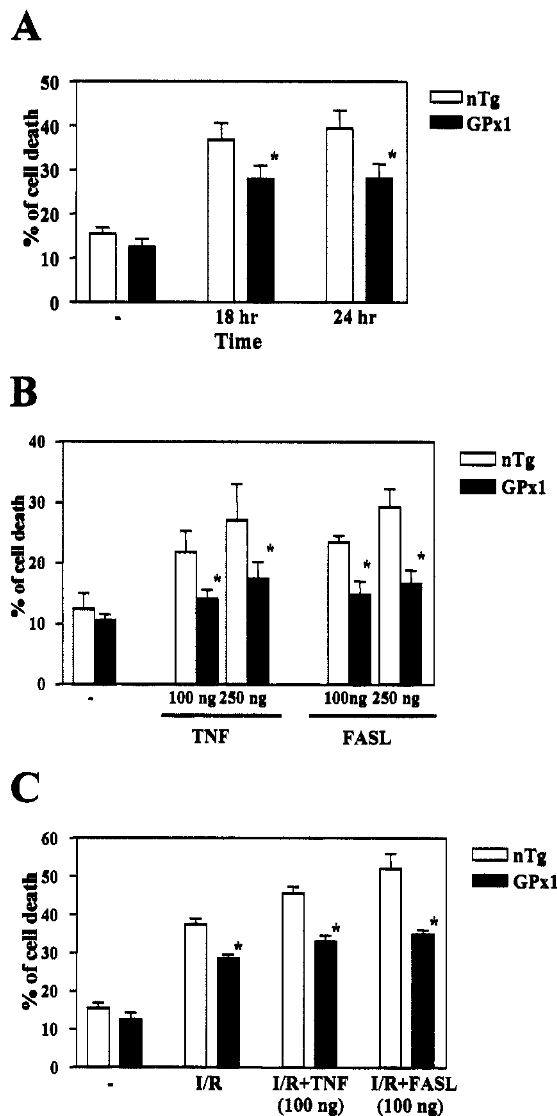

FIGURE 3. Cortical neuron cell death induced by hypoxia/reoxygenation and by TNF-α and FasL. Primary cortical neurons from normal and transgenic mice were exposed to hypoxia in medium without glucose for 4 h. After exposure medium was replaced with normal growth medium without (*A*) or with 100 ng/ml TNF-α/FasL (*C*). In a separate experiment cells were treated with 100 and 250 ng/ml TNF-α or FasL without hypoxic exposure (*B*). LDH was measured at 18 (*A*) and 24 h. Values represent the mean ± SD from three independent experiments, each conducted in quadruplicate. *, $p < 0.05$ vs nontransgenic mice.

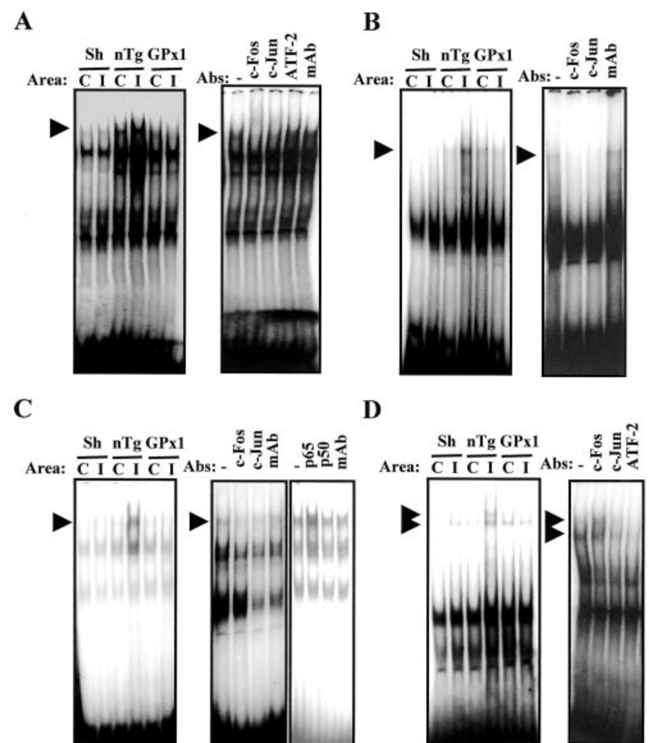

FIGURE 4. I/R-induced DNA binding activity of the proteins in nuclear extracts from normal (nTg) and GPx1 (GPx1) mice. Extracts from sham-operated mice (Sh) and mice after I/R were incubated with ^{32}P-labeled oligonucleotides corresponding to AP-1 consensus site (*A*) and FasL promoter sequences (*B*, −282 to −252; *C*, −233 to −215; *D*, −119 to −103), and then EMSA was performed. At least five animals were analyzed per group. C and I, Contralateral and ipsilateral cortexes, respectively. No differences were observed in DNA binding in extracts from both brain sides of nontransgenic and GPx1 sham-operated animals; therefore, only one sample is shown per assay. Binding activities of representative animals are shown. For identification of binding proteins, extracts were incubated with c-Fos, c-Jun, ATF-2, p65, and p50 Abs (Abs) or with normal serum (mAb). Bands corresponding to identified complexes are shown by arrows.

region (http://transfac.gbf.de/TRANSFAC/; Braunschweig, Germany). Results are shown in Fig. 4. All four tested oligonucleotides revealed significantly enhanced binding in damaged brain regions of nontransgenic mice after I/R. Induction was very low in corresponding samples from GPx1 mice. An Ab supershift assay was used to analyze transcription factors involved in complex formation. One of the bands in three oligonucleotides (consensus AP-1, −282 to −252 and −119 to −103) contained c-Fos and c-Jun, whereas two bands in DNA binding with oligonucleotide −233 to −215 contained c-Jun and ATF-2, but not c-Fos. Because oligonucleotide −119 to −103 also contains the binding site of NF-κB identified by Matsui et al. (23) as a factor critically involved in FasL activation in T cells, we tested anti-p65 and p50 Abs. As shown in Fig. 4*C*, the results eliminated the possibility of involvement of this transcription factor in complex formation with oligonucleotide −119 to −103. The existence of two major complexes in DNA binding with oligonucleotide −233 to −215 is in agreement with the data reported by Faris et al. (19), who first identified this stress-responsive element and showed its binding with c-Jun and ATF-2, but not c-Fos. None of the described complexes was supershifted with normal serum (Abs; see Fig. 4). The specificity of the binding was verified in the presence of competitive and noncompetitive oligonucleotides (data not shown). Several additional bands not shifted with tested Abs (Fig. 4, *A* and *C*) are currently under investigation.

Recently, Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) activation was shown to be critical for the induction of death-inducing ligands in neuronal cells during focal cerebral ischemia in vivo as well as in neuroblastoma cells treated with doxoribicin (24, 25). Phosphorylation of ATF-2 and c-Jun by JNK/SAPKs was linked to activation of FasL, TNF-α, and probably TRAIL, which induce neuronal cell death. Because the level of activation of those transcription factors was sensitive to GP overexpression in GPx1 transgenic mice, we analyzed phosphorylation of SEK1, which is upstream of the JNK/SAPK, as well as the level of phospho-c-Jun by Western blot using Abs specific for phosphorylated forms of the proteins. Results are shown in Fig. 5. The data indicate that the level of phospho-c-Jun was significantly diminished in brain regions of transgenic mice. The level of phosphorylated SEK1 was dramatically decreased in the core region of

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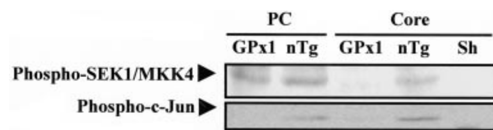

FIGURE 5. Phosphorylation of SEK1/MKK4 and c-Jun in pyriform cortex (PC) and core of the infarct from normal (nTg), GPx1 mice (GPx1), and sham-operated animals (Sh) at 1 h after I/R. Western blots were probed with phospho-SEK1/MKK4 (Thr[261]) and phospho-c-Jun (Ser[63]) Abs (Cell Signaling Technology). The results from representative animals from three independent experiments are shown.

the infarct and was only slightly affected in pyriform cortex. This result might indicate differences in sensitivity of various levels of SAPK/JNK signal transduction mechanisms in brain regions undergoing predominantly apoptotic or necrotic cell death in transgenic mice after I/R compared with normal animals. It is important to note that this effect was observed very early, before the development of histologically evident cell damage. This result is in agreement with a role of ROS in activation of the JNK/SAPK cascade, which is well documented (26).

Discussion

ROS are thought to be involved in the initiation of immediate cell damage during I/R as well as in the modulation of cellular signaling and control of gene expression. Therefore, antioxidant enzymes controlling ROS production possess the ability to effectively modulate the development of brain injury during I/R. Indeed, as shown by us and others, transgenic mice overexpressing antioxidant enzymes demonstrate significant protection, whereas knockout animals with reduced antioxidant defense show increased sensitivity to brain I/R injury (5, 27, 28). To investigate possible mechanisms of protection demonstrated by GPx1, we analyzed the inflammatory response in normal and transgenic mice after I/R.

In wild-type mice brain injury was accompanied by infiltration by neutrophils. In contrast, brains of transgenic mice showed decreased neutrophil migration. One possibility for this effect is the difference between transgenic and wild-type mice in the ability to produce chemoattractant factors. Neutrophils in brain ischemia respond to a variety of chemoattractant signals, including chemokines from injured cells. We observed dramatic differences in the production of several key CXC and CC chemokines (such as MIP-2, MIP-1α, MIP-1β, IP-10, and MCP-1) attracting neutrophils and macrophages in Gpx1 transgenic and normal mice as early as 3 h of reperfusion. This effect preceded the appearance of migrating leukocytes in tissue parenchyma. It is now becoming clear that chemokines and their receptors might have additional roles during I/R other than chemotaxis, such as gliosis, regulation of neuronal sprouting, etc. (29). The role of ROS in chemokine activation is well recognized (30). Another possible target for regulation by GP-sensitive pathways includes adhesion molecules, which play key roles in leukocyte recruitment and are known to be sensitive to oxidative stress (30, 31).

Chemokines may diffuse through the neuropil to induce neutrophil margination in blood vessels; however, such attraction may not be sufficient to induce neutrophils to traverse the vascular wall. For example, direct intracerebral injection of neutrophil-activating protein-1/IL-8 caused neutrophil margination, but no infiltration into the perivascular space, indicating an intact vascular barrier (32). To enter the brain parenchyma, inflammatory cells must either traverse the endothelial cells cytoplasm or pass between cells, a process impeded by endothelial tight junctions. In wild-type mice, compromise of vascular integrity was indicated by the appearance of perivascular space containing edema fluid, as we reported previously (5). Transgenic animals manifested less vascular permeability, possibly due to an increased constitutive ability of GPx1-overexpressing cells to withstand oxidative stress and toxic metabolites. A dramatic effect of GP overexpression on leukocyte behavior has been previously reported in two other inflammation-related injury models, kidney I/R and LPS-induced septic shock (6, 7). Significant resistance to injury in both models correlated with the level of key inflammatory mediators.

Measurement of mRNA for several cytokines after brain I/R indicates significant effects of GP on the regulation of their expression. Levels of TNF-α, FasL, and especially IL-6 mRNA as well as receptors for TNF-α and FasL (TNF Rp55 and FasL) were down-regulated after 3 h of reperfusion. There is strong evidence implicating several inflammatory cytokines and their receptors in exacerbation of focal I/R damage, although protective effects for TNF-α and IL-6 also were also reported. Levels of IL-6 had a strong correlation with neurologic function and infarct size in animal experimental ischemia as well as in stroke patients (33, 34). It was also demonstrated that the IL-6 system plays an important role in local inflammatory reactions by amplifying leukocyte recruitment and changing endothelial permeability (35). The ability of GPx1 to modulate the level of IL-6 is most likely due to the inhibitory effect on ROS production, which is known by several investigators to stimulate transcriptional activation of IL-6 (36, 37).

Increased production of TNF-α during permanent and transient ischemia in experimental models and clinical studies is well documented (38). Its inhibition by pharmacological agents, neutralizing Abs, or soluble receptors has a protective effect. Despite several reports of beneficial effects of this cytokine from in vitro studies and knockout mice, endogenous production of TNF-α in I/R brain models is deleterious (38). TNF-α is a potent activator of necrosis and/or apoptosis through TNF Rp55 receptor depending on the cell type and/or the intracellular ATP concentration (15).

Involvement of other members of the death receptor family, Fas and FasL, in active neuron killing during I/R has attracted significant attention (39, 40). Both molecules were activated during brain I/R. FasL-induced apoptosis in primary neurons and most importantly in *lpr* mice expressing dysfunctional Fas was more resistant to reversible cerebral artery occlusion (25, 41). According to the first report, FasL in the ischemic penumbra was exclusively expressed in neurons, whereas the second report shows positive staining in cells of neuronal and microglial origin. A role for FasL expression in the control of leukocyte extravasation as well as maintenance of vascular integrity was also demonstrated (42). Our data indicate that the expression of TNF-α and TNFR-1 as well as Fas and FasL during I/R was significantly affected by GPx1 overexpression. Therefore, important mechanisms of protection in GPx1 transgenic mice may include regulation of expression of executors of ischemia-mediated neuronal cell death as well as cellular sensitivity to these molecules. Several transcription factors were shown to be important for up-regulation of Fas and FasL expression. Their activation during the cellular response to I/R may be mediated by several signal transduction mechanisms, including the JNK/SAPK pathway. The DNA binding activity of AP-1-binding transcription factors as well as SEK1/MKK4 and c-Jun phosphorylation were very sensitive to GP overexpression in transgenic mice. Among other possible direct targets of redox regulation are transcription factors themselves or other regulatory redox-sensitive proteins, such as thioredoxin or GST. Proteasome activity, which plays an important role in transcription factor activation, was shown to be affected by the level of GPx1 activity in transgenic and knockout mice (43). Recently, another group (22) reported the ability of Cu,Zn-superoxide dismutase overexpression

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to reduce focal I/R-induced AP-1 activation, although no specific targets of this regulation were demonstrated.

The behavior of AP-1-related transcription factors was examined in the present report. As previously noted, involvement of other transcription factors, especially NF-κB, in the mediation of FasL activation was previously demonstrated. NF-κB constitutes an important component of cellular response to oxidative stress. We as well as others have previously reported the ability of antioxidant enzymes in transgenic animals to inhibit activation of this transcription factor during I/R (6, 44). Characterization of the behavior of other redox-sensitive transcription factors and the genes that they regulate is currently in progress.

We also tested whether protection may be provided by the increased antioxidant potential of neurons themselves. In vitro studies show increased protection of purified cortical neurons from transgenic mice to hypoxia/reoxygenation-induced injury, to treatment by death-inducing cytokines, as well as to combined treatment by both insults. These results support the hypothesis that ROS are implicated in Fas-induced apoptosis of neurons, as demonstrated in other type of cells (45, 46).

The mechanism of the GPx1 protective role during brain I/R injury was recently a focus of a report by Crack et al. (47). These authors demonstrated that GPx1 knockout mice display increased infarct size and exacerbated apoptosis compared with normal animals after MCA occlusion and reperfusion. Those data correlate with the high resistance of our GPx1 transgenic mice as well as neurons from those animals in cell culture to inducers of cell death. Importantly, GPx1 knockout mice revealed significant increases in caspase-3 activation as early as 8 h of reperfusion, in contrast to normal mice. Caspase-3 is one of the effector caspases that may be activated by oxidative stress (48). Therefore, the authors suggested that ROS sensitive to GPx1 play an important role in regulation of the apoptosis cascade. The effect of modulation of the level of Cu,Zn-superoxide dismutase on caspase-8 activation in transgenic mice was also reported (49). Interestingly, at 8 h of reperfusion we already observed in our model a significant difference in FasL expression, which represents one of the key initiator of apoptosis. It most likely influences the level of activation of executor apoptotic mechanisms, including caspases. Which of those death-initiating or -executing mechanisms is more sensitive to ROS needs to be further investigated.

In conclusion, GPx1 overexpression in transgenic mice provides significant protection against focal I/R injury by multiple mechanisms. By modulating the activity of cells producing inflammatory mediators and by decreasing the number of injured brain cells, GPx1 significantly attenuated the postischemic inflammatory reaction. On a molecular level, early transcriptional activation of cytokines, including several classes of chemokines and families of death-inducing cytokines and their receptors was a target of GPx1-sensitive ROS. This effect was mediated in part through stress-responsive kinases and transcription factors. The data suggest that GPx1 is able to interfere efficiently not only with major neuronal death pathways, but also with postischemic inflammation. Recent studies with GPx1 mimetic BXT-51072 show a significant neuroprotective effect against oxidative as well as excitotoxic-neuronal cell death (50). Therefore, GPx1 and compounds with similar activity represent promising tools for future therapeutic interventions designed to prevent or regulate pivotal elements of brain damage following ischemic stroke.

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