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Original Contribution

A global transcriptomic view of the multifaceted role of glutathione peroxidase-1 in cerebral ischemic–reperfusion injury

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ABSTRACT

Transient cerebral ischemia often results in secondary ischemic/reperfusion injury, the pathogenesis of which remains unclear. This study provides a comprehensive, temporal description of the molecular events contributing to neuronal injury after transient cerebral ischemia. Intraluminal middle cerebral artery occlusion (MCAO) was performed to induce a 2-h ischemia with reperfusion. Microarray analysis was then performed on the infarct cortex of wild-type (WT) and glutathione peroxidase-1 (a major antioxidant enzyme) knockout ($Gpx1^{-/-}$) mice at 8 and 24 h postreperfusion to identify differential gene expression profile patterns and potential alternative injury cascades in the absence of Gpx1, a crucial antioxidant enzyme, in cerebral ischemia. Genes with at least ±1.5-fold change in expression at either time point were considered significant. Global transcriptomic analyses demonstrated that 70% of the WT-MCAO profile overlapped with that of $Gpx1^{-/-}$-MCAO, and 28% vice versa. Critical analysis of the 1034 gene probes specific to the $Gpx1^{-/-}$-MCAO profile revealed regulation of additional novel pathways, including the p53-mediated proapoptotic pathway and Fas ligand (CD95/Apo1)-mediated pathways; downplay of the Nrf2 antioxidative cascade; and ubiquitin–proteasome system dysfunction. Therefore, this comparative study forms the foundation for the establishment of screening platforms for target definition in acute cerebral ischemia intervention.

Stroke causes 9% of all deaths around the world and is the second most common cause of death after heart disease [1]. The prevalence of stroke increases exponentially from 30 years of age, with advanced age presenting as one of the most significant risk factors [2]. Because of the aging population, this burden is expected to increase during the next 20 years. Existing literature suggests that ischemic stroke triggers severe focal hypoperfusion accompanied by deprivation of oxygen and glucose to the cerebral tissue, together with loss of ATP, depolarization of neurons, and elevated extracellular potassium concentration, which subsequently leads to excitotoxicity, as well as increased oxidative stress promoting microvascular injury, blood–brain barrier deregulation, postischemic inflammation, and eventually the consequential neurological deficit. A number of biochemical cascades including the production and release of reactive oxygen species (ROS) [3], inflammatory changes [4], and necrotic and apoptotic cell death pathways [5] have been documented in the ischemic core and surrounding penumbra [6]. Despite the current view that oxidative stress and inflammation are the major pathophysiological mechanisms implicated in ischemic stroke, a detailed and global examination of the molecular events linking these pathways is lacking.

Glutathione peroxidase (Gpx) is a selenium-containing enzyme that catalyzes the reduction of a variety of biological peroxides at the expense of reduced glutathione. Gpx1 is the most abundant isoform and its role has been implicated in neurodegenerative disorders such as Parkinson disease, dementia with Lewy bodies [7], and traumatic brain injury [8]. Because of its high abundance, deletion of the Gpx1 allele would lower overall Gpx activity in the brain significantly. Gpx1 knockout ($Gpx1^{-/-}$) mice do not show overt phenotypic differences,

Abbreviations: DAVID, Database for Annotation, Visualization, and Integrated Discovery; GEO, Gene Expression Omnibus; Gpx, glutathione peroxidase; $Gpx1^{-/-}$, glutathione peroxidase knockout; Hsp, heat shock protein; MCAO, middle cerebral artery occlusion; NTC, no-template control; PCR, polymerase chain reaction; ROS, reactive oxygen species; TNF, tumor necrosis factor; UPS, ubiquitin–proteasome system; WT, wild type.

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but all indications suggest that these mice are in a chronic "pro-oxidant" state [9]. Indeed, a recent study from our laboratory illustrated that the absence of Gpx1 exacerbated stroke injury via increased ROS production and vascular permeability [10]. Furthermore, Gpx1$^{-/-}$ mice demonstrated an increase in caspase-3 activation and greater infarct volume [11]. As such, it has been proposed that selenium dietary supplementation may provide cytoprotection against neurodegenerative and cardiovascular disorders through the maintenance of Gpx1 activity and other detoxifying selenoenzymes such as thioredoxin reductase and selenoproteins [12]. Despite extensive studies demonstrating exacerbation of neuronal injury after stroke in its absence, the molecular events mediated by Gpx1 after cerebral ischemia require further elucidation.

The aim of this study was to decipher the temporal recruitment of signaling mechanisms deregulated and/or affected in the course of cerebral ischemia-mediated neuronal death in the presence or absence of Gpx1 from a global transcriptomic perspective. This study of global gene profiling analyses revealed a substantial number of genes with significant minimum transcriptional regulation of ±1.5-fold difference at at least one of the reperfusion time points in postischemic Gpx1$^{-/-}$ mice (1456 probe sets) compared to the WT counterparts (572 probe sets). A mere 422 gene probes are common to postischemic WT and Gpx1$^{-/-}$ mice, accounting for over 70% of the WT middle cerebral artery occlusion (MCAO) gene profile. This signifies substantial modulation of biological pathways in the absence of Gpx1 upon cerebral ischemia. Intriguingly, inter-time-point fold-change differences within each model did not reveal any stark contrast compared across models. The overall data from this study imply that the absence of Gpx1 induces activation of compensatory signaling mechanisms as a means to reduce oxidative stress, without potentiating the transduction of existing activated pathways.

Materials and methods

Animals

All animal procedures were approved by The University of Melbourne Animal Ethics Committee. Male WT and Gpx1$^{-/-}$ mice on a C57BL/6J background of 8–10 weeks were employed in this study. Gpx1$^{-/-}$ mice demonstrate no overt phenotype and there exist no differences in the cerebral vascular anatomy between the Gpx1$^{-/-}$ mice and their WT counterparts [9,11].

Mouse focal cerebral ischemia model

WT and Gpx1$^{-/-}$ mice underwent the MCAO model of cerebral ischemia–reperfusion injury as previously mentioned [11]. Briefly, mice were anesthetized intraperitoneally with a cocktail consisting of ketamine hydrochloride (200 mg/kg; Pfizer, West Ryde, NSW, Australia) and xylazine (10 mg/kg; Troy Laboratories, Smithfield, NSW, Australia). After ligation of the right proximal common carotid artery, a 6-0 nylon monofilament with a silicone rubber-coated tip diameter of 0.21–0.23 mm (Doccol Corp., Redlands, CA, USA) was introduced into the distal internal carotid artery and was advanced 12 mm distally to the carotid bifurcation where it occluded the middle cerebral artery. In experiments examining biochemical end-points and infarct size, a 2-h ischemic period was used. Reduction in cerebral blood flow upon intraluminal suture insertion was confirmed with laser Doppler. Control sham animals were subjected to the initial anesthetic and neck incision only. All animals were put back onto a 37 °C heating pad postsurgery to recover from anesthesia. After a 2-h ischemic period, the animals underwent reperfusion by withdrawal of the suture under anesthesia and return to the heating pad until euthanized at the designated time points of 2, 8, and 24 h.

Preparation of tissue extracts

At selected time points postreperfusion, WT and Gpx1$^{-/-}$ mice were deeply anesthetized and then decapitated. Brains were removed quickly and the cortex was dissected from each of the hemispheres. The whole cortex was frozen immediately in liquid nitrogen and stored at −80 °C.

Total RNA extraction and isolation

Total RNA from homogenized cortex samples was extracted using the conventional phenol–chloroform extraction method with Trizol reagent (Sigma–Aldrich). The obtained RNA samples were further purified using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. All pipette tips used were RNase-free and filtered. The RNA samples were aliquotted at 1.5 μl for spectrophotometric quantification using Nanodrop ND-1000 version 3.2.1 and 1 μl for RNA quality analysis using an E-gene HDA-GT12 genetic analyzer.

Real-time polymerase chain reaction (PCR)

Reverse transcription was carried out according to steps specified by the manufacturer (Applied Biosystems TaqMan reverse transcription reagents). Each cDNA sample was duplicated with two no-template controls (NTCs) for each probe used. Twenty microliters of the TaqMan master mix was pipetted into the bottom of each well of the optical 96-well fast reaction plate. Five microliters of cDNA or water (NTC) was added to the designated reaction well. The plate was then read by the 7000 Fast Real-Time PCR System with conditions according to the manufacturer's protocol.

Microarray analysis

Global transcriptomic profiling was performed on Illumina Mouse Ref8 version 2 arrays for the right-hemisphere injured cortex samples of WT and Gpx1$^{-/-}$ sham controls and 2, 8, and 24 h after reperfusion. Four biological replicates were obtained for the sham controls and each of the three time points for each mouse strain. Each biological replicate from individual animals was run on an array. Total RNA samples of 500 ng were brought up to an initial start volume of 11 μl. RNA was reverse transcribed using an Illumina TotalPrep RNA amplification kit. The yield of cRNA was quantitated using the NanoDrop ND-1000. Seven hundred fifty nanograms of cRNA prepared in 5 μl RNase-free water was mixed with 10 μl hybridization buffer. The hybridization process using streptavidin–Cy3 labeling was carried out according to the manufacturer's instruction (Illumina, Inc.) The bead chip was then ready for scanning on the Illumina scanner using Bead Studio software at a scan factor of 0.8.

Microarray data collection and analysis

Initial analysis of the scanned images was performed using BeadScan (Illumina). Signal data generated from the Illumina Bead Studio software was analyzed on GeneSpring version 7.3 software. All differentially expressed genes in this study were selected based on the following parameters: (1) a minimum of ±1.5-fold change at at least one of the reperfusion time points and (2) the gene passed the statistical screening test of one-way analysis of variance (ANOVA; $p<0.05$) and Benjamini–Hochberg false discovery rate correction. Genes that were differentially expressed are annotated according to the Gene Ontology biological process provided by the online bioinformatics resource Database for Annotation, Visualization, and Integrated Discovery (DAVID) 6.7 (http://david.abcc.ncifcrf.gov/) [13,14]. This exploratory, computational *cum* statistical tool of clustering and enrichment permits the identification of biological processes most pertinent to the biological phenomena of interest. All