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Ischemia-Reperfusion-Related Repair Deficit after Oxidative Stress: Implications of Faulty Transcripts in Neuronal Sensitivity after Brain Injury

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Abstract

Diseases of the heart are the No.1 killer in industrialized countries. Brain injury can develop as a result of cerebral ischemia-reperfusion due to stroke (brain attack) and other cardiovascular diseases. Learning about the disease is the best way to reduce disability and death. We present here whether gene repair activities are associated with neuronal death in an ischemia-reperfusion model that simulates stroke in male Long-Evans rats. This experimental stroke model is known to induce necrosis in the ischemic cortex. Cerebral ischemia causes overactivation of membrane receptors and accumulation of extracellular glutamate and intracellular calcium, which activates neuronal nitric oxide synthase, causing damage to lipids, proteins, and nucleic acids, and reduces energy sources with consequent functional deterioration, leading to cell death. Restoration processes normally repair genes with few errors. However, ischemia elevates oxidative DNA lesions despite these repair mechanisms. These episodes concurrently occur with the induction of immediate-early genes that critically activate other late genes in the signal transduction pathway. Damage, repair, and transcription of the *c-fos* gene are presented here as examples, because Fos peptide, one of the components of activator protein 1, activates nerve growth factor and repair mechanisms. The results of our studies show that treatments with 7-nitroindazole, a specific inhibitor of nitric oxide synthase known to attenuate nitric oxide, oxidative DNA lesions, and necrosis, increase intact *c-fos* mRNA levels after stroke. This suggests that the accuracy of gene expression could be accounted for the recovery of cellular function after cerebral injury.

Keywords

Gene repair; Head injury; Immediate-early genes; Mutagenesis; Neuroregeneration; Oxidative stress; Plasticity; Signal transduction; Stroke; Transcription

Stroke induces cerebral lesions through the combined action of several mechanisms, including excitotoxicity, free radical production, inflammation, and apoptosis. Excitotoxicity results from excessive release of glutamate, the main excitatory neurotransmitter from dead or dying neurons. Elevated glutamate levels trigger the death of neighboring neurons that survived the initial insult, but cannot survive such a massive activation under restricted glucose and oxygen supply. Glutamate acts through ion-channel-mediated receptors (NMDA and AMPA-kainate receptors) and metabotropic receptors. Activation of these membrane receptors can be mediated via different G proteins, leading to the activation of phospholipases, phosphoinositide

hydrolysis, and the formation of inositol triphosphate and diacylglycerol. This event leads to Ca^{2+} influx and activation of protein kinase C. Other membrane receptors may change adenylate cyclase and cAMP production. It is likely that membrane receptor activation leads to activation of MAP kinase which regulates activator protein 1 (AP-1; a transcription factor that is neurotoxic in cell culture studies). The major component of AP-1 encoded in the *c-fos* gene is expressed at a minimal level under normal conditions, but is activated immediately after stroke and injury to the nerve [2,35]. Recent studies showed that *c-fos* mRNA is transcribed, when its nuclear gene contains oxidative DNA lesions (ODLs) [2,12]. The effect of ODLs on neurotoxicity is not clear, but the presence of excess ODLs requires DNA repair processes, including the expression of poly(ADP-ribose) polymerase (PARP). We will review what we know about gene damage in the brain after cerebral oxidative stress and its relationship to the fidelity of gene transcription from genes that are infested with oxidative damage before they can be completely repaired.

Nucleic Acid Lesions

The brain is particularly vulnerable to oxidative stress because of its high oxygen consumption. The brain has low levels of antioxidant enzymes (catalase, glutathione peroxidase) and high levels of substrates for oxidative reaction (iron, membrane polyunsaturated fatty acids). Ischemia-reperfusion injury perturbs oxygen supply and energy metabolism of the brain, generating free radicals [28,53]. Free radicals interact with all molecules of the cell, causing damage to proteins, lipids, membrane, and nucleic acids. With the exception of oxidative RNA lesions (ORLs) which are not repaired, gene repair processes normally repair ODLs with few errors. Ischemia-reperfusion induces a significant elevation of reactive oxygen species (ROS), ODLs and ORLs in the brain [31,61,70]. To maintain gene expression of the correct message, the brain would have to contain an extremely accurate mechanism that removes and repairs ODLs, such that oxidative damage that occurs in an oxygen-utilizing organ will be kept to a minimum. The levels of two repair enzymes that remove some forms of ODLs, 8-oxo-2'-deoxyguanosine glycosylase and endonuclease APE/Ref-1, are elevated after brain injury [31,54]. During the first hours of initial injury after cerebral ischemia, however, the level of gene damage exceeds the capacity to repair by the brain (fig. 1a). While tissue repair via neurogenesis in the brain is limited to a certain population of cells in the subgranular zone of the dentate gyrus and in the rostral subventricular zone in the third ventricle of the brain [3, 34,43,44,58,59,89,100], investigations showed that different regions of the brain may have different rates of repair [5]. Neuroinflammatory processes in response to the initial injury may further initiate ODLs and delay recovery or cause brain cell death [29,49]. Relatively little is known about the effect of ODLs during the primary insult.

Recent studies have demonstrated an elevation of ODLs by DNA single-stranded breaks, base modifications, and loss of a base (AP site) from DNA in the brain after stroke in rats [62]. Unlike DNA fragmentation that is observed in late apoptosis, base modifications in genomes occur early during reperfusion, but can be removed and resynthesized by DNA repair processes. Knowledge of the process of gene repair in the brain mainly comes from studies using single replication cells. Information about repair in surviving brain is not available. If the resulting ODLs are not repaired, however, there are both early and late consequences for the cell. The presence of ODLs, before repair, results in errors in transcription and translation which may delay or prevent the expression of proteins, with beneficial roles in recovery. In actively dividing cells, when neurogenesis is possible, ODLs most likely inhibit DNA replication, except in the case when bypass replication is permitted. The recent demonstration of bypass replication by certain DNA polymerases, if existing in the brain, may have errorprone consequences [30]. During repair, PARP can be activated in acute response and initiates processes that may result in early energy depletion despite reperfusion. A delicate balance exists between the two that ensures proper recovery.

Our hypothesis is that newly transcribed mRNA from genes that contain ODLs may be faulty and contributes to neurosensitivity. Enhancing accurate products that are involved in cell viability may augment the response to overcome oxidative stress (fig. 2). We will present background evidence that supports this hypothesis.

Experimental Stroke Model

The focal cerebral ischemia-reperfusion (FCIR) model that is used in this presentation involves a bilateral carotid occlusion (BCO) and unilateral occlusion of the middle cerebral artery (MCAO) for 30–120 min using suture ligation, followed by a release of BCO/MCAO to allow blood reperfusion for various time intervals. The FCIR model is characterized by severe transient ischemia (blood flow reduced by 88–92%) in the core area of the right cerebral cortex irrigated by the right middle cerebral artery [9,64,98]. Brain injury using FCIR models has been shown to induce ROS [56]. The FCIR model of the rat elicits oxidative stress during early reperfusion in only one hemisphere, thereby leaving the other hemisphere for comparison of results as a nonischemic control [2,11,13]. The advantages of the rat model also include the ability to use monoclonal antibodies of murine origin and a well-studied profile of the expression of the immediate-early gene after ischemia and reperfusion [1,82]. Ischemia for 30 min or less results in minimal ischemic brain injury in this model (infarct volume < 10 mm³), but induces DNA fragmentation in the core after 4 days of reperfusion [22,32]. BCO/MCAO for 90 min results in significant injury (infarct volume <90 mm³) and DNA fragmentation within 24 h [2,55].

Because humans and rats generally have a functional circle of Willis, the rat FCIR model may reflect more closely the molecular pathophysiology of stroke and other ischemic injuries than does the mouse model. We have used the FCIR model in male Long-Evans hooded rats to study gene damage and repair [12,13] and gene regulation in the brain [11,14,60,79]. The mitochondria and nuclear genes are prone to DNA damage [8,12-14,26,40,61,70]. DNA repair removes ODLs in the mitochondrial and in the nuclear genes [7,19,54,61,67]. In addition to mitochondrial DNA [13], we have detected ODLs in nuclear genes [12,70]. We have focused on nuclear genes as a critical target for ODLs, because the essential genes are present in one to two copies in each cell. Evidence shows that if the ODLs were not repaired at the time of active transcription at the onset of FCIR, it would alter the transcripts [66,92]. Recent data support this hypothesis [14].

Gene Damage

Nuclear genes in the rat brain are targets of ROS after stroke. We have detected that FCIR induces damage to three nuclear genes (c-fos, actin, and DNA polymerase β) [12,70]. Gene damage occurs in both astrocytes and neurons [13]. A summary of gene damage after FCIR is presented in figure 1a. This observation confirms those found using other methods [74]. The repair activity in the brain actively removes gene damage within 45 min of reperfusion after FCIR (fig. 1 b). After 45 min of reperfusion, approximately 20% of ODLs are not repaired.

Recent studies show that nitric oxide (NO) and superoxide anion, through the formation of peroxynitrite, may participate in nuclear gene damage in cerebral ischemia [12,13,40]. Brain injury induces an elevated level of extracellular glutamate which activates neuronal NO synthase (nNOS) via calcium influx [16,86]. This is supported by an increase in the NO level in brain tissue following cerebral ischemia-reperfusion, traumatic head injury, and spinal cord injury [10,45,51,102]. The level of superoxide ion is also elevated [49]. The NO radical has dual effects: (1) to combine with superoxide anion to form peroxynitrite and (2) to interfere with superoxide dismutase by reducing its antioxidant effect [27,57]. 3-Nitrotyrosine, a marker of peroxynitrite, is elevated in disease state and is neurotoxic [24,81,88]. Mice with a knockout

of nNOS and animals treated with 7-nitroindazole (7NI), an inhibitor of nNOS, are protected against neuronal death after experimental stroke [24,39,46,72,73,99]. Although it is not known if ODLs are elevated in chemical-induced brain injury, 7NI also prevents energy depletion in a model of chemical-induced brain injury [77]. The neuroprotective effect in the absence of nNOS activity may also involve the effect of other NOS activities [41,80]. Nevertheless, the fact that treatments with nNOS inhibitors in rat stroke models and mouse cardiac arrest models reduce ODLs and cell death supports the notion that ODL elevations are associated with neuronal death [12,13,40].

DNA single-stranded breaks activate PARP 2 h after stroke [91]. Injury to the ischemic core is directly related to energy depletion [23,25]. NO probably induces DNA breaks, which activate PARP and lead to energy depletion and necrosis in permanent occlusion models [69, 97,101]. On the other hand, a direct inhibition of PARP using 3-aminobenzamide in a transient global ischemia model of stroke causes an accumulation of single-stranded breaks in the brain, perhaps by reducing DNA ligation at the last step of gene repair. Therefore, an increase in apoptotic cell death is observed [71]. These observations suggest that there are diversified pathways after the initial injury.

Gene Transcription

A major role of gene repair is to ensure faithful transcription of mRNA from each gene for protein translation. Expression of functional proteins may be pivotal in maintaining the neuronal viability [38]. Energy depletion is proposed to be responsible for a lack of gene expression in the ischemic core [37]. On the other hand, spreading depression or depolarization in tissue surrounding the core, in combination with neurotransmitter release, intracellular calcium, or NOS activation, induces gene expression [20,47,68,87,95]. Examples include an elevated expression of immediate-early genes (c-fos, heat shock proteins, c-jun, cAMP-responsive element binding), neurotrophin genes (nerve growth factor, brain-derived neurotrophic factor, neurotrophin 3), and cell cycle regulator genes (bcl-2, p53) [1,2,31,78, 79,83], but no significant increase in the β -actin gene. It has been reported that heat shock proteins, hypoxia-inducible factor, and heme oxygenases are neuroprotective [6,21,76,84,85, 90,96]. Expression of AP-1, of which Fos protein is a major component, is thought to be neurotoxic after stroke. If the expression of the *c-fos* gene is neurotoxic, a limited expression of Fos will provide neuroprotection. Yet, a knockdown of *c-fos* mRNA leading to no Fos expression in whole animals either enhances necrosis significantly [60,103] or without statistical significance (infarct volume elevation at $p = 0.05$) [Hsu and Liu, unpubl. data]. The role of Fos expression as an immediate-early gene is not as simple as it appears to be.

Expression of Immediate-Early Genes

AP-1 is a transcription factor composed of gene products from the *c-fos* and/or other immediate-early genes (such as *c-jun*) or itself. During normal physiological conditions, very low levels of Fos and *c-fos* mRNA are detected in the brain. The elevation of *c-fos* mRNA is an endogenous response of the cells according to several models of brain injury [33,65,75, 94]. An elevated *c-fos* mRNA level is most likely derived from the new transcription of its nuclear gene. AP-1 transactivates a large number of late effector genes, including nerve growth factor and fibroblast growth factor [11,50]. While nerve growth factor is required for neuroregeneration, fibroblast growth factor 2 is required for neurogenesis after stroke [100]. Fos/AP-1 has a function as a gene activator, but with numerous effects: some may be neuroprotective [17,103], some are essential for learning processes [52,93], while others show *c-fos* and *c-jun* to be neurotoxic [4,18,33].

The initial clue that suggests that Fos may be neuroprotective comes from the observation showing the absence of the intact (normal) *c-fos* transcript in the core where necrosis is

developed. Figure 3 shows that the expression of actin (control) is unchanged in both ipsilateral and contralateral cortices before and after FCIR, although there is a visible decrease in the ischemic core (arrows in figure 3, lower panels) at 15 min of reperfusion. Because of preexisting actin mRNA, we do not know whether the newly transcribed actin mRNA is sensitive to RNaseA.

The expression of c-fos mRNA after FCIR is limited to the ischemic cortex, including core and penumbra at the end of cerebral ischemia (fig. 3, 0 min reperfusion, upper panels). The expression of c-fos mRNA occurs when ODLs are present in its nuclear genes (fig. 2a). This observation suggests that energy depletion due to blood flow occlusion is less likely to be associated with c-fos mRNA transcription. On the other hand, at 15 min of reperfusion, distribution of c-fos mRNA is observed only in the penumbral areas (including ipsilateral hippocampus), but is absent in the ischemic core where it is irrigated by the middle cerebral artery. The presence of penumbral c-fos mRNA is supported by the spread depression theory. The absence of c-fos mRNA in the core during the first 15–30 min of reperfusion indicates that the newly transcribed c-fos mRNA is sensitive to this assay or that the absence of intact c-fos mRNA at early stage and the development of necrosis in the core may be linked. Indeed, in preliminary studies, the ischemic cortex contained mutant c-fos mRNA (fig. 4a-c) [14]. Although it is a sensitive assay, it does not detect base substitutions that generate mismatches.

3Br7NI Increases FCIR-induced c-fos mRNA

Many investigators have reported that 7NI is neuro-protective by reducing necrosis in stroke models. We have reported that 3Br7NI reduces gene damage after stroke [12,40]. Here we show that treatment with 3Br7NI, an analog of 7NI, causes enhancement of early expression in intact and normal c-fos mRNA in ipsilateral cortex and dentate gyrus of rat brains after FCIR (fig. 5). It appears that NO inhibition using 7NI attenuates ODLs. Therefore, energy is conserved from the necessity for repair and activation of PARP. These events lead to enhanced transcription or elevated intact c-fos mRNA for translation. The finding further suggests, and needs supporting data, that the expression of certain mRNA, if expressed in time after reperfusion, may be neuroprotective and that ROS inhibition with a specific reference to nNOS activity reduction could be a new therapeutic target.

Discussion and Summary of Preliminary Studies

We noticed that the induction of ODLs and gene expression of immediate-early genes occurs at the start of reperfusion in our studies (fig. 1a, 3) and that ODLs may cause the presence of mutant transcripts (fig. 4). We have reported that gene repair activity is elevated after cerebral ischemia [54]. In a very preliminary study using crude extract of the brain after cerebral ischemia, we observed in animals treated with 3Br7NI (0, 30, and 60 mg/kg) that there was a further increase in the activity that removes oh8dG [63]. Our observation of an elevation in c-fos mRNA by 3Br7NI (fig. 5), however, is not consistent with the theory that nNOS-mediated spreading depression induces gene expression. Treatments with nNOS inhibitors in the brain, in which the formation of ODLs is not demonstrated, reduce immediate-early gene expression [36]. According to this theory, we expected a reduced c-fos mRNA expression when the nNOS activity was inhibited by 3Br7NI in our study. We did not observe it, however. 3Br7NI may reverse energy depletion, possibly via attenuation of NO and ODLs, resulting in a null induction of PARP. Therefore, there is an implication on the role of PARP in gene repair or energy depletion during early reperfusion.

We have proposed the hypothesis that faulty mRNA is transcribed when the c-fos gene contains ODLs during the first hours of reperfusion [62]. This hypothesis predicts that transcription from genes that contain ODLs (base modifications) would contain altered sequences

(mutations) in the transcript due to altered coding properties in base modification. In assays using in situ hybridization, the hybrid that is formed between the cRNA probe made from normal cDNA of the *c-fos* gene [60] and the target transcript may contain mismatches due to the presence of mutations. The cRNA probe will be removed by RNaseA that is used in this assay. Resulting from RNaseA treatments, the signal is decreased in the tissue where ODLs are present in the nuclear genes (fig. 3,4), but the signal will be present or elevated according to the copies of *c-fos* transcript, when ODLs in the *c-fos* gene are reduced (fig. 5).

The long-term goal of stroke research is to reduce neuronal loss and to improve functional recovery after cerebral ischemia-reperfusion. Success in this long-term goal will require an understanding of the molecular processes that contribute to neuronal loss after stroke. The immediate goals are to gain knowledge about which of these processes can cause neuronal dysfunction and to identify the principal molecular processes that promote effective repair functions that are already in the brain and that will reduce injury and/or cell death. The ultimate benefit of our work will be to assist in the development of genetic, biochemical, and pharmacological interventions for use immediately following the onset of stroke.

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Fig. 1.

a Presence of ODLs in three nuclear genes after FCIR [data from refs. 12,70]. **b** Rate of DNA repair in three nuclear genes. The percentage of repair is calculated as: $(a - b)/a \times 100$, where a = the number of ODLs at the end of ischemia (0 min of reperfusion) and b = the ODLs at various time points of calculation. [Reproduced with permission from ref. 70].

Fig. 2.

Hypothesis of ODLs causing faulty gene transcript and the failure to activate late effector genes. One particular pathway presented here is the activation of neurotrophin by the Fos/ AP-I complex, products of *c-jun* and *c-fos* supergene families. Damaged *c-fos* gene can be reduced via gene repair or inhibitors/scavengers that reduce ROS [62]. The importance of nerve growth factor in neuronal viability was recently reviewed by Isacson et al. [42]. NTGs = Neurotrophin genes; FGF = fibroblast growth factor. For explanation of the other abbreviations see text.

Fig. 3.

The expression of intact *c-fos* and actin mRNA using in situ hybridization (with RNase A treatment) is shown before and after FCIR [15]. The transcription of intact full-length *c-fos* mRNA begins immediately after cerebral ischemia (0 min reperfusion), but is absent in the ischemic core during the next 30 min of reperfusion, while intact *c-fos* mRNA remains elevated in the penumbral region when a ³³P-cRNA probe is used. (The ³³P-cRNA probe produces defined signals as compared with the ³²p-cRNA probe.) Little *c-fos* mRNA transcript is detected in sham-operation or contralateral cortices. [Reproduced with permission from ref. 12.)

Fig. 4.

Partial *c-fos* cDNA is amplified from figure 3 (at 30 min of reperfusion) using RTPCR (**a, b**) and resolved in HPLC (**c**) using Wave DNA fragment analysis (Transgenomic, Inc.). The arrow shows the position of normal *c-fos* cDNA from a normal *c-fos* gene using similar RTPCR amplification (retention time = 4.58 min, data not shown). The cDNA from the ischemic cortex contains two fragments: one with a retention time of 4.05 min, containing three-nucleotide deletion mutations from the normal; the other fragment was one with a retention time of 4.62 min. The cDNA from the nonischemic cortex showed no significant amplification of the *c-fos* mRNA. A size marker is shown at the retention time of 3.31 min. (Courtesy by Dr. S. Lileberg, Transgenomic, Inc., San Diego, Calif., USA.)

Fig. 5.

Early expression of intact full-length c-fos mRNA in the brain with 3Br7NI (FCIR = 60115) (**a**) as compared with that without 3Br7NI (**b**). The neuronal NOS inhibitor that reduces oxidative DNA lesions and neuronal sensitivity increases the expression of intact c-fos transcripts after brain injury. [Reproduced from ref. 14, with permission.]