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Targeting Microglial Activation in Stroke Therapy: Pharmacological Tools and Gender Effects

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Abstract

Ischemic stroke is caused by critical reductions in blood flow to brain or spinal cord. Microglia are the resident immune cells of the central nervous system, and they respond to stroke by assuming an activated phenotype that releases cytotoxic cytokines, reactive oxygen species, proteases, and other factors. This acute, innate immune response may be teleologically adapted to limit infection, but in stroke this response can exacerbate injury by further damaging or killing nearby neurons and other cell types, and by recruiting infiltration of circulating cytotoxic immune cells. The microglial response requires hours to days to fully develop, and this time interval presents a clinically accessible time window for initiating therapy. Because of redundancy in cytotoxic microglial responses, the most effective therapeutic approach may be to target the global gene expression changes involved in microglial activation. Several classes of drugs can do this, including histone deacetylase inhibitors, minocycline and other PARP inhibitors, corticosteroids, and inhibitors of TNF α and scavenger receptor signaling. Here we review the pre-clinical studies in which these drugs have been used to suppress microglial activation after stroke. We also review recent advances in the understanding of sex differences in the CNS inflammatory response, as these differences are likely to influence the efficacy of drugs targeting post-stroke brain inflammation.

Keywords

Brain; corticosteroid; female; HDAC; inflammation; ischemia; minocycline; PARP

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CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

INTRODUCTION

Ischemic stroke is caused by critical reductions in blood flow to one or more arteries of the brain or spinal cord, and is a leading cause of morbidity and mortality worldwide. The only clinically-validated treatment for stroke now available is acute thrombolysis, and the utility of this approach is constrained by the need to initiate treatment within 3–6 hours of symptoms and the risk of causing cerebral hemorrhage. These factors have limited the number of stroke patients receiving this treatment to less than 5%, even in areas where this treatment modality is readily available. It is therefore important to develop new approaches that can be used for a much larger fraction of stroke patients.

Ischemic brain injury results from a cascade of events initiated by energy depletion and culminating in cell death. Contributing factors in this cascade include glutamate excitotoxicity, oxidative stress, and inflammation. Interventions targeting glutamate excitotoxicity and oxidative stress (or their sequelae) have been shown to reduce acute ischemic cell death in animal models of stroke [1], but these approaches are generally ineffective if not initiated very soon after ischemia onset. Inflammation, however, requires many hours to days to fully develop, and is thus a much more practical target for treating a large fraction of stroke patients [2].

Microglial cells are the resident immune cells in the central nervous system [3]. These cells continuously scan their environment with highly motile processes and are thought to be the immediate sensors of brain pathology [4]. After ischemia, microglia undergo phenotypic transformation to an “activated” phenotype [5]. Acutely activated microglia produce factors such as reactive oxygen species, cytokines, and proteases that may kill neighboring cells and disrupt the blood–brain barrier [6–9]. This initial innate immune response occurs with almost any disruption in brain homeostasis, and it is thought to be teleologically adapted as a first line defense against infection. In stroke, however, this initial response may be maladaptive.

Signals released by microglia also trigger infiltration of circulating immune cells to the injured area. The effects of these circulating immune cells and of the systemic immune response in general on stroke outcome are complex. Infiltrating neutrophils and T-lymphocytes can aggravate acute injury [10–12], circulating B-lymphocytes may exert a countering, anti-inflammatory effect [13], and all of these cell types can in turn affect microglial activation [14]. Moreover, infiltrating macrophages in brain can be difficult to distinguish from activated microglia. These and other effects of the systemic immune response on stroke are beyond the scope of this review; however, it should be recognized that because of these interactions, drugs affecting post-stroke microglial activation may act in part through effects on the systemic immune system.

Prior comprehensive reviews have examined the many different signaling pathways that trigger microglial activation, their resulting genomic responses, and the several effector mechanisms by which microglia can have cytotoxic effects on surrounding cells [5, 9, 15, 16]. Drugs targeting any of these processes could in principle have salutary effects, but the multiplicity of signaling pathways and effector mechanisms renders it unlikely that targeting

any single one would be optimally effective. An alternative approach is to target the coordinated gene expression changes that underlie microglial activation. Here we review the preclinical (animal model) data supporting efficacy of drugs targeting this aspect of microglial activation after stroke. A summary of representative studies is provided as (Table 1). In evaluating this literature we have distinguished between the different stroke models employed because they produce cell death by somewhat different mechanisms, and they have varying fidelity to the clinical condition of stroke.

PRECLINICAL STROKE MODELS

Variations of four stroke models are commonly employed. In “focal ischemia”, the middle cerebral artery is occluded to produce a focal infarct, as occurs in clinical ischemic stroke. The vessel may be permanently occluded, (“permanent ischemia”), or re-opened after an interval of 30–90 minutes (“transient ischemia” or “focal ischemia-reperfusion”). The latter has become popular because it produces a mild injury in which is easier to identify neuroprotective effects; however it is only rarely true in human stroke that circulation is reestablished within such a short time interval. “Hypoxia-ischemia” is a variation on this model, in which focal ischemia-reperfusion is coupled with hypoxia. This is most commonly used in neonatal animals as a model of perinatal birth asphyxia. In “global ischemia”, animals are subjected to a brief (5 – 20 minute) reduction of blood flow to either the entire brain or just the forebrain, typically by a combination of bilateral carotid artery occlusion and hypotension. This is not a model of stroke per se, but more closely replicates the brain injury produced by cardiac arrest or prolonged hypotension. Global ischemia does not cause infarction (cell necrosis), but instead leads to selective neuronal death of certain vulnerable neuronal populations in the hippocampus and cerebral cortex. All four of these stroke models produce robust post-ischemic microglial activation, but the biological drivers and the effects of the inflammatory response may differ among these models.

PHARMACOLOGICAL AGENTS USED TO SUPPRESS POST-STROKE MICROGLIAL ACTIVATION

a. Corticosteroids and Non-steroidal Anti-inflammatory Drugs

Acute immunosuppression is typically accomplished in non-CNS tissues by the use of corticosteroids or nonsteroidal anti-inflammatory agents. Corticosteroids have been shown to inhibit microglial activation in several neurological disease models including spinal cord injury, traumatic brain injury, and acute stress [17–19]. It is likely that corticosteroids would similarly suppress microglial activation after stroke, but surprisingly only a single published study has addressed this question, and in that study corticosteroids were administered prior to ischemia [20]. The lack of research in this area may reflect the fact that corticosteroids have well-recognized systemic effects that may have impair stroke recovery, such as hyperglycemia, muscle wasting, osteoporosis, and increased vulnerability to infections. It has also been reported that corticosteroids have a direct proapoptotic effect on certain neuronal populations [21], and in some settings they can paradoxically exacerbate inflammation [22, 23]. In (uncontrolled) clinical trials, corticosteroid treatment did not affect

either the mortality or aggregated functional outcomes of patients with acute ischemic stroke [24].

Whether nonsteroidal anti-inflammatory drugs can block post-ischemic microglial activation also remains uncertain. However, the drug indomethacin was shown to increase the survival of progenitor cells and allow a higher fraction to differentiate into oligodendrocytes and neurons, with a reduction of inflammatory cells after focal cerebral ischemia [25]. It was not established in that study whether these effects are attributable to reduced microglial activation, or to direct effects of the drug on the progenitor cells.

b. PARP Inhibitors

Poly (ADP-ribose) polymerases (PARPs) catalyze the transfer of ADP-ribose units from NAD^+ to target proteins including histones and transcriptional factors. The resulting poly(ADP-ribosyl)ation of these substrate proteins regulates chromatin structure, DNA metabolism, and gene expression [26, 27]. In particular, PARP regulates the activity of NF- α B and other transcription factors, which in turn regulate many aspects of the inflammatory response [28, 29]. PARP inhibitors are small molecules which compete with NAD^+ at the enzyme catalytic site. Many PARP inhibitors have good CNS penetration, and some of them have entered clinical trials for cancer and other diseases [30].

Cell culture studies have shown that PARP inhibitors block NF- α B transcriptional activity and block the upregulation in microglial iNOS expression, MMP9 release, morphological changes, and neurotoxicity that is otherwise induced by lipopolysaccharide and other pro-inflammatory mediators [28, 31]. Several studies have examined the effects of PARP inhibitors after stroke and have found that they suppress microglial activation and improve neuronal survival [32–35]. However, the mechanistic interpretation of these results is complicated by the fact that PARP inhibitors also have acute neuroprotective effects that are mediated by a mechanism unrelated to their anti-inflammatory effects [36], such that reduced inflammation seen with these agents could be secondary to reduced cell death rather than vice-versa. However, these neuroprotective effects, like those of almost all neuroprotective agents, require administration within 3 to 6 hours after onset of ischemia, and drugs given after this time interval can be assumed to act by mechanisms other than acute neuroprotection. Studies performed in this manner, with PARP inhibitor therapy initiated 8–48 hours after ischemia and continued for several days, showed reduced microglial activation and improved histological and behavioral outcomes assessed up to 8 weeks after ischemia [32, 33, 37].

c. Minocycline

Like the PARP inhibitors, minocycline and related tetracycline derivatives such as doxycycline have both anti-inflammatory and neuroprotective effects in cell culture preparations [38–41]. They similarly have both antiinflammatory and neuroprotective effects in animal models of stroke [8, 38, 42–47]. The beneficial effects of minocycline on stroke outcome have been attributed to a bewildering variety of biochemical processes, including upregulation of mitochondrial bcl-2 expression [48], reduced mitochondrial calcium uptake, calcium-induced mitochondrial swelling, calcium-induced cytochrome-C

release and mitochondrial permeability transition [49–51], direct scavenging of reactive oxygen species [52], and inhibition of mitogen activated protein kinases [53, 54]. However, the biochemical mechanisms by which minocycline affects these processes have not been established, and many may be secondary effects. Indeed, the fact that minocycline, like PARP inhibitors, has both neuroprotective and anti-inflammatory effects may stem from the fact minocycline is itself a potent PARP inhibitor [55]. Minocycline, like other competitive PARP inhibitors, contains an aromatic ring-linked carboxamide group or carbamoyl group analogous to the NAD⁺ substrate of PARP (Fig. 1).

Only a few studies have initiated minocycline therapy at time points greater than 6 hours after ischemia, i.e. at times that limit the likelihood that observed anti-inflammatory effects are secondary to neuroprotective effects. Treatment initiated 1 day after focal ischemia and continued for 13 days resulted in reduced microglial activation, improved scores on behavioral tests, and improved survival rate [56]. Similarly, minocycline treatment begun 4 days after transient focal ischemia and continued for 4 weeks improved motor and cognitive function, in association with reduced microglia activation and enhanced neurogenesis [57].

Minocycline crosses the blood-brain-barrier and is clinically well-tolerated at anti-inflammatory doses [58]. In a clinical trial, ischemic stroke patients given 200 mg of minocycline for 5 days, beginning 6 – 24 hours after onset of stroke symptoms, showed better functional outcomes relative to placebo-treated patients [59].

d. HDAC Inhibitors

Gene expression is regulated in part by the enzymatic addition and removal of acetyl groups at specific lysine residues by histone acetyltransferase (HAT) and histone deacetylases HDACs [60]. Emerging evidence suggests that HDAC activity may have a master regulatory role in diverse biological activities, including inflammation [61–66]. HDAC inhibition suppresses the inflammatory response in part by inhibiting transcription factor binding at promoter sites of pro-inflammatory genes such as iNOS and COX [67]. HDAC inhibition has been shown to reduce neuronal injury, alleviate post-stroke inflammation, and improve functional outcome in multiple models of focal ischemia [68–71]. A direct effect on inflammation is supported by studies showing that post-insult treatment with the HDAC inhibitors valproic acid and sodium butyrate suppressed microglial activation, reduced the number of microglia, and inhibited other inflammatory markers in the ischemic brain [72]. HDAC inhibitors can also enhance memory and synaptic plasticity in the CNS, suggesting that in addition to salvaging tissue, HDAC inhibition might also be a useful strategy for promoting functional recovery [73–75]. However, the enthusiasm for pan-HDAC inhibition in treating neurological conditions is tempered by their toxicity toward many CNS cell types [68, 76, 77]. Accordingly, an aim of ongoing research is to selectively target specific HDAC isoforms.

e. Inhibitors of Scavenger Receptor and Toll-Like Receptors (TLRs)

After ischemia, danger-associated molecular pattern molecules such as high mobility group box 1 (HMGB1) are released from dead cells and activate pattern recognition receptors, including toll-like receptors (TLRs) and the scavenger receptor CD36, which are key

molecular sensors for the innate immune response in brain [78–81]. TLR-mediated intracellular signaling pathways converge to activate NF- κ B and c-Jun N-terminal kinases (JNKs), which induce transcription of many genes important in the inflammatory response. The cooperative signaling of TLR2 heterodimers (TLR2/1) and CD36 is a critical factor in the inflammatory response and tissue damage evoked by cerebral ischemia [80]. Microglial expression of CD36 protein is increased in the ischemic brain, and CD36-null mice show attenuated microglial activation, reduced NF- κ B activation [81], and reduced infarct size [82]. Drugs that block CD36 signaling are not yet available, but a peptide that blocks CD36 upregulation was found to attenuate ischemic brain injury [83]. Drugs targeting TLRs are also not yet available, but studies using TLR2^{-/-} mice suggest this could be an effective approach for suppressing post-stroke inflammation. TLR2^{-/-} mice have reduced microglial activation and proliferation after stroke, and decreased brain levels of monocyte chemoattractant protein-1 [78].

f. Tumor Necrosis Factor-alpha (TNF- α) Receptor Antagonists

TNF- α is a potent stimulator of microglial activation [84], and effective TNF α antagonists are now clinically available. TNF- α exerts its effects by binding to two cell-surface receptors, the p55 and p75 TNF receptors (TNFR), commonly referred to as TNFR1 and TNFR2, which are expressed on both neurons and microglia. The expression of both TNF- α and TNF- α receptors is increased in the ischemic injury zone within hours after stroke onset [85]. Several different strategies for inhibiting TNF- α signaling by pharmacologic agents, neutralizing antibodies or soluble receptors have been reported to reduce infarct volume in preclinical models [86–91]. Some of these studies also reported reduced microglial activation, but it is not possible to ascertain whether the reduced microglial activation reduced neuronal death, or vice versa. It should be noted, however, that TNF- α antagonists can also have deleterious effects on stroke outcome. Hippocampal neurogenesis evaluated two weeks after the ischemic onset after ischemic injury was abolished by anti-TNF- α antibodies administered between day 8 and 14 after stroke [92]. These effects may be attributable to pro-survival effects of TNFR2 activation. A therapeutic strategy that selectively targets TNFR1-mediated signaling, which promotes apoptosis and inflammation, while retaining TNFR2-mediated pro-surviving signaling may lessen adverse effects of anti-TNF α therapies [93]. Antibody to TNF- α or based on TNF- α receptors are now clinically available and in use for autoimmune disorders, but have not yet been used in controlled trials for stroke.

SEXUAL DIMORPHISM IN STROKE AND POSTSTROKE INFLAMMATION

Females exhibit smaller infarcts and better outcomes after experimental brain ischemia than males [94–96]. Sex differences can result from differences in sex steroid hormone levels, sex steroid-induced developmental differences, or hormone-independent differences specified by XX versus XY chromosomal gene dosage [97]. The steroid hormones 17 β -estradiol and progesterone influence brain development, and the number and morphology of microglia and the expression of several cytokines, chemokines and their receptors vary with sex and age [98]. Sex steroid receptors are expressed on neurons, astrocytes, and oligodendrocytes in addition to microglia [99–102]. Results of cell culture studies indicate

that both estrogens [99] and XX genotype [103] reduce death in cultured neurons exposed to ischemia-like conditions, indicating effects independent of inflammation. However, treatment with 17 β -estradiol and progesterone can suppress microglia-mediated neurotoxic effects in culture [101, 104–107], suggesting that an anti-inflammatory effect may also contribute to the neuroprotective effects observed *in vivo*.

Evidence also suggests that sexual dimorphism exists in the post-stroke inflammatory response with respect to microglial activation, peripheral immune cell infiltration, and expression of pro-inflammatory factors such as cyclooxygenase-2, NADPH oxidase, and vascular cell adhesion molecule-1 [108–111]. In a rat focal ischemia-reperfusion model, 17 β -estradiol and progesterone reduced infarct size and improved behavioral function in males and ovariectomized females to a similar extent [112]. These effects were associated with attenuated microglial activation and down-regulated pro-inflammatory cytokines and chemokines (IL-6, CCL2 and CCL5) [112]. In a rat permanent focal ischemia model, progesterone inhibited the expression of Iba1 and COX-2 after stroke *in vivo*, suggesting that progesterone can exert neuroprotective action by inhibiting the activation of microglia and the over expression of COX-2 after stroke [104].

SEXUAL DIMORPHISM IN RESPONSE TO ANTIINFLAMMATORY TREATMENTS

Given the known differences in male and female inflammatory responses, it might be expected that males and females would respond differently to therapeutic interventions targeting these inflammatory responses. The few studies done in this area point to large differences. The protective effect of PARP-1 inhibitors (including minocycline) is strikingly dependent on sex, with males preferentially protected compared with females [113–116]. Unpublished work from our lab suggests this dichotomy extends also to the effects of these agents on the post-stroke inflammatory response [117]. The reason for these differences has not been established, but one study suggests that estrogen may anchor PARP to ER- α and to the DNA and prevent its recognition of DNA strand breaks and following PARP activation [118]. It remains possible however that sex differences in brain inflammation and in PARP effects on the brain inflammatory response result from developmental rather than hormonal mechanisms [115].

CONCLUSIONS AND CAVEATS

Microglia undergo activation in response to brain ischemia. Cell culture studies show that activated microglia can kill neighboring neurons, and the *in vivo* studies reviewed here strongly suggest that this occurs *in vivo* as well. The relatively long time interval (many hours) between ischemia onset and a fully developed microglial activation state makes targeting of this microglial response clinically feasible, and several pharmacologic agents are now available that can effectively block microglial activation at a global, gene-expression level. Together these observations lend credence to the idea that targeting microglial activation after stroke may provide an effective way to limit brain injury caused by stroke.

However, caveats should be noted with respect to extrapolation of these findings to the clinical realm. First, almost all of the preclinical studies in this area have been done with male animals. As noted above, the brain inflammatory response may differ in fundamental ways between males and females, and the effect of anti-inflammatory interventions may likewise differ between males and females. To add to this complexity, the degree to which these differences may persist in post-menopausal females, which is the group most prone to stroke, is unknown.

A second caveat is that microglial “activation” is not a univalent state; the morphological and gene expression changes associated with microglial activation vary enormously with the nature, strength, and duration of the stimulus [119], and activated microglia are very difficult to distinguish from infiltrating peripheral macrophages. Evidence also suggests that brain microglial populations are heterogeneous, and may respond differently to similar stimuli [120]. Activated microglia may be classified as M1 or M2 phenotypes on the basis of surface markers and other differences [121], although hybrid and other phenotypes also occur. The M1 phenotype is characterized by the expression of high levels of pro-inflammatory cytokines and aggravation of inflammatory responses, while M2 macrophages have antiinflammatory functions and promote tissue remodeling [122]. Markers for both phenotypes increase during the first few days after stroke, but their rates of later decline may vary [123]. M2 and possibly other microglial phenotypes can also support neuronal survival [123] and recruit endogenous neural stem cells to the lesion site [124], effects that may be impaired by non-specific anti-inflammatory agents. Microglia similarly play a crucial role in brain recovery after injury through their effects on debris clearance, angiogenesis, and neurite outgrowth [125–127]. For these reasons, the efficacy of anti-inflammatory treatment after stroke may be critically influenced by the timing and duration of this treatment approach.

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Fig. (1). Structure / activity relationships of tetracycline PARP-1 inhibitors

A. An aromatic ring-linked carboxamide group (circled) or carbamoyl group built in a polyaromatic heterocyclic skeleton is shared by the natural PARP-1 substrate NAD⁺, the competitive PARP-1 inhibitors nicotinamide, DPQ, and PJ34, and the tetracycline derivatives. Nicotinic acid is not a PARP-1 inhibitor and lacks this amide group. **B.** Activity of isolated, recombinant PARP-1 in the presence of these agents. Studies were performed in the presence of 210 μ M NAD⁺ substrate, as described [55].

Abbreviations: PARP-1, poly(ADP-ribose) polymerase-1; Mc, minocycline; Doxy, doxycycline; Demeclo, demeclocycline; Chlortet, chlortetracycline; DPQ, 3, 4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone; PJ34, N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N, N-dimethylacetamide hydrochloride. Figure modified from [55].

Table 1

Drug Treatment Effects on Post-Ischemic Inflammation

Target	Drug	Dose ¹ (mg/kg)	Route	Time to First dose	Treatment Duration	Stroke Model ²	Species	Inflammatory Markers	Other Outcomes	Reference
PARP	PI34	15	i.p.	8 hours	3 days	GI/R	Rat	CD11b(+) cells	Cell death	[32]
		15	i.p.	2 days	7 days	GI/R	Rat	CD11b(+) cells	Cell death, behavior	[33]
PARP and others	Minocycline	45	i.p.	2 hours	second dose at 4 hours	FI/R	Rat-pup	IL-1 β , CINC-1, IL-18, MCP-1; ED1(+) cells	Cell death	[128]
		50 then 25	i.p.	4 days	4 weeks	FI/R	Rat	ED1(+) cells	Neurogenesis, behavior	[57]
		45	i.p.	0.5 hours	2 days	FI/R	Rat	CD11b(+) and MPO(+) cells, 5- LOX	Cell death, behavior	[129]
		20 then 10; 45 then 23	i.p.	2 hours	6 days	HI/R	Rat-pup	Iba1(+) cells	O1(+), O4(+), MBP(+) cells	[130]
		10	i.p.	1 day	13 days	FI/R	Mouse	Iba1(+) cells, HMGB1	Survival, cell death, behavior	[56]
		10	i.p.	5 days	9 days	FI/R	Mouse	Iba1(+) cells, plasma HMGB1	Survival, behavior	[131]
		45 then 22.5	i.p.	2 hours	5 days	HI/R	Rat pup	P2X ₄ R, and Iba1		[43]
		45 then 22.5	i.p.	2 hours	5 days	HI/R	Rat pup	Iba-1(+) cells, TNF α , IL-1 β	5-HT and SERT expression	[44]
HDAC	SB	300	s.c.	0 hours	second dose at 12 hours	FI	Rat	OX42(+) cells, iNOS, COX2	Behavior	[72]
	VPA	300	s.c.	0 hours	second dose at 12 hours	FI	Rat	OX42(+), ED1(+), iNOS(+) cells	Behavior	[72]
		300	i.p.	0 hours	6 days	GI/R	Rat	OX42(+); IL-1 β , TNF α expression	Cell death, behavior	[132]
CD36	SS31	2	i.p.	0 hours	2 days	FI/R	Mouse	CD36 expression	Cell death	[83]
TNF α	3,6'-DT	56	i.p.	1 or 3 hours	3 days	FI/R	Mouse	Iba-1(+) and MPO(+) cells; TNF α , IL-1 β and iNOS expression	Cell death	[87]

The table excludes studies in which drugs were given prior to ischemia.

Abbreviations: i.p., intraperitoneal injection; s.c., subcutaneous injection; PARP, poly(ADP-ribose) polymerase; HDAC, histone deacetylase; SS31, D-Arg-Dmt-Lys-Phe-NH₂; MPO, myeloperoxidase; TNF α , tumor necrosis factor alpha; IL-18, interleukin 18; MCP-1, monocyte chemoattractant protein 1; VPA, valproic acid; SB, sodium butyrate; ICE, IL-1 β -converting enzyme; COX, cyclooxygenase-2; PGE₂, prostaglandin E₂; IL-1 β , interleukin 1beta; CINC-1, cytokine-induced chemoattractant protein 1; 3,6'-DT, 3,6'-Dithiothalidomide; 5-LOX, 5-lipoxygenase; iNOS, inducible nitric oxide synthase; 5-HT, serotonin; SERT, serotonin transporter.

¹In some studies the initial dose was higher than subsequent doses.

²Stroke models are G/I/R, global ischemia/reperfusion; F/I/R, focal ischemia/reperfusion; HI/R, hypoxia-ischemia reperfusion; FI, focal ischemia, without reperfusion. See text for details.