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SYMPOSIUM: Role of Inflammation Following Stroke and Neurotrauma

Inflammation and Stroke: Putative Role for Cytokines, Adhesion Molecules and iNOS in Brain Response to Ischemia

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Ischemic stroke is a leading cause of death and disability in developed countries. Yet, in spite of substantial research and development efforts, no specific therapy for stroke is available. Several mechnism for neuroprotection have been explored including ionchannels, excitatory amino acids and oxygen raicals yet none has culminated in an effective therapeutic effect. The review article on "inflammation and stroke" summarizes key data in support for the possibility that inflammatory cells and mediators are important contributing and confounding factors in ischemic brain injury. In particular, the role of cytokines, endothelial cells and leukocyte adhesion molecules, nitric oxide and cyclooxygenase (COX-2) products are discussed. Furthermore, the potential role for certain cytokines in modulation of brain vulnerability to ischemia is also reviewed. The data suggest that novel therapeutic strategies may evolve from detailed research on some specific inflammatory factors that act in spatial and temporal relationships with traditionally recognized neurotoxic factors. The dual nature of some mediators in reformatting of brain cells for resistance or sensitivity to injury demonstrate the delicate balance needed in interventions based on anti-inflammatory strategies.

Ischemia and reperfusion induced cerebral injury

The initiation of cellular inflammation in the brain by ischemic injury takes place within the microvasculature. Cerebral microvessels are the interface at which PMN leukocytes first adhere, and then pass into the underlying neuropil (32, 34, 45). One commonly held view is that reperfusion, following occlusion of a cerebral artery, increases cerebral injury. However, little data so far exist to support this claim. Kontos *et al*. and Suematsu *et al*. have demonstrated evidence of oxygen-free radical generation *in vivo*, the former in the pial microvasculature following transient global ischemia (83, 140). Limited data have been provided that, under exclusive circumstances, reperfusion may increase evidence of ischemic injury, which is dependent upon protein synthesis (2). However, as in other organ systems, reperfusion more commonly effects a reduction in infarct volume. Clinical studies, and experimental work in middle cerebral artery occlusion (MCAO) provide support for this view (98, 100, 160). An alternative viewpoint suggests that a number of processes are initiated in the microvasculature early following MCAO, which are not recovered during reperfusion (Figure 1). One result of this early commitment of microvascular responses to MCAO is the impression that reperfusion may induce injury of its own. Central to this viewpoint is the demonstration that microvascular reactivity in response to initiators of ischemia is both dynamic and rapid. Heretofore, it has been considered that in experimental systems microvascular reactivity following MCAO was slow compared to neuron injury, supporting the notion that the vasculature is an inert conduit.

Microvascular ultrastructure

Cerebral microvessels are ternary structures consisting of an endothelial cell lining, basal lamina, which is an extension of the extracellular matrix (ECM), surrounded by the end-foot processes of astrocytes (35). This structural arrangement implies a ready transfer of nutrients between the microvascular lumen and neurons, via astroglial processes. Rather than inert conduits, the microvasculature responds dynamically to flow disturbances, free-radical exposure, and cytokine generation associated with focal ischemia.

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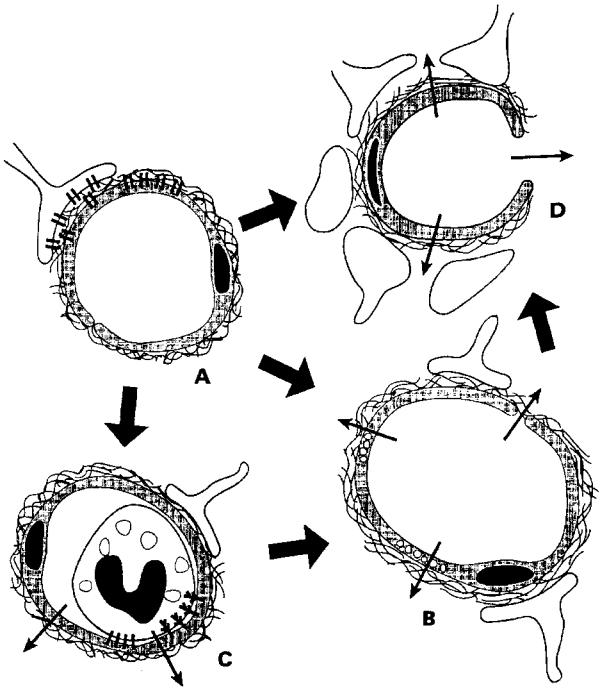

Figure 1. Responses of normal cerebral microvessels (A) to focal ischemia, as with middle cerebral artery occlusion (MCAO), include increases in permeability of the endothelial cell component of the blood brain barrier (B), adhesion of polymorphonuclear (PMN) leukocytes and platelets to endothelial cell receptors expressed in sequence (C) (see text), and loss of integrin-matrix attachments of endothelial cells and astrocyte end-feet which accompany loss of the basal lamina (D).

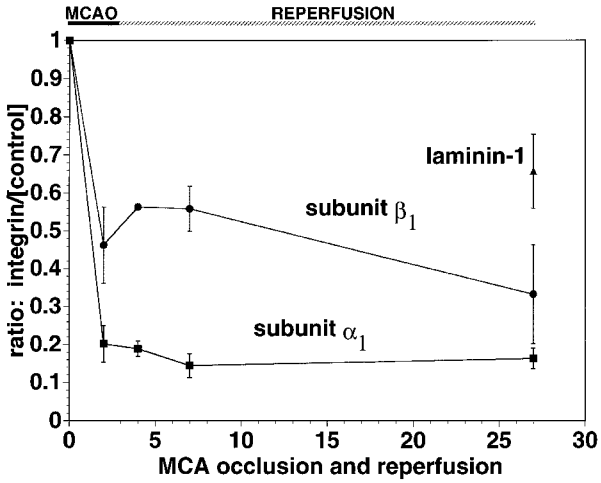

Figure 2. Effect of MCAO and reperfusion on integrin $\alpha_1\beta_1$ expression (hours).

Onset*	Antigen
0 - 90 min	P-selectin
2 - 4 hr	ICAM-1
7 - 24 hr	E-selectin
* following MCAO	

Table 1. Microvascular endothelial-leukocyte adhesion receptors.

Leukocyte adhesion receptor expression

Tagaya *et al* first demonstrated significant accelerated cellular injury in the striatum of non-human primates compared to that of rodents undergoing reversible MCAO (141). The leukocyte adhesion receptors P-selectin, ICAM-1, and E-selectin are expressed in sequence by microvascular endothelium within the ischemic territory (61, 114, 152, 153) (Table 1). P-selectin is seen within 60-90 minutes following MCAO (114), indicating the rapid reactivity of microvascular endothelium to the ischemic insult. P-selectin and E-selectin receptors are continually expressed within the ischemic territory, and are not altered by reperfusion at 3 hours MCAO in this model. Furthermore, E-selectin expression may appear in non-ischemic tissue by 24 hours following MCAO (61). In addition to providing the bases for cellular inflammation following focal cerebral ischemia, their expression indicates dynamic responses of the microvascular endothelium. Of importance, E-selectin expression occurs in those microvessels with intact basal lamina (61).

Matrix integrity during focal cerebral ischemia

The microvascular basal lamina is an extension of the ECM, which functions as a barrier to cellular transit (e.g., erythrocytes) into the neurophil. It functions further as an anchoring scaffold for both endothelial cells and astrocyte end-feet in cerebral microvessels. Following MCAO, degradation of the basal lamina occurs, which is most prominent in regions of hemorrhagic transformation (58). The underlying mechanisms of vascular matrix dissolution in the central nervous system are not understood, but metalloproteinases (MMPs), plasminogen activators (PAs), and various serine proteases have been implicated. Heo *et al*. have demonstrated the rapid expression of MMP-2 in the primate striatum within 1 hour following MCAO, in concert with evidence of neuron injury (65). A significant relationship between MMP-2 activity and cerebral injury has been observed. MMP-9 expression is most associated with evidence of hemorrhagic transformation, at any

endothelial cell	↓integrin $\alpha_1\beta_1$
	↓integrin $\alpha_3\beta_1$
astrocyte	↓integrin $\alpha_6\beta_4$
	↓integrin $\alpha_1\beta_1$
smooth muscle cell	↑integrin $\alpha_V\beta_3$

Table 2. Microvascular endothelial-leukocyte adhesion receptors.

time point (65). MMP-2 transcripts associated with the ischemic microvasculature appear in the same time frame (T. Abumiya, G. J. del Zoppo; unpublished data). Those observations confirm that microvascular responses to ischemia are temporally and topographically linked to neuron injury following MCAO in the non-human primate.

Microvascular integrity

Adhesion of endothelial cells and astrocytes to the intervening matrix is mediated by integrin receptors. In the adult primate, expressions of the integrin heterodimers $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_4$, and $\alpha_6\beta_1$ are confined to the microvasculature along with their ligands laminins-1 and -5, collagen IV, and fibronectin (60) (Figures 2, 3). MCAO initiates a rapid downregulation of integrin $\alpha_6\beta_4$ and $\alpha_1\beta_1$, associated with the astrocyte end-feet and select astrocyte fibers, respectively (149). Similar responses to endothelial cell integrin receptors have been observed, which are both topographically and temporally linked to neuron injury (33). Within 2 hours a highly significant relation to neuron injury is apparent. The downregulation of these receptors is unaffected by reperfusion at 3 hours MCAO in this model, having reached nadir several hours prior. In contrast, the integrin $\alpha_V\beta_3$, is rapidly upregulated (within 1-2 hours MCAO) in select noncapillary microvessels (113). Integrin $\alpha_V\beta_3$ is linked to an equally rapid and coordinate expression of vascular endothelial growth factor (VEGF) and evidence of microvascular activation (PCNA) (1). Although the latter observations may represent initial steps in microvascular remodeling, their true intent is unknown. Nonetheless, it is evident (Table 2) that alterations in integrin-matrix interactions are initiated by MCAO and appear simultaneous with evidence of neuron injury. Those changes are not altered by reperfusion, but may be construed as evidence of ischemic injury.

In summary, with the cessation of cerebral blood flow which causes symptomatic stroke, leukocyte adhesion receptors are expressed in cerebral microvessels. These lead to occlusion of the ischemic microvasculature by activated PMN leukocytes and platelets (99). Rapid decrease in expression of integrin receptors on endothelial cells and astrocyte end-feet, together with changes in matrix structure, are initiated within minutes of MCAO in the non-human primate striatum. These changes in receptor expression coincide with the earliest evidence of neuron injury (113), matching both the temporal course and extent of non-vascular cell injury. These changes are not altered by reperfusion of the occluded MCA even within hours of the initial occlusion. Given the heterogeneous nature of the microvascular response, it is likely that injuries caused, in part, by inflammatory cells lead to confluent destruction of cerebral tissue in the ischemic territory. However, the early microvascular responses to ischemia, including leukocyte adhesion and transmigration, together with structural damage caused by the rapid loss of integrin-matrix adhesion, may impact neuron injury.

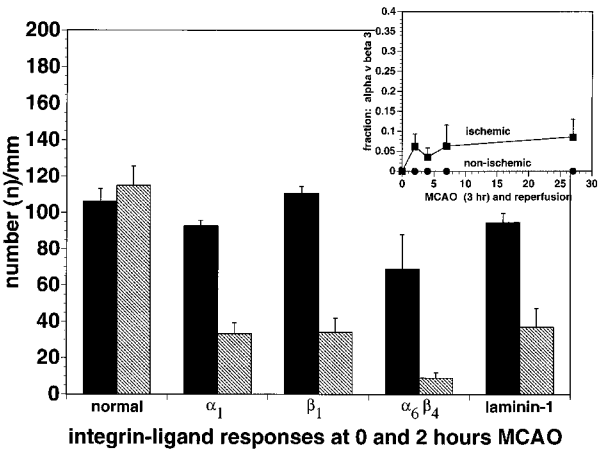

Figure 3. Changes in expression of the integrins $\alpha_1\beta_1$, $\alpha_6\beta_4$, and $\alpha_V\beta_3$ in relation to the matrix ligand laminin-1 by 2 hours MCAO.

Nitric oxide and cyclooxygenase products in post-ischemic inflammation

Evidence accumulated over the past several years suggests that nitric oxide (NO) and cyclooxygenase (COX) products play an important role in ischemic brain injury.

Nitric oxide: neuromodulator and neurotoxin

NO is a molecular mediator that is involved in a wide variety of physiological and pathological processes. NO is synthesized by the enzyme NO synthase (NOS) from oxidation of the guanidino nitrogen of L-arginine (53).

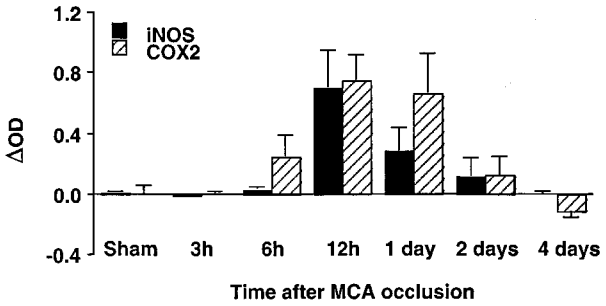

Figure 4. Time course of iNOS and COX-2 mRNA expression in brain following transient focal cerebral ischemia produced by occlusion of the rat MCA. mRNA was detected by RT-PCT and expressed as relative optical density (OD)(110).

Description	iNOS	COX-2
Expressed after focal ischemia	yes	yes
Species	Mouse Rat Human	Mouse Rat Human
Cellular site of expression	Neutrophils Vascular cells	Neurons Neutrophils Vascular cells Microglia (?)
Peak time of expression	12-24 hours	12-24 hours
Effect of pharmacological inhibition	Reduction in infarct volume	Reduction in infarct volume
Effect of gene deletion in mice	Reduction in infarct volume	Not known

Table 3. Comparison between iNOS and COX-2 after focal cerebral ischemia.

Three isoforms of NOS have been described: neuronal NOS, (nNOS), endothelial NOS (eNOS), and inducible or immunological NOS (iNOS). nNOS is localized in a selected group of neurons, whereas eNOS is found mainly in endothelial cells and in some neurons as well. eNOS and nNOS are expressed constitutively and their activity is regulated by intracellular calcium. Therefore, NO production by nNOS and eNOS is believed to occur in small bursts only when intracellular calcium concentration increases. In contrast, iNOS is not normally present in most cells but its expression is induced in pathological states, typically those associated with inflammation (105). At variance with nNOS and eNOS, iNOS is not regulated by intracellular calcium, and produces NO continuously and in large amounts (147). Therefore, iNOS expression and NO production are thought to participate in the deleterious effects of inflammation (55).

Nitric oxide and ischemic brain injury

There is substantial evidence that NO is involved in ischemic brain injury. The role of NO has been studied most extensively in rodent models of cerebral ischemia produced by MCAO. The evidence suggests this mediator can have both beneficial and deleterious effects, depending on the cellular compartment in which NO is generated, and on the stage of evolution of ischemic brain injury (68, 127). Immediately after induction of ischemia, the vasodilator effect of NO, produced mainly by eNOS, protects the brain by limiting the degree of flow reduction produced by the arterial occlusion (66). However, after ischemia develops, NO produced by nNOS and, later, by iNOS, contributes to the evolution of the brain injury (68). Because iNOS is expressed in the setting of the inflammatory reaction that accompanies cerebral ischemia, its role in ischemic brain injury will be discussed in some detail in the following sections.

iNOS and ischemic brain injury

As discussed earlier in this paper, focal cerebral ischemia is associated with a marked inflammatory reaction that contributes to the evolution of the tissue injury. Expression of iNOS occurs in the setting of such inflammatory reaction. Following permanent or transient MCA occlusion in rodents, iNOS message, protein, and enzymatic activity are expressed in the post-ischemic brain (52, 71, 72, 75) (Figure 4). The expression peaks 12-48 hours after ischemia and occurs in inflammatory cells infiltrating the injured brain and in cerebral blood vessels (Figure 4, Table 3). A recent study, in which iNOS expression was investigated in patients who died within 24 hours after a major stroke, reported that iNOS immunocytochemistry is present in neutrophils and vascular cells in the injured brain (42). Interestingly, cells containing iNOS immunoreactivity were also positive for the nitrotyrosine, a relatively-specific marker of NO-derived peroxynitrite (27). This finding suggests that iNOS is catalytically active also in the post-ischemic human brain. The observation that iNOS is present in the human brain after stroke supports the hypothesis that iNOS expression is also involved in the mechanisms of cerebral ischemia in humans.

To explore the role of iNOS in ischemic stroke systemic administration of relatively selective iNOS inhibitors, such as aminoguanidine or 1400GW were used (74, 115). Because iNOS is expressed many hours

after induction of ischemia, the inhibitors were administered starting 12-24 hours after MCA occlusion, at the time when iNOS was present. It was found that iNOS inhibition reduced infarct volume by 30-40% (72, 74, 115). Importantly, the reduction in histological damage was associated with an improvement of the neurological deficits produced by the infarct (103). The effect could not be attributed to changes in body temperature, plasma glucose, hematocrit, arterial pressure or blood gases. Furthermore, aminoguanidine did not influence cerebral blood flow (CBF), suggesting that the protection is not related to the preservation of post-ischemic blood flow (74).

To rule out the possibility that the protection exerted by iNOS inhibition was related to non-specific effects of the inhibitors, mice lacking the iNOS gene (90) were used in brain following MCA occlusion (73). It was found that iNOS null mice have smaller infarcts (-30%) and better neurological outcome than wild-type littermates (73). The reduction in infarct volume was more marked in homozygous than in heterozygous iNOS mice (164). This observation is consistent with a gene-dosing effect of iNOS deletion. The protection could not result from cerebrovascular effects of iNOS deletion, because the reduction in CBF produced by MCA occlusion did not differ between iNOS null mice and controls (73). Furthermore, the reduction in infarct volume could not be attributed to effects on the cellular reaction that occurs after ischemia, because the degree of neutrophilic infiltration and astrocytic activation was comparable in iNOS null mice and controls (73).

Interestingly, the magnitude of the protection exerted by deletion of the iNOS gene is dependent on the age of the mice. Thus, the reduction in infarct volume was greater in 1-2 month-old mice than in 6 month-old mice (102).

iNOS as a therapeutic target for stroke

The findings reviewed above, collectively, provide strong evidence in favor of a major role of NO produced by iNOS in the mechanisms of the evolution of cerebral ischemic injury. Furthermore, the fact that iNOS is expressed also in the human brain after ischemia, strengthens the argument that iNOS is a valuable therapeutic target in human stroke. The extended therapeutic window of iNOS inhibitors (12-24 hours) would permit to treat stroke patients that do not qualify for treatment with modalities, such as thrombolysis or glutamate receptor inhibition, that are effective only in the early stages of the damage (37). Therefore, inhibition of iNOS expression or activity would be a valuable therapeutic strategy to selectively target the delayed phase of the damage.

Cyclooxygenase gene expression and inflammation

Another gene that is expressed during inflammation is cyclooxygenase-2 (COX-2). Cyclooxygenases are rate limiting enzymes in the synthesis of prostaglandins and thromboxanes. Two isoforms of COX have been described: COX-1 and COX-2. COX-1 is expressed in many cells and is thought to play a role in platelet aggregation, gastric secretion, and renal function (136). COX-2 is constitutively expressed in excitatory neurons, wherein it is localized to dendritic spines (79, 158). In many organs, COX-2 expression is upregulated by a wide variety of stimuli, such as inflammatory mediators and mitogens (137). In models of inflammation, COX-2 reaction products are believed to be destructive and contribute to cytotoxicity possibly due to production of reactive oxygen species and toxic prostanoids (129).

COX-2 and cerebral ischemia

Following cerebral ischemia, COX-2 mRNA and protein are upregulated peaking 12-24 hours after ischemia (95, 109, 110, 119) (Figure 4). COX-2 is expressed in neurons and vascular cells located at the border of the ischemic territory (95, 110) (Table 3). In neurons, COX-2 is expressed in cells that exhibit ischemic changes, as well as in neurons that appear structurally normal (110). Recently, COX-2 has been found to be expressed also in the human brain after ischemic stroke (69).

The role of COX-2 in the mechanisms of cerebral ischemia has not been completely elucidated. Initial data suggest that the relatively selective COX-2 inhibitor NS-398 reduces infarct volume by 20-30% in a model of focal ischemia (110). Furthermore, COX-2 inhibition also reduces neuronal damage in a model of global cerebral ischemia (104). These observations raise the possibility that COX-2 reaction products contribute to the evolution of ischemic damage. The fact that delayed administration of NS-398 (6 hours after MCA occlusion) reduces the damage supports the notion that COX-2 is involved in the late stages of ischemic injury. However, evidence that NS-398 acts exclusively on COX-2 activity is lacking. NS-398, like other COX inhibitors, may also have effects on gene transcription which may play a role in its protective effect (54, 80).

Another line of evidence, suggestive of a pathogenic role of COX-2 in the mechanisms of cerebral ischemia, is provided by studies in which the interaction between NO produced by iNOS and COX-2 was investigated.

Following cerebral ischemia, iNOS and COX-2 are expressed with a similar time-course and in cells that are close to each other (109) (Figure 4, Table 3). The spatial and temporal proximity of iNOS and COX-2 suggests that NO produced by iNOS could activate COX-2 and enhance the toxic output of the enzyme (126). This possibility is supported by studies demonstrating that selective inhibition of iNOS reduces COX-2 reaction products in the post-ischemic brain (109). Furthermore, COX-2 reaction products are reduced in iNOS null mice (109), which do not produce iNOS-derive NO after ischemia. These data, collectively, suggest that NO produced by iNOS may "drive" COX-2 activity in the post-ischemic brain and increase COX-2 reaction products.

COX-2 as a therapeutic target for stroke

While the interaction between iNOS and COX-2 provides additional evidence that COX-2 activity may be deleterious to the ischemic brain, the role of COX-2 in ischemic brain injury is far from clear. It has long been known that COX reaction products contribute to the regulation of the cerebral circulation (118). COX-2 inhibition might have effects on cerebrovascular regulation that could alter the outcome of cerebral ischemia. These effects need to be characterized and their contribution to ischemic injury remains to be defined. Furthermore, COX-2 is expressed in excitatory neurons, wherein it is likely to play a role in synaptic transmission and plasticity (79). Therefore, COX-2 inhibition may have effects on regenerative and processes involved in functional recovery after stroke. Further studies addressing these issues are required to clarify in full the role of COX-2 in ischemic brain injury.

Molecular mechanisms of expression of iNOS and COX-2

The molecular mechanisms of iNOS and COX-2 expression after cerebral ischemia are not fully understood. In a variety of cell systems, cytokines are known to induce expression of both iNOS and COX-2. Furthermore, hypoxia has been reported to elicit iNOS expression via activation of the hypoxia inducible factor-1 (94). Transcription factors that are involved in the expression of inflammation related genes include NFκB and interferon regulatory factor-1 (5, 108). These transcription factors are induced after ischemia (20, 70) and they are likely to contribute to the expression of iNOS and COX2, as well as other genes expressed after cerebral ischemia.

Inflammatory cytokine in brain ischemia and trauma

Direct trauma, deprivation of oxygen and nutrients (ischemia), neurotoxicity, viral infection or immunological challenge produce a well-defined response of "gliosis" (117). The activation, proliferation and hypertrophy of cells derived from the mononuclear phagocytic system (e.g., macrophages and microglia) are the hallmark of this reaction. Originally, this response was thought to mediate repair, restoration of blood supply, re-establishing the integrity of the blood brain barrier and promoting general homeostasis at the site of injury (111, 112). Since cytokines activate glial cells (39) which then produce cytokines (128), a close relationship appears to exist between inflammation, cytokine production and gliosis. Indeed, gliosis can be induced by TNF-α, IL-1β and interferon α (IFNα) (6).

Several cell types within the brain are able to secrete cytokines, including microglia, astrocytes, endothelial cells and neurons; in addition, there is also evidence to support the involvement of peripherally derived cytokines in brain inflammation. Peripherally derived mononuclear phagocytes, T-lymphocytes, natural killer (NK) cells and PMN's which produce and secrete cytokines, can all contribute to CNS inflammation and gliosis. In support of this, irradiation of the bone marrow or treatment *in vivo* with colchicine, attenuate gliosis, wound repair, neovascularization and generalized inflammation (47).

The inflammatory response to brain injury has been studied systematically following focal stroke by several investigators. The early accumulation of neutrophils in ischemic brain damage has been clearly demonstrated based upon histopathological (25, 43, 56), biochemical (9), and ^{111}In-labeled leukocyte studies (38). Ameboid microglia, a form of activated microglia, can be identified within 2 hours of ischemia. Unlike normal brain microvessels that are clear of inflammatory cell brain microvessels from ischemic zones are filled with leukocytes and a significant zone of edema surrounds them. Many of the leukocytes, primarily neutrophils, found in vessels within ischemic tissue are adherent to the endothelium, a situation not normally observed in intact brain microvessels. Some of these neutrophils migrate outside the vascular walls into the focal ischemic cortex. Brain injury is associated with the expression of inflammatory mediators, e.g., inflammatory cytokines (IL-1 and TNF-α) and chemokines, (IL-8 for neutrophils, MCP-1, RANTES, IP-10) while up-regulation of adhesion receptors (ICAM-1, selectins) support leukocyte adherence to the endothelium (41). TNF-α and IL-1β pre-dispose or "prime" endothelium for cellular adher-

ence. Additionally, adhesion molecules such as CD11/CD18 integrins are also thought to be pivotal in this inflammatory process. The importance of leukocyte infiltration in the pathogenesis of brain injury has been reviewed previously (41). This inflammatory reaction not only contributes to lipid-membrane peroxidation, but also exacerbates the degree of tissue injury due to the rheologic effects of "sticky" leukocytes in the blood vessels (i.e., an interference with normal microvascular perfusion due to vascular plugging in an already compromized ischemic tissue bed), and also due to the release of cytotoxic products from these activated leukocytes (i.e., by generation and release of oxygen radicals and cytotoxic products that are cytodestructive to the already compromised tissue; 82). The exact nature of the signaling mechanisms in brain inflammation still remains to be elucidated but undoubtedly involves TNF-α and IL-1β, chemotactic cytokines (e.g., chemokines such as IL-8) as well as the expression of adhesion molecules and proteinases that together promote both recruitment of adherent leukocytes and infiltration, and enhanced permeability of brain endothelium. For example, for a neutrophil to adhere to the endothelium and then migrate unidirectionally into the tissue, adhesion molecules have to be up-regulated. In order to upregulate adhesion molecule expression, injury must up-regulate specific cytokines, such as TNF-α and IL-1β. This has to happen very rapidly, and proteins also have to be rapidly translated. In addition, when those cytokines are expressed and translated, they have to up-regulate adhesion molecules and produce chemokines that will induce chemoattraction to drive neutrophils into the tissue.

Roles of TNF-α in Traumatic Brain Trauma and Stroke

TNF-α is a pleotrophic cytokine released by many cell types upon diverse stimulation. TNF-α exerts a diverse array of biological activities including secretions of acute phase proteins and vascular permeability. TNF-α and its receptors are present in the CNS (135). In addition, several clinical studies have shown a distinct relationship between elevated levels of cytokines, including TNF-α, neurodegenerative disorders, and brain injury.

Elevated TNF-α has been repeatedly demonstrated in various experimental models of brain injury. Systemic kainic acid administration induces within 2-4 hours TNF-α mRNA levels in cerebral cortex, hippocampus and hypothalamus. Systemic or intracerebroventricular administration of lipopdysaccharide endotoxin (LPS) has also been shown to increase brain TNF-α levels as determined by bioassay (134). In a model of closed head injury, Shohami *et al*. (133) reported an early increase in TNF-α peptide at the site of the focal insult. Also, in rat traumatic head injury, TNF-α mRNA and protein levels are rapidly elevated (40). Furthermore, in mice challenged with particles of charcoal injected into the hippocampus, an increase in striatal levels of TNF-α mRNA was observed (133). Elevated serum TNF-α was also observed following severe head injury in man (49).

Elevated expression of TNF-α mRNA and protein occurs shortly (1-3 hours) following middle cerebral artery occlusion (MCAO) in rats (87, 154). In ischemic cortex, TNF-α mRNA levels are elevated as early as 1 hr post-occlusion (i.e., prior to significant influx PMN) peaked at 12 hours and persisted for about 5 days. The early expression of TNF-α mRNA preceding leukocyte infiltration suggests that TNF-α may be involved in this response. Double-labeling immunofluorescence studies localized the de novo synthesized TNF-α to neurons but not astroglia. At 5 days following the ischemic insult, neuronally-associated TNF-α was diminished, and TNF-α immunoreactivity was localized in the inflammatory cells. The significance of TNF-α expression in the brain was studied by microinjection of TNF-α into the rat cortex; TNF-α induced leukocyte adhesion to the capillary endothelium, but no evidence for neurotoxicity at the site of injection was found. Buttini *et al*. (18) identified a rapid upregulation of TNF-α mRNA and protein in activated microglia and macrophages following focal stroke, again suggesting that TNF-α is part of an intrinsic inflammatory reaction of the brain following ischemia. TNF-α may exert a primary effect on microvascular inflammatory response as reflected by TNF-α-induced neutrophil adhesion to brain capillary endothelium (87). Furthermore, intracerebroventricular injection of TNF-α 24 hr prior to MCAO exacerbates the ischemia induced tissue injury (8). This effect was reversed by ventricular administration of anti-TNF-α mAb in the contralateral ventricle. Further evidence for the involvement of TNF-α in stroke-induced injury is supported by findings that spontaneously hypertensive rats that are stroke prone have higher levels of TNF-α production in the brain as compared with normotensive rats (134). These data suggest that TNF-α may prime the brain for subsequent damage by activating capillary endothelium to a pro-adhesive state.

IL-1β in brain trauma and stroke

IL-1β is produced in the CNS by various cellular elements including microglia, astrocytes, neurons and endothelium (124). Like TNF-α, IL-1β has many pro-inflammatory properties , and receptors for this cytokine have been demonstrated in the CNS. Increase in IL-1β

mRNA expression has been shown to occur following several types of injury to the brain including kainate excitotoxicity (96) and LPS (19). Furthermore, mechanical damage following implantation of a microdialysis probe has been shown to induce expression of IL-1β. Following fluid percussion brain trauma in the rat, a rapid increase in IL-1β mRNA expression has been reported. Microglial IL-1α expression has been observed in human head injury. IL-β mRNA expression has been shown to increase following transient brain ischemia in the rat (97). The exacerbation of ischemic brain injury due to exogenous IL-1β administered into the brain has been observed (159). A rapid (3-6 hr post ischemia) increase in IL-1β mRNA following MCAO peaked at 12 hours but returned to basal values at 5 days (88, 154). Early IL-1β expression following focal stroke has also been demonstrated using in situ hybridization. The recent development of tools such as specific antibodies to rat IL-1β has permitted the identification (by immunohistochemistry) of IL-1β peptide in cerebral vessels, microglia and macrophages following focal stroke.

Interleukin-1 receptor antagonist (IL-1ra), a 23-25 kDa-glycosylated protein, is a naturally occurring inhibitor of IL-1 activity that competes with IL-1 for occupancy of IL-1RI without inducing a signal of its own. IL-1ra is produced by many different cellular sources including monocytes/macrophages, endothelial cells, fibroblasts, neurons and glial cells. The expression of IL-1ra and IL-1R mRNA following focal stroke were also reported (150). The level of IL-1ra mRNA was markedly increased in the ischemic cortex at 6 hours, then reached a significantly elevated level from 12 hours to 5 days following MCAO. The presence of IL-1ra in the normal brain and the upregulation of IL-1ra mRNA after ischemic injury suggest that IL-1ra may serve as a defense system to attenuate the IL-1-mediated brain injury. It is interesting to observe that the temporal induction profile of IL-1ra following MCAO virtually parallels that of IL-1β as demonstrated previously (88), except that IL-1ra mRNA exhibited prolonged elevation beyond that of IL-1β. Thus, the balance between the levels of IL-1β and IL-1ra expressed post ischemia may be more critical to the degree of tissue injury than IL-1 levels per se.

The mediators responsible for IL-1ra induction after focal stroke are not known. However, previous studies indicate that some cytokines such as IL-1, TNF, IL-6 and TGFβ are inducers of IL-1ra. Ischemia-induced expression of IL-1ra mRNA could originate from monocytes/macrophages, endothelial cells, fibroblasts, neurons and glial cells as observed previously under normal conditions. The same cellular sources may be responsible for IL-1β and IL-1ra production based upon the close temporal, and perhaps functional coupling of these two genes after focal stroke.

Anti-Leukocyte Strategies for Neuroprotection

Matsuo *et al*. (92) have used the RP-3 monoclonal antibody that selectively depletes leukocytes in the rat (about 90-95%) and reported a dramatic reduction in both neutrophil accumulation in focal ischemic brain tissue and infarct size (decreased by 45-50%). However, some controversy exists (62).

Anti-adhesion molecule antagonists for neuroprotection

Another attractive approach is the inhibition of endothelial interactions with the leukocyte. Chen *et al*. (21) treated MCAO rats intravenously with an antibody against MAC-1, the leukocyte counterpart of ICAM-1 binding and demonstrated reduction in infarct size by 45-50% in a rat transient MCAO model. Zhang *et al*. (162) used the i.v. administration of an anti-ICAM-1 antibody to demonstrate a 40% reduction of infarct size in a similar model. Blocking adhesion molecules can also reduce apoptosis induced by focal ischemia (23). Other studies verified these effects but also illustrated that these antibodies could not reduce infarct size when the ischemia was permanent (15, 24, 26, 77, 161). However, the strategy may work if both leukocyte and endothelial adhesion proteins are blocked in permanent focal stroke. The combination of t-PA and anti-CD 18 provides significantly improved outcome, and may increase the therapeutic time window in stroke (163). In a rabbit embolic model of stroke, anti-ICAM-1 antibody was shown to increase the amount of clot necessary to produce permanent damage (15). In addition, in a baboon model of transient focal ischemia anti-CD 18 monoclonal anti-body administered 25 min prior to reperfusion led to increase in reflow in microvessels of various sizes (99). However, in contrast to the demonstrated anti-ischemic effect of anti-adhesion molecules in animal models, the recent failure of the murine anti-ICAM mAb (enlimomab) in human stroke (31) and its ability to activate human neutrophils (148) demonstrate the difficulties in extrapolating encouraging data derived from animal models to clinical reality.

Neuroprotection by cytokine inhibition supports anti-inflammatory approach

An alternate possibility to modulate inflammation is

to aim directly for cytokine and chemokine suppressive agents. While proof for a role of TNF-α and IL-1β in ischemia brain damage has not been definitively established, the availability of selective and potent antagonists of cytokine production may aid in reaching this goal. Many studies have demonstrated the protective effects of IL-1ra in brain injury. Thus, intracerebroventricular administration of recombinant IL-1ra produced a marked reduction in brain damage induced by focal stroke (89, 120, 125), or brain hypoxia (91). This neuronal protective effect of IL-1ra in focal stroke was further supported by a recent study using an adenoviral vector that over-expressed IL-1ra in the brain (12). The excess of IL-1ra significantly reduced infarct size following focal stroke. While such modes of IL-1ra delivery are impractical in clinical terms, the studies point out a potential therapeutic remedy if delivery of IL-1ra can be achieved in a timely fashion. In addition, IL-1ra expression increases following ischemic preconditioning in a manner that parallels the development of brain ischemic tolerance (11). Of interest is data showing that peripheral administration of IL-1ra reduces brain injury (120), suggesting a potential use of IL-1ra as a neuroprotective agent in human stroke and/or neurotrauma. Likewise, several studies have shown that blocking TNF-α results in improved outcome in brain trauma and stroke. Pentoxifyline (a methylxanthine that reduces TNF-α production at the transcriptional level) or soluble TNF receptor I (which acts by competing with TNF-α at the receptor) improves neurological outcome, reduces the disruption of the blood brain barrier and protects hippocampal cells from delayed cell death following closed head injury in the rat (131). In rat focal ischemia, an anti-TNF-α monoclonal antibody (mAb) and the soluble TNF receptor I were neuroprotective (8). In the latter studies, TNF-α was blocked by repeated i.c.v. administrations before and during focal stroke which significantly reduced infarct size. In murine focal stroke, topical application of soluble TNF receptor I on the brain surface significantly reduced ischemic brain injury (106, 107). In addition, in another study evaluating TNF blockade on focal stroke in hypertensive rats, soluble TNF receptor I administered i.v. pre- or post-MCAO significantly reduced the impairment in ischemic cortex microvascular perfusion and the degree of cortical infarction, strongly suggesting an inflammatory/vascular mechanism for TNF-α in focal stroke (30).

The detrimental effects of TNF-α and its role as a mediator of focal ischemia may involve several mechanisms. For example, TNF-α increases blood brain barrier permeability and produces pial artery constriction that can contribute to focal ischemic brain injury; furthermore, a direct toxic effect of TNF-α on the capillaries were also noted (48). Furthermore, by stimulating the production of matrix-degradating metalloproteinase (gelatinase B) (122, 123), TNF-α may further exacerbate capillary integrity. TNF-α also causes damage to myelin and oligodendrocytes (121), and increases astrocytic proliferation thus potentially contributing to demyelination and reactive gliosis . In addition, TNF-α activates the endothelium for leukocyte adherence and procoagulation activity (i.e., increased tissue factor, von Willebrand factor and platelet activating factor) that can exacerbate ischemic damage. Indeed, increased TNF-α in the brain and blood in response to lipopolysaccharide appears to contribute to increased stroke sensitivity/risk in hypertensive rats (10, 41, 134). TNF-α activates neutrophils increases leukocyte-endothelial cell adhesion molecule expression, leukocyte adherence to blood vessels, and subsequent infiltration into the brain (41).

Interference with either IL-1 or TNF-α has now been shown repeatedly to result in reduced deficits in focal stroke and head trauma models. The evaluation of additional potent and specific anti-cytokine therapeutics in proper models of brain injury is clearly warranted. Much evidence has accumulated that indicates TNF-α production is regulated at both transcriptional and translational levels (144). Thus, TNF-α mRNA synthesis inhibitors such as rolipram (130), a phosphodiesterase IV inhibitor, could be of benefit in the treatment of brain inflammation. Other novel classes of drugs include highly specific protein kinase C (PKC) inhibitors such as calphostin C (81), which has been shown to potently inhibit LPS-stimulated TNF-α production from human monocytes *in vitro* as well as LPS and viral stimulated TNF-α production in astrocytic cell lines (85). However, due to the ubiquitous nature of PKC, non selective inhibitors may result in toxic consequences. The utility of blocking inflammatory cytokines in conjunction with thrombolysis using tPA has been discussed recently (44).

Cytokines and brain pre-conditioning

When exposed to single or repetitive episodes of sublethal ischemic stress, brain cells acquire resistance to subsequent, otherwise lethal ischemic insults (for review see 22). As with other types of stress, ischemic stress causes a number of biochemical changes in the cell which trigger activation of multiple signaling pathways. In turn this leads to expression of new genes and/or down regulation of currently active genes. Some of these changes are beneficial such as redistribution of

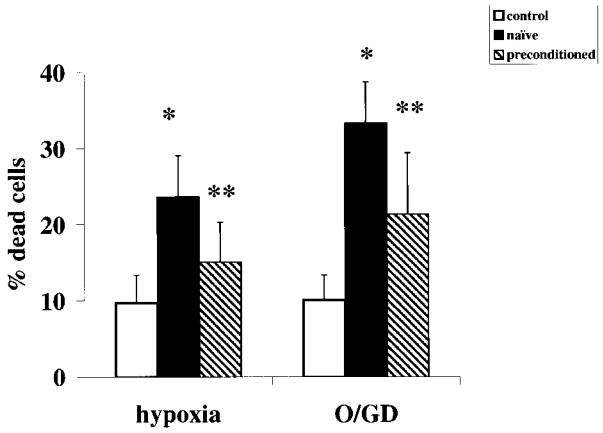

Figure 5. *Cell Death in Neuronal Cultures Subjected to Hypoxia or to Oxygen/Glucose Deprivation (O/GD).* Neuronal cultures grown in 24 well plates, either naïve or preconditioned with 20 min hypoxia were subjected to 2.5 hours of hypoxia or to 2.5 hours of O/GD and percentage of dead cells was measured at 8 hours after reoxygenation by means of ethidium homodimer exclusion fluorescent assay. Dead cells were also measured in control, untreated but sham-washed cultures. Each measurement was performed in eight wells and averaged. Each bar represents mean ± SD of 4 experiments * and ** denotes significant difference from control and from hypoxia or (O/GD), respectively.

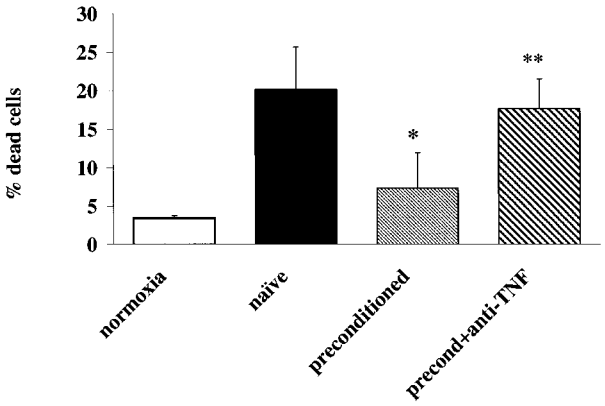

Figure 6. *The role of TNF-α in hypoxic preconditioning of neurons against hypoxia-induced neuronal injury.* Neuronal cultures, were preconditioned with 20 min hypoxia in the absence or presence of TNF-α neutralizing antibody, and then subjected to 2.5 hours of hypoxia. Measurements of the dead cell number were performed at 8 and 24 hours after cell reoxygenation. Each measurement was performed in 8 wells and averaged. Each bar represents mean ± SD of 4 experiments. * and ** denote significant difference from hypoxia-induced injury in naïve and preconditioned cells, respectively.

energy, activation of alternative metabolic pathways, production of antioxidants, and activation of DNA repair mechanisms. Others, like release of cytokines, upregulation of adhesion molecules, and induction of apoptotic machinery, exacerbate brain injury. In response to the stressful situation, another wave of biochemical and genetic changes occurs which attempts to resist the stress and to return the brain to its original condition. Thus, the same stress stimuli could cause both cell protection and cell death and also could elicit feed back and/or feed forward reactions that would quench either of these responses. This quenching reaction consists of at least of two mechanisms: either cytoprotective genes are activated to neutralize the effect of cytotoxic gene products (e.g. anti-inflammatory cytokines vs. proflammatory cytokines or anti-apoptotic genes vs. pro-apoptotic genes) or expression of harmful genes is simply shut off by newly synthesized or activated inhibitors of transcription and/or translation. We suggest that ischemic tolerance occurs because of this "after stress" homeostatic correction. Indeed, development of the tolerant state takes time, usually 24-72 hours (11). It is quite possible that although sublethal stress causes no visible damage, it still initiates anti-stress responses that proceed during the latent period and result in a transient stress-resistant phenotype of brain cells. Within this theoretical framework, a molecular mediator which triggers ischemic preconditioning could be the same agent that triggers ischemic injury; only in the case of preconditioning it is not harmful because the intensity of the effect is held below the threshold of cytotoxicity and/or because its effects are not exacerbated by severe stress environment. The response to such a mediator affects expression of multiple genes in the various types of brain cells.

TNF-α, a cytokine with pleiotropic activity, is a universal agonist for many different types of cells. Thus, whole brain, neurons (57, 87), microglia, astrocytes (145), and brain endothelium (14) are not only capable of TNF-α synthesis in response to stress but also express TNF-α receptors and amplify this response through paracrine and autocrine mechanisms (135). Further, TNF-α has been implicated in both, detrimental (3) and neuroprotective (93) actions on brain cells depending on the experimental conditions. Our recent studies demonstrated a mild but significant induction of TNF-α mRNA in ischemic brain tolerance, suggesting a potential neuroprotective role of this cytokine (151). The dual function of TNF-α has been also revealed *in vivo* (reviewed in 132). Neutralization of TNF-α by TNF-α-binding protein had a protective effect against focal ischemia

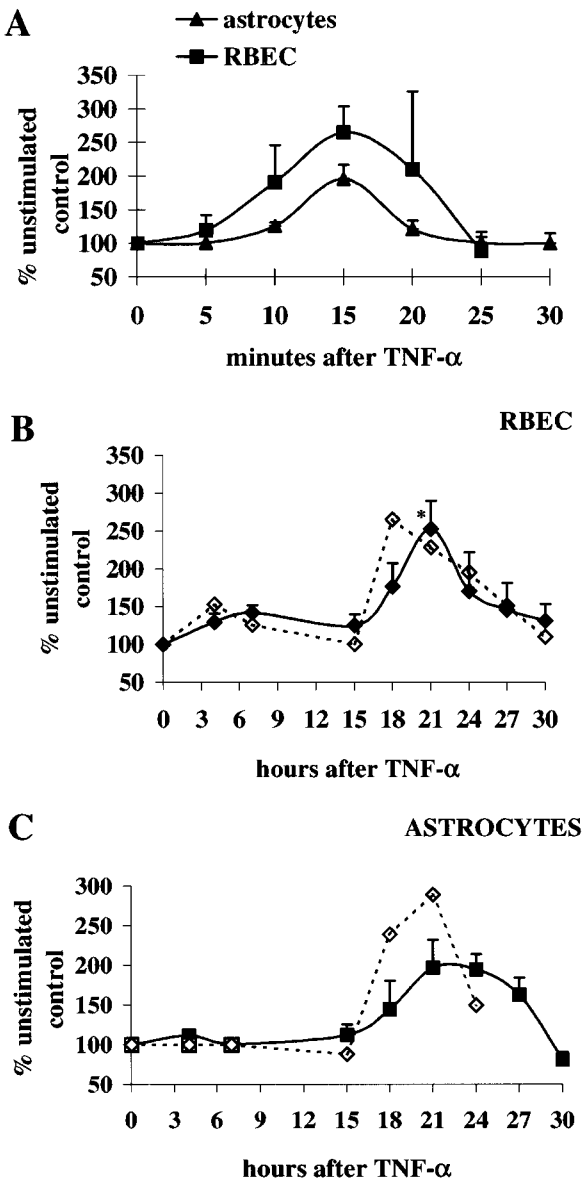

Figure 7. *TNF-α Pretreatment Induces a Biphasic Ceramide Response in Brain Cells.* Cell lipids were extracted with chloroform: methanol mixture (1:2), and ceramide was subjected to hydrolysis in 1N KOH in CH_3OH at 95°C to release deacylated ceramide (sphingosine) which was then derivatized with the fluorescent reagent orthophtaldialdehyde and quantitated by means of reversed HPLC. **A.** TNF-α-induced early ceramide increase in endothelial cells (closed squares, mean ±SD; n=3; peak $p<0.01$) and astrocytes (closed triangles; mean ±SD; n=4; peak $p<0.025$). The results are presented as a % increase over concentrations in unstimulated cells (0.17±0.07 and 0.11±0.03 pmol/nmol lipid phosphate for endothelial cells and astrocytes respectively). **B and C:** Cells were preconditioned with TNF-α for 4 hours and ceramide levels were measured at 4, 7, 15, 18, 21, 24, 27, and 30 hours after addition of TNF-α. **B.** Endothelial cells; a statistically significant peak was observed at 21 hours (closed diamonds) (mean ±SEM; n=5); a representative experiment is also included (open diamonds). **C.** Astrocytes; a statistically significant peak was observed between 21 and 24 hours (closed diamonds) (mean ±SEM; n=7); a representative experiment is also included (open diamonds).

(30, 106). On the other hand, transgenic mice, lacking TNF-α receptors developed significantly larger infarcts in the model of permanent MCAO (16). A possible inference is that TNF-α has the potential to function as a stresser or as a molecule with a homeostatic function perhaps as a preconditioning stimulus. In support of the preconditioning stimulus possibility, recent studies show that intravenous pretreatment of SHR rats with the TNF-α-inducing agent, lipoplysaccharide (142), or intracisternal pretreatment of mice with TNF-α (107) can substitute for ischemic preconditioning by protecting animals from ischemic injury in the MCAO model.

We have recently developed an *in vitro* model of ischemic preconditioning where cultured cortical neurons from 2-day-old Sprague-Dawley rats when preconditioned with mild hypoxia (8% oxygen in the medium for 20 minutes) become more resistant to severe hypoxia (2-5% oxygen for 2.5 hours) or to ischemia (similar hypoxia plus no glucose in the medium) applied 24 hours later (Figure 5). Similar to animal studies, TNF-α pretreatment of cultured cells (24 ng/mL for 24 hours) protected neurons to the same degree as did hypoxic preconditioning (about 50% protection). In addition this *in vitro* paradigm of adaptation to hypoxic/ischemic stress permitted direct demonstration that TNF-α is a key mediator of tolerance; when preconditioning was performed in the presence of an antibody known to neutralize TNF-α biological activity, no tolerance occurred (Figure 6).

Preconditioned neurons released measurable amounts of TNF-α into the medium (6.2 pg/2.5×105 cells according to the TNF-a ELISA). Increase of TNF-α concentrations was observed as early as 4 hours after preconditioning. It is likely that TNF-α which is released during preconditioning desensitizes neuronal cells against cytotoxic effects of TNF-α demonstrated in animal models of ischemia (87) and in neuronal cell cultures subjected to severe hypoxia (36). Indeed in another paradigm of TNF-α-induced tolerance, pretreatment of brain cells with TNF-α resulted in their unresponsiveness to subsequent TNF-α activation (46). In this model, pro-inflammatory effects of TNF-α have been studied in cultured rat astrocytes and brain endothelial cells. These cells failed to upregulate ICAM-1 adhesion ligand in response to TNF-α activation if they were pre-

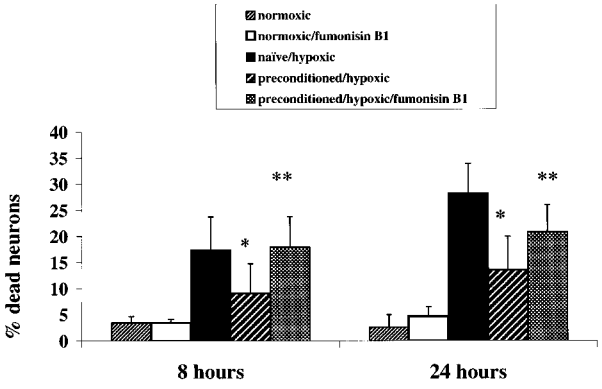

Figure 8. *The role of ceramide on in hypoxic preconditioning of neurons against hypoxia-induced neuronal injury.* Neuronal cultures, were preconditioned with 20 min hypoxia in the absence or presence of 50 μM fumonisin B_1, and then subjected to 2.5 hours of hypoxia. Each measurement was performed in 8 wells and averaged. Each bar represents mean +SD of 3 experiments. * and ** denote significant difference from naïve and preconditioned cells, respectively.

treated with TNF-α for only 4 hours the day before. In support of the universal character of TNF-α preconditioning, it also protected brain endothelial cells from upregulation of ICAM-1 induced by hypoxia. Similarly, Barnes *et al*. (7) have demonstrated that the same schedule of pre-treatment with TNF-α (or IL-1β) inhibited TNF-α (or IL-1β) activated synthesis of RANTES in astrocytes. While neuronal death depends on multiple mechanisms, synthesis of an adhesion receptor or a cytokine molecule could be traced to its gene expression. We have shown that TNF-α pre-treatment affects transcription of the ICAM-1 gene.

Astrocytes and endothelial cells acquired tolerance after preincubation with TNF-α for only 4 hours. Neuronal cells released TNF-α early in the course of hypoxic preconditioning. These observations suggested that tolerance (which requires at least 24 hours to develop) is triggered upon TNF-α binding to TNF-α receptor and then achieved through a chain of signal transduction events. Our goal was to identify the downstream signaling steps most proximal to the development of the tolerance.

The pleiotropic nature of TNF-α is reflected in the multiple signaling pathways initiated upon TNF-α binding to its p55 receptor. This includes activation of various intermediate molecules including two cascades of mitogen-activated protein kinases (MAPK), extracellular-regulated protein kinases (ERK) and stress-activated protein kinases (SAPK), protein phosphatases, reactive oxygen intermediates, phospholipase A_2 (PLA_2), phosphatidylcholine specific phospholipase C (PLC), PK-C, proteases, neutral and acid sphingomyelinases, and transcription factors NFκB and AP-1 (for review see 29). The sphingolipid, ceramide, has been implicated as a second messenger in almost all of these pathways thus participating in many of the effects caused by TNF-α (51). Although the role of ceramide in TNF-α-triggered apoptosis has attracted the most attention and has been extensively studied in tumor cell lines (116, 138), ceramide effects in non-tumor cells are more diverse and range from cell cycle arrest (84), to cell proliferation (4), to neuronal survival upon selective induction of the PKC-zeta/JNK pathway (155), and to selective activation of the ERK pathway (139). There is evidence that ceramide mediates TNF-α-induced protection (50, 76, 86). Thus, ceramide is similar to TNF-α in that it can trigger both pro-apoptotic as well as protective signals, which makes it well suited for signaling TNF-α-induced tolerance.

Two types of ceramide kinetics in response to TNF and other agents have been observed. In most cellular models, the ceramide response occurs within minutes after cell activation and quickly subsides (59). In some cells a delayed and persistent (12-24 hours) ceramide accumulation has been detected (67). Similar ceramide kinetics have been observed in our models of preconditioning. TNF-α pretreatment caused a biphasic increase of ceramide levels in astrocytes and brain microvascular endothelial cells; an early peak occurred at 15-20 minutes, when ceramide levels increased 1.9-fold in astrocytes and 2.7-fold in endothelial cells, and a delayed 2- to 3-fold ceramide increase was observed between 18 and 24 hours after addition of TNF-α (Figure 7). The later ceramide peak coincided with the state of unresponsiveness to TNF-α activation, and could be attenuated by Fumonisin B_1, an inhibitor of ceramide synthase. These results suggested that delayed ceramide increase in TNF-α-pretreated glial and endothelial cells was responsible for induction of tolerance to pro-inflammatory effects of TNF-α in these cells. In support of this hypothesis, the ceramide analog, N-acetylceramide (C-2 ceramide), inhibited TNF-α-activated ICAM-1 upregulation in both cell types when added 30 minutes before or even 1 hour after TNF-α, but not 24 hours prior to TNF-α addition. Strikingly, hypoxic preconditioning of neuronal cells against ischemic stress was also accompanied by a delayed ceramide increase which reached a maximum at 24 hours and then quickly subsided. As in the case of astrocytes and endothelium, ceramide increase in neurons was also sensitive to Fumonisin B_1. Furthermore, Fumonisin B_1 completely abolished the

effect of preconditioning (Figure 8). C-2 ceramide was able to protect neurons against the cytotoxic effects of severe hypoxia much as it protected astrocytes and endothelium against inflammation.

Interestingly in all three types of brain cells studied, neither addition of exogenous C-2 ceramide, nor upregulation of endogenous ceramide levels resulted in apoptosis although delayed ceramide upregulation has been commonly associated with an apoptotic signal (143) that could be mimicked by exogenous ceramide analogs (59). In most of these studies, cytokine- or stress-induced ceramide release was measured in undifferentiated cells or in tumor cell lines, but normal and malignant astrocytes, for example, exhibit differential sensitivity to TNF-α (28), and in order to induce apoptosis in endothelial with TNF-α or ceramide, inhibitors of protein synthesis need to be present (78, 157). We have recently shown that the cytotoxic effect of TNF-α in brain endothelial cells is drastically exacerbated by the presence free radicals resulting in inhibition of NF-kappa B activation (I, Ginis, M. Spatz, J.M. Hallenbeck, and E. Shohami, manuscript in preparation). NF-kappa B has been shown to protect against apoptosis in several models (for review see 146). We speculate that ischemic injury, which is accompanied by inhibition of protein synthesis (17), by production of free radicals (101) and by loss of NF-kappa B activity (13), provide pathological environment in which TNF-α and other effectors exert cytotoxicity. But during preconditioning these mediators only erform their homeostatic role by eliciting an anti-stress response. In this context, ceramide acts as a messenger of life, rather than of programmed cell death.

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