Molecular Neurobiology

ISSN0893-7648/05/32(1): 51–57/$30.00

Cortical Spreading Depression Modifies Components of the Inflammatory Cascade

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Abstract

As more information becomes available regarding the role of inflammation following stroke, it is apparent that some inflammatory mediators are detrimental and others are beneficial to the progression of ischemic injury. Cortical spreading depression (CSD) is known to impart some degree of ischemic tolerance to the brain and to influence the expression of many genes. Many of the genes whose expression is altered by CSD are associated with inflammation, and it appears likely that modulation of the inflammatory response to ischemia by CSD contributes to ischemic tolerance. Understanding which inflammatory processes are influenced by CSD may lead to the identification of novel targets in the effort to develop an acute treatment for stroke.

Index Entries: Inflammation; stroke; cortical spreading depression; gene expression.

Introduction

It is increasingly apparent that inflammation is an important factor in the delayed progression of injury following ischemia *(1,2)*. It is also becoming evident that variations in the inflammatory cascade are not always harmful, because inflammation appears to play a role in the repair and recovery phases following a stroke *(3)*. During focal ischemia, cells experiencing the most severe reduction in blood flow undergo rapid necrotic cell death, whereas cells in the surrounding regions may remain viable for a period of time, available for rescue but eventually undergoing a form of programmed cell death *(4)*. Therefore, it is important to understand and to attempt to modulate the inflammatory response during and after ischemia, because various inflammatory processes are known to continue for many weeks after the ischemic episode and are believed to influence the resolution of the infarct *(1,5)*. Wang and Feuerstein *(2)*

Received July 20, 2004; Accepted November 23, 2004.
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recently reviewed the role of immune and inflammatory mediators in central nervous system (CNS) injury.

Cortical Spreading Depression Confers Neuroprotection Against Ischemic Injury

One means of modulating the brain's propensity to ischemic injury is through the prior imposition of spreading depression (SD). Kawahara et al. *(6)* first demonstratedthat SD induces ischemic tolerance in 1995 in hippocampal CA1 neurons in rat brain. Since then, several other studies have confirmed this observation for cortical neurons *(7–13)* and have further characterized the protective effects of SD. A period of time is required for ischemic tolerance to develop in the brain, with some authors reporting a delay of up to 12 d *(11)* and others reporting a delay as little as 24 h *(7,9)*. Additionally, the protection conferred by cortical spreading depression (CSD) is a transient phenomenon, lasting up to 15 d following 48 h of CSD *(11)*. Following transient focal ischemia, the cortical infarct volume in rats subjected to CSD before ischemia is about 50% of that found in rats subjected to sham CSD surgery before ischemia *(8,10,11,13)*. Yanamoto et al. *(14)* measured improvement in infarct volumes 14 d after ischemia, indicating that the CSD induces long-lasting neuroprotection and does not simply delay the process of programmed cell death in cells destined to die.

The hypothesis justifying this article is that understanding the influence CSD has on the brain will allow better identification of the molecules that may be damaging, as well as those that may be protective, in the setting of ischemia. Part of that understanding is the influence CSD has on the inflammatory cascade.

CSD has been shown to profoundly affect gene expression in the brain, and to date, over 40 genes have been reported to be upregulated by CSD. Although it is not likely that all of these genes are of equal importance in the induction of ischemic tolerance, it has become apparent that many factors are involved in changing the brain to a more tolerant phenotype. Therefore, we explored the genomic response of the brain to CSD by DNA microarray analysis. The preliminary results obtained in this manner must be interpreted with caution, because a change in messenger RNA (mRNA) expression does not necessarily imply a similar change in functional protein expression. Additionally, the method yields no information concerning which cell types in the brain are affected, and the functional significance of very large-fold changes in mRNA levels from genes initially showing very low levels of expression might not be very important. Nonetheless, our work shows that the expression of many genes associated with inflammation is affected by CSD. Therefore, it appears likely that CSD may modulate the inflammatory response to subsequent ischemia.

Methodology

All surgical procedures followed the guidelines of the Canadian Council for Animal Care and were approved by the Animal Care Committee of the University of Ottawa. CSD was induced as described previously *(15)*. Briefly, animals were anesthetized and mounted in a stereotaxic frame. The skull was exposed, and a 2-mm diameter burr hole was drilled over the left occipital cortex without breaking the dura. CSD was elicited by placement of a cotton pledget soaked in 0.5 *M* of KCl directly over the dura. A fresh KCl pledget was reapplied every 15 min for a 2-h period, after which the rat was either sacrificed under deep anesthesia or allowed to recover and survive for 48 h. Rats typically have 10 to 14 waves of spreading depression during the 2-h period, but electrical recordings were not made to avoid inflammation and altered gene expression around the site of the recording wire. After sacrifice, the brain was removed and placed in a rat brain matrix, and a 1-mm thick coronal section was cut at approx bregma (–1.3 mm). The remaining left cortex was then

quickly frozen in liquid nitrogen and stored at –80ºC prior to RNA extraction. Control animals were treated in an identical manner, with the exception that 0.5 *M* of NaCl was used, rather than 0.5 *M* of KCl.

Immunohistochemistry was used to verify the occurrence of CSD. The 1-mm thick coronal sections from each rat were fixed in 4% paraformaldehyde, cryoprotected in 10% sucrose, and frozen on dry ice using liquid CO_2 expansion. Free-floating sections of 20 μm were cut, and those from rats sacrificed immediately after CSD were immunostained for the phosphorylated form of extracellular signal-regulated kinase (ERK) 1/2 *(16)*. Sections from rats sacrificed 48 h after CSD were stained for glial fibrillary acidic protein (GFAP) *(17)*. When GFAP-like or phosphorylated ERK 1/2-like immunoreactivity was clearly elevated in the ipsilateral cortex relative to the contralateral cortex (which was not exposed to CSD), CSD was determined to have occurred.

For each condition and time point, the RNA from three animals was pooled for micro-array analysis. RNA was extracted from the left cortices of CSD-treated rats by conventional means, and the Affymetrix™ Rat U34A Neurobiology Genechip was used to assess the expression levels of 8799 transcripts.

The expression of the inflammatory genes described in this study was not validated further. However, other have reported the induction expression of interleukin (IL)-1β and cyclooxygenase (COX)-2, as well as that of tumor necrosis factor (TNF)-α *(18,19)*. Kunkler et al. *(20)* recently reported the upregulation of IL-1α, -1β, -2, -6, and -10; interferon-γ; and TNF-α at the protein level following spreading depression in the hippocampus.

Effect of Cortical Spreading Depression on Elements in the Inflammatory Cascade

There were four patterns of changes in the mRNA concentrations of the inflammatory mediators. The two major patterns consisted of persistent elevation or depression in the mRNA of inflammatory mediators both immediately and at 48 h post-CSD. Far fewer molecular species changed in opposite directions at these two time intervals. Our data are presented in Table1 (molecules showing at least a fivefold change compared to baseline, at either interval after CSD, are in bold).

Our data show that CSD causes profound and usually persistent changes in the inflammatory cascade. IL-1α, IL-1β, IL-6, IL-13, IL-2 receptor α-chain, IL-1β converting enzyme, IL-1 receptor accessory protein, and IL-1 receptor-related protein were found to be upregulated at at least one time point following CSD, whereas IL-2, IL-10, and IL-12 were significantly downregulated both immediately and 48 h after CSD. Cell adhesion molecule mRNAs are also influenced by CSD, with vascular cell adhesion molecule (VCAM)-1 overexpressed and neural cell adhesion molecules (NCAMs)-1 and -2 suppressed. Macrophage inflammatory proteins are strongly activated, whereas B-integrin is suppressed. TNF-α mRNA was expressed at a low level and was only slightly affected by CSD, but the expression of TNF-β, TNF receptor, TNF receptor type II, and TNF-α converting enzyme mRNA was altered twofold or more at at least one time point. Some inflammatory species did not seem much affected by CSD—notably, neuronal nitric oxide synthase (NOS); E-selectin; IL-4, -5, and –7; and several molecules associated with cell adhesion and leucocyte infiltration. COX-2 expression was also elevated at both post-CSD intervals, but only by about twofold.

Discussion

The assumption in studying the effect of CSD on the inflammatory cascade is that the changes brought about by CSD (in their overall impact) would be desirable, because CSD results in smaller subsequent infarcts. However, it must be understood that our microarray results indicate that CSD causes changes

Table 1
CSD Influences the Expression of Many Genes Associated With Inflammation

A ↑0 h ↑48 h		B ↓0 h ↓48 h		C ↓0 h ↑48 h		D ↑0 h ↓48 h	
GenBank no.	Name	GenBank no.	Bank	GenBank no.	Bank	GenBank no.	Bank
D00403	IL-1α	**M22899 S82489**	**IL-2**	U49066	IL-1 receptor-x related protein	AF015719	IL-15
M98820	IL-1β	**L02926 X60675**	**IL-10**	L23088	P-selectin	U22414	MIP-1α
M26744	**IL-6**	**U16674 S82489**	**IL-12**	X52498	TGF-β1	**L00981**	**TNF-β**
M55049	IL-2 receptor α-chain	D44591 D83661	iNOS	**M96643**	**TGF-β2**		
U34684	**IL-1β converting enzyme**	AF110508	eNOS	**U55849**	**TNF receptor type II**		
U48592	IL-1 receptor accessory protein	**U11031**	**NCAM BIG-1**				
L26913	IL-13	U35371	NCAM BIG-2				
M84488	**VCAM-1**	**U81035**	**Ankyrin binding cell adhesion molecule neurofascin**				
U20194	Complement C8-β	AB003042	C5a receptor				
U86379	Anaphylatoxin C3a receptor	**U42719**	**C4 complement protein**				
U06434	MIP-1β	AF010466	IFN-γ				
M63122	TNF receptor	**S44606**	**β-integrin**				
S67722	COX-2	**S58528**	**Integrin αv-subunit**				
L26267	**NF-κB p105 subunit**	AF016900	Latent TGF-β binding protein				
U46958	Hyaluronan binding protein CD44i						
AJ012603	TNF-α converting enzyme						

Abbreviations: CSD, cortical spreading depression; IL, interleukin; MIP, macrophage inflammatory protein; TGF, transforming growth factor; TNF, tumor necrosis factor; iNOS, inductible nitric oxide synthase; eNOS, epithelial nitric oxide synthase; NCAM, neural cell adhesion molecule; VCAM, vascular cell adhesion molecule; COX, cyclooxygenase; NF-κB, nuclear factor-κB.

A, Genes whose expression was elevated both immediately following 2 h of CSD and 48 h later. **B,** Genes whose expression was decreased immediately and 48 h later. **C,** Genes with decreased expression immediately following CSD and elevated expression 48 h later. **D,** Genes with elevated expression immediately following CSD and decreased expression 48 h later. All genes listed showed at least a twofold change in expression. Genes showing at least a fivefold change in expression are listed in **bold**.

in hundreds of genes, so its influence on the inflammatory cascade may or may not be a major factor in providing the subsequent neuroprotection. Several studies have described the influence of CSD on the expression of specific genes, and some of these genes are associated with inflammation. Included in the list of genes reported to be upregulated by CSD are several adaptor protein-1 and zinc-finger transcription factors, heat shock proteins, growth factors, and many proteins associated with neurotransmitter systems and second messenger pathways. Several genes involved in inflammatory pathways have also been found to be upregulated by CSD, including nNOS (*21*), TNF-α *(18)*, IL-1β *(18)*, COX-2 *(19)*, and matrix metalloproteinase-9 *(22)*.

More specifically, our purpose in performing the studies was threefold. First, we wished to identify inflammatory molecular species (whose expression is modulated by CSD) that might be expected to have a positive or negative influence on outcome in the setting of ischemia. For example, our data indicate that CSD stimulates IL-6 mRNA immediately, and this activation is maintained at 48 h. IL-6 has been reported to be neuroprotective: its application rescued neurons and oligodendrocytes that were exposed to *N*-methyl-D-aspartate *(23)*.

Second, we sought a more comprehensive description of the inflammatory changes consequent to CSD. In this regard, it is interesting that our data show that the changes in the inflammatory cascade brought about by CSD have not always been reported in the literature as neuroprotective. For example, our data show that CSD suppressed IL-10, yet infarct volume was 30% larger in IL-10 knockout mice *(24)*. Similarly, CSD strongly activated the expression of VCAM-1, which has been found to be upregulated in response to ischemia in vitro *(25)*, and its inhibition has been reported to be neuroprotective *(26)*. CSD also suppressed the expression of the NCAMs BIG-1 and BIG-2. Cell adhesion molecules have been reported as important for neurite outgrowth during recovery from ischemia, with the expression of NCAM persisting after reperfusion *(27)*. Additionally, the expression of nuclear factor-κB, which contributes to infarction after permanent brain ischemia *(28)*, is strongly activated by CSD. Therefore, CSD causes several changes in the inflammatory cascade that may not appear to be protective to the brain at first blush, but it is not known if their expression in the CNS may set secondary neuroprotective responses in motion. It is also important to remember that our definition of which inflammatory species may be protective arises from work in ischemia, whereas the changes caused by CSD come about without significant changes in blood flow. Finally, the importance of the reported changes in the context of the hundreds of other genes affected by CSD is difficult to judge.

The third and final goal of our work was to develop hints at new therapeutic targets. For example, IL-12 is strongly suppressed by CSD, and the suppression persists over time. IL-12 is known to cooperate with IL-18 in the induction of interferon-γ expression *(29)* and is involved in the progression of ischemic injury in the liver *(30)*; however, little has been reported about this IL in the setting of ischemia in the brain. Similarly, the expression of C4 complement protein mRNA is strongly suppressed by CSD. The complement system is known to be involved in ischemia/reperfusion injury in some tissues *(31)*, and C4 expression is elevated following focal ischemia in mouse brain *(32)*; however, little else is known about role of C4 in the progression of ischemic damage in the brain. The results of this study suggest that further characterization of the role of IL-12, C4, and several other molecules might prove fruitful.

In conclusion, CSD may be a useful tool in exploring novel neuroprotective strategies, but data from studies of its modulation of the molecular cascades in the brain must be interpreted cautiously.

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