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Expression of COX-1, COX-2, iNOS and p38 in Human Brain with Stroke Lesions

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Abstract: The expression profile of COX-1, COX-2, iNOS and p38, in both the normal and post-ischemic human brain was studied. Focal cerebral ischemia is associated with a marked inflammatory reaction that contributes to the evolution and progression of brain tissue injury. Studies employing anti-inflammatory compounds and transgenic mouse models have suggested that both cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) mediate the deleterious effects of ischemic brain injury. A potential role for the mitogen-activated protein kinase (MAPK) p38 in cytokine production following stroke has been hypothesized. In order to evaluate the expression profiles of COX-1, COX-2, iNOS and p38 in normal and post-ischemic human brain, we evaluated the brain tissue from 12 patients with a pathological diagnosis of cerebrovascular disease (CVD) or cerebrovascular accident (CVA) for expression of COX-1, COX-2, p38 and iNOS via immunohistochemistry (IHC) and *in situ* hybridization (ISH). Corresponding brain sections from six normal patients served as controls. COX-1, COX-2 and iNOS were all present in the normal brain. However, in infarcted brains, an increase in iNOS and COX-2 expression was observed, with no concomitant change in COX-1 staining or p38 noted. Our data demonstrate up-regulation of both iNOS and COX-2, but not p38 or COX-1, in infarcted brains, bolstering the hypothesis that iNOS, COX-2 and their reaction products contribute to the progression of post-ischemic cerebral injury via cytotoxic, rather than cerebrovascular mechanisms.

Key words: Cerebral ischemia, cyclooxygenase, iNOS, MAPK

INTRODUCTION

Focal cerebral ischemia is associated with a marked inflammatory reaction that contributes to the evolution and progression of brain tissue injury (Iadecola and Alexander, 2001; Weston *et al*., 2007). Many of the pathophysiologic features of cerebral ischemia have been studied using animal models of middle cerebral artery occlusion (MCAO) (Huang *et al*., 2006; Tureyen *et al*., 2008). Studies employing anti-inflammatory compounds and transgenic mouse models, have suggested that both cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) mediate the deleterious effects of ischemic brain injury (Piao *et al*., 2003; Nagayama *et al*., 1999). A potential role for the mitogen-activated protein kinase (MAPK) p38 enzyme in cytokine production following stroke has been hypothesized (Barone *et al*., 2001; Piao *et al*., 2003; Sawe *et al*., 2008).

The cyclooxygenase (COX) enzymes catalyze the committed enzymatic step in the conversion of arachidonic acid to prostaglandins (PGs). COX exists in two related, but unique, isoforms: COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and catalyzes the PG synthesis believed to support physiologic functions, including platelet aggregation. The COX-2 isoform catalyzes the synthesis of pro-inflammatory PGs and is predominantly induced by bacterial endotoxins, cytokines and growth factors and minimally constitutive in select tissues (e.g., brain neurons) (Brone and Parsons, 2000; Harris *et al*., 1994; Iadecola, 1997; Kaufmann *et al*., 1997). In the adult brain, COX-1 is most abundantly expressed in the forebrain, where it may be involved in complex integrative functions; whereas COX-2 is primarily present in the cortex, hippocampus and hypothalamus and is highly induced by the modulators of specific neuronal responses (i.e., ischemia, structural brain damage and toxic insult) (Seibert *et al*., 1995; Xiang *et al*., 2007). Although COX-1 expression has been identified in endothelial cells of the aorta, microvasculature of the heart and in the central nervous system (CNS), it has not been implicated in the generation of post-ischemic brain injury (Harris *et al*., 1994; Stanfield *et al*., 2001). In contrast, COX-2 expression is up-regulated following cerebral ischemia in both animal models of MCAO and in brain specimens from human patients (Iadecola *et al*., 1999; Nogawa *et al*., 1997;

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Xiang *et al*., 2007). Furthermore, COX-2$^{-/-}$ mice exhibit a reduction in brain injury following MCAO, implicating COX-2 in the pathophysiology of focal ischemic brain injury (Iadecola *et al*., 2001).

Similar to COX-2, iNOS is not constitutively expressed, but is induced by cytokines under inflammatory conditions (Nathan, 1997). iNOS expression has been identified in inflammatory neutrophils that infiltrate the injured brain, as well as, in the cerebral blood vessels and has been shown to be up-regulated after stroke in both humans and animal models of MCAO (Brown *et al*., 2007; Iadecola, 1997; Samdani *et al*., 1997). Studies with iNOS$^{-/-}$ mice and those using an iNOS inhibitor, have demonstrated a reduction in both infarct volume and histological damage, suggesting that iNOS mediates the deleterious effects of post-ischemic brain injury (Iadecola *et al*., 1996, 2000; Zhao *et al*., 2000).

No connection has been identified between p38 and focal ischemic brain injury. And while expression of p38 has been associated with inflammatory cytokine production following cellular stress, p38 inhibitor treatment reduced the infarct size in *in vitro* brain preparations (Nagayama *et al*., 1999). However, little is known about the type of cells expressing p38 in either the normal or post-ischemic brain tissue.

Herein, we report the expression of COX-1, COX-2, iNOS and p38, in both the normal and post-ischemic human brain.

MATERIALS AND METHODS

Tissues: The study was conducted between 2001 to 2003. Formalin-fixed brain tissue specimens were obtained from the Human Brain and Spinal Fluid Resource Center (Veterans Administration Medical Center (VAMC), Los Angeles, CA, USA), which is sponsored by NINDS/NIMH, National Multiple Sclerosis Society (VA Greater Los Angeles Healthcare System, Los Angeles, CA, USA) and Veterans Health Services and Research Administration, Department of Veterans Affairs. Brain sections from 12 patients with a pathological diagnosis of cerebrovascular disease (CVD) or cerebrovascular accident (CVA) were analyzed for the expression of COX-1, COX-2, iNOS and p38 via immunohistochemistry (IHC) and *in situ* hybridization (ISH). Corresponding brain sections of six normal patients served as controls. Patients age ranged from 53-90 years of age. The autolysis interval ranged from 3.5-26 h.

Immunohistochemistry (IHC): Standard immunohistochemical procedures and commercially available assay kits were used for the immunolocalization of COX-1, COX-2, iNOS and p38, as previously described, with minor modifications (Iadecola *et al*., 2001). In general, formalin-fixed, paraffin-embedded brain specimens were cut into 4-5 μm sections and mounted on positively charged slides (Erie Scientific Co., Portsmouth, NH, USA). Sections were then dewaxed, rehydrated in xylenes and descending alcohols and then blocked for endogenous avidin/biotin. All tissues were permeabilized in 0.3% triton/0.2% saponin/1% bovine serum albumin (Sigma, St Louis, MO, USA) in phosphate buffered saline containing 10% normal goat serum (Vector Laboratories, Inc., Burlingame, CA, USA) and then incubated in primary antibody for 30 min at 4°C.

COX-1: COX-1 primary antibodies were monoclonal mouse anti-human (Cayman Chemical, Ann Arbor, MI, USA). The antibody was diluted 1:100 in antibody diluent (DAKO andS0809).

COX-2: COX-2 primary antibodies were PGHS-2 polyclonal rabbit anti-human antibodies (Oxford Biomedical Research, Inc., Oxford, MI, USA). The antibody was diluted 1:100 in antibody diluent (DAKO, Carpenteria, CA, USA).

iNOS: The iNOS primary antibodies were polyclonal rabbit anti-human (Oxford Therapeutic Antibody Centre, Oxford, England). The antibody was diluted 1:1000 in antibody diluent (DAKO).

p38: The p38 primary antibodies were polyclonal rabbit anti-human (Cell Signal Technology, Inc, Danvers, MA). The antibody was diluted 1:100 in antibody diluent (DAKO). Immunoreactive complexes were detected via an enhanced streptavidin-biotin affinity system (DAKO) and visualized with diaminobenzidine (DAKO). Slides were counterstained briefly in hematoxylin-1 (Richard-Allan Scientific, Kalamazoo, MI, USA). Negative control slides were incubated with either biotinylated goat anti-rabbit IgG or rabbit anti-mouse IgG (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA), at comparable dilutions to the positive slides.

***In-situ* Hybridization (ISH):** The nucleotide sequence for the COX-1, COX-2 and iNOS were obtained from the GenBank database. A computer program, Oligo 5.0 (NBI, Plymouth, MN, USA), was used to obtain compatible primer pairs. COX-1 primers based on a human template were: 5'TGG GGG CAG GAA CAT 3 and 5'CGG CAC ACG GAA GGA3', for the upper and lower primers, respectively. COX-2 primers were:

5'TGGGGGCAGGAACAT and 5'CGGCACACGGAAGGA, for the upper and lower primer, respectively. iNOS primers were: 5CAA TAA CCT GAA GCC CGA AGA 3 and 5'GAA AAG ACC GCA CCG AAG AT 3', for the upper and lower primer, respectively. Due to technical limitations, *in situ* hybridization assessments were not performed for p38. The templates for each generated probe were obtained by RT-PCR and cloned into the pCRII plasmid using a TA cloning kit (Invitrogen, Carlsbad, CA, USA). Competent INVαF' cells were transformed with this construct and screened for presence of the plasmid with insert. Insert orientation was confirmed by both restriction enzyme analysis and DNA sequencing. Plasmids were linearized and the RNA probe was generated with an *in vitro* transcription reaction utilizing ^{33}P-UTP and appropriate RNA polymerases. The percent incorporation of ^{33}P was determined in a scintillation counter and the probe quality was evaluated using denaturing polyacrylamide gel electrophoresis. Sections, approximately 4 μm in thickness, prepared from paraffin blocks, were deparaffinized with xylene, rehydrated in a graded series of ethanol and fixed in 4% paraformaldehyde (10 min, 4°C). The sections were then digested with Proteinase K (5 μg mL^{-1}) for 10 min at 37°C, washed and incubated in hybridization buffer (50% formamide, 2 X SSC, 10% dextran sulfate) for two hours at 42°C and then hybridizied overnight at 55°C with buffer containing tRNA (50 μg mL^{-1}) and appropriately labeled with probe (2×10^6 cpm/slide). Autoradiographic detection of the hybrids was carried out by coating the slides with photographic emulsion (NTB-2, Eastman Kodak, Rochester, NY, USA), followed by development at 3 and 5 weeks post-exposure. Tissue sections were then counterstained with hematoxylin and eosin and examined by bright- and dark-field microscopy.

RESULTS

Marked differences in the expression patterns between COX-1, COX-2 and iNOS were observed, while no expression of p38 was observed in either normal or pathological brain specimens (Table 1).

COX-1 expression: COX-1 expression was evident in all of the normal brain specimens and localized to the endothelial cells of the cerebral vasculature, as confirmed by both IHC and ISH. No change in COX-1 expression was observed in the brains from patients with CVD or CVA.

COX-2 expression: Minimal COX-2 expression was observed in the normal brain tissues. Expression was localized to neurons and some cerebral blood vessels. However, COX-2 expression was up-regulated in the brains of patients with CVA or CVD. Marked COX-2 expression was observed in neurons, cerebral blood vessels, glial cells and inflammatory cell in areas surrounding the infarcted areas.

iNOS expression: Marked iNOS expression was observed in 2/6 normal brains and was localized to neuronal tissue and vascular smooth muscle cells. A moderate increase in this expression intensity was evident in the infracted brains. In one case of CVD, minimal staining was observed in gitter cells.

Table 1: Expression of COX-1, COX-2, iNOS and p38 in human brain from Normal and CVA and CVD subjects

Age	Sex	Tissue	Diagnosis	COX-1	COX-2	iNOS	p38
85	M	Caudate/Internal capsule	Normal	+++	-	-	-
62	F	Parietal cortex	Normal	+++	+	-	-
87	F	Cerebellar cortex	Normal	+++	-	-	-
90	F	Occipital cortex	Normal	+++	-	-	-
78	M	Pons	Normal	+++	-	+ [a]	-
63	M	NAWM/NAGM	Normal	+++	+	+ [c]	-
53	M	Parietal cortex	CVA, infarction, (large, intermediate)	+++	+++	+++ [a]	-
53	M	Parietal cortex	CVA, infarction, (large, intermediate)	+++	+++	+++ [c]	-
71	M	Occipital cortex	Infarction	+++	+++	+++ [a]	-
71	M	Occipital cortex	CVD	+++	+++	+++ [c]	-
55	M	White Matter	CVD	+++	+++ [a]	-	-
55	M	NAWM/NAGM	CVD	+++	+++ [a]	+	-
84	F	Caudate/Internal capsule	CVD	+++	-	+ [d]	-
84	F	Caudate/Internal capsule	CVD	+++	+ [b]	+	-
82	M	Cerebellar cortex	CVD	+++	+++	-	-
82	M	Cerebellar cortex	CVD	+++	+++	+	-
79	M	Pons	CVD	+++	-	+	-
79	M	Pons	CVD	+++	-	-	-

- = No staining; += Minimal staining; +++ = Marked staining; Abbreviations: NAWM = Normal appearing white matter; NAGM = Normal appearing gray matter; CVA = Cerebro vascular accident; CVD = Cerebro vascular disease. [a]Staining present in neuronal tissue; [b]Staining present in neuronal tissue and vasculature; [c]Staining present in neuronal tissue and vascular smooth muscle cells; [d]Staining present in neuronal tissue, vascular smooth muscle and gitter cells

p38 expression: No p38 expression was observed in the normal or infarcted brain specimens.

DISCUSSION

Present results demonstrate a marked up-regulation in COX-2 and iNOS expression in the brain of patients with cerebral infarction. This up-regulation was prominent in the neuronal tissues surrounding the infarct, which further validates previous reports of both COX-2 and iNOS induction in response to infarction (Candelario-Jalil *et al*., 2007; Nogawa *et al*., 1998). As expected, constitutive COX-1 expression was observed in both normal and infarcted brains, supporting the hypothesis that COX-1 is not associated with inflammatory responses, but rather functions in homeostasis. However, no p38 staining was evident in either normal or infarcted human brains, indicating that p38 may not have a prominent role in normal or infarcted brain pathophysiology.

The inflammatory cytokines produced shortly after ischemic injury are believed to contribute to the progression of the brain injury following stroke (del Zoppo *et al*., 2000). After induction of an ischemic event, an increase in permeability of the endothelium component of the blood brain barrier occurs, followed by the adhesion of neutrophils within the microvasculature and subsequent loss of endothelial cells and astrocyte end-feet of the neurons. The inflammatory reactions with the accumulated leukocytes in the microvasculature are thought to cause tissue injury via interference with normal microvascular perfusion, in addition to the release of oxygen radicals and cytotoxic products (Candelario-Jalil *et al*., 2007; del Zoppo *et al*., 2000; Veldhuis *et al*., 2003). A majority of these accumulated neutrophils are adherent to the endothelium, with some migrating outside the vascular walls to the area of focal ischemia. Therefore, structural damage to microvasculature, leukocytic transmigration and the production of inflammatory cytokines all impact the subsequent neuronal injury following an ischemic event (Candelario-Jali *et al*., 2007; del Zoppo *et al*., 2000).

In several models of focal ischemia, up-regulation of both iNOS and COX-2 has been demonstrated, with peak expression occurring between 12 and 24 h post-ischemia (Iadecola *et al*., 1996; Zhao *et al*., 2000; Nogawa *et al*., 1998). In the post-ischemic brain, iNOS localized to neutrophils, microvessels, macrophages within the ischemic core and astrocytes of the borderzone (Niwa *et al*., 2001). Observed reduction in infarct size upon treatment with an iNOS inhibitor in a rat model of MCAO and smaller infarcts in iNOS$^{-/-}$ mice than wild-type littermates, suggests a direct role for iNOS in the progression of post-ischemic brain injury. Similar results were noted in studies of MCAO using COX-2 inhibitors and COX-2$^{-/-}$ mice. COX-2 is induced globally in infarcted human brain (Sairanen *et al*., 1998). Treatment with a COX-2 inhibitor reduced post-ischemic cerebral infarction size, with COX-2$^{-/-}$ animals exhibiting a decreased extent of post-ischemic cerebral injury (Candelario-Jalil *et al*., 2007; Iadecola *et al*., 2001; Sasaki *et al*., 2004). These results imply that COX-2, like iNOS, is involved in post-ischemic brain injury.

Many cytokines (e.g., TNF-α, IL1-β, IL-6) have been implicated in the generation of stroke-related brain injury (del Zoppo *et al*., 2000; Zhang *et al*., 2008). In an effort to identify therapeutic targets for the alleviation of stroke-induced brain injury, inhibitors of cytokine production are being studied for their potential to reduce post-ischemic brain damage p38 has been shown to be up-regulated in response to stress and linked to the production of cytokines during inflammation (Brone and Parsons, 2000). A study using a p38 inhibitor demonstrated the reduction of infarction extent in *in vitro* brain preparations; however, this study failed to detect p38 in either normal or infarcted human brain tissue (Barone *et al*., 2001).

The mechanism by which iNOS and COX-2 contribute to cerebral damage is not well understood. It has been postulated that the potential cerebrovascular effects of both enzymes might explain their participation in the generation of brain injury; however, the reduction in infarct volume among iNOS$^{-/-}$ mice could not be attributed to the effects on neutrophilic infiltration or astrocytic activation since these parameters were similar to wild-type control animals (Zhao *et al*., 2000). Further, the known cytotoxic effects of COX-2 and its localized expression to neurons render a cerebrovascular effect unlikely.

A possible hypothesis to explain the function of these enzymes in the progression of post-ischemic brain injury may be that iNOS activates COX-2; thereby enhancing the toxic output of iNOS (Seibert *et al*., 1995; Nogawa *et al*., 1998; Salvemini and Masferrer, 1996; Seibert *et al*., 1994). This theory is supported by the observed reduction of COX-2 in iNOS$^{-/-}$ mice and COX-2 localized expression to neurons within close proximity of iNOS-expressing neutrophils surrounding the infarction (Nogawa *et al*., 1998). Furthermore, the potential for deleterious effects of COX-2 on the brain has been suggested by the observed up-regulation of COX-2 in several neurological conditions (i.e., Alzheimer's disease) (Kotilinek *et al*., 2008; Yasojima *et al*., 1999), coupled with demonstration of COX-2-derived prostanoid cytotoxicity

(Seibert *et al*., 1995). Present data demonstrate the up-regulation of iNOS and COX-2 in infarcted brains and provide support for the hypothesis that these enzymes contribute to the progression of post-ischemic cerebral injury.

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