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EXPRESSION AND LOCALIZATION OF INTER-ALPHA INHIBITORS IN RODENT BRAIN

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Abstract—Inter-alpha Inhibitor Proteins (IAIPs) are a family of related serine protease inhibitors. IAIPs are important components of the systemic innate immune system. We have identified endogenous IAIPs in the central nervous system (CNS) of sheep during development and shown that treatment with IAIPs reduces neuronal cell death and improves behavioral outcomes in neonatal rats after hypoxic–ischemic brain injury. The presence of IAIPs in CNS along with their exogenous neuroprotective properties suggests that endogenous IAIPs could be part of the innate immune system in CNS. The purpose of this study was to characterize expression and localization of IAIPs in CNS. We examined cellular expressions of IAIPs *in vitro* in cultured cortical mouse neurons, in cultured rat neurons, microglia, and astrocytes, and *in vivo* on brain sections by immunohistochemistry from embryonic (E) day 18 mice and postnatal (P) day 10 rats. Cultured cortical mouse neurons expressed the light chain gene Ambp and heavy chain genes Itih-1, 2, 3, 4, and 5 mRNA transcripts and IAIP proteins. IAIP proteins were detected by immunohistochemistry in cultured cells as well as brain sections from E18 mice and P10 rats. Immunoreactivity was found in neurons, microglia, astrocytes and oligodendroglia in multiple brain regions including cortex and hippocampus, as well as within both the ependyma and choroid plexus. Our findings suggest that IAIPs are endogenous proteins expressed in a wide variety of cell types and regions both *in vitro* and *in vivo* in rodent CNS. We speculate that endogenous IAIPs may represent endogenous neuroprotective immunomodulatory proteins within the CNS.

Key words: cell culture, fetal, Inter-alpha Inhibitors, neonate, neurons, rodent.

INTRODUCTION

Endogenous proteases are found in all organisms from microbes to mammals and participate in various physiological and pathological processes including coagulation, immune responses, and cancer metastasis (Ranasinghe and McManus, 2013). In the central nervous system (CNS), a subfamily of proteases, serine proteases, and their inhibitors have been identified in both neurons and glia (Choi et al., 1990; Weinstein et al., 1995; Vivien and Buisson, 2000). Accumulating evidence suggests that serine proteases play important roles in neuronal development, plasticity, and pathology (Turgeon and Houenou, 1997) and that their inhibitors modulate neuronal cell death and exert neuroprotective properties in brain injury (Vivien and Buisson, 2000; Reuther et al., 2014).

Inter-alpha Inhibitor Proteins (IAIPs) are a group of serine protease inhibitors that have been detected in many tissues including liver, intestine, kidney, stomach, placenta and brain signifying their diverse biological functions (Itoh et al., 1996; Daveau et al., 1998; Takano et al., 1999; Spasova et al., 2014). Although the liver is the primary site of IAIP synthesis and the major source of the IAIPs found in blood, our recent findings suggest that IAIPs are also present in the brain and other organs during development (Spasova et al., 2014).

In hepatocytes, IAIP molecules are encoded by an α1-microglobulin/bikunin precursor (Ambp) light chain gene and at least five structurally associated inter alpha-trypsin inhibitor heavy chain genes (Itih 1–5) (Salier et al., 1996; Zhuo et al., 2004). The genes of Ambp and Itih 1–5 code for the polypeptide precursors of the light chain (AMBP) and heavy chains (H1P, H2P, H3P, H4P, and H5P), which subsequently undergo post-translational processing including removal of signal peptides and trimming of C-terminal ends (Kaumeyer et al.,

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Abbreviations: AMBP, polypeptide precursors of light chain; Ambp, α1-microglobulin/bikunin precursor light chain mRNA transcript; BSA, bovine serum albumin; DAB, 3,3′-diaminobenzidine; DMEM, Dulbecco's modified eagle medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; HC, mature polypeptide of heavy chain; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HP, polypeptide precursors of heavy chain; HRP, horseradish peroxidase; *Ial*, Inter-alpha Inhibitor; IAIPs, Inter-alpha Inhibitor Proteins; Itih, alpha-trypsin inhibitor heavy chain mRNA transcripts; LC, mature polypeptide of light chain; MAP-2, microtubule-associated protein 2; NeuN, Fox3/neuronal nuclei; P/S, penicillin/streptomycin; *Pal*, Pre-alpha Inhibitor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PIS, pre-immune serum; TBS, Tris-buffered saline.

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1986; Salier et al., 1987, 1996; Bratt et al., 1993; Zhuo and Kimata, 2008). The core mature polypeptide of the light chain (LC) is called bikunin because it contains two Kunitz-type domains making it a serine protease inhibitor (Potempa et al., 1989). Bikunin is covalently linked to one or two mature polypeptide(s) of the heavy chains (HCs) by a glycosaminoglycan forming a bikunin–HC complex before secretion by liver into blood (Jessen et al., 1988; Zhuo and Kimata, 2008). There are two major bikunin–HC complexes found in blood: Inter-alpha Inhibitor (*IaI*, 250 kDa), in which bikunin is linked to HC-1 and HC-2 and Pre-alpha Inhibitor (*PaI*, 120 kDa), in which bikunin is linked to HC-3 (Enghild et al., 1989; Odum et al., 1989). In these complexes, bikunin remains inactive until it is cleaved by partial proteolytic degradation (Fries and Blom, 2000).

Currently, IAIPs are gaining increasing attention because of their potential therapeutic beneficial effects. Administration of exogenous bikunin and IAIPs has been shown to attenuate systemic inflammation (Lim et al., 2003; Singh et al., 2010) and inhibit tumor metastasis (Kobayashi et al., 2003; Suzuki et al., 2004). IAIPs exert beneficial effects in sepsis and systemic inflammatory disorders both in neonates and in adults (Lim et al., 2003; Singh et al., 2010). Very little information is available regarding IAIPs in CNS. We recently have identified endogenous *IaI* and *PaI* proteins in the cerebral cortex, choroid plexus and cerebral spinal fluid of sheep during development and have shown that exogenous treatment with IAIPs reduces neuronal cell death and improves behavioral outcomes in neonatal rats exposed to hypoxic–ischemic brain injury (Spasova et al., 2014; Threlkeld et al., 2014; Gaudet et al., 2016). Others have shown beneficial effects of the light chain, bikunin, in cerebral ischemia–reperfusion injury and experimental autoimmune encephalomyelitis (Yano et al., 2003; Koga et al., 2010; Shu et al., 2011). Although IAIPs in blood are mainly hepatic in origin, our recent findings have suggested that IAIPs in the brain and other tissues may be endogenously produced (Spasova et al., 2014). Even though it is plausible that IAIPs might cross the blood–brain barrier via a yet-to-be-demonstrated specific transport mechanism (Banks, 2015), their ability to readily traverse the blood–brain barrier seems unlikely given the large size of the IAIPs and the early development of blood–brain barrier integrity in the fetus (Stonestreet et al., 1996). It is far more likely that endogenous IAIPs (Spasova et al., 2014) are produced within specific brain cells. Hence, the major goal of the current study was to determine the presence of IAIP mRNA and protein within specific cell types of the immature rodent brain.

In contrast to the increasing number of studies examining the exogenous effects of IAIPs, the endogenous localization, specific cellular expression, and regional distribution of IAIPs in CNS have not been previously examined, particularly in fetal and neonatal subjects. In addition, the information that is available regarding localization of IAIPs in CNS remains controversial. Some studies report the presence of the bikunin gene in neurons but not in astrocytes (Takano et al., 1999), whereas, others report bikunin immunoreactivity in astrocytes, but not in neurons (Yoshida et al., 1991a, 1994). Although we identified both the *IaI* and *PaI* proteins in the cerebral cortex of sheep (Spasova et al., 2014), more detailed information regarding the expression and localization of IAIPs in different cell types in the fetal and neonatal brain is lacking. Expression of endogenous IAIPs in cortical and hippocampal neurons may be of great importance given our previous observation that exogenous treatment with IAIPs attenuated cortical neuronal loss, improved behavioral outcomes, and decreased the number of basal dendrites per CA1 pyramidal neuron in the hippocampus of neonatal rats exposed to hypoxic–ischemic brain injury (Threlkeld et al., 2014; Gaudet et al., 2016). Therefore, we reasoned that it would also be important to determine whether endogenous IAIPs were expressed by neurons in these critical brain regions.

The objective of the current study was to establish the presence and expression of IAIPs in fetal and neonatal CNS in an effort to begin to elucidate the cellular and regional distribution of endogenous IAIPs in the immature CNS. The current study also begins to explore the concept that endogenous IAIPs could be locally produced in diverse cell types and brain regions and potentially have paracrine/autocrine modes of action. Therefore, we examined the cellular expression of IAIPs *in vitro* in cultured cortical mouse neurons, in cultured rat neurons, microglia, and astrocytes, and *in vivo* in brain sections by immunohistochemistry from embryonic day 18 (E18) mice and postnatal day 10 rats (P10).

EXPERIMENTAL PROCEDURES

Experimental animals

Wild-type C57BL/6 mice and Wistar rats were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA) and Charles River Laboratories (Wilmington, MA, USA), respectively. The present study was performed with approval by the Institutional Animal Care and Use Committees of the Alpert Medical School of Brown University and Women & Infants Hospital of Rhode Island and in accordance with the National Institutes of Health Guidelines for the use of experimental animals.

Primary cortical neuron, microglia, and astrocyte cultures

Mice on embryonic day 18 (E18) and rats on postnatal day 1–2 (P1–2) were used for the primary cortical neuronal cultures. The mouse brain samples used in this study were available from terminal mouse studies that did not use the CNS. The techniques for the neuronal cultures were performed as previously described in detail (Beaudoin et al., 2012). Briefly, the mice or rats were decapitated and the brains immediately transferred into a dissection medium [97.5% Hank's balanced salt solution (Invitrogen, Frederick, MD, USA, 0.11 mg/ml sodium pyruvate (Invitrogen), 0.1% glucose, 10 mM HEPES (pH 7.3, Sigma–Aldrich), and 1000 U/mL penicillin/streptomycin (P/S, Invitrogen)]. The meninges were removed and the cerebral cortices dissected.