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Acid-sensing ion channel 1 and nitric oxide synthase are in adjacent layers in the wall of rat and human cerebral arteries

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ABSTRACT

Extracellular acidification activates a family of proteins known as acid-sensing ion channels (ASICs). One ASIC subtype, ASIC type 1 (ASIC1), may play an important role in synaptic plasticity, memory, fear conditioning and ischemic brain injury. ASIC1 is found primarily in neurons, but one report showed its expression in isolated mouse cerebrovascular cells. In this study, we sought to determine if ASIC1 is present in intact rat and human major cerebral arteries. A potential physiological significance of such a finding is suggested by studies showing that nitric oxide (NO), which acts as a powerful vasodilator, may modulate proton-gated currents in cultured cells expressing ASIC1s. Because both constitutive NO synthesizing enzymes, neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS), are expressed in cerebral arteries we also studied the anatomical relationship between ASIC1 and nNOS or eNOS in both rat and human cerebral arteries. Western blot analysis demonstrated ASIC1 in cerebral arteries from both species. Immunofluorescent histochemistry and confocal microscopy also showed that ASIC1-immunoreactivity (IR), colocalized with the smooth muscle marker alpha-smooth muscle actin (SMA), was present in the anterior cerebral artery (ACA), middle cerebral artery (MCA), posterior cerebral artery (PCA) and basilar artery (BA) of rat and human. Expression of ASIC1 in cerebral arteries is consistent with a role for ASIC1 in modulating cerebrovascular tone both in rat and human. Potential interactions between smooth muscle ASIC1 and nNOS or eNOS were supported by the presence of nNOS-IR in the neighboring adventitial layer and the presence of nNOS-IR and eNOS-IR in the adjacent endothelial layer of the cerebral arteries.

Introduction

Acid-sensitive ion channels (ASICs) are a class of proton-gated cation channels that are widely distributed in the central and peripheral nervous system (CNS and PNS, respectively) where they are activated in response to decreases in extracellular pH as may occur with inflammation, ischemic stroke, traumatic brain injury and epileptic seizure (Lingueglia, 2007; Waldmann, 2001). The ASIC channel consists of three homomeric or heteromeric subunits encoded by four genes (Chu and Xiong, 2013). There are seven types of ASIC subunits (1a, 1b1, 1b2, 2a, 2b, 3 and 4), and each type of ASIC subunit consists of two transmembrane domains (TM1 and TM2) and a large cysteine-rich loop (Chu and Xiong, 2013). In addition to their acting as chemosensors responding to extracellular acidosis, some ASIC channels are also implicated in mechanotransduction (Chung et al., 2010).

ASIC type 1 (ASIC1) is the most broadly expressed channel of the ASIC family in CNS areas including the olfactory bulb, cerebral cortex, hippocampus, basolateral amygdaloid nuclei, subthalamic nuclei and cerebellum (Alvarez et al., 2003). Although the function of ASIC1 in the CNS has not been established, studies have shown

Abbreviations: ACA, anterior cerebral artery; ASIC, acid-sensitive ion channel; ASIC1, acid-sensitive ion channel type1; BA, basilar artery; CNS, central nervous system; eNOS, endothelial nitric oxide synthase; IR, immunoreactivity; MCA, middle cerebral artery; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; PCA, posterior cerebral artery; PBS, phosphate buffered saline; PGP9.5, protein gene product 9.5; PNS, peripheral nervous system; RECA-1, rat endothelial cell antigen; RRX, rhodamine red X; SDS, sodium dodecyl sulphate; SMA, alpha-smooth muscle actin; SNAP, S-nitroso-N-acetyl-penicillamine.

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that homomeric ASIC1a plays an important role in normal brain functions such as synaptic plasticity, learning/memory, and fear conditioning (Ziemann et al., 2009). In addition, ASIC1 channels may contribute to development of a number of pathological conditions (Pignataro et al., 2011; Xiong et al., 2004). For example, loss of ASIC1a function after intracerebroventricular injection of an ASIC1a blocker or in ASIC1a gene knockout animals protects the brain from ischemic injury (Xiong et al., 2004). Also, pre- or post-conditioning modulates expression of ASIC1a during ischemia (Pignataro et al., 2011). Therefore, it is plausible that ASIC1 may play a role in the development of acidosis-mediated ischemic brain damage in stroke. ASIC1 is predominantly expressed in axons, axon terminals and cell bodies in the CNS, PNS and cultured neurons (Zha, 2013) but also may be found in vascular smooth muscle cells where it may regulate muscle cell migration and influence vascular tone (Drummond et al., 2008b; Grifoni et al., 2008).

A vascular and neuronal origin for ASIC is shared with nitric oxide (NO), a short-lived mediator that plays an important role in modulation of cerebral blood flow and may provide tonic vasodilatory influences on cerebral vessels (McCarron et al., 2006; Talman et al., 2007). Neuronal nitric oxide synthase (nNOS), the enzyme responsible for neuronal synthesis of NO, is found in nerve fibers that surround and innervate cerebral arteries in experimental animals and in humans (Nozaki et al., 1993; Taktakishvili et al., 2010). NO may modify the function of a wide variety of proteins, including ASICs (Wang et al., 2012), by two major pathways (Ahern et al., 2002). One pathway involves activation of soluble guanylate cyclase, which produces cGMP and through it activates protein kinase G (PKG) to affect other proteins (Potter, 2011). Another pathway directly modifies the tertiary structure of proteins by S-nitrosylation of the thiol side chains of neighboring cysteine (Marozkina and Gaston, 2012). One such S-nitrosylated product, S-nitroso-N-acetyl-penicillamine (SNAP), itself an NO donor, has been shown to modulate ASIC1 activity and to potentiate proton-gated currents in rat cultured dorsal root ganglion neurons and in Chinese hamster ovary cells expressing ASIC1a or ASIC1b (Cadiou et al., 2007).

Although ASIC1 (both ASC1a and ASIC1b) expression has been found in isolated mouse cerebral artery smooth muscle cells (Chung et al., 2010), there have been no studies to determine if ASIC1 is present in intact cerebral arteries from rats or humans and, if so, in what layers the ASIC1 may be found. We hypothesized that ASIC1 is present in cerebral arteries and that it is expressed in close proximity to nNOS expressing nerve fibers that are found in the adventitial layer of the arteries. In this study, utilizing an antibody that recognizes both ASIC1a and 1b, we performed Western blot analyses and immunofluorescent histochemistry with confocal microscopy in the human and rat anterior cerebral artery (ACA), middle cerebral artery (MCA), posterior cerebral artery (PCA) and basilar artery (BA). To determine if there is an anatomical basis for an interaction between ASIC1 and nNOS in cerebral arteries, we performed multiple-labeling immunofluorescent histochemistry for ASIC1 and nNOS in cerebral arteries. Because endothelial NOS (eNOS) is a major source of NO in blood vessels, we also performed multiple-labeling immunofluorescent histochemistry for ASIC1 and eNOS in these cerebral arteries.

Experimental procedures

Animals and tissue preparation

All procedures conformed to standards established in the *Guide for Care and Use of Laboratory Animals* (National Academy Press, Washington, DC 2011). The Institutional Animal Care and Use Committees of the University of Iowa and Department of Veterans Affairs Medical Center, Iowa City reviewed and approved all protocols. Both institutions are accredited by AAALAC, International. All efforts were made to minimize the number of animals used and to avoid their experiencing pain or distress. As the studies on human tissues were performed on post mortem material obtained through the Autopsy Service at the University of Iowa Hospitals and Clinics, approval was not required from the Institutional Review Board (IRB).

For studies utilizing Western blot analysis of rat cerebral vessels to validate the ASIC antibody, we euthanized adult male Sprague-Dawley rats (280–330 g) under deep pentobarbital (150 mg/kg) anesthesia as we have previously described (Lin et al., 2011). The brains were then removed and placed on ice. The ACA, MCA, PCA and BA from six rats were carefully dissected from surrounding tissue. Because the amount of vascular tissue from any single rat was insufficient for analysis, we divided the six rats into two groups of three rats per group, pooled together tissues from each group, and homogenized the pooled tissue for Western blot analysis (see below). Although the majority of the dissected tissue consisted of ACA, MCA, PCA and BA, it also contained part of the basal vein, which runs along part of the ACA and part of the PCA (Greene, 1970), and small veins that run along these cerebral arteries. A piece of parietal cortex (approximately 50 mg) was also removed from one rat in each group and was homogenized for Western blot analysis to provide comparison between ASIC1 in predominantly cortical tissue vs. predominantly vascular tissue.

For immunofluorescent staining of rat cerebral arteries, we euthanized and perfused adult male Sprague-Dawley rats (280–330 g, $n = 5$) under pentobarbital (50 mg/kg) anesthesia according to procedures described in our earlier publications (Lin et al., 2007, 2011; Lin and Talman, 2005a). The brain was then removed, post-fixed in 4% paraformaldehyde for 2 h and then cryo-protected for 2 days in 30% sucrose in phosphate buffered saline (PBS) at 4 °C. Frozen 20 μm coronal sections were cut with a cryostat and mounted on Colorfrost Plus microscope slides (Fisher Scientific, PA, USA). Brain sections that contained the ACA, MCA, PCA and BA were processed for immunofluorescent staining as will be described later.

Preparation of human cerebral arteries

Cerebral arteries were collected from five patients at necropsy approximately 15–22 h after each patient's death. One patient had died of lung cancer (age 74), three of septic shock in the setting of enterococcal bacteremia, coagulopathy, and pneumonia (age 75, 63 and 16, respectively) and one of heart failure associated with mitral valve prolapse (age 84). We obtained an un-fixed 1 cm segment from each of the ACA, MCA, PCA and BA. Tissue was fixed in 4% paraformaldehyde for 1 h at 4 °C and then cryo-protected for 1 h in 30% sucrose at 4 °C. Frozen 30 μm cross sections were cut with a cryostat and processed for immunofluorescent staining. We also obtained a 0.5 cm un-fixed segment from each of the ACA, MCA, PCA and BA from the same subjects and processed each piece of tissue from each subject for Western blot analysis (see below).

Western blot analysis of ASIC1

Procedures like those described in our previous publications (Lin et al., 2012; Lin and Talman, 2005b) were used for Western blot analysis of ASIC1. In brief, we homogenized tissue in homogenization buffer containing 2% sodium dodecyl sulphate (SDS), 1 mM phenylmethylsulfonyl flouride, 1 mM dithiothreitol and 1 mM EDTA in Tris buffered saline, pH 7.4, using disposable polypropylene pestles in 1.5 ml microcentrifuge tube (Kimble Chase, NY, USA). The volume of homogenization buffer was 120 μl for pooled rat cerebral arteries, 300 μl for 50 mg rat cortex, and 150 μl for each segment of 0.5 cm human cerebral arteries. After centrifugation, protein concentration of the supernate was determined by means of Bio-Rad DC Protein Assay (Bio-Rad Laboratories, CA, USA). Supernate containing 10 μg (rat) or 50 μg (human) protein was separated alongside Bio-Rad Precision Plus Proteins Standards (Bio-Rad Laboratories) by 7.5% SDS-polyacrylamide gel electrophoresis (Ready Gel, Bio-Rad Laboratories) using the Mini Protein II System (Bio-Rad Laboratories) according to Laemmli (1970). The separated proteins were transferred to nitrocellulose membrane (Bio-Rad Laboratories) using the Mini Trans-Blot Cell (Bio-Rad Laboratories). The blot was blocked in 10% milk in PBS and then incubated with ASIC1 antibody (1:1000 dilution, Santa Cruz Biotechnology, TX, USA) at 4 °C for 24 h. After thorough washes, the blot was incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:10,000 dilution, Jackson ImmunoResearch Lab., PA, USA) at 25 °C for 4 h. Protein bands were visualized with ECL Plus™ Western Blotting Reagents (GE Healthcare/Amersham Biosciences, South San Francisco, CA, USA) and exposed to X-ray films. Because ASICs are assembled from homomultimeric or heteromultimeric subunits and because the exact subunit combination of ASICs in native tissue is not clear, there are no accepted molecular weights of ASIC1a and 1b. Therefore, molecular weights of visualized bands were compared with known (Chung et al., 2010; Grifoni et al., 2008; Jahr et al., 2005) molecular weights of ASIC1 protein.

Immunofluorescent histochemistry

Procedures similar to those described in our previous publications (Lin et al., 2007; Lin and Talman, 2005a, 2006) were used for immunofluorescent staining of rat and human tissue sections. Immunofluorescent histochemistry for ASIC1 was performed with or without biotin-streptavidin amplification. Sections were incubated in rabbit anti-ASIC1 antibody (1:100 dilution with biotin-streptavidin amplification, 1:10 dilution without biotin-streptavidin amplification, Santa Cruz Biotechnology, catalog number SC-28756) in 10% donkey normal serum for 24 h in a